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PREPARATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO PREGNENOLONE 16-α-CARBONITRILE INDUCIBLE RAT LIVER CYTOCHROME P-450*

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Abstract—Hybridomas were prepared from mouse myeloma cells and spleen cells derived from BALB/c female mice immunized with purified rat hepatic pregnenolone 16-α-carbonitrile (PCN) induced cytochrome P-450 2a/PCN-E. The monoclonal antibodies (MAbs) thus obtained were screened for binding to the purified P-450 2a/PCN-E by radioimmunoassay. Eleven independent hybrid clones produced MAbs, each of which was of a single mouse immunoglobulin subclass of the IgG1, IgG2a or IgG2b type. Each of the MAbs produced by the eleven individual hybrid clones bound strongly to P-450 2a/PCN-E as assessed by radioimmunoassay and immunoprecipitation of P-450 2a/PCN-E in Ouchterlony double-immunodiffusion plates. Of the eleven MAbs, three also bound strongly to the phenobarbital-inducible rat liver cytochrome P-450 PB-4. Thus, two classes of MAbs were obtained, one class specific for P-450 2a/PCN-E and a second class that bound to both PCN- and phenobarbital-inducible P-450 forms. The reactivities of one MAb from each class toward eight highly purified rat hepatic cytochromes P-450 were examined using solid phase enzyme-linked immunosorbent analyses. The MAb designated C2 was found to be specific for P-450 2a/PCN-E and did not cross-react with seven other P-450 forms. This MAb was shown to be an effective probe for monitoring, by Western blotting, the induction of microsomal P-450 2a/PCN-E by PCN and phenobarbital. The MAb designated C1 reacted both with P-450 2a/PCN-E and with the two major phenobarbital-inducible P-450 forms, PB-4 and PB-5. None of the MAbs was inhibitory towards P-450 2a/PCN-E-dependent aryl hydrocarbon hydroxylase, benzphetamine *N*-demethylase, ethoxycoumarin *O*-deethylase or ethylmorphine *N*-demethylase activity, indicating that the epitopes recognized by these MAbs are not directly associated with catalytic activity. The strong reactivities of three of the MAbs with both P-450 2a/PCN-E and P-450s PB-4 and PB-5 indicate that these two structurally quite different cytochrome P-450 families share at least one common epitope. These new MAbs are additions to our library of MAbs to different cytochromes P-450 and should help further our understanding of the relationship of cytochrome P-450 phenotype and multiplicity to inter-individual differences in drug and carcinogen metabolism and sensitivity.

Cytochromes P-450 (P-450¶) are key components of the mixed-function oxidase system which metabolizes a broad spectrum of xenobiotics including various drugs, insecticides, and carcinogens as well as endobiotics** such as fatty acids, prostaglandins and steroids [1–3]. Recent studies have demonstrated a multiplicity of P-450 forms or isozymes, which differ in their subunit molecular weights, spectral properties, electrophoretic mobilities, substrate and inducer specificities, immunogenic properties, amino acid sequences, and substrate regiospecificities [3–21]. Drugs and xenobiotics are detoxified as well as activated to toxic metabolites, mutagens and carcinogens by P-450 [22, 23]. Specific forms of P-450 may direct substrate flow into alternative metabolic pathways and thus determine the consequences of interactions between environmental chemicals and the individual [24–31].

Monoclonal antibodies (MAbs) are made by hybridomas formed from myeloma cells fused with single spleen cells previously primed for a specific antigenic determinant (epitope). Thus, MAbs are pure reagents specific for single epitopes on the antigen, and the hybridomas producing them are potentially immortal [32]. We have reported pre-

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¶ Abbreviations: P-450, cytochrome P-450; MAbs, monoclonal antibodies; PB, phenobarbital; MC, 3-methylcholanthrene; BNF, β-naphthoflavone; PCN, pregnenolone 16-α-carbonitrile; P-450-PB, P-450-MC, P-450-BNF, or P-450-PCN, cytochrome P-450 induced by PB, MC, BNF, or PCN; HAT, hypoxanthine, aminopterin and thymidine; and PBS, phosphate-buffered saline.

** The word endobiotics from the Greek meaning "inner" or from within is used to describe endogenous substrates, in contrast to xenobiotics for foreign compounds.

viously preparations of panels of MAbs raised to two rabbit P-450s [33, 34] and to a 3-methylcholanthrene-inducible rat liver P-450 [35]. The latter MAb inhibits the aryl hydrocarbon hydroxylase (AHH) and ethoxycoumarin *O*-deethylase activities of a 3-methylcholanthrene-inducible P-450 and can be used to identify a type of P-450 present in the tissues of humans [36, 37] as well as those from mouse, guinea pig and hamster [38]. We have also reported the preparation and characterization of MAbs to phenobarbital-inducible P-450 of rat liver [39]. Several of the MAbs to the phenobarbital-inducible P-450 immunoprecipitate and strongly inhibit enzymatic activity [39]. We have used the MAbs raised to different forms of P-450 [33–39] to detect and quantitate epitope specific P-450 forms by radioimmunoassay [40, 41], and for P-450 immunopurification [42–44]. The enzyme inhibitory MAbs have been used to reaction phenotype, i.e. to determine the contributions of specific P-450s to the total reaction in a tissue [36–38, 45]. In this study we report the production and characterization of two classes of MAbs to a pregnenolone 16-α-carbonitrile-inducible P-450 form designated P-450 2a/PCN-E. One class of MAbs binds specifically to P-450 2a/PCN-E, while a second class recognizes an epitope common to both P-450 2a/PCN-E and two forms of PB-inducible P-450 designated PB-4 and PB-5. These additions to our library of anti-P-450 MAbs should prove useful in phenotyping both tissues and individuals for specific forms of P-450 and for furthering our understanding of the relationship between P-450 phenotype and individual differences in drug metabolism and sensitivity to drugs and carcinogens.

MATERIALS AND METHODS

Preparation of cytochrome P-450 and microsomes. Liver microsomes and purified preparations of cytochrome P-450 isolated from rats treated with phenobarbital (designated P-450 PB-4 or PB-B and P-450 PB-5 or PB-D), 3-methylcholanthrene (P-450 MC-B), β-naphthoflavone (P-450 BNF-B) and pregnenolone 16-α-carbonitrile (P-450 2a/PCN-E) were prepared as described [5, 12, 14, 16]. The fraction designated P-450 PCN-E [12] was used both as the antigen to prepare the MAbs reported in this study and also to screen the MAb-producing hybridomas.

Media and cells. Dulbecco's modified Eagle's medium and horse serum were purchased from the Grand Island Biological Co., Grand Island, NY. Fetal calf sera were obtained from both GIBCO and HyClone Lab., Logan, UT. The myeloma cell line, SP2/OAg14 [46], resistant to 6-azaguanine and a non-producer of immunoglobulins, was grown in Dulbecco's modified Eagle's medium with 25 mM glucose and 4 mM glutamine, and supplemented with 10% fetal calf serum, 10% horse serum and 50 μg gentamicin per ml (complete medium). Mouse spleen hybridomas were selected in the complete medium containing 100 μM hypoxanthine (H), 0.4 μM aminopterin (A), and 16 μM thymidine (T) (HAT medium) [34]. Dulbecco's modified Eagle's medium with 5.6 mM glucose plus 10 mM 4-(2-hydroxymethyl)-1-piperazine ethanesulfonic acid, pH 7.4, was used as washing medium in the preparation of hybridomas [34, 35, 39].

Immunization of mice and production of hybridomas. Female Balb/c mice were immunized by intraperitoneal injection weekly for 4 weeks with 10 μg P-450 2a/PCN-E emulsified in 0.2 ml of Freund's complete adjuvent (Miles). To enhance the immune response, the mice were further immunized with the antigen in Dulbecco's phosphate-buffered saline (PBS, pH 7.4) 3–4 days before being killed, and the spleens were removed as previously described [34, 35, 39]. The fusion of myeloma cells with the primed, dissociated spleen cells was carried out essentially as described [34, 35, 39]. Washed myeloma cells (10^7) and spleen cells (10^8) were placed in a 50-ml plastic centrifuge tube and sedimented by centrifugation at 613 *g*. The pellet was loosened and treated with polyethylene glycol 1,000 (50% in 1 ml washing medium) for 1 min and then gradually diluted with 21 ml of washing medium for a period of 5 min. The polyethylene glycol treated cells were harvested by centrifugation at 260 *g*, resuspended in 100 ml HAT medium, and dispensed in 0.2-ml aliquots directly into each of 96 wells of five microtiter plates.

Radioimmunoassay. [^{35}S]Methionine-labeled MAbs to mouse κ light chains were used for detection of MAbs in the screening of hybridoma cells in a direct solid phase radioimmunoassay [39, 47].

Preparation of MAbs in serum-free culture fluids and in ascites. Hybrid cells producing MAbs were grown in flasks containing complete medium plus hypothanxine and thymidine (HT medium), harvested by centrifugation, transferred to Dulbecco's modified Eagle's medium with only HT and antibiotics (serum-free medium), and placed at 37° in an incubator for 2–3 days. The culture fluids were cleared by centrifugation, then filtered through a 0.45 μm filter set and concentrated on an Amicon 15 membrane (mol. wt cut off 15,000). Alternatively, HT medium cultures were collected by centrifugation, resuspended in PBS, and inoculated into female BALB/c mice i.p. (5×10^6 cells in 0.2 ml PBS). Ascites fluid was collected after 2–3 weeks from the peritoneal cavity with a syringe. The ascites fluid was kept at room temperature for 30 min and clarified by two successive centrifugations, at 2,300 *g* for 15 min and at 53,800 *g* for 1 hr. The ascites fluid was kept frozen at −80° until use.

Measurement of protein concentration. Protein concentrations in culture fluids and in ascites fluid were measured by the method of Lowry *et al.* [48].

Double-immunodiffusion analysis. The Ouchterlony double-immunodiffusion technique was utilized to measure the presence and nature of antibodies in the mouse sera, culture fluids, and ascites fluids. Various antigen or antibody solutions (20-μl samples) were placed in 1% agarose disc gel wells (Cappel), incubated at room temperature, and observed for precipitin bands, which appeared after different incubation periods. For a clear demonstration of precipitin bands, the disc gel was removed from the plate and stained as described previously [39].

Measurement of the effect of MAbs on enzyme activities of microsomes and reconstituted cytochrome

P-450 systems. Aryl hydrocarbon hydroxylase activity was determined by measuring the conversion of benzo[*a*]pyrene to phenolic products equivalent to 3-OH-benzo[*a*]pyrene [19], ethoxycoumarin *O*-deethylation by the method of Greenlee and Poland [49] as modified [14], and formaldehyde release resulting from benzphetamine *N*-demethylase and ethylmorphine *N*-demethylase activities by the method of Nash [50].

Enzyme-linked immunosorbent assay (ELISA). The isozymic cross-reactivities of MAbs were determined by an ELISA assay. Purified P-450 forms were diluted to 1 μg/ml with 10 mM potassium phosphate buffer (pH 7.4), 0.9% NaCl, bound to a microtiter plate for 16 hr at 22° and their reactivities with MAbs then determined as described [16]. Saturating amounts of each MAb (1.3 μg ascites protein per ml of PBS and containing 1 mg/ml bovine serum albumin and 0.5 mg/ml Tween 20) were incubated for 60 min at 22°, followed by the binding of a second antibody (affinity purified goat anti-mouse horseradish peroxidase conjugate, diluted 1/3000; BioRad Lab.) for 60 min at 22°. P-450-form cross-reactivities are expressed as a percentage of the A_{415} value measured for each heterologous P-450 relative to the homologous P-450 after a 9-fold dilution into 0.5% sodium dodecyl sulfate. Measured A_{415} values for P-450 2a/PCN-E were 0.9 to 1.0 and are defined as 100%.

Western blot analysis. Liver microsomes (4 μg/well) prepared from adult male and female rats that were either untreated or induced with PB, PCN or BNF were electrophoresed on SDS-gels, transferred to nitrocellulose, and probed with MAb C2 at a concentration of 80 μg MAb/ml. Microsomal P-450 2a/PCN-E was detected using horseradish peroxidase-coupled goat anti-mouse IgG (BioRad) and H_2O_2 + 3,3′-diaminobenzidine (Sigma) as peroxidase substrates, as previously described [15].

RESULTS

Identification and classification of MAbs. Three series of hybridizations were carried out with spleen cells from BALB/c female mice immunized with P-450 2a/PCN-E and myeloma cells, SP2/OAg14 [46]. Mice having immune sera that bound to P-450 2a/PCN-E at levels eight to ten times higher than normal sera (determined after a 100-fold dilution of each serum) were used as the donors of spleen cells for the hybridizations. Two of the immunization series yielded the desired MAbs. Representative hybrid clones from the successful hybridizations are shown in Table 1. Each corresponds to an independent hybrid clone that originated from a single well and was subcloned three times at a density probability of 0.4 cells per microtiter plate well. The nomenclature used for hybrid clones and mouse immunoglobulin classification has been described in a previous report [39]. The first number of the hybrid clone indicates the experiment number; second, well number; third, clone number; and p, number of cell passages. The latter may be important in tracing the chromosomal segregation and stability of hybrid clones. In addition, a trivial designation of C1 to C11 is given to each hybrid clone, and will be used in the text of this paper. Non-specific MAbs used in these studies are designated NS1 to NS4 and include NBS hybrid clones derived from the hybridization between myeloma cells and spleen cells from unimmunized BALB/c female mice [39] (MAbs NS1, NS3) and HyHel-9p10, a hybridoma which produces an IgGl MAb to chicken lysozyme (MAb NS4) [51]. As shown in Table 1, each individual hybrid clone produced a single MAb subclass which was either IgG1, IgG2a or IgG2b. Each of the hybridomas produced MAbs that bound to P-450 2a/PCN-E at least to a 17-fold greater extent than did the nonspecific MAbs, NS1 and NS2. MAbs from three of the hybrid clones bound to both P-450 2a/PCN-E and P-450 PB-4. The remaining hybrid clones produced MAbs that bound only to P-450 2a/PCN-E. None of the MAbs derived from the eleven hybridomas bound to cytochrome P-450 MC-B (equivalent to P-450 BNF-B) or to β-galactosidase, both of which were used as negative controls. Precipitin bands between P-450 2a/PCN-E and all MAbs tested (C1, C2, C7, C9, C10, C11) were observed upon Ouchterlony immunodiffusion followed by staining with Coomassie blue (Fig. 1). The precipitin reactions were specific as no reaction was observed with non-specific MAbs such as NS3 and NS4. Other unreactive MAbs used as controls for these experiments included those raised to the isosafrole-inducible P-450 ISF-G (data not shown). The immunoprecipitations observed in these experiments may have been due to the binding of MAbs to cytochrome P-450 aggregates since precipitation did not occur in the presence of detergent.

Specificity of MAbs. MAbs are probes directed against a specific epitope of an antigenic molecule. As shown in Table 1, three of the eleven MAbs raised to P-450 2a/PCN-E (MAbs C1, C3, C4) bound to both P-450 2a/PCN-E and to P-450 PB-4 while the remaining eight MAbs bound only to P-450 2a/PCN-E. The isozymic cross-reactivity of one MAb from each group was characterized in greater detail by ELISA under conditions of saturating MAb (Fig. 2). MAb C2 was found to bind selectively to P-450 2a/PCN-E with no binding detected between MAb C2 and seven other highly purified rat hepatic P-450s. By contrast, MAb C1 bound equally well to P-450s 2a/PCN-E, PB-4, and PB-5. Thus, the epitopes recognized by MAb C1 and MAb C2 are distinct and non-interacting. Neither MAb bound rat hepatic P-450 forms PB-1, 2c, 2d, 3 [16] or BNF-B. The cross-reactivity of MAb C1 with P-450 forms 2a/PCN-E and forms PB-4 and PB-5 indicates that all three P-450s share a common epitope which presumably corresponds to a region of either primary, secondary or tertiary structural homology.

Effect of MAbs on aryl hydrocarbon hydroxylase and benzphetamine demethylase activity of purified P-450 2a/PCN-E. The effects of culture fluid concentrates derived from eleven anti-P-450 2a/PCN-E MAbs on the AHH activity of purified and reconstituted P-450 2a/PCN-E are shown in Table 1. None of the eleven MAbs was a strong inhibitor of AHH activity. MAbs C9 and C10 were the most inhibitory, reducing AHH activity to 40 or 50% of control, with little or no inhibition effected by the other nine MAbs. Ascites fluids were prepared from three of the MAbs, and these were then tested for their

Table 1. Characteristics of monoclonal antibodies prepared to purified pregnenolone-16-α-carbonitrile-induced rat liver cytochrome P-450

Source of MAbs*	Ig class	Binding† (RIA)				Immunoprecipitation by double-immunodiffusion	% Control AHH activity‡
		Purified cytochrome P-450					
		2a/PCN-E	PB-4	MC-B	β-Galactosidase	Purified cytochrome P-450 2a/PCN-E	
Non-specific MAbs							
NS1 NBS 1-14-1p6	IgG1	155	280	275	130		
NS2 P3X63 (Myeloma)	IgG1	105	100	100	NT		
NS3 NBS 1-48-5p24	IgG2a					−	100
NS4 HyHel-9p10	IgG1					−	
P-450 2a/PCN-E specific MAbs							
C1 2-3-2p4	IgG1	17340	15540	560	150	+	104
C2 2-13-1p4	IgG1	17645	300	340	145	+	74
C3 2-14-1p4	IgG1	16140	12135	505	145	NT§	118
C4 2-14-2p4	IgG1	19670	11695	600	120	NT	166
C5 3-1-58p3	IgG1	7520	110	125	NT	NT	96
C6 3-2-20p3	IgG1	3900	60	100	NT	NT	104
C7 3-3-53p3	IgG2a	6110	425	190	NT	+	76
C8 3-3-63p3	IgG2b	5010	490	280	NT	NT	96
C9 3-3-78p3	IgG1	2535	130	140	NT	+	38
C10 3-7-78p3	IgG1	5255	200	95	NT	+	54
C11 3-10-24p3	IgG2b	7010	445	380	NT	+	70

* Culture fluids were used as the source of MAbs for testing of binding by RIA and for immunoglobulin classification. Ascites fluids were used for double-immunodiffusion analysis.

† Purified liver microsomal cytochrome P-450 (10 μg protein) from rats treated with pregnenolone-16-α-carbonitrile, phenobarbital or 3-methylcholanthrene was precoated overnight at 4° on wells of 96-well microtiter plates, and solid phase radioimmunoassays were carried out as described in Materials and Methods. The input radioactivity of ^{35}S-labeled rat anti-mouse κ chain was 83,340 cpm/4.7 μg sample for the experiments which included β-galactosidase and the activity for others was 74,900 cpm/13 μg sample.

‡ Purified P-450 2a/PCN-E (12 μg) was incubated with serum-free culture fluid concentrates (200 μg) at room temperature for 15 min, and AHH activities were measured in the reconstituted systems containing NADPH-cytochrome P-450 reductase (10.6 μg) and phospholipid (30 μg) at 37° for 20 min. The specific activity of the reconstituted system in the presence of the non-specific MAb NS3 was 50 pmoles 30H-BP/nmole P-450 2a/PCN-E/min.

§ Not tested.

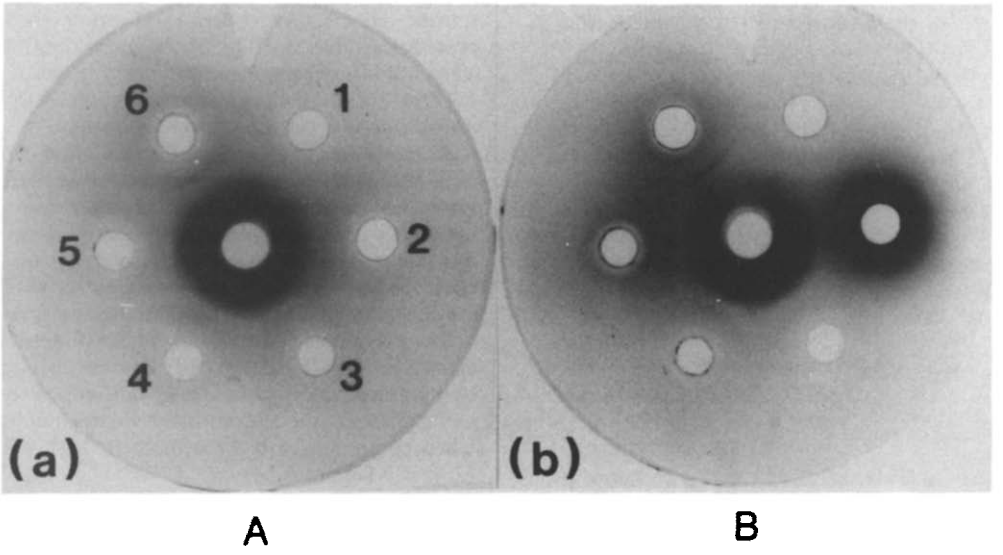

Fig. 1. Double-immunodiffusion analysis of the specificity of monoclonal antibodies to purified P-450 2a/PCN-E. Immunodiffusion plates were incubated for 9 days at room temperature and stained with Coomassie blue. Center wells of A and B: P-450 2a/PCN-E (20 μl of 0.34 mg/ml). Outer wells of A: 1, 2, 3, 4, 5, and 6 contained 20 μl ascites of non-specific MAbs NS3 (52.5 mg/ml), and NS4 (32.5 mg/ml), MAbs to P-450 2a/PCN-E C1 (74.5 mg/ml), C2 (7.8 mg/ml), C7 (45.5 mg/ml), and C9 (5.5 mg/ml). Outer wells of B: 1 and 2 contained 20 μl serum-free culture fluids of specific MAbs to isosafrole-inducible rat liver cytochrome P-450 1-4-10 (5.5 mg/ml) and 1-19-2 (4.4 mg/ml) (unpublished data); wells 3, 4, 5 and 6 contained 20 μl of the specific MAbs C1 (62.5 mg/ml), C2 (61.3 mg/ml), C9 (49 mg/ml) and C11 (50 mg/ml).

inhibition of the benzphetamine *N*-demethylase activity of purified and reconstituted P-450 2a/PCN-E (Table 2). This second substrate was used in these inhibition experiments to assess the possibility that one or more of the MAbs might subtly discriminate between the approaches of different substrates to the active site of the P-450. These experiments suggested partial inhibition of benzphetamine demethylase activity by MAb C1. The absence of significant inhibition of the purified cytochrome P-450 suggests that the epitopes to which the MAbs are directed are not directly associated with the catalytic site of P-450 2a/PCN-E. In the case of the purified and reconstituted P-450 2a/PCN-E, addition of non-specific IgG caused some inhibition, suggesting that inhibitory activities of less than 50% may be related to non-

Table 2. Effect of MAbs on benzphetamine *N*-demethylase (BPDM) activity of purified P-450 2a/PCN-E and ethylmorphine *N*-demethylase (EMDM) activity of dexamethasone-induced rat liver microsomes

Source of MAbs (ascites: 200 μg)	BPDM (pmoles HCHO/ nmole P-450/min)	% Control	EMDM (nmoles HCHO/ mg/min)	% Control
Non-specific MAbs				
NS3	54.3	100	28.5	100
NS4			25.5	90
P-450 2a/PCN-E specific MAbs				
C1	23.3	43	29.4	103
C2	69.9	129	25.6	90
C11	50.4	93		

Purified P-450 2a/PCN-E (4.8 μg) was incubated with ascites fluid (200 μg) for 15 min at room temperature and then assayed for BPDM activity in a reconstituted system containing NADPH–cytochrome P-450 reductase (10.6 μg) and phospholipid (30 μg) at 37° for 60 min. Microsomes (10 μg protein) prepared from male rats treated with dexamethasone (50 mg/kg rat) were preincubated with ascites (200 μg), and EMDM activity was measured as described in Materials and Methods. The microsomes from dexamethasone-treated rats were induced ~2 fold with respect to their P-450 2a/PCN-E-dependent EMDM activity as compared to microsomes from untreated rats [52].

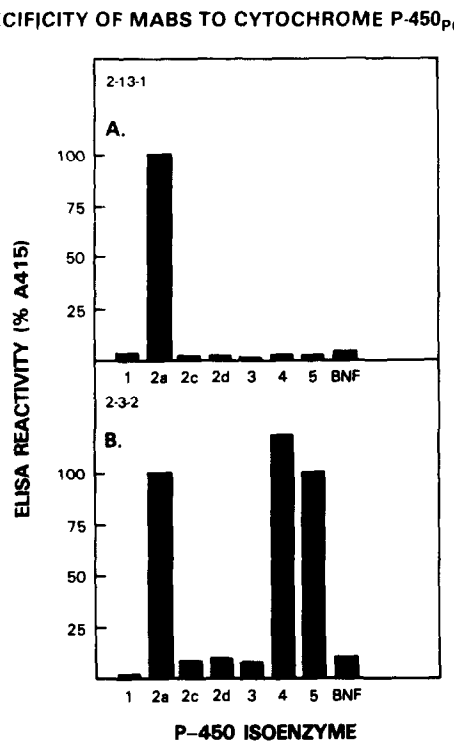

Fig. 2. Isozymic cross-reactivities of MAbs. Purified P-450 forms PB-1, 2a/PCN-E, 2c, 2d, 3, PB-4, PB-5 and BNF-B were diluted to 1 μg/ml with 10 mM potassium phosphate buffer (pH 7.4), 0.9% NaCl, bound to a microtiter plate for 16 hr at 22°, and their reactivities with MAb C2 (Panel A) and C1 (Panel B) were determined by ELISA [16]. Cross-reactivities of the MAbs with each P-450 form are expressed as the ratio of the A_{415} value obtained with each P-450 relative to the A_{415} value for P-450 2a/PCN-E and expressed as a percentage. The measured A_{415} value for the homologous P-450 2a/PCN-E was 0.9 to 1.0. The procedures are described in detail in Materials and Methods.

specific effects. By contrast, in other studies we previously reported that MAbs were obtained that were inhibitory to more than 90% [35, 39].

Effect of MAbs on enzyme activity of dexamethasone-induced rat liver microsomes. Dexamethasone has been reported to be a good inducer of P-450 2a/PCN-E as are pregnenolone 16-α-carbonitrile and phenobarbital [53]. We examined the inhibitory effects of MAbs C1 and C2 on ethylmorphine *N*-demethylase activity of liver microsomes isolated from male rats treated with dexamethasone (Table 2). These microsomes exhibited about two times the ethylmorphine *N*-demethylase activity and P-450 2a/PCN-E content as compared to uninduced microsomes [12]. Neither MAb exerted any significant inhibition of the microsomal activity when compared with the effects of the non-specific MAbs, NS3 and NS4.

Effect of anti P-450 2a PCN-E MAbs on the ethoxycoumarin O-deethylase activity of purified P-450 PB-4. As shown in Fig. 2, MAb C2 bound P-450 2a/PCN-E selectively. By contrast MAb C1 bound well to both P-450s PB-4 and PB-5, in addition to P-450 2a/PCN-E. Therefore, it was of interest to examine the effects of these MAbs on the catalytic activities of purified and reconstituted P-450 PB-4. In addition to MAbs C1 and C2, raised to P-450 2a-PCN-E, the effect of MAb 4-29-5, a strong inhibitor of phenobarbital-inducible P-450 [39], and of MAbs 1-7-1 and 1-31-2, which inhibit 60–90% of the aryl hydrocarbon hydroxylase activities of cytochrome P-450 MC-B or P-450 BNF-B [35], were also examined (Table 3). MAb 4-29-5 inhibited 99% of the ethoxycoumarin *O*-deethylase activity of P-450 PB-4. No inhibition was effected by MAb C1 even though this MAb was fully cross-reactive with P-450 PB-4. Furthermore, no inhibition was observed when using MAbs 1-7-1, 1-31-2 and C2. Taken together, these results indicate that the epitopes on P-450 2a/PCN-E to which our MAbs are directed are not directly associated with

Table 3. Effect of monoclonal antibodies to P-450 2a/PCN-E on the ethoxycoumarin *O*-deethylase activity of P-450 PB-4

Source of MAbs (ascites: 200 μg)	Ig class	Activity (nmoles 70H-C/nmole P-450/min)	% Control
Non-specific MAb			
NS4	IgG1	15.7	100
P-450 specific MAbs			
PCN-P-450 C1	IgG1	15.0	96
C2	IgG1	15.4	98
PB-P-450 4-29-5p6	IgG2b	0.1	<1
MC-P-450 1-31-2p4	IgG1	16.8	107
1-7-1p6	IgG1	17.6	112

Purified liver microsomal cytochrome P-450 isolated from phenobarbital-induced rats (P-450 PB-4, 30 nM) was reconstituted with saturating NADPH-cytochrome P-450 reductase and dilauroyl phosphatidylcholine (5 μg/ml) in a volume of 45 μl for 10 min at 22–25°. Samples were then preincubated with ascites (200 μg) at room temperature for 30 min, then diluted to a final volume of 0.4 ml with 0.1 M potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, 20% glycerol (v/v) containing 1 mM 7-ethoxycoumarin (Aldrich Chemical Co.) and 1% methanol (final concentrations). 7-Ethoxycoumarin *O*-deethylase activity was then determined as described previously [14].

catalytic activity related to the metabolism of benzo-[*a*]pyrene, benzphetamine, ethylmorphine or ethoxycoumarin.

Western blot analysis using MAb C2. We have shown previously [15] that P-450 2a/PCN-E is male-specific in adult rat liver (♂/♀ = ~20) as a consequence of its developmental reduction in female rats going through puberty. This sex-specificity is, however, abolished upon administration of chemicals such as phenobarbital and pregnenolone 16-α-carbonitrile which induce P-450 2a/PCN-E about 3- to 5-fold in males and up to 70-fold in females, as demonstrated immunochemically, using polyclonal anti-P-450 2a/PCN-E antibodies, and catalytically, using microsomal androstenedione 6-β-hydroxylase activity as a specific monitor for P-450 2a/PCN-E activity levels [15]. These findings were confirmed by Western blot analysis using MAb C2. Liver microsomes prepared from adult male and female rats that were either untreated or induced with phenobarbital, pregnenolone 16-α-carbonitrile or β-naphthoflavone were electrophoresed on SDS-gels, transferred to nitrocellulose, and then probed with MAb C2. The blots were then reacted with a peroxidase-coupled goat anti-mouse IgG followed by a peroxidase substrate to visualize the MAb-detected microsomal P-450 2a/PCN-E. The results obtained (Fig. 3) confirm the male specificity of P-450 2a/PCN-E in the adult animals and demonstrate a marked induction of this P-450 form in both sexes by pregnenolone 16-α-carbonitrile and, to a lesser extent, phenobarbital. A small decrease in immunoreactive P-450 2a/PCN-E was observed upon β-naphthoflavone administration. These findings demonstrate the utility of form-specific anti-P-450 MAbs such as C2, which, although not inhibitory, can be used to assess the effects of various chemicals on the expressed levels of specific P-450 forms.

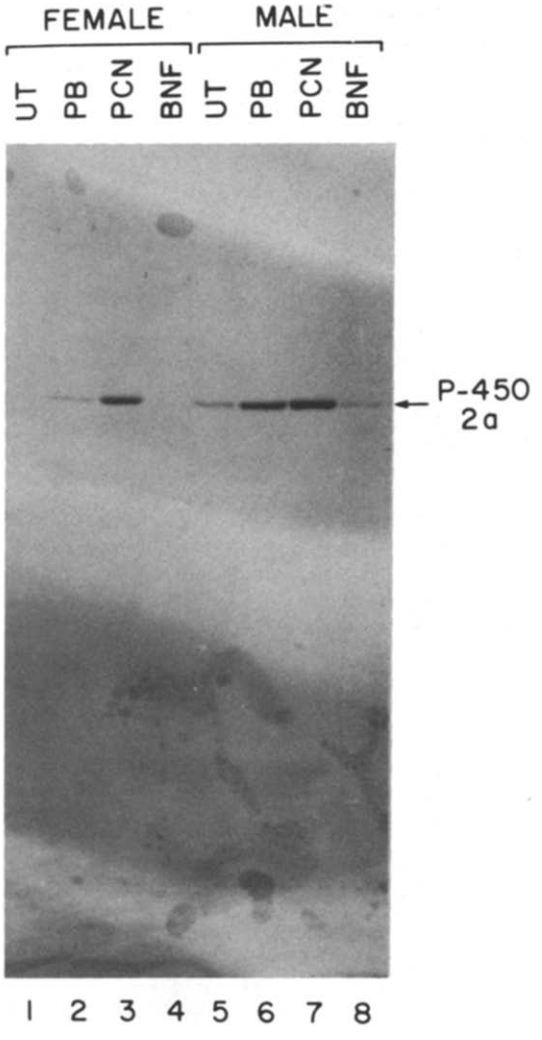

Fig. 3. Western blot analysis using MAb C2. Liver microsomes prepared from adult rats of both sexes that were either untreated (UT) or induced with phenobarbital (PB), pregnenolone 16-α-carbonitrile (PCN) or β-naphthoflavone (BNF) were subjected to SDS-gel electrophoresis (4 μg microsomes/well) and Western blotting [15]. MAb C2 was incubated with the Western blot at a concentration of 80 μg MAb/ml, and microsomal P-450 2a/PCN-E was detected using horseradish peroxidase-coupled goat anti-mouse IgG and H_2O_2 + 3,3′-diaminobenzidine as peroxidase substrates. The electrophoretic mobility of purified P-450 2a/PCN-E [16] was as indicated.

DISCUSSION

Several laboratories have successfully purified various constitutive forms of cytochrome P-450 as well as P-450 forms induced in several species treated with different inducers [3–16]. Identification and characterization of the purified cytochromes P-450 are necessary both for the elucidation of P-450 multiplicity and its relationship to metabolic differences in tissues and organs as well as for the understanding of the molecular basis of inter-individual differences in drug metabolism and sensitivity to drugs and carcinogens. Pregnenolone 16-α-carbonitrile is an effective inducer of at least one specific form of P-450 which is clearly distinct from the forms induced by phenobarbital and polycyclic hydrocarbons respectively [53–56]. MAbs are highly specific probes that can be used for the purification and characterization of cytochromes P-450. We have constructed recently a library of panels of MAbs to different forms of cytochrome P-450 in order to study cytochromes P-450 and their functions [33–35, 39]. MAbs to both phenobarbital- and 3-methylcholanthrene-inducible P-450 forms including some MAbs that are inhibitory to catalytic activity were thus obtained. Other laboratories have prepared MAbs to hepatic P-450s isolated from rats [57–59] and rabbits [60, 61]. In the current study, eleven MAbs to P-450 2a/PCN-E were obtained, none of which was specifically inhibitory to enzyme activity. The lack of inhibitory potency is not surprising since MAbs are directed to many different epitopes of an antigenic molecule including segments not related to the active site of the enzyme. MAb C2 specifically recognized an epitope on P-450 2a/PCN-E that is absent from the seven other highly purified rat hepatic P-450s examined. The high specificity of this MAb makes it an ideal reagent for immunochemical analysis of P-450 2a/PCN-E in crude liver fractions, as demonstrated by Western blot analysis (Fig. 3). MAb C1, however, recognized an epitope common to P-450 2a/PCN-E and to the

major phenobarbital-inducible P-450 forms, PB-4 and PB-5. These later results indicate that there exist MAb-detectable homologies between P-450s 2a/PCN-E, PB-4 and PB-5 despite the fact that there is only about 30% sequence homology between these P-450 forms [62]. We have used MAbs for reaction phenotyping [36–38, 45], detection, and identification of P-450s by radioimmunoassay [40, 41], and immunopurification [42–44] of cytochromes P-450. MAbs have also been useful in the identification of mRNA translation products and in the preparation of cDNA complementary to the mRNA [63]. Identification and characterization of different P-450 forms by ELISA, radioimmunoassay, and immunopurification using MAbs should be useful for furthering our understanding of the role of cytochromes P-450 in individual differences in drug metabolism and sensitivity to chemical carcinogens.

REFERENCES

1. A. H. Conney, *Pharmac. Rev.* **19**, 317 (1967).
2. H. V. Gelboin, *Physiol. Rev.* **60**, 1107 (1980).
3. A. Y. H. Lu and S. B. West, *Pharmac. Rev.* **31**, 277 (1980).
4. D. A. Haugen, T. A. van der Hoeven and M. J. Coon, *J. biol. Chem.* **250**, 3567 (1975).
5. F. P. Guengerich and M. V. Martin, *Archs Biochem. Biophys.* **205**, 365 (1980).
6. D. E. Ryan, P. E. Thomas, D. Korzeniowski and W. Levin, *J. biol. Chem.* **254**, 1365 (1979).
7. D. E. Ryan, P. E. Thomas and W. Levin, *J. biol. Chem.* **255**, 7941 (1980).
8. D. E. Ryan, S. Iida, A. W. Wood, P. E. Thomas, C. S. Lieber and W. Levin, *J. biol. Chem.* **259**, 1239 (1984).
9. E. Le Provost, J. P. Flinois, P. Beaune and J. P. Leroux, *Biochem. biophys. Res. Commun.* **101**, 547 (1981).
10. G. F. Fisher, H. Fukushima and J. L. Gaylor, *J. biol. Chem.* **206**, 4388 (1981).
11. N. Harada and T. Omura, *J. Biochem., Tokyo* **89**, 237 (1981).
12. F. P. Guengerich, G. A. Dannan, S. T. Wright, M. V. Martin and L. S. Kaminsky, *Biochemistry* **21**, 6019 (1982).
13. K. Cheng and J. B. Schenkman, *J. biol. Chem.* **257**, 2378 (1982).
14. D. J. Waxman and C. Walsh, *J. biol. Chem.* **257**, 10446 (1982).
15. D. J. Waxman, G. A. Dannan and F. P. Guengerich, *Biochemistry* **24**, 4409 (1985).
16. D. J. Waxman, *J. biol. Chem.* **259**, 15481 (1984).
17. P. E. Thomas, A. Y. H. Lu, D. Ryan, S. B. West, J. Kawlek and W. Levine, *Molec. Pharmac.* **12**, 746 (1976).
18. W. L. Dean and M. J. Coon, *J. biol. Chem.* **252**, 3255 (1977).
19. J. Deutsch, J. C. Leutz, S. K. Yang, H. V. Gelboin, Y. L. Chiang, K. P. Vatsis and M. J. Coon, *Proc. natn. Acad. Sci. U.S.A.* **75**, 3123 (1978).
20. J. Deutsch, P. Okano and H. V. Gelboin, in *Carcinogenesis: Fundamental Mechanisms and Environmental Effects* (Eds. B. Pullman, P. O. P. Ts'o and H. V. Gelboin), p. 125. D. Reidel, Hingham, MA (1980).
21. L. S. Kaminsky, M. J. Fasco and F. P. Guengerich, *J. biol. Chem.* **255**, 85 (1980).
22. A. W. Wood, P. G. Wislocki, R. L. Chang, W. Levin, A. Y. H. Lu, H. Yagi, O. Henandez, D. M. Jerina and A. H. Conney, *Cancer Res.* **36**, 3358 (1976).
23. E. Huberman, L. Sachs, S. K. Yang and H. V. Gelboin, *Proc. natn. Acad. Sci. U.S.A.* **73**, 607 (1976).
24. D. W. Nebert and H. V. Gelboin, *Archs Biochem. Biophys.* **134**, 76 (1969).
25. G. Kellerman, C. R. Shaw and A. Kellerman-Luyten, *New Engl. J. Med.* **289**, 934 (1973).
26. G. Kellerman, M. Kellerman-Luyten and C. R. Shaw, *Am. J. hum. Genet.* **25**, 327 (1973).
27. H. V. Gelboin, *New Engl. J. Med.* **297**, 384 (1977).
28. T. Okuda, E. S. Vesell, V. Plokin, R. Tarone, R. C. Bast and H. V. Gelboin, *Cancer Res.* **37**, 3904 (1977).
29. F. P. Guengerich, P. Wang and N. K. Davidson, *Biochemistry* **21**, 1698 (1982).
30. G. P. Vlasuk, J. Ghrayeb, D. E. Ryan, L. Reik, P. E. Thomas, W. Levin and F. G. Walz, Jr., *Biochemistry* **21**, 789 (1982).
31. R. E. Kouri, C. E. McKinney, D. J. Slomany, D. R. Snodgrass, N. P. Wray and T. L. McLemore, *Cancer Res.* **42**, 5030 (1982).
32. G. Köhler and C. Milstein, *Nature, Lond.* **256**, 495 (1975).
33. S. S. Park, S. J. Cha, H. Miller, A. V. Persson, M. J. Coon and H. V. Gelboin, *Fedn Eur. Biochem. Soc. Lett.* **116**, 231 (1980).
34. S. S. Park, S. J. Cha, H. Miller, A. V. Persson, M. J. Coon and H. V. Gelboin, *Molec. Pharmac.* **21**, 248 (1982).
35. S. S. Park, T. Fujino, D. West, F. P. Guengerich and H. V. Gelboin, *Cancer Res.* **42**, 1978 (1982).
36. T. Fujino, S. S. Park, D. West and H. V. Gelboin, *Proc. natn. Acad. Sci. U.S.A.* **79**, 3682 (1982).
37. T. Fujino, K. Gottlieb, D. K. Manchester, S. S. Park, D. West, H. L. Gurtoo and H. V. Gelboin, *Cancer Res.* **44**, 3916 (1984).
38. T. Fujino, D. West, S. S. Park and H. V. Gelboin, *J. biol. Chem.* **259**, 9044 (1984).
39. S. S. Park, T. Fujino, H. Miller, F. P. Guengerich and H. V. Gelboin, *Biochem. Pharmac.* **33**, 2071 (1984).
40. B. J. Song, T. Fujino, S. S. Park, F. K. Friedman and H. V. Gelboin, *J. biol. Chem.* **259**, 1394 (1984).
41. B. J. Song, F. K. Friedman, S. S. Park, G. C. Tsokos and H. V. Gelboin, *Science* **228**, 290 (1985).
42. F. K. Friedman, R. C. Robinson, S. S. Park and H. V. Gelboin, *Biochem. biophys. Res. Commun.* **116**, 859 (1983).
43. K. Cheng, H. C. Krutzsch, S. S. Park, P. H. Grantham, H. V. Gelboin and F. K. Friedman, *Biochem. biophys. Res. Commun.* **123**, 1201 (1984).
44. K. C. Chung, F. K. Friedman, B. J. Song, S. S. Park and H. V. Gelboin, *J. biol. Chem.* **259**, 12279 (1984).
45. F. J. Wiebel, S. S. Park, F. Kiefer and H. V. Gelboin, *Eur. J. Biochem.* **145**, 455 (1984).
46. M. Shulman, C. D. Wilde and G. Köhler, *Nature, Lond.* **276**, 269 (1978).
47. D. E. Yelton, C. Sesaymard and M. D. Scharff, *Hybridoma* **1**, 5 (1981).
48. O. H. Lowry, N. J. Rosebrough, A. L Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
49. W. F. Greenlee and A. Poland, *J. Pharmac. exp. Ther.* **205**, 596 (1976).
50. T. Nash, *Biochem. J.* **55**, 416 (1953).
51. S. Smith-Gill, A. C. Wilson, M. Potter, E. M. Prager, R. J. Feldmann and C. R. Mainhart, *J. Immun.* **128**, 314 (1982).
52. T. Shimada and F. P. Guengerich, *Molec. Pharmac.* **28**, 215 (1985).
53. D. M. Heuman, D. J. Gallagher, J. L. Barwick, N. A. Elshourbagy and P. S. Guzelian, *Molec. Pharmac.* **21**, 735 (1982).
54. M. Pasanen and O. Pelkonen, *Biochem. biophys. Res. Commun.* **103**, 1310 (1981).
55. A. Y. H. Lu, A. Somogi, S. West, R. Kuntzman and

A. H. Conney, *Archs Biochem. Biophys.* **152**, 457 (1972).
56. N. A. Elshourbagy and P. S. Guzelian, *J. biol. Chem.* **255**, 1279 (1980).
57. F. Latawe-Goujin, P. Kremers, P. Beaune, M. Paye and J. K. Gielen, *Biochem. biophys. Res. Commun.* **119**, 744 (1984).
58. P. E. Thomas, L. M. Reik, D. E. Ryan and W. Levin, *J. biol. Chem.* **259**, 3890 (1984).
59. P. E. Thomas, J. Reidy, L. M. Reik, D. E. Ryan, E. R. Koop and W. Levin, *Archs Biochem. Biophys.* **235**, 239 (1984).
60. K. M. Lewis, A. R. Boobis, M. B. Slade and D. Davis, *Biochem. Pharmac.* **31**, 1851 (1982).
61. I. Reubi, K. J. Griffin, J. L. Raucy and E. F. Johnson, *J. biol. Chem.* **259**, 5887 (1984).
62. F. J. Gonzalez, D. W. Nebert, J. P. Hardwick and C. B. Kasper, *J. biol. Chem.* **260**, 7435 (1985).
63. J. B. Fagan, J. V. Pastewka, S. S. Park, F. P. Guengerich and H. V. Gelboin, *Biochemistry* **21**, 6574 (1982).