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Monoclonal Antibodies to Rabbit Liver Cytochrome P-450$_{LM2}$ and Cytochrome P-450$_{LM4}$

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SUMMARY

Monoclonal antibodies were prepared from hybridoma clones isolated by the fusion of myeloma cells and spleen cells derived from mice immunized with either purified rabbit liver microsomal cytochrome P-450$_{LM2}$ or cytochrome P-450$_{LM4}$. Seven hybridoma clones produced three kinds of monoclonal antibodies to P-450$_{LM2}$. The first class bound, precipitated, and inhibited the enzyme activity of P-450$_{LM2}$ for both benzo[a]pyrene hydroxylation and 7-ethoxycoumarin deethylation. The other two classes either bound and precipitated or only bound the enzyme. These monoclonal antibodies to P-450$_{LM2}$ showed a precipitin reaction and inhibition of enzyme activity that was specific for cytochrome P-450$_{LM2}$. Thus, they did not react with or inhibit the enzyme activity of the other isozyme cytochrome P-450$_{LM4}$, Fraction 1 or Fraction 7. All of the monoclonal antibodies formed against P-450$_{LM2}$ were mouse immunoglobulin (Ig) subclass IgG$_1$. The most effective monoclonal antibody strongly inhibited the formation of oxygenated metabolites of benzo[*a*]pyrene at various positions as well as the deethylation of 7-ethoxycoumarin. Four hybridomas were isolated which produced monoclonal antibodies to P-450$_{LM4}$. One of the four was of the IgM class and three were of the IgG$_1$ type. The four monoclonal antibodies bound to P-450$_{LM4}$ but did not precipitate the enzyme, and did not bind to P-450$_{LM2}$. The monoclonal antibody P-450$_{LM4}$ complexes interacted with protein A, and the enzyme activity for benzo[*a*]pyrene hydroxylation could be removed by centrifugation. The high specificity and monoclonality of these antibodies suggest their potential usefulness for studying the genetics, regulation, and roles of the different isozymes of P-450$_{LM}$ in drug and carcinogen metabolism.

INTRODUCTION

The key enzyme systems that metabolize xenobiotics, including drugs, carcinogens, and certain endogenous substrates such as steroids, are the mixed-function oxidases which contain various forms of cytochrome P-450 (1–3). The mixed-function oxidases thus represent the primary metabolic interface between environmental chemicals, including carcinogens and the individual organisms. In this role, these enzyme systems may be the key determinants in individuals of the rates of drug metabolism (2), carcinogen susceptibility (3–5), and drug and chemical toxicity.

A major class of environmental carcinogens to which humans are exposed comprises the polycyclic aromatic hydrocarbons, the most common and a prototype of which is BP[3] (6). BP is oxygenated by the cytochrome P-450-containing mixed-function oxidase, AHH, to several epoxides, phenols, and quinones. Metabolically related enzymes, such as epoxide hydrolases and several transferases, subsequently hydrate or conjugate the metabolites to dihydrodiols, diol epoxides, and water-soluble conjugates of glutathione, glucuronate, and sulfate (7). Thus, there are more than 40 known oxygenated metabolites of BP. The metabolic pathway leading to the BP diol epoxides is believed to be a primary pathway of carcinogen activation (7), whereas many of the other pathways resulting in conjugate formation are thought to be routes of detoxification.

Following the resolution of the liver microsomal system into its components, P-450$_{LM}$, NADPH cytochrome P-450 reductase, and phospholipid (8), several laborato-

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[3] The abbreviations used are: BP, benzo[*a*]pyrene; AHH, aryl hydrocarbon hydroxylase; HPLC, high-pressure liquid chromatography; PBS, phosphate-buffered saline; RIA, radioimmunoassay; Ig, immunoglobulin; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

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ries reported the isolation and characterization of different forms of cytochrome P-450, as recently reviewed (9, 10). For example, P-450$_{LM2}$[4] and P-450$_{LM4}$ have been purified to homogeneity (11–13), as has the constitutive form, P-450$_{LM3b}$ (14), and other forms have been partially purified from rabbit liver (11). The highly purified P-450$_{LM2}$, P-450$_{LM4}$, and P-450$_{LM3b}$ exhibit different biochemical and kinetic properties (14, 15). Furthermore, each isozyme of P-450 exhibits stereoselectivity in substrate choice and product formation with respect to both BP metabolism and the conversion of the (−)-*t*-7,8-diol of BP to the highly mutagenic BP 7,8-diol-9,10-epoxides (16–18). Thus the level and activity of each isozyme of cytochrome P-450 may govern metabolic pathway choice and thus be instrumental in determining the balance between carcinogen activation to carcinogenic forms and detoxification. This balance may relate to biochemical individuality to carcinogen susceptibility and to individual differences in rates of drug metabolism.

Koehler and Milstein (19) have developed the technique of producing mouse "hybridomas" that secrete pure and specific monoclonal antibodies against either purified antigens or mixtures of antigens in either semipurified or unfractionated preparations. The clonal selection and immortality of the hybridoma cell lines represent a continuous source of antibodies that are monoclonal and specific to single antigenic sites. Using this system, we have prepared monoclonal antibodies to purified rabbit liver cytochrome P-450$_{LM2}$ and P-450$_{LM4}$. Some of the preliminary results have been reported (20). The various classes of monoclonal antibodies obtained exhibit different activities in respect to binding and precipitation of P-450$_{LM2}$ and P-450$_{LM4}$ and in the inhibition of BP hydroxylation measured by AHH activity or by HPLC, and of ethoxycoumarin deethylation. The antibodies to P-450$_{LM2}$ do not interact with or inhibit the activity of the other isozymes of cytochrome P-450, i.e., Fraction 1, P-450$_{LM4}$, and Fraction 7. The antibodies to P-450$_{LM4}$ do not interact with P-450$_{LM2}$. Thus, the monoclonal antibodies show specificity for antigenic sites on the individual cytochromes which are specific for each form and may therefore be useful in studying the content, function, and mechanism of action of the various isozymes of the P-450s. The preparation of monoclonal antibodies to the various cytochrome P-450s and metabolically related enzymes may be, accordingly, helpful in determining the genetic and other factors that govern biochemical individuality in response to drug and carcinogen action.

EXPERIMENTAL PROCEDURES

Preparation of cytochrome. The various forms of cytochrome P-450 were isolated from rabbit liver microsomes. P-450$_{LM2}$ is inducible by phenobarbital but present in only trace amounts in microsomes from untreated or 5,6-benzoflavone-treated rabbits, whereas P-450$_{LM4}$ is inducible by 5,6-benzoflavone and is present at a lower but significant levels in microsomes from uninduced or drug-induced animals. The other forms of P-450$_{LM}$ are present in all of the microsomal preparations used and are not known to be inducible. Electrophoretically homogeneous P-450$_{LM2}$ (17.5 nmoles/mg of protein) was isolated from phenobarbital-induced microsomes, and the two P-450$_{LM4}$ preparations (14.0 and 12.9 nmoles/mg of protein, respectively) were isolated from phenobarbital and 5,6-benzoflavone-induced microsomes (12, 13). Partially purified Fraction 1 (2.2 nmoles/mg of protein) was obtained from phenobarbital-induced microsomes, and Fraction 7 (1.2 nmoles/mg of protein) was obtained from microsomes of untreated animals (11, 21). Since the fractions tentatively called P-450$_{LM1}$ and P-450$_{LM7}$ earlier (16, 18) lose all heme on extensive purification, some doubt remains whether the activity is indeed due to such cytochromes; in the present paper we are referring to these preparations simply as Fraction 1 and Fraction 7, respectively. Owing to heme loss, the cytochrome preparations contain varying amounts of apoenzyme, which is not distinguishable from the holoenzyme upon gel electrophoresis. Partially purified NADPH-cytochrome P-450 reductase from phenobarbital-induced rabbit liver microsomes (22) had a specific activity toward cytochrome *c* at 30° of 7.4–17.0 μmoles reduced per minute per milligram of protein and was entirely free of P-450$_{LM}$. Dilauroylglyceryl-3-phosphorylcholine was obtained from Serdary Research Laboratories (London, Ont., Canada).

Media and cells. Dulbecco's modified Eagle's medium, fetal calf serum, and horse serum were purchased from Grand Island Biological Company (Grand Island, N. Y.). The myeloma cell line RGNS-1, which was azaguanine-resistant and a nonproducer of immunoglobin, was obtained from Dr. John D. Minna (National Cancer Institute, Bethesda, Md.) and grown in Dulbecco's modified Eagle's medium with 4.5 g of glucose per liter and 0.584 g of glutamine per liter supplemented with 10% fetal calf serum, 10% horse serum and gentamicin, 50 μg/ml (complete medium). Mouse spleen myeloma hybridomas were grown in complete medium containing 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT medium) (23). Dulbecco's modified Eagle's medium with 1.0 g of glucose per liter plus 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) was used as the 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 μg of purified P-450$_{LM2}$ or P-450$_{LM4}$ emulsified in 0.2 ml of Freund's complete adjuvant. The following week they were further immunized by an i.v. injection of 12 μg of P-450$_{LM2}$ or P-450$_{LM4}$ in Dulbecco's PBS, pH 7.4 (Grand Island Biological Company). Three days after the i.v. injection, five mice were killed 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, then at 4° overnight, and centrifuged at 1000 × *g* for 30 min to obtain the clear serum. The sera were stored at −90°.

[4] The isozymes of rabbit liver microsomal cytochrome P-450 are numbered according to their electrophoretic mobilities; P-450$_{LM2}$ and P-450$_{LM4}$ are induced by phenobarbital and 5,6-benzoflavone or 3-methylcholanthrene, isosafrole, or tetrachlorodibenzo-*p*-dioxin, respectively.

Production of hybridoma cells. The fusion of the myeloma cells with spleen cells was carried out essentially as described by Koehler and Milstein (19) except that we used polyethylene glycol for the fusion (24). The spleens from five immunized mice were cut into small pieces, and the cells were dissociated by squeezing the tissue through a sterile mesh (No. 80) 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 of 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 0.83% NH_4Cl-buffered 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (pH 7.2) and kept in a 37° water bath for 2 min with shaking to break red blood cells. The NH_4Cl-treated cells were transferred to a 15-ml conical plastic centrifuge tube containing 2 ml of fetal calf serum and centrifuged 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, 1×10^7 RGNS-1 cells and 1×10^8 spleen cells were combined in a 50-ml plastic conical centrifuge tube and centrifuged at 1500 rpm for 15 min. The cell pellets were loosened, and 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; the mixture was diluted gradually with 1 ml 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 two Costar plates, and incubated in the CO_2 incubator at 37° overnight. The following day, 1 ml of the supernatant fluid was 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 2–3 days. After 2–3 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 cloned in HAT medium in 96-well microtiter plates (Costar) and distributed in a 0.2-ml cell suspension per well with a cell distribution probability of 0.4 cell in a single well. The cells grown into clones were transferred to the wells of a 24-well Costar plate and further grown to confirm their production of monoclonal antibodies. Clones were subcloned to different extents to determine the stability of the antibody-producing cells.

Preparation of monoclonal antibodies in mouse peritoneal ascites fluid. Hybrid cells producing antibodies were grown in flasks containing HAT or HT medium and collected by centrifugation, resuspended in PBS, and inoculated i.p. into BALB/c female mice (5×10^6 cells in 0.2 ml of PBS). After 2–3 weeks, the ascites fluids were collected with a syringe or by puncturing the abdomen and clarified as described above for serum.

RIA. Rabbit anti-mouse IgG specific for both heavy and light chain (Cappel) was labeled with ^{125}I in a 0.26 ml of reaction mixture containing 100 μg of rabbit anti-mouse IgG, 150 μl of 0.5 M phosphate buffer (pH 7.5), 10 μl of 0.5–1 mCi of ^{125}I-labeled sodium (Amersham), and 0.1 mg of chloramine T for 1 min. The reaction was stopped by the addition of 200 μl of sodium metabisulfite solution (12 mg/5 ml in 0.05 M phosphate buffer, pH 7.5). ^{125}I-Labeled rabbit anti-mouse IgG was separated from free ^{125}I by passing the reaction mixture through a 5-ml column of Dowex 1-X8, chloride form, 200 mesh (Bio-Rad). The column was pretreated with 1 ml of normal rabbit IgG (1 mg/ml) to prevent nonspecific binding of protein to the Dowex resin. After this treatment the column was washed with 0.15 M NaCl. The fractions of ^{125}I-labeled rabbit anti-mouse IgG were pooled and mixed with equal amounts of PBS containing 3% BSA and 2% sodium azide (3% BSA/PBS/azide) and stored at 4°.

The solid-phase RIA was carried out with modifications previously described (25, 26). The wells of a microtiter plate (flexible, polyvinyl chloride; Dynatech) were coated with P-450_{LM2} or P-450_{LM4} by incubation with 100 μl of the purified cytochrome in PBS (0.5 mg/ml) for 2 hr at 37°. The remaining nonspecific sites were covered with BSA by replacing the cytochrome solution with 3% BSA/PBS/azide and incubating for 30 min. The wells were washed with PBS three times, and the cytochrome P-450 bound to the walls was incubated for 2 hr with the culture fluids of monoclonal antibodies. The monoclonal antibodies bound to enzymes were then incubated with ^{125}I-labeled rabbit anti-mouse IgG overnight and washed five 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.

Antibody-cytochrome P-450 incubation. Each dilution of antibody in 420 μl was mixed with 30 μl of P-450_{LM2} (1–10 μg of protein) and 50 μl of PBS and incubated at room temperature for 15 min with gentle shaking. For enzyme inhibition assay of the monoclonal antibodies to P-450_{LM4}, the antibody-P-450_{LM4} mixtures were placed at 4° overnight, and then for 1 hr with protein A-Sepharose 4B, and subjected to centrifugation at 15,000 × *g* for 5 min. The supernatants were used for the determination of residual enzyme activity.

Measurement of cytochrome P-450 enzyme activity. Arylhydrocarbon (BP) hydroxylase (AHH) was assayed with the reconstituted mixed-function oxidase system by measuring the fluorescence of alkali-extracted phenols with an Aminco Bowman spectrofluorometer as described previously (27). Cytochrome P-450 (1–10 μg of protein) in 0.5 ml, or 0.5 ml of the incubation mixture of cytochrome P-450 and ascites fluid, was combined with 0.49 ml of a mixture containing 250 μl of Tris-HCl (0.2 M, pH 8.3), 30 μl of dilauroylglyceryl-3-phosphorylcholine (1 mg/ml), 30 μl of NADPH-cytochrome P-450 reductase (3.36 μg of protein), 50 μl of NADPH (0.17 mg), 30 μl of $MgCl_2$ (0.1 M), and 100 μl of water. The reaction was started by the addition of 10 μl of BP (2 mM). The incubation was carried out at 37° for 20 min. Cytochrome P-450-catalyzed BP metabolism was measured by HPLC as previously described (16). The same procedure as in the AHH assay was used to measure the enzyme-catalyzed formation of BP metabolites, except that the sub-

strate consisted of 50 nmoles of [^{14}C]BP 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 Spectro-Physics Model 3500 liquid chromatograph with a DuPont Zorbax octadecyltrimethoxysilane column (6.2 mm inner diameter × 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 co-chromatographed with the samples. Cytochrome P-450-catalyzed 7-ethoxycoumarin deethylation was measured according to the method of Greenlee and Poland (28) except that the substrate was added in methanol (500 nmoles) to the reconstituted enzyme system described for the AHH assay above.

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 usually appeared after 2–3 days.

Labeling of cells and polyacrylamide gel electrophoresis. Hybrid cells (0.2–1 × 10^6 cells) were labeled with 5 μCi of ^{14}C-labeled leucine in 0.2 ml of Dulbecco's leucine-free medium (Grand Island Biological Company) containing 5% dialyzed horse serum. After incubation overnight (14–15 hr at 37°), the cells were pelleted, and aprotinin (Sigma Chemical Company) and SDS were added to the supernatants to obtain final concentrations of 330 units of aprotinin per milliliter and 3.3% SDS,

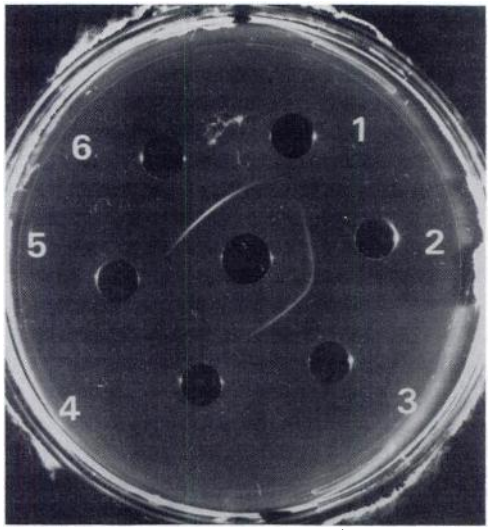

FIG. 1. *Double-immunodiffusion analysis of sera of mice immunized with P-450$_{LM2}$*

Wells were filled with 20 μl of the following samples: *center, P-450$_{LM2}$* (0.35 mg/ml); *1*, IgG (1 mg/ml) of rabbits treated with phenobarbital; *2*, *3*, *6*, sera from mice immunized for 2, 3, and 4 weeks, respectively, with P-450$_{LM2}$; *4*, mouse IgG (1 mg/ml); and *5*, serum of normal mice.

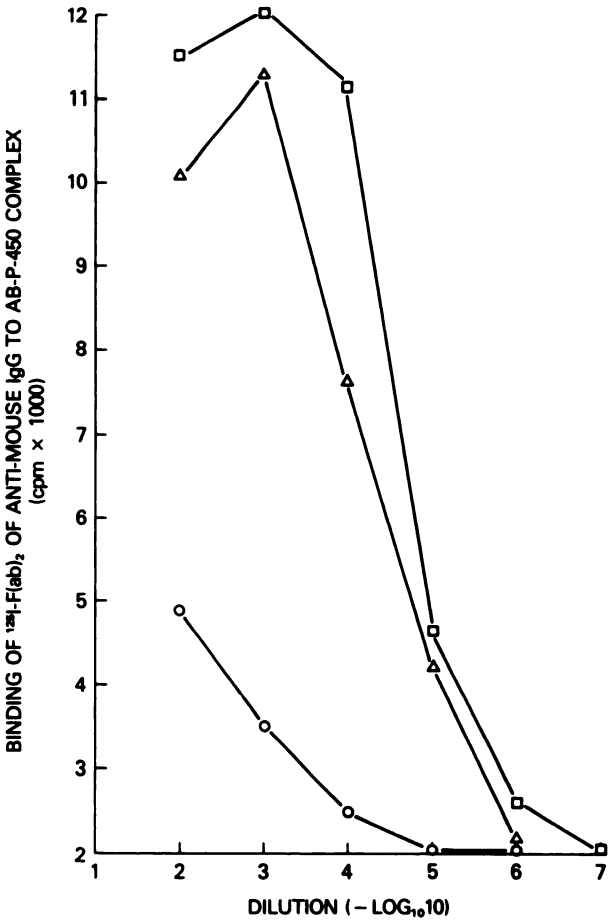

FIG. 2. *RIA of sera of mice immunized with rabbit liver microsomal cytochrome P-450$_{LM4}$ to P-450$_{LM4}$*

The protein concentration of sera from the unimmunized animals (○) as well as those immunized with P-450$_{LM4}$ for 3 weeks (△) and for 4 weeks (□) was adjusted to 58 mg/ml and subjected to solid-phase RIA with ^{125}I-labeled F(ab')$_2$ of anti-mouse IgG (specific activity: 31 μCi/μg).

respectively. The supernatants were dialyzed at room temperature for 5 hr against 3 liters of 0.1% SDS, lyophilized, and dissolved in 100 μl of 10% SDS/10% (v/v) glycerol/0.1 M Tris-HCl (pH 6.8)/0.3 M β-mercaptoethanol/0.002% bromophenol blue (29). The samples were then boiled for 2 min, and 20-μl portions were subjected to polyacrylamide gel electrophoresis on three 20% gradient slab gels (1 hr at 5 mamps and 16 hr at 15 mamps). After electrophoresis, the gels were stained with 0.002% Coomassie blue/10% (v/v) isopropanol/10% glacial acetic acid solution, and destained in 10% (v/v) isopropanol/10% (v/v) glacial acetic acid for 8 hr and then 10% (v/v) acetic acid solution overnight. The gels were soaked in dimethyl sulfoxide for 30 min twice and 22% (w/v) 2,5-diphenyloxazole/dimethyl sulfoxide solution (29–32), dried, and placed in contact with XR-2 Kodak film.

RESULTS

Mouse serum antibodies. Mice immunized for 4 consecutive weeks with highly purified rabbit liver P-450$_{LM2}$ produced serum antibodies which bound to the cytochrome as detected by RIA (25, 26). The mouse serum antibodies bound the cytochrome, and the complex was

detected by its binding to anti-mouse IgG-^{125}I. The presence of antibodies to P-450$_{LM2}$ in serum of mice immunized for 2, 3, and 4 weeks was also detected by double-immunodiffusion precipitation of the serum with P-450$_{LM2}$. Precipitin bands between the cytochrome and anti-P-450$_{LM2}$ mouse sera, but not between the cytochrome and IgG from the sera of rabbits induced with phenobarbital, normal mouse IgG, or normal mouse serum (Fig. 1). The presence of antibody to P-450$_{LM4}$ in the serum of mice which were immunized with this cytochrome was detected by RIA (Fig. 2) and also by double immunodiffusion (Table 2).

Monoclonal antibodies: RIA and double-immunodiffusion analysis. Fusion of 1×10^7 myeloma cells with 1×10^8 spleen cells derived from mice immunized with P-450$_{LM2}$ resulted in the growth of hybrid cells in 36 of 48 wells containing the selective HAT medium (23). The HAT medium inhibits the growth of azaguanine-resistant myeloma cells, and unhybridized spleen cells were eliminated in subcultures because of their inability to grow in the culture media. Of the 36 wells containing hybrid cells, 15 were positive for mouse Ig production when tested with ^{125}I-labeled anti-mouse IgG (heavy- and light-chain specific).

The hybrid cells producing antibody were cloned into plates containing 96 wells, grown, and subcloned into Costar plates, each of which contained 24 wells. After initial growth for 2 weeks in HAT medium in the 96-well plates, antibody formation was examined by RIA of the cell culture medium. Of the 206 independent hybrid clones examined, seven were positive for the production of antibodies that bind to P-450$_{LM2}$ as indicated by a positive RIA (Table 1). The supernatant culture fluid of the control parent myeloma cells and the remaining 199 hybrid cell clones did not contain any mouse Ig that bound to P-450$_{LM2}$. The hybridomas producing the P-450$_{LM2}$ antibodies were further grown in HAT medium, and the culture fluids were concentrated 30–40 times. The antibody content of these concentrates was examined by double-immunodiffusion analysis.

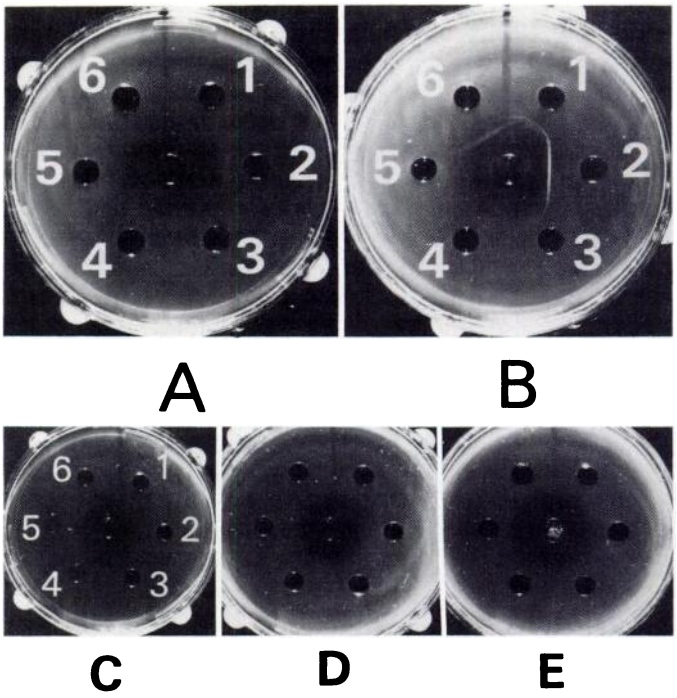

FIG. 3. *Double-immunodiffusion analysis of Ig secretion and monoclonal antibody to purified P-450$_{LM2}$*

Culture media from myeloma RGNS-1p72 and cloned hybrid cells 1-26-11p3 were concentrated 30 times and applied to central wells.

A. *Center,* RGNS-1p72; *1,* rabbit anti-mouse IgG (heavy- and light-chain specific, 4 mg/ml); *2,* goat anti-mouse IgG (heavy-chain specific, 5 mg/ml; *3,* rabbit anti-mouse κ-chain (1 mg/ml); *4,* goat anti-mouse 1gM (60–70 mg of total protein); *5,* goat anti-mouse IgA (5.5 mg/ml); *6,* purified P-450$_{LM2}$ (1.1 mg/ml).

B. *Center,* 1-26-11p3; 1–6 as in A. All wells were filled with 20-μl samples.

Center wells of C, D, and E were filled with 20 μl of concentrates (25 times) from the culture fluids of parent myeloma RGNS-1p73 (nonproducer), hybridoma 1-26-2p3, and hybridoma 1-31-1p3. Outer wells *1, 2, 3, 4, 5,* and *6* were filled with 20 μl of rabbit anti-mouse IgG$_1$, rabbit anti-mouse IgG$_{2a}$, rabbit anti-mouse IgG$_{2b}$, rabbit anti-mouse IgG$_3$, goat anti-mouse IgM, and rabbit anti-mouse IgA, respectively.

TABLE 1

Characteristics of hybridoma monoclonal antibodies to P-450$_{LM2}$

Antibody source[a]	^{125}I-Labeled anti-IgG binding to Ig-P-450$_{LM2}$ (culture fluid)	Precipitation of P-450[b] (ascites)				Ig subclass (culture fluid)
		Fraction 1	P-450$_{LM2}$	P-450$_{LM4}$	Fraction 7	
Myeloma parent[c] RGNS-1p59	–	–	–	–	–	–
Hybridoma						
P-450-1-7-15p5	–	–	–	–	–	–
P-450-1-31-1p3	+	–	+	–	–	γ1
P-450-1-31-2p3	+	–	+	–	–	γ1
P-450-1-31-3p3	+	–	–	–	–	–
P-450-1-31-4p8	+	–	–	–	–	–
P-450-1-31-5p5	+	–	–	–	–	–
P-450-1-26-2p3	+	–	+	–	–	γ1
P-450-1-26-11p3	+	–	+	–	–	γ1
Normal mouse serum	–	–	–	–	–	
Anti-P-450$_{LM2}$ mouse serum	+	–	+	–	–	

[a] Cells (5×10^6) were inoculated i.p. into BALB/c female mice and ascites fluid was collected after 2 weeks for hybridoma antibody production.
[b] Ouchterlony double-immunodiffusion technique.
[c] Myeloma parent cells which were used for the hybridization and the preparation of ascites fluid for control.

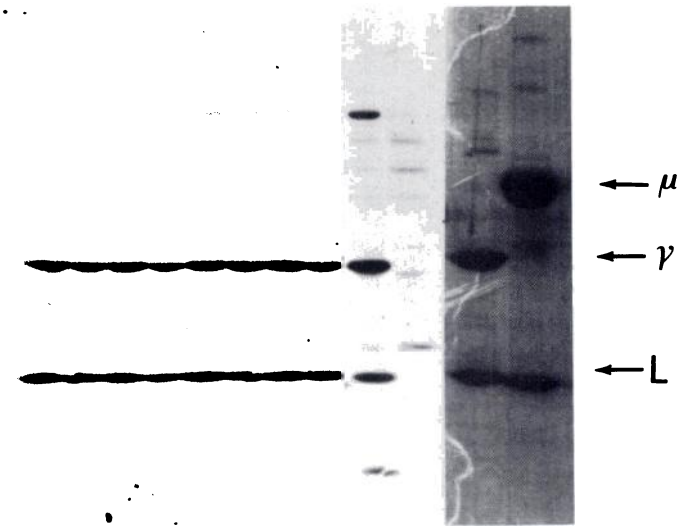

FIG. 4. *SDS polyacrylamide gel electrophoresis of reduced Ig secreted by the hybrids*

^{14}C-Labeled L-leucine was incorporated into the secreted proteins of the parental and hybrid cells. The secreted proteins were treated with SDS and 2-mercaptoethanol and submitted to electrophoresis on 3%–20% gradient slab gels with 3% spacer gel. *Lanes 1* through *4*, proteins secreted by hybrids 1-26-2 subclones, s19, s21, s23, and s24; *5* through *8*, proteins secreted by hybrids 1-26-11 subclones, s14, s21, s23, and s27; *9*, secretion by myeloma P3-X-63, IgG$_1$ (IgGκ) producer; *10*, nonsecretion by parent myeloma RGNS-1 (κ, nonsecreting); and *11* and *12*, markers of IgG$_1$ (IgF$_c$, MOPC21) and IgM (IgMλ$_1$, MOPC 104E) (*arrows*).

Figure 3A shows that the parent myeloma cell supernatant RGNS-1P72 did not secrete detectable levels of Ig, no reaction being observed with any of the anti-IgGs added, i.e., anti-IgG, anti-IgM, anti-IgA, or anti-mouse κ-chain. The hybridoma clone 1-26-11p3 formed from the spleen cells of P-450$_{LM2}$-immunized mice and RGNS-1 myeloma cells produced monoclonal antibodies which formed a clear precipitin band between P-450$_{LM2}$ and anti-mouse IgG specific for mouse IgG heavy and light chains and mouse IgG specific for the heavy chain only (Fig. 3B). The concentrated hybridoma fluids also showed a faint precipitin band with anti-mouse κ-chain serum. The culture fluids did not form a precipitin band with the other rabbit anti-mouse Ig sera, anti-IgM and anti-IgA. The concentrates of culture fluids of hybrids 1-26-2p3, 1-26-11p3, 1-31-1p3, and 1-31-2p3 were also subjected to further Ouchterlony double-immunodiffusion analysis with anti-mouse immunoglobulins IgG$_1$, IgG$_{2a}$, IgG$_{2b}$, IgG$_3$, IgM, and IgA. Precipitin bands were observed only between the wells containing the culture fluids and anti-mouse IgG$_1$ (Table 1 and Fig. 3D and E). Thus, all of the hybrid clones forming antibodies that precipitated P-450$_{LM2}$ were of the subclass IgG$_1$.

Monoclonal antibodies, being directed to a single antigenic site, do not generally precipitate the protein antigens to which they bind. The microsomal cytochrome P-450$_{LM2}$, being a membrane-type protein, tends to aggregate in the absence of detergent. Thus, the precipitation of the antigen-antibody complex in the double-immunodiffusion assay is likely due to binding of monoclonal antibody to the cytochrome P-450$_{LM2}$ that has either aggregated prior to or after interaction with the antibody. This view is supported by our finding that, under certain conditions in the presence of the detergent Emulgen 913, the monoclonal antibodies do not precipitate the cytochrome P-450$_{LM2}$ whereas precipitates are formed by the ordinary mouse serum antibodies to the cytochrome P-450$_{LM2}$.

The hybridoma cells 1-26-2 and 1-26-11 were passaged 13 times, subcloned, and then grown in the presence of [^{14}C]leucine in L-leucine-free media. These hybrid clones synthesized Ig exclusively and secreted it into the media. Fig. 4 shows a gel electrophoresis of the hybridoma-produced Ig. Both the heavy chain and light chain were evident upon reduction of the disulfide bonds and electrophoresis. These were identical in size with those of myeloma P3-X63 and MOPC 21 protein (Litton Bionetics). The parent myeloma RGNS-1 did not secrete any significant amounts of biosynthetically labeled Ig.

When the mice were immunized with purified P-450$_{LM4}$ and their spleen cells hybridized with myeloma cells,

TABLE 2

Characteristics of monoclonal antibodies to P-450$_{LM4}$

Antibody source	IgG subclass culture fluid	^{125}I-Labeled anti-IgG binding[a] to Ig P-450$_{LM4}$		Precipitation[b] of cytochrome P-450 (ascites fluid)	
		Culture fluid	Ascites (1:1024)	LM2	LM4
Myeloma parent RGNS-1p59	–	1167	2035	–	–
Hybridoma, nonspecific[c]					
NBS 1-3-1p7	γ2b		1712	–	–
NBS 1-48-5p22	γ2a			–	–
Hybridoma, specific[d]					
LM4 1-1-8p8	μ	5788	5523	–	–
LM4 1-4-3p8	γ1	7898	5398	–	–
LM4 1-6-1p8	γ1	9614		–	–
LM4 1-14-3p8	γ1	7569	6610	–	–
Normal mouse serum				–	–
Anti-P-450$_{LM4}$ mouse serum				–	+

[a] ^{125}I-labeled F(ab')$_2$ of anti-mouse IgG (specific activity, 31 μCi/μg) was used in the solid-phase RIAs.

[b] Ouchterlony double-immunodiffusion technique.

[c] Hybridoma between myeloma and normal mouse spleen cells.

[d] Hybridoma between myeloma and spleen cells of mice immunized with cytochrome P-450$_{LM4}$.

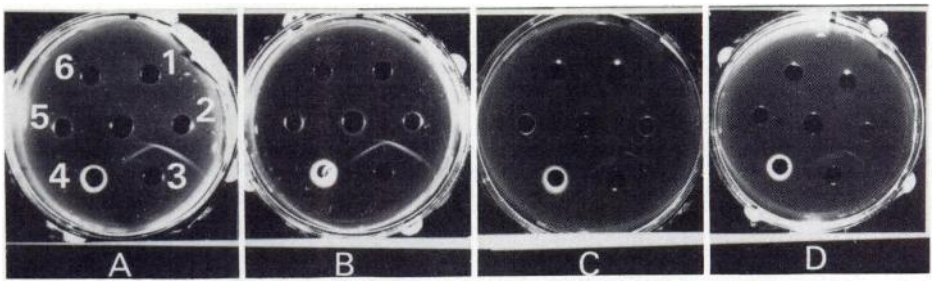

FIG. 5. *Double-immunodiffusion analysis of the specificity of monoclonal antibodies to purified isozymes of P-450$_{LM2}$*
The plates were incubated for 2 days at room temperature.
A. *Center,* monoclonal antibody to P-450$_{LM2}$, produced by hybrid 1-31-1p3; 1, Fraction 1 (0.49 mg/ml); *2* and *5*, anti-P-450$_{LM2}$ mouse serum from mice immunized for 3 weeks. *3, 4,* and *6*, P-450$_{LM2}$ (0.48 mg/ml), P-450$_{LM4}$ (0.67 mg/ml), and Fraction 7 (0.56 mg/ml), respectively.
B, C, and D. *Centers,* monoclonal antibodies to P-450$_{LM2}$ produced by hybrids 1-31-2p3, 1-26-2p3, and 1-26-11p3; *1–6* same as in A.

upon cell culture we observed the growth and appearance of hybrid cells in 45 among 48 wells. Of the 45 wells of supernatant fluids, 8 were positive for the production of monoclonal antibodies against P-450$_{LM4}$ as indicated by a positive RIA. After cloning, we obtained the 40 of the 264 clones that were positive for antibody production when screened against P-450$_{LM4}$ by RIA. Table 2 summarizes some of the data obtained with the monoclonal antibodies to P-450$_{LM4}$. Three of the antibodies were of the subclass IgG$_1$, and one was of class IgM. Both the cell culture fluid and the ascites fluid formed from positive hybridomas bound the enzyme as shown by a positive RIA.

Specificity of monoclonal antibodies to rabbit cytochrome P-450$_{LM2}$. Each of the seven hybridoma clones producing mouse Ig against P-450$_{LM2}$ which were detected by RIA were grown i.p. in 11-week-old mice for 2–3 weeks. The ascites fluid was withdrawn and analyzed for its ability to precipitate P-450$_{LM2}$ in a double-immunodiffusion assay. Of the seven samples, four (1-31-1p3, 1-31-2p3, 1-26-2p3, and 1-26-11-p3) precipitated P-450$_{LM2}$ as indicated in a double-immunodiffusion assay (Fig. 5). The remaining three failed to precipitate detectable amounts of P-450$_{LM2}$, although they were producing Ig that bound to P-450$_{LM2}$ as detected by RIA.

The patterns of precipitation, shown in Fig. 5, indicate the presence of two groups of monoclonal antibodies. The monoclonal antibodies secreted by hybrids 1-26-2p3 and 1-26-11p3 were identical (Fig. 5C and D) with the antibodies present in anti-P-450$_{LM2}$ mouse serum. However, the antibodies secreted by hybrids 1-31-1p3 and 1-31-2p3 were different from those present in the mouse serum (Fig. 5A and B). No precipitation was observed between the monoclonal antibodies and Fraction 1, P-

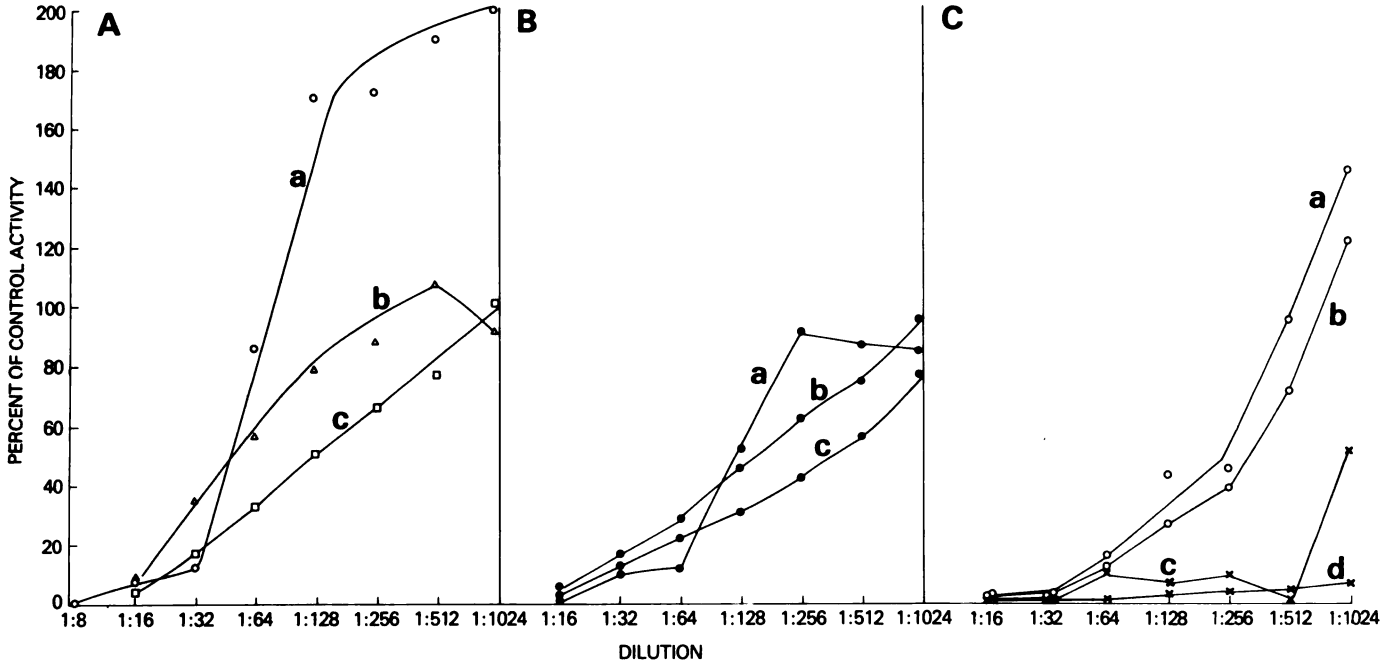

FIG. 6. *Effect of monoclonal antibodies to P-450$_{LM2}$ on AHH activity*
Cells, 5 × 10^6 of parent myeloma, RGNS-1p59, and hybridomas were grown in BALB/c mice intraperitoneally and ascites fluid was collected for AHH assay after 2–3 weeks.
A. *a* and *c*, Ascites fluids by RGNS-1p59 and hybrid 1-17-5p5; *b*, BSA.
B. *a*, *b*, and *c*, Ascites fluids by hybrids 1-31-5p5, 1-31-4p8, and 1-31-3p3.
C. *a*, *b*, *c*, and *d*, Ascites fluids by hybrids 1-31-1p3, 1-31-2p3, 1-26-11p3, and 1-26-2p3. A 420-μl sample of each dilution of ascites fluid (2.18 mg at 1:8 dilution) was incubated with 3 μg of P-450$_{LM2}$ in 80 μl of PBS at room temperature for 15 min and assayed for AHH activity using 20 nmoles of BP as the substrate in a reconstituted system.

450_{LM4}, or Fraction 7, indicating that they lack the antigenic site of P-450_{LM2} to which the monoclonal antibody is directed.

Monoclonal antibody inhibition of P-450_{LM2}-catalyzed BP metabolism. The addition of high concentrations of proteins from the cell culture medium or ascites fluid from either myeloma control cells or antibody-producing hybridomas inhibited the AHH activity of the reconstituted system containing a purified P-450_{LM2}. This inhibition was nonspecific and was also observed with purified proteins such as BSA. Since both the culture medium and the ascites fluids contain such proteins, some inhibition of AHH was observed in their presence. The hybridoma cells formed from spleen cells of unimmunized mice produced antibodies which did not bind (RIA) or precipitate P-450_{LM2} (Ouchterlony). The nonspecific inhibition by these control culture and ascites fluids was eliminated by their dilution. The specific monoclonal antibodies in the culture medium and ascites fluids of the positive hybridoma cells retained their inhibitory activity of AHH activity even at high dilution.

Figure 6A shows the inhibitory effect on enzyme activity of high concentrations of control myeloma cells (*a*) or the hybridoma 1-7-15p-5 (*c*), a nonproducer clone. Upon dilution of these preparations, the inhibitory activity was lost. Figure 6B shows the effect of the ascites fluids from mice that received injections of hybridomas which produced antibodies that bound to P-450_{LM2} in the RIA but not precipitate this cytochrome in the Ouchterlony double-immunodiffusion assays. These hybridomas showed AHH inhibition curves similar to those of ascites fluids from control hybridomas shown in Fig. 6A. Figure 6C shows the effect of ascites fluids from mice that received injections of hybridomas which produced monoclonal antibodies that could bind and precipitate the P-450_{LM2}. Two of the monoclonal antibodies (*a* and *b*) from these hybridomas behaved similarly to control hybridomas in respect to inhibition of AHH activity. However, the antibodies of two other hybrids (*c* and *d*) inhibited the AHH at high dilutions and low protein concentrations (0.04–0.28 mg/ml). These ascites fluids, from the hybrids 1-26-2p3 (*d*) and 1-26-11p3 (*c*), almost completely inhibited P-450_{LM2} AHH activity at protein concentrations at which the ascites fluid formed from control myeloma cells and control hybridomas had either no effect or stimulated AHH activity (Fig. 6A, *a* and *c*). At high dilutions of culture fluid and ascites fluid, we often observed some stimulation of AHH activity. This phenomenon is not understood.

TABLE 3

Effect of monoclonal antibodies to P-450_{LM2} on AHH activity of isozymes of cytochrome P-450

Enzyme preincubation with ascites fluids[a]	AHH enzyme	AHH activity (pmoles 3-OH-BP formed/20 min/nmole P-450)	% Control
None	P-450_{LM2}	179.0	100.0
Myeloma, RGNS-1p59	P-450_{LM2}	232.9	130.0
Hybridoma, 1-7-15p5	P-450_{LM2}	390.8	218.3
Hybridoma, 1-26-11p3	P-450_{LM2}	1.4	0.8
None	Fraction 1	300.2	100.0
Myeloma, RGNS-1-59	Fraction 1	199.4	66.4
Hybridoma, 1-7-15p5	Fraction 1	342.3	114.0
Hybridoma, 1-26-11p3	Fraction 1	150.4	50.1
None	P-450_{LM4}	5.2	100.0
Myeloma, RGNS-1-59	P-450_{LM4}	4.4	84.6
Hybridoma, 1-7-15p5	P-450_{LM4}	6.4	123.1
Hybridoma, 1-26-11p3	P-450_{LM4}	6.6	126.9
None	Fraction 7	630.0	100.0
Myeloma, RGNS-1-59	Fraction 7	506.3	80.4
Hybridoma, 1-7-15p5	Fraction 7	443.5	70.4
Hybridoma, 1-26-11p3	Fraction 7	438.8	69.7

[a] Ascites fluids were obtained as described under Experimental Procedures by injecting RGNS-1 myeloma cells, non-monoclonal antibody-producing hybridoma 1-7-15p5, or the monoclonal antibody-producing hybridoma 1-26-11p3.

TABLE 4

Effect of monoclonal antibodies to P-450_{LM4} on AHH activity of P-450_{LM4} and P-450_{LM2}

Antibody[a]	AHH activity			
	P-450_{LM4}		P-450_{LM2}	
	Amount[b]	% Control	Amount[b]	% Control
Control	1.65	100	73.5	100
Hybridoma, nonspecific				
NBS 1-48-5p8	1.61	98	50.4	96
Hybridomas, specific				
LM4 1-1-8p8	0.27	16	69.0	94
LM4 1-4-3p8	0.16	10	60.6	83
LM4 1-14-3p8	0.07	4	63.0	86

[a] Cytochrome P-450 (3 μg of P-450_{LM2} and 7.8 μg of P-450_{LM4} in 80 μl of PBS) was incubated with each antibody (ascites 62.5 μg in 420 μl of water at 4°C overnight and subjected to centrifugation at 15,000 rpm for 5 min after an additional 1-hr incubation with 20 μl of protein A-Sepharose 4B suspension (18.75 μg for 3 μg of P-450_{LM2} and 75 μg for 7.8 μg of P-450_{LM4}). The AHH assay was carried out on the supernatant with 20 nmoles of BP as the substrate in a reconstituted system at pH 7.6 with NADPH-cytochrome P-450 reductase (3.6 μg with P-450_{LM2} and 9.7 μg with P-450_{LM4}).

[b] Picomoles of phenols equivalent to 3-OH-BP/nanomole of P.450 per minute.

Thus, of the seven RIA-positive clones, four were also positive for cytochrome P-450 precipitation; of the latter four hybridomas, two also strongly inhibited the AHH activity of P-450_{LM2}. Table 3 summarizes the specificity for AHH inhibition of the monoclonal antibody produced by hybridoma 1-26-11p3. This monoclonal antibody completely inhibited P-450_{LM2} at a dilution of 1:512 protein concentration but did not inhibit the other P-450 isozymes, Fraction 1, P-450_{LM4}, and Fraction 7. The small inhibition observed with some of the latter proteins is nonspecific and was also observed with fluids from the parent myeloma and nonproducer hybrid cells 1-7-15p5.

Monoclonal antibody binding to cytochrome P-450_{LM4} and inhibition of AHH activity. The monoclonal antibodies formed to P-450_{LM4} failed to inhibit AHH activity after a short preincubation of antibody with P-450_{LM2} or P-450_{LM4}. The monoclonal antibodies did bind to P-450_{LM4} and, after an overnight incubation, the addition

of protein A to the complex permitted removal of AHH activity from the solution by centrifugation. Table 4 and Fig. 7 show the effect of this binding and removal of the complex on the residual enzyme activity of P-450$_{LM4}$ and P-450$_{LM2}$. The antibodies from the control hybridoma had no effect on AHH activity of either P-450$_{LM4}$ or P-450$_{LM2}$. The ascites fluids from the hybridomas producing monoclonal antibodies to P-450$_{LM4}$ formed a complex which, when removed, eliminated 84–96% of the P-450$_{LM4}$ AHH activity but did not significantly affect the P-450$_{LM2}$ AHH activity. In contrast to monoclonal antibodies to cytochrome P-450$_{LM2}$, monoclonal antibodies to cytochrome P-450$_{LM4}$ failed to precipitate cytochrome P-450$_{LM4}$. However, anti-P-450$_{LM4}$ mouse serum precipitated cytochrome P-450$_{LM4}$ (Table 2).

HPLC analyses of BP metabolism: inhibition by monoclonal antibodies. Table 5 shows the distribution of metabolites formed from BP by a reconstituted mixed-function oxidase system containing P-450$_{LM2}$. Four phenol fractions (unknowns 1 and 2, 1-OH, and 3-OH), three quinones (1,6-, 6,12-, and 3,6-quinones), and an unknown metabolite were formed to varying extents by the P-450$_{LM2}$-reconstituted system. The addition of ascites fluid containing nonspecific antibodies formed from hybridomas of unimmunized mice (NBS-1-3-1p7) altered the ratio of metabolites formed to only a slight extent. The addition of ascites fluid, adjusted to contain identical amounts of protein, from the monoclonal antibody-producing hybridoma 1-26-11p35 caused almost complete inhibition of the formation of all the major known metabolites. The antibody thus appears to bind to a site required for the formation of each of the metabolites. This may be the active site for the formation of each of the shown metabolites or a different site required for enzyme activity.

TABLE 5

Effect of monoclonal antibodies to P-450$_{LM2}$ on BP metabolism

P-450$_{LM2}$ (0.28 nmole; 13.5 μg) was incubated with 297 μg of monoclonal antibody (NBS 1-3-1p5, nonspecific, or 1-26-11p35, specific to P-450$_{LM2}$) in 0.5 ml of PBS for 15 min and transferred to a reaction mixture for AHH assay or HPLC determination in a final volume of 1 ml. ^{14}C-Labeled BP (50 nmoles) was used as substrate.

BP metabolites	Specific activity (pmoles/nmole of P-450$_{LM2}$/min)			% Monoclonal antibody inhibition
	P-450$_{LM2}$ (control)	P-450$_{LM2}$ + NBS 1-3-1p7	P-450$_{LM2}$ + 1-26-11p35	
Unknown 1	4	18.1	3.2	28
Unknown 2	10	8.7	ND[a]	100
1-OH	40	22.7	ND	100
3-OH	53	44.3	2.2	96
1,6-Quinone	20	38.6	1.9	90
6,12-Quinone	17	19.6	0.9	95
3,6-Quinone	9	18.3	ND	100
Total	155	170.4	8.2	95

[a] ND, Not detectable.

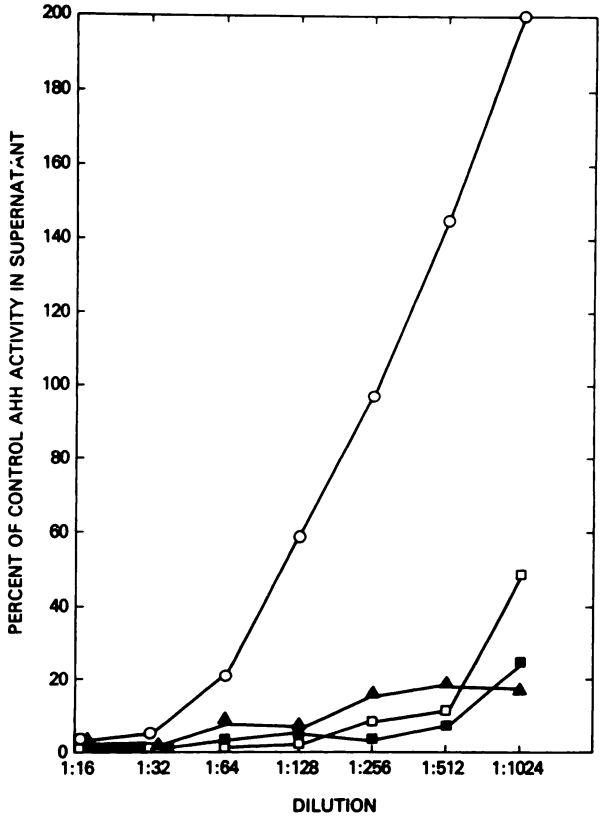

FIG. 7. *Effect of monoclonal antibodies to P-450$_{LM4}$ on AHH activity*

Monoclonal antibody (32 mg/ml) was diluted in 420 μl of water and incubated with 7.8 μg of P-450$_{LM4}$ in 80 μl of PBS at 4° overnight. The mixture was then incubated for 1 hr with 20 μl of protein A-Sepharose 4B (75 μg). The mixture was then centrifuged for 5 min at 15,000 × *g*. AHH activity of the supernatant was measured with 20 nmoles of BP as the substrate in a reconstituted system with NADPH-cytochrome P-450 reductase (9.7 μg) at 37° for 20 min in a 1-ml final volume. ○, Monoclonal antibody, nonspecific, NBS 1-48-5p22. Specific monoclonal antibodies: ■, P-450$_{LM4}$-1-1-8p8; □, P-450$_{LM4}$ 1-4-3p8; ▲, P-450$_{LM4}$ 1-14-3p8.

Effect of monoclonal antibodies to cytochrome P-450$_{LM2}$ on 7-ethoxycoumarin deethylase activity. Table 6 shows the effect of monoclonal antibodies on the P-450$_{LM2}$ activity directed to BP hydroxylation and 7-ethoxycoumarin deethylation. The control ascites fluids from mice that received injections of either myeloma cells or the control hybridomas from nonimmunized mice caused inhibition of both activities at high protein concentration and very little inhibition at lower protein concentration. However, the monoclonal antibody from hybridoma 1-26-11p35 inhibited both activities very strongly at both low and high dilutions of the ascites fluids. At 8-fold dilutions, the antibodies inhibited both the AHH and 7-ethoxycoumarin activities by about 90%. Thus, this monoclonal antibody exhibits very similar inhibitory activity toward both enzymatic activities of the cytochrome P-450, indicating that there is a common antigenic site necessary for both the hydroxylation of BP and the deethylation of coumarin.

Stability of hybridoma-producing monoclonal antibodies to cytochrome P-450$_{LM2}$. Since the loss of capacity of hybridomas to produce monoclonal antibodies is often observed, we examined the stability of the hybridomas with respect to specific monoclonal antibody production. We subcloned the four hybridomas which produced anti-

TABLE 6

Effect of monoclonal antibodies to P-450$_{LM2}$ on AHH and 7-ethoxycoumarin deethylase activities

Ascites fluid		AHH activity		7-Ethoxycoumarin deethylase activity	
Source	Amount of protein[a] (dilution)	Amount[b]	% Control	Amount[c]	% Control
Control	—	108	100	2150	100
Normal spleen cell hybrid NBS 1-3-1p7	1:64	31	20	898	41
	1:512	83	77	1431	66
Myeloma parent RGNS-1p99	1:64	35	32	845	39
	1:512	102	94	1679	78
Hybrid cells 1-26-11p35	1:64	4	3	101	4
	1:512	13	12	266	12

[a] Each dilution of 1:64 and 1:512 contains 297 and 37.1 μg of total protein.
[b] Picomoles of 3-OH-BP/nanomole of P-450$_{LM2}$ per minute.
[c] Picomoles of 7-OH-coumarin/nanomoles of P-450$_{LM2}$ per minute.

bodies that precipitated P-450$_{LM2}$. All of the subclones of 1-31-1 passaged 13 times (24 of 24) and 1-31-2 passaged 13 times (7 of 7) either partially or fully lost their capacities to produce antibodies that bound to P-450$_{LM2}$. All 24 subclones of 1-26-2p11 and all 72 subclones of 1-26-11p8 remained positive for production of monoclonal antibodies to P-450$_{LM2}$ as tested by RIA. The hybridoma 1-26-11 was passaged 68 times, and ascites fluids were prepared by injecting the cells into BALB/c mice. Our examination of the ascites and culture fluids indicated a stability of antibody production with respect to both AHH inhibition and precipitation of cytochrome P-450$_{LM2}$.

DISCUSSION

The mixed-function oxidase enzyme systems contain multiple forms of cytochrome P-450 which determine the substrate specificity as well as the regiospecificity of product formation from BP (16, 17). P-450$_{LM2}$ is the predominant form in phenobarbital-induced rabbit liver microsomes and P-450$_{LM4}$ in normal and β-naphthoflavone-induced rabbit liver microsomes (12). Similarly, different forms are predominant in rat liver microsomes induced by methylcholanthrene from those induced by phenobarbital (33, 34). Other inducers have been reported to induce still other forms of cytochrome P-450, and different tissues appear to display different amounts of the various cytochrome P-450 isozymes. The amounts of AHH inducibility in humans varies among individuals, suggesting the occurrence of individual differences in levels of basal and inducible cytochrome P-450s (4). Thus tissue, species, and individual differences in respect to drug and carcinogen metabolism and activation may be a function of the profile of cytochrome P-450 isozymes. The new and powerful technique of monoclonal antibody production is likely to offer a new and useful method for investigating the multiplicity and genetic control of these cytochromes.

In our studies, monoclonal antibodies to purified rabbit liver microsomal P-450$_{LM2}$ from four independent hybridomas were able specifically to bind and precipitate P-450$_{LM2}$ but not Fraction 1, P-450$_{LM4}$, and Fraction 7. The Ig class produced in the culture medium of the four hybridomas was IgG$_1$. Two of the latter four monoclonal antibodies not only were able to bind and precipitate P-450$_{LM2}$, but also interfered with the function of the active enzyme site as seen by inhibition of the enzyme activity of P-450$_{LM2}$. The antibodies did not inhibit the enzyme activity of Fraction 1, P-450$_{LM4}$, or Fraction 7. Thus, P-450$_{LM2}$ exhibits distinctive antigenic determinants which are different from those of the other cytochromes. The monoclonal antibodies were similar in their properties of precipitating and inhibiting the enzyme activity of P-450$_{LM2}$, but the degree of enzyme inhibition and patterns of immunoprecipitation were different. The monoclonal antibodies produced by two of the four hybridomas, 1-26-2p3 and 1-26-11p3, inhibited enzymatic activity of P-450$_{LM2}$ very strongly and precipitated the P-450$_{LM2}$ with a precipitin pattern identical with that between P-450$_{LM2}$ and anti-rabbit P-450$_{LM2}$ mouse serum. On the other hand, the monoclonal antibodies produced by two other hybridomas, 1-31-1p3 and 1-31-2p3, inhibited the P-450$_{LM2}$ enzyme activities only moderately, and the immunoprecipitation patterns were not identical with those of anti-P-450 mouse serum reaction with P-450$_{LM2}$. Besides the latter hybridomas producing four precipitating antibodies, three hybridomas produced monoclonal antibodies capable of binding to P-450$_{LM2}$ which were detected by RIA but not able to precipitate P-450$_{LM2}$. Therefore, it appeared that the different groups of monoclonal antibodies were primed against different antigenic determinants.

The enzyme-inhibiting activity of the monoclonal antibody from hybridoma 1-26-11p35 toward P-450$_{LM2}$ was equally effective with respect to hydroxylation at the different positions on the BP ring and for 7-ethoxycoumarin deethylation. Therefore, the antigenic determinants for this antibody appear to be necessary for hydroxylation at several positions of BP hydroxylation as well as ethoxycoumarin deethylation. The monoclonal antibodies for P-450$_{LM4}$ were of both the IgG and IgM types. They did not precipitate this cytochrome, but bound sufficiently strongly to form a complex with protein A, which caused a loss of enzyme activity from the solution upon centrifugation. Thus, this class of antibodies appears to be directed at antigenic sites on the cytochrome P-450 that do not interfere with enzyme activity or cause precipitation of the enzyme.

The stability of the hybridomas is dependent upon many factors such as segregation of chromosomes and loss or repression of immunoglobulin gene expression. However, the two hybridomas we isolated (1-26-2 and 1-26-11) were very stable, and the production of monoclonal antibodies was observed even after 68 passages in culture medium. The hybridomas can also be grown in the intraperitoneal cavity of the mouse and thus the monoclonal antibodies can be obtained in high titer.

Our studies suggest that monoclonal antibodies will be a very useful tool not only for the investigation of different forms of cytochrome P-450 with respect to molecular structure and function related to catalytic activities, but also for studies of their relative distributions in various tissues, species, and individuals. In addition, monoclonal antibodies should be most useful in studying the genetic and regulatory aspects of cytochrome P-450 content and function and their relationship to carcinogen susceptibility and rates of drug metabolism.

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REFERENCES

1. Gelboin, H. V. Carcinogens, enzyme induction and gene action. *Adv. Cancer Res.* **10**:1–81 (1967).
2. Conney, A. H. Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev.* **19**:317–366 (1967).
3. Gelboin, H. V., N. Kinoshita, and F. J. Wiebel. Microsomal hydroxylases: induction and role in polycylic hydrocarbon carcinogenesis and toxicity. *Fed. Am. Soc. Exp. Biol.* **31**:1298–1302 (1972).
4. Okuda, T., E. S. Vesell, V. Plotkin, R. Tarone, R. C. Bast, and H. V. Gelboin. Interindividual and intraindividual variations in arylhydrocarbon hydroxylase in monocytes from monozygotic twins. *Cancer Res.* **37**:3904–3911 (1977).
5. Gelboin, H. Cancer susceptibility and carcinogen metabolism. *N. Engl. J. Med.* **297**:384–387 (1977).
6. Baum, E. J. Occurrence and surveillance of polycyclic aromatic hydrocarbons, in *Polycyclic Hydrocarbon and Cancer* (H. V. Gelboin and P. O. Ts'o, eds.), Vol. 1. Academic Press, New York, 45–70 (1978).
7. Gelboin, H. V. Benzo(*a*)pyrene metabolism, activation, and carcinogenesis: role and regulation of mixed-function oxidases and related enzymes. *Physiol. Rev.* **60**:1107–1165 (1980).
8. Lu, A. Y. H., and J. M. Coon. Role of hemeprotein P-450 in fatty acid ω-hydroxylation in a soluble enzyme system from liver microsomes. *J. Biol. Chem.* **243**:1331–1332 (1968).
9. Lu, A. Y. H., and S. B. West. Multiplicity of mammalian microsomal cytrochrome P-450. *Pharmacol. Rev.* **31**:277–295 (1980).
10. Coon, M. J., and R. E. White. Cytochrome P-450, a versatile catalyst in monooxygenation reactions, in *Metal Ion Activation of Dioxygen* (T. G. Spiro, ed.). Wiley, New York, 73–123 (1980).
11. Haugen, D. A., T. A. van der Hoeven, and M. J. Coon. Purified liver microsomal cytochrome P-450: Separation and characterization of multiple forms. *J. Biol. Chem.* **250**:3567–3570 (1973).
12. Haugen, D. A., and M. J. Coon. Properties of electrophoretically homogeneous phenobarbital-inducible and β-naphthoflavone-inducible forms of liver microsomal cytochrome P-450. *J. Biol. Chem.* **251**:7929–7939 (1976).
13. Coon, M. J., T. A. van der Hoeven, S. B. Dahl, and D. A. Haugen. Two forms of liver microsomal cytochrome P-450, P-450$_{LM2}$ and P-450$_{LM4}$ (rabbit liver). *Methods Enzymol.* **52**:109–117 (1978).
14. Koop, D. R., and M. J. Coon. Purification and properties of P-450$_{LM3b}$, a constitutive form of cytochrome P-450, from rabbit liver microsomes. *Biochem. Biophys. Res. Commun.* **91**:1075–1081 (1979).
15. Dean, W. L., and M. J. Coon. Immunochemical studies on two electrophoretically homogeneous forms of rabbit liver microsomal cytochrome P-450, P-450$_{LM2}$ and P-450$_{LM4}$. *J. Biol. Chem.* **252**:3255–3261 (1977).
16. Deutsch, J., J. Leutz, S. K. Yang, H. V. Gelboin, Y. L. Chiang, K. P. Vatsis, and M. J. Coon. Regio- and stereoselectivity of various forms of purified cytochrome P-450 in the metabolism of benzo(*a*)pyrene and (−)-trans-7,8-dihydroxy-y,8-dihydrobenzo(*a*)pyrene as shown by product formation and binding to DNA. *Proc. Natl. Acad. Sci. U. S. A.* **75**:3123–3127 (1978).
17. Deutsch, J., K. P. Vatsis, M. J. Coon, J. C. Leutz, and H. V. Gelboin. Catalytic activity and stereoselectivity of purified forms of rabbit liver microsomal cytochrome P-450 in oxygenation of the (−) and (+) enantiomers of *trans*-7,8-dihydroxl-78-dihydrobenzo(*a*)pyrene. *Mol. Pharmacol.* **16**:1011–1018 (1979).
18. Wiebel, F. J., J. K. Selkirk, H. V. Gelboin, D. A. Haugen, T. A. van der Hoeven, and M. J. Coon. Position-specific oxygenation of benzo(*a*)pyrene by different forms of purified cytochrome P-450 from rabbit liver. *Proc. Natl. Acad. Sci. U. S. A.* **72**:3917–3920 (1975).
19. Koehler, G., and C. Milstein. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* (*Lond.*) **256**:495–497 (1975).
20. Park, S. S., A. V. Persson, M. J. Coon, and H. V. Gelboin. Monoclonal antibodies to rabbit liver cytochrome P-450$_{LM2}$. *F. E. B. S. Lett.* **116**:231–235 (1980).
21. Coon, M. J., J. L. Vermillion, K. P. Vatsis, J. S. French, W. L. Dean, and D. A. Haugen. Biochemical studies on drug metabolism: isolation of multiple forms of liver microsomal cytochrome P-450, in *Concepts in Drug Metabolism* (D. M. Jerina, ed.). American Chemical Society Symposium Series 44, Washington, D. C., 46–71 (1971).
22. French, J. S., and M. J. Coon. Properties of NADP-cytochrome P-450 reductase purified from rabbit liver microsomes. *Arch. Biochem. Biophys.* **195**: 565–577 (1979).
23. Littlefield, J. W. Selections of hybrids from matings of fibroblasts *in vitro* and their presumed recombinants. *Science* (*Wash. D. C.*) **145**:709 (1964).
24. Galfre, G., S. C. Howe, C. Milstein, G. W. Butcher, and J. C. Howard. Antibodies to major histocompatibility and antigens produced by hybrid cell lines. *Nature* (*Lond.*) **266**:550–552 (1977).
25. Farr, R. J. A quantitative immunological measures of the primary interaction between I*-BSA and antibody. *J. Infect. Dis.* **103**:239–262 (1958).
26. Hopper, J. E., A. B. McDonald, and A. Nisonoff. Quantitative investigation of idiotypic antibodies. II. Nonprecipitating antibodies. *J. Exp. Med.* **131**:42–56 (1970).
27. Nebert, D. W., and H. V. Gelboin. Substrate-inducible microsomal arylhydrocarbon hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. *J. Biol. Chem.* **243**:6242–6249 (1968).
28. Greenlee, W. F., and A. Poland. An improved assay of 7-ethoxycoumarin *O*-deethylase activity: induction of hepatic enzyme activity in C57BL/6J and DBA/2J mice by phenobarbital, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J. Pharmacol. Exp. Ther.* **205**:596–605 (1978).
29. Laskov, R., K. J. Kim, and R. Asofsky. Induction of amplified synthesis and secretion of IgM by fusion of murine B lymphoma with myeloma cells. *Proc. Natl. Acad. Sci. U. S. A.* **76**:915–919 (1979).
30. Bonner, W. M., and R. A. Laskey. A film detection method for tritium-labeled proteins and nucleic acids in polyarylamide gels. *Eur. J. Biochem.* **46**:83–88 (1974).
31. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (*Lond.*) **227**:680–685 (1970).
32. Takacs, B. Electrophoresis of proteins in polyacrylamide slab gels, in *Immunological Methods* (I. Lefkovits and B. Pernis, eds.). Academic Press, New York, 81–103 (1979).
33. Guengerich, F. P. Separation and purification of multiple forms of microsomal cytochrome P-450. *J. Biol. Chem.* **253**:7931–7939 (1978).
34. Kumar, A., and G. Padmanaban. Studies on the synthesis of cytochrome P-450 and cytochrome P-448 in rat liver. *J. Biol. Chem.* **255**:522–525 (1980).

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