MONOCLONAL ANTIBODIES TO RABBIT LIVER CYTOCHROME P450 LM2

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1. Introduction

The mixed-function oxidases containing cytochrome P450 are the primary systems that metabolize xenobiotics including drugs and carcinogens and endogenous substrates such as steroids [1–3]. The mixed-function oxidases may be key determinants of the rates of drug metabolism [2] and carcinogen susceptibility [3–5].

A major class of carcinogens to which humans are exposed is the polycyclic aromatic hydrocarbons. A prototype and most common hydrocarbon of this class is benzo[a]pyrene (BP) [6]. Benzo[a]pyrene is oxidized by the mixed-function oxidase, aryl hydrocarbon hydroxylase (AHH) and metabolically related enzymes to >40 oxygenated metabolites which include simple epoxides, phenols, quinones, dihydrodiols, diol epoxides and water-soluble conjugates of glutathione, glucuronide and sulfate [7]. The pathway leading to the benzo[a]pyrene diol epoxides is believed to be a primary pathway of carcinogen activation [7].

Different forms of cytochrome P450 have been isolated and characterized (reviewed [8]). Cytochromes P450 LM2 and LM4 have been purified to homogeneity [9–11] and P450 LM1, LM3 and LM7 have been partially purified [9] from rabbit liver. The highly purified LM2 and LM4 exhibit different biochemical, immunological and kinetic properties [12]. Furthermore each isozyme of P450 exhibits stereoselectivity in substrate choice and product formation with respect to both benzo[a]pyrene metabolism and the conversion of (−)*t*-7,8-diol benzo[a]pyrene into the highly mutagenic benzo[a]pyrene 7,8-diol 9,10-epoxides [13,14].

Immunoglobulin genes derived from mouse-spleen cells primed with specific antigens are expressed and functional in hybrids formed from spleen cells and myeloma cells [15]. Using this system we prepared monoclonal antibodies to purified cytochrome P450 LM2. These monoclonal antibodies bind, and precipitate cytochrome P450 LM2 and inhibit the benzo[a]-pyrene hydroxylation (AHH) activity. These antibodies did not interact with or inhibit the activity of the other isozymes of cytochrome P450 LM1, LM4 and LM7. Thus, monoclonal antibodies show specificity for isozymes of cytochrome P450 and may be useful in the determination of the content and function of the different cytochromes P450 in different tissues, species and individuals.

2. Materials and methods

Rabbit-liver cytochromes P450 LM2, LM4, LM1 and LM7 were purified as in [8]. The LM4 was prepared from phenobarbital-induced rabbits. Eleven-week-old female mice were immunized 4 consecutive weeks with 12 μg cytochrome P450 LM2 emulsified in 0.2 ml Freund's complete adjuvant and in 0.2 ml Dulbecco's phosphate-buffered saline pH 7.4 (PBS). Three days after the last immunization 5 mice were sacrificed and the spleens removed. Myeloma cells (RGNS-1), which are azaguanine resistant and are non-producers of immunoglobulins were grown in Dulbecco's modified Eagle's medium (DMEM) with 4500 mg glucose/l and 584 mg/l glutamine, supplemented with 10% fetal bovine calf serum, 10% horse serum and 50 μg/ml gentamycin. The myeloma cells were fused with spleen cells derived from the mice immunized with purified rabbit-liver microsomal cytochrome P450 LM2 [15]. For the fusion we used polyethyleneglycol (PEG-1000). The PEG-treated cells were seeded into 24-well Costar plates, and

hybrids were selected in growth medium containing 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT medium) [19]. Hybrids producing mouse immunoglobulins were screened by radioimmunoassay (RIA) [16,17] and grown in the culture medium for the classification of mouse immunoglobulins or were injected intraperitoneally into mice for further growth and production of antibody in the ascites fluid. The cell culture or ascites fluids were used for analysis of antibody production by Ouchterlony immunodiffusion and radioimmunoassay and by their effects on enzyme activity. Aryl hydrocarbon hydroxylase activity was determined by measuring the amount of benzo[a]pyrene conversion to phenolic products equivalent to 3-OH-BP as in [18]. The reaction mixture in the reconstituted mixed-function oxidase system contained 250 μl Tris–HCl (0.2 M, pH 8.3), 30 μl dilauryl glyceryl-3-phosphoryl choline (1 mg/ml), 30 μl NADPH cytochrome P450 reductase (3.36 μg), 50 μl NADPH (0.17 mg) and 30 μl $MgCl_2$ (0.1 M), and 10 μl BP (2 mM) in 1.0 ml total vol. For analysis of antibody inhibition of enzyme activity, 3–8 μg cytochrome P450 in 80 μl PBS was incubated with 420 μl antibody fluids for 15 min at room temperature and then the mixture was analyzed for AHH activity.

3. Results and discussion

Upon fusion of 10^7 myeloma cells with 10^8 spleen cells derived from the mice immunized with purified rabbit microsomal cytochrome P450 LM2, the growth of hybrid cells occurred in 36 of 48 wells containing the selective HAT medium [19]. Of the 36 wells containing hybrid cells, 15 wells were positive for mouse immunoglobulin 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 and grown again in Costar plates containing 24 wells. After growth for 2 weeks in HAT medium of the 96 well plates, the formation of clones was observed and the production of antibody by these clones was examined by radioimmunoassay. Seven of the 206 independent hybrid clones were positive for antibody production as indicated by radioimmunoassay. The culture fluid of the parent myeloma cells and the remaining hybrid cells did not contain any mouse immunoglobulin that bound to rabbit P450 LM2.

The cytochrome P450 LM2 antibody producing hybridoma, 1-26-11p3 was further grown in HAT medium and the culture fluid concentrated 30 times. This concentrate was examined by double immunodiffusion analysis and showed a clear single precipitation band with the cytochrome P450 LM2 (fig.1B). Fig.1B also shows the precipitin reaction between the hybrid 1-26-11p3 supernatant fluid and the wells containing rabbit anti-mouse IgG specific for mouse heavy and light chains and heavy chain only. A faint precipitation band was also detected between the wells containing the culture fluid and anti-mouse *K*-chain serum. There were no detectable precipitation bands between the culture fluid and any of the other rabbit anti-mouse immunoglobulin sera, anti-IgM and anti-IgA. The myeloma control well shown in fig.1A did not react with anti-IgM, anti-IgA, anti-IgG or cytochrome P450 LM2. Our other studies showed that all 4 of the antibody producing clones produced mouse IgG1. The 7 hybridomas producing mouse IgG against cytochrome P450 LM2 which was detected by RIA were grown intraperitoneally in 11-week-old mice for 2–3 weeks, and the ascites fluid withdrawn and clarified. Among the 7 samples, only 4 (1-31-1p3, 1-31-2p3, 1-26-2p3 and 1-26-11p3) precipitated P450 LM2 as indicated in a double immunodiffusion assay. All of the samples however contained detectable amounts of mouse IgG that bound to P450 LM2 as detected by RIA.

The monoclonal antibodies examined in double immunodiffusion formed a precipitate band with P450 LM2 but showed no precipitin reaction with P450 LM4, P450 LM1 or P450 LM7 (fig.2). The cytochrome P450 LM2 also reacted positively with anti-cytochrome P450 LM2 mouse serum.

In examining the effect of the supernatant or ascites fluids of myeloma control cells on AHH activity of the purified P450 LM2 reconstituted system we observed a non-specific inhibition of AHH activity. This non-specific inhibition was observed with proteins such as bovine serum albumin (BSA) and with BSA-containing supernatants from parent myeloma control cells or ascites fluids from control cells. Fig.3 shows this inhibitory effect at high concentrations of proteins (0.55–2.18 mg/ml) from the ascites fluid of both the myeloma and the antibody producing hybrid. However, at high dilutions and low protein concentrations (0.04–0.28/ml) the ascites fluid from the hybrid I-26-11P3 almost completely inhibited P450 LM2 AHH activity while the ascites fluid from

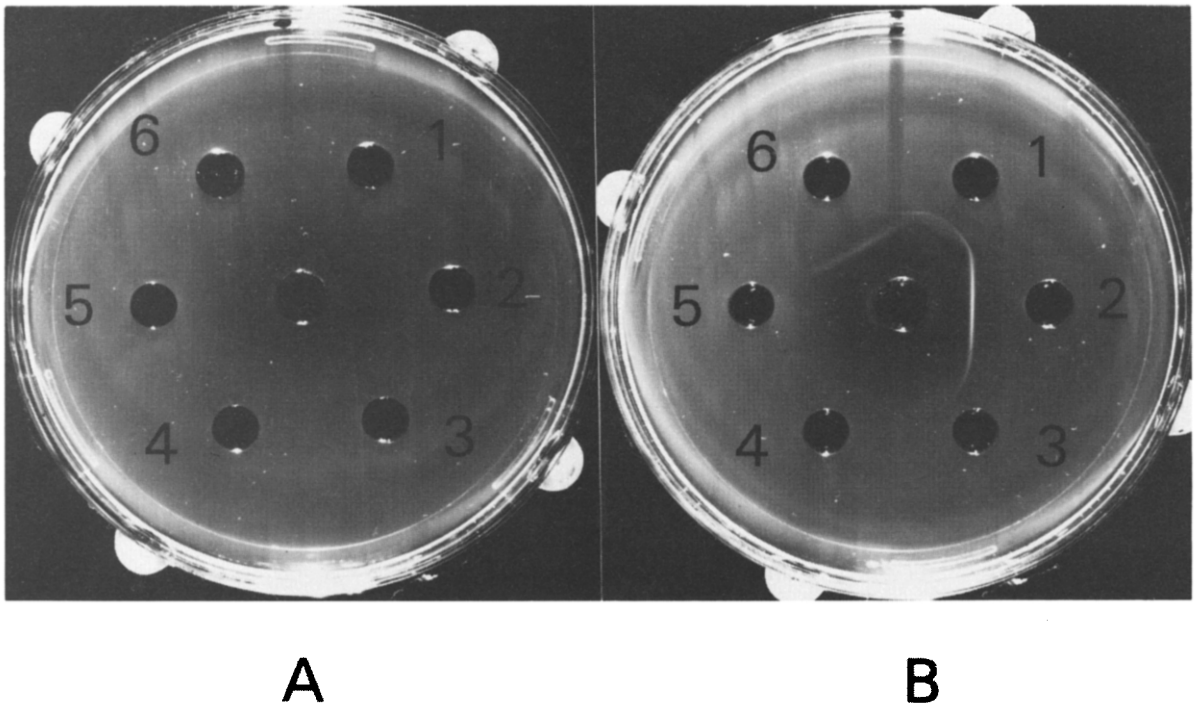
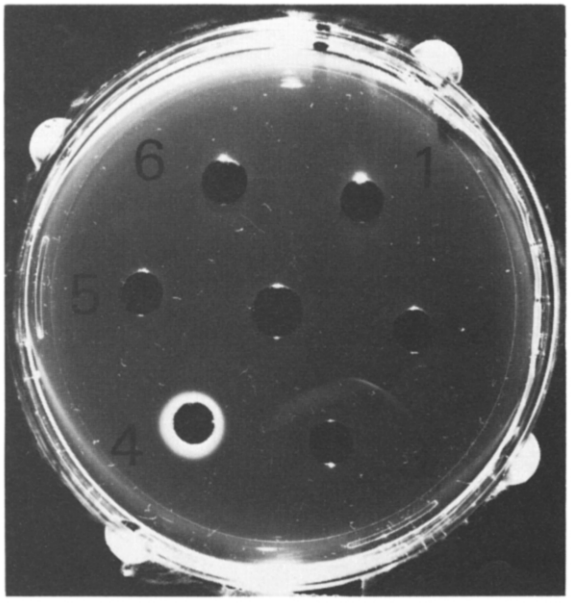


Fig.1. Double immunodiffusion analysis of Ig secretion and monoclonal antibody to purified rabbit cytochrome P450 LM2. Culture media from myeloma RGNS-1p72 and cloned hybrid cells, 1-26-11p3 were concentrated 30 times and applied to central wells. (A) center: RGNS-1p72; (1) rabbit anti-mouse IgG (H and L chain specific, 4 mg/ml); (2) goat anti-mouse IgG (H chain specific, 5 mg/ml); (3) rabbit anti-mouse *K* (1 mg/ml); (4) goat anti-mouse IgM (60–70 mg total protein); (5) goat anit-mouse IgA (5.5 mg/ml); (6) purified rabbit P450 LM2 (1.1 mg/ml). (B) Center: 1-26-11p3; (1–6) as in (A). All wells were filled with 20 μl samples.



control myeloma cells had either no effect or stimulated AHH activity. Table 1 shows the specificity of the monoclonal antibodies produced by hybridoma I-26-11P3. The monoclonal antibodies completely inhibited cytochrome P450 LM2 at a dilution of 1:512 and essentially did not inhibit the isozymes P450 LM1, P450 LM4 and P450 LM7. The small inhibition observed with some of the latter proteins is non-specific and is also observed with fluid from the parent myeloma cells.

Fig.2. Double immunodiffusion analysis of the specificity of monoclonal antibody to purified rabbit cytochrome P450 LM2. Center: monoclonal antibody to rabbit P450 LM2, 1-26-11p3; (1) rabbit P450 LM1 (0.49 mg/ml); (2,5) anti-rabbit P450 LM2 mouse serum; (3) rabbit P450 LM2 (0.48 mg/ml); (4) rabbit P450 LM4 (0.66 mg); (6) rabbit P450 LM7 (0.56 mg/ml). All wells were filled with 20 μl samples.

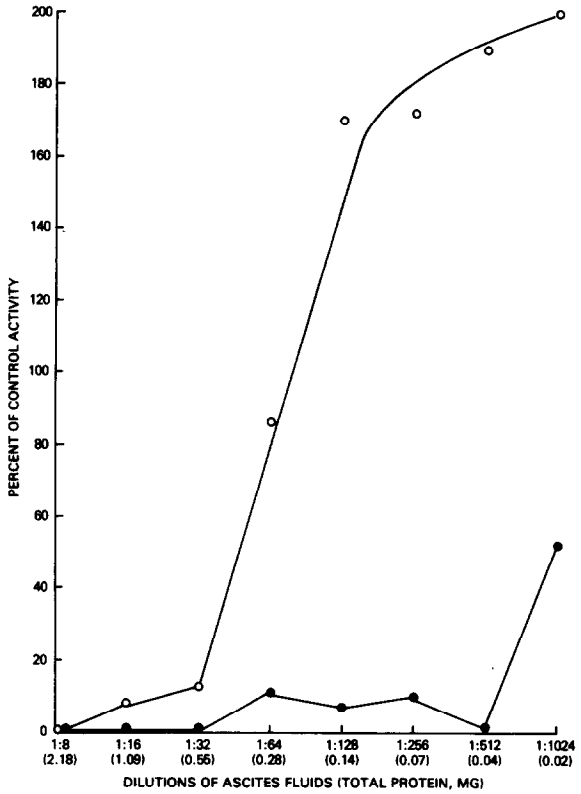


Fig.3. Effect of monoclonal antibody to purified rabbit liver microsomal cytochrome P450 LM2. Cells, 5 × 10^6, of parent myeloma, RGNS-1p59 (–○–) and hybridoma producing monoclonal antibodies, 1-26-11p3 (–●–) were grown in BALB/c mice intraperitoneally and ascites fluid was collected for AHH assay after 2–3 weeks. A 420 μl sample of each dilution of ascites fluid was incubated with 3 μg P450 LM2 in 80 μl PBS at room temperature for 15 min and assayed for AHH activity in a reconstituted system.

Since the loss of capacity of hybridomas to produce monoclonal antibodies is often observed, we examined the stability of the antibody-producing hybrids. We subcloned 4 hybrids which produced antibodies that precipitated and inhibited the AHH activity of cytochrome P450 LM2. All of the subclones of 1-31-1p12 (24/24) and 1-31-2p12 (7/7) either partially or fully lost their capacities to produce antibodies that bound to cytochrome P450 LM2. All 72 subclones of 1-26-11p8 and all 24 subclones of 1-26-2p11 remained positive for production of monoclonal antibodies to cytochrome P450 LM2 as tested by radioimmunoassay.

In our studies, monoclonal antibodies to purified

Table 1
Effect of monoclonal antibody to cytochrome P450 LM2 on aryl hydrocarbon hydroxylase (AHH) activity of different cytochromes P450

P450	Antibody source[a] (ascites)	AHH activity (pmol 3OH-BP . 20 min^{-1} nmol P450 LM^{-1})	% Control
LM2	None (control)	179	100
	Myeloma, RGNS-1p59 (non producer)	232	130
	Hybridoma, 1-26-11p3 (producer)	1	<1
LM1	None	300	100
	RGNS-1p59	199	66
	1-26-11p3	150	50
LM4	None	5	100
	RGNS-1p59	4	84
	1-26-11p3	6	126
LM7	None	630	100
	RGNS-1p59	506	80
	1-26-11p3	438	69

[a] Ascites fluid (420 μl at 1:512 dilution; 0.035 mg total protein) by non-producer myeloma RGNS-1p59 or producer hybridoma, 1-26-11p3 was incubated with P450 LM in 80 μl PBS at room temperature for 15 min and assayed for AHH activity in 1 ml reconstituted systems using benzo[a]pyrene as a substrate at 37°C for 20 min

rabbit-liver microsomal cytochrome P450 LM2 from four independent hybridomas were able to specifically bind and precipitate P450 LM2 but not P450 LM1, P450 LM4 and P450 LM7. The class of the immunoglobulins produced in the culture medium of the 4 hybridomas was IgG1. The monoclonal antibodies were not only able to bind and precipitate P450 LM2 but also interfered with the function of the active enzyme site as seen by inhibition of the enzyme activity of P450 LM2. The antibody did not inhibit the enzyme activity of P450 LM1, LM4 or LM7. Thus, rabbit cytochrome P450 LM2 exhibits distinctive antigenic determinants which are different from those of cytochrome P450 LM1, LM4 and LM7. Our studies suggest that monoclonal antibodies will be a useful tool for the investigation of different forms of the cytochromes P450 for molecular studies of enzyme structure and function and for the determination of enzyme type, content and role in carcinogen and drug metabolism.

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