Use of a Monoclonal Antibody Specific for Rabbit Microsomal Cytochrome P-450 3b To Characterize the Participation of This Cytochrome in the Microsomal 6β- and 16α-Hydroxylation of Progesterone†

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ABSTRACT: A monoclonal antibody was developed that is specific for the 3b electrophoretic class of rabbit liver microsomal cytochrome P-450 as judged by immunoprecipitation and subsequent electrophoretic analysis. The antibody is inhibitory of catalytically distinct, variant forms of P-450 3b prepared from New Zealand White or IIIVO/J rabbits, respectively. Peptide mapping of the immunopurified P-450 3b from NZW and IIIVO/J microsomes indicates that a characteristic difference between the variant forms is exhibited by the antigen. In addition, a competitive assay indicates that the binding properties of the antibody do not differ substantially toward the variant forms of P-450 3b. The inhibitory antibody was used to examine the contribution of P-450 3b to the microsomal 16α- and 6β-hydroxylation of progesterone. The antibody inhibits 40–70% of the 16α-hydroxylase activity of microsomes from either New Zealand White or IIIVO/J rabbits. In contrast, it does not inhibit 6β-hydroxylation catalyzed by microsomes prepared from strain IIIVO/J but does inhibit this reaction as catalyzed by microsomes from most New Zealand White rabbits. The antibody also inhibits the increased 16α-hydroxylase activity of IIIVO/J microsomes observed in the presence of 5β-pregnane-3β,20α-diol, an allosteric effector of this variant form of P-450 3b. Use of this monoclonal antibody provides a link between the observed properties of the purified, variant forms of P-450 3b and microsomal metabolism. These results indicate that the antibody can be used to phenotype variant forms of P-450 3b in microsomal fractions.

The microsomal cytochrome P-450 system encompasses a group of structurally diverse and multifunctional enzymes. Early work on the characterization of the various forms that comprise the rabbit P-450 system resulted in the description of a number of electrophoretically distinct and homogeneous forms of P-450 (Lu & West, 1980). This in turn led to a nomenclature based on electrophoretic mobility (Haugen et al., 1975; Johnson, 1979). Although electrophoretic homogeneity has been widely used to characterize the purity of P-450 preparations, it does not preclude structural and functional polymorphism within an electrophoretic type of P-450. Previous work in this laboratory indicates that there are two or more electrophoretically similar, yet catalytically distinct, forms of liver microsomal P-450 3b, as judged by the catalytic and structural dissimilarities between P-450 3b isolated from rabbit strain IIIVO/J and from outbred New Zealand White (NZW) rabbits (Dieter & Johnson, 1982). Microsomes and purified reconstituted P-450 3b prepared from IIIVO/J rabbits exhibit a low efficiency, high K_m progesterone 6β-hydroxylase activity and also a low efficiency 16α-hydroxylase activity that can be stimulated directly by a variety of progesterone derivatives (Johnson et al., 1983). In contrast, P-450 3b prepared from most outbred NZW rabbits exhibits a 16α-hydroxylase activity that displays the kinetic properties of a mixture of two hydroxylases differing in K_m and a relatively low K_m, high efficiency 6β-hydroxylase activity (Dieter & Johnson, 1982).

The observed catalytic variation associated with P-450 3b, whether due to differences in expression of two distinct gene products or posttranslational modification, is suggestive evidence for microheterogeneity. Monoclonal antibody technology was chosen to extend these studies, and in this report we describe the development and characterization of a monoclonal antibody that recognizes a nonrepeating epitope, restricted to the 3b electrophoretic class of P-450 that is shared by the catalytically distinct, variant forms of P-450 3b. This antibody inhibits the high efficiency, low K_m progesterone 6β-hydroxylase as well as the high efficiency and pregnanediol-stimulated low efficiency 16α-hydroxylase activities, previously linked to P-450 3b. This antibody has been used to determine the contribution of P-450 3b to the microsomal metabolism of progesterone.

Experimental Procedures

Materials. Dulbecco's modified Eagle's medium, fetal calf serum, glutamine, and pyruvate were obtained from M. A. Bioproducts. Hypoxanthine, aminopterin, thymidine, 2-amino-6-mercaptopurine, rabbit anti-mouse alkaline phosphatase, phosphatase substrate, protein A–agarose, glutaraldehyde, lysine, and alkaline phosphatase were purchased from Sigma. Rabbit anti-mouse IgG subclass antisera and the IgG fraction of rabbit anti-mouse IgG were produced by Miles Laboratories. Penicillin–streptomycin was obtained from Gibco and 30% bovine serum albumin (BSA)[1] was purchased from Armour Pharmaceuticals. Costar, Falcon, and Corning tissue culture plastic ware was used. Microtiter test plates, 96-well, were manufactured by Nunc or Dynatech. Reagents for SDS–polyacrylamide gel electrophoresis were obtained from Bio-Rad. Cyanogen bromide was purchased from Pierce. Sepharose CL-4B and purified protein A were obtained from Pharmacia. Pristane (2,6,10,14-tetramethylpentadecane) was purchased from Aldrich. [^{14}C]Progesterone was a product of Amersham and was used with a specific activity of 56 mCi/mmol. Na^{125}I was obtained from New England Nuclear.

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[1] Abbreviations: PBS, phosphate-buffered saline, 10 mM sodium phosphate, pH 7.4, and 150 mM NaCl; BSA, bovine serum albumin; 3% BSA, PBS containing 3% BSA; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTE, dithioerythritol.

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The serum-free medium, HB101, is a product of Hana Biologicals.

New Zealand White rabbits were obtained from local rabbit breeders. IIIVO/J rabbits were obtained from Jackson Laboratories. Balb/c mice were purchased from the mouse breeding colony maintained at this Institute.

Production and Purification of Antibodies. Two 6-week-old female Balb/c mice were injected intraperitoneally with 40 μg of a purified preparation of P-450 3b emulsified in complete Freund's adjuvant. Three months later each mouse was injected with 50 μg of the same antigen in PBS intraperitoneally as well as subcutaneously with 20 μg in incomplete Freund's adjuvant. Three days later spleen cells were collected aseptically, mixed 7:1 with SP2/0 myeloma cells (Shulman et al., 1978), and fused by using 30% polyethylene glycol 1000, pH 7.8. The fusion procedure is described elsewhere (Kennett, 1979) and was used with minor modifications. After fusion, the cells were plated at a density of 1.45×10^5 cells/well in five 96-well plates containing feeder layers of peritoneal macrophages [$(1.5–3.0) \times 10^4$ cells/well] prepared the day before. The cultures were maintained as described (Galfre & Milstein, 1981; Schreier et al., 1980). Twelve days after the fusion procedure, all cultures were tested for production of antibodies recognizing purified P-450 3b. Selected positive cultures were cloned twice at a density of <0.5 cells/well prior to injection into pristane-primed Balb/c mice. Ascites fluid was collected 10–14 days after injection. Immunoglobulins were precipitated from ascites fluid by ammonium sulfate precipitation and purified with protein A–agarose (Oi & Herzenberg, 1980). The appropriate column fractions were dialyzed against 50 mM sodium phosphate, pH 7.4, and assayed for contaminating protease activity (Bjerrum et al., 1975). For labeling of antibody, 200 μg of IgG purified from serum-free cell culture supernatant was reacted with 1 mCi of $Na^{125}I$ by using Chloramine T (McConahey & Dixon, 1966). Resulting specific activities ranged from 3×10^6 to 6×10^6 cpm/μg of protein.

Immunoadsorbent Preparation. Sepharose Cl-4B was activated with cyanogen bromide (March et al., 1974). Purified antibody in 0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl was mixed with freshly activated Sepharose in a ratio of 8–13 mg of IgG/mL of Sepharose and processed as described (Pharmacia Fine Chemicals, 1979). The immunoadsorbent was washed and equilibrated with the appropriate buffer before use. Preparations of antibody-coupled Sepharose routinely contained 5–8 mg of antibody/mL and bound 20–30 pmol of P-450/μL.

Alkaline Phosphatase–P-450 3b Conjugate. Three milligrams of alkaline phosphatase, dissolved in 3.2 M $(NH_4)_2SO_4$, pH 7, and stabilized with 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$, was dialyzed for 3 h against phosphate-buffered saline (PBS), pH 6.8. One milligram of purified P-450 3b was added, and the combined proteins were then dialyzed in PBS, pH 6.8, overnight at 4 °C. Glutaraldehyde was added (final concentration 0.2%), and the reaction mixture was incubated at room temperature under constant agitation for 2 h. A total of 0.3 mL of 1 M lysine was then added and the incubation continued for an additional hour. After extensive dialysis, the mixture was applied to a column containing Sephadex G-100 (1.6 × 30 cm) equilibrated with PBS, pH 8.0, in order to separate the conjugate from unreacted alkaline phosphatase. The final sample was kept in PBS, pH 8.0, containing 0.5 mM $MgCl_2$, 1% BSA, 20% glycerol, and 0.02% NaN_3.

P-450 Purification, Microsome Preparation, and Solubilization. Microsomes and individual forms of cytochrome P-450 were prepared as described previously (Dieter & Johnson, 1982; Johnson et al., 1983; Dieter et al., 1982) with minor modifications. Microsomes were diluted to 14 mg/mL with 50 mM potassium phosphate, pH 7.4, containing 20% glycerol and solubilized by the addition of 0.62 mg of sodium cholate and 1.24 mg of the nonionic detergent Nonidet P-40 per mg of microsomal protein. The mixture was stirred at 4 °C for 1 h and then centrifuged at 105000*g* for 1 h to remove insoluble material. The supernatant was collected and subsequently used.

Results

Initial Characterization of Hybridomas. Hybridoma culture supernatants were examined for the production of antibodies directed toward P-450 3b purified from an NZW rabbit by using a solid-phase assay. In this assay, the immunization antigen is adsorbed to the wells of a test plate, and aliquots of culture supernatants are then incubated in each well overnight at room temperature. After washing with PBS, a second antibody conjugated with alkaline phosphatase or labeled with ^{125}I is used to detect whether the adsorbed antigen had sequestered antibody from the culture media. Those hybrids that exhibited rapid growth and strong positive production of antibodies recognizing this P-450 3b were cloned at a density of <0.5 cell/well. This process was repeated to ensure a monoclonal origin for each presumed cell line.

Initial characterization of 23 positive cultures indicated that they all secreted antibodies that recognized P-450 3b prepared from either NZW rabbits or strain IIIVO/J, indicating that the antibodies all recognized the variant form expressed in both rabbit strains that lacks the high efficiency 6β-hydroxylase activity. In order to assess whether any of the antibodies recognized the 6β-hydroxylase, we sought antibodies that could inhibit the 6β-hydroxylase activity of P-450 3b. This required the isolation and the purification of the immunoglobulin, and we therefore selected five cultures that secreted immunoglobulins that could be easily purified by affinity chromatography on protein A–agarose.

Characterization of the purified antibodies indicated that four were specific for P-450 3b when tested for reactivity with other forms of P-450 purified in our laboratory. Of these, only one was inhibitory, and the corresponding cell line was subsequently cloned a third time. Double immunodiffusion studies indicate that the cloned cell line secretes an IgG 2b with κ light chains. As shown in Figure 1, the antibody 8-27 inhibits the catalytic activity of the two variant forms of P-450 3b prepared from New Zealand White or IIIVO/J rabbits.

Comparison of the Reactivity of the Antibody toward Variant Forms of P-450 3b in a Competitive Assay. The reactivity of the antibody toward P-450 3b prepared from either NZW or IIIVO/J rabbits was examined by using a competitive assay system. In this assay P-450 3b was labeled by conjugation to alkaline phosphatase with glutaraldehyde. Although this procedure probably leads to the production of a heterogeneous population of P-450–phosphatase conjugates, the conjugate preparation is used in excess of the binding capacity of the antibody, and the specificity of the antibody should select only those conjugates for which the antigenicity is preserved. Purified monoclonal antibody was adsorbed to the wells of microtiter plates, and mixtures of unlabeled preparations of P-450 and the P-450 3b–phosphatase conjugate were incubated in these antibody-coated wells. The amount of conjugate bound was determined by measuring phosphatase activity. To preclude the possibility that individual components of the assay system might affect the results, each component or various combinations thereof were excluded, and in each

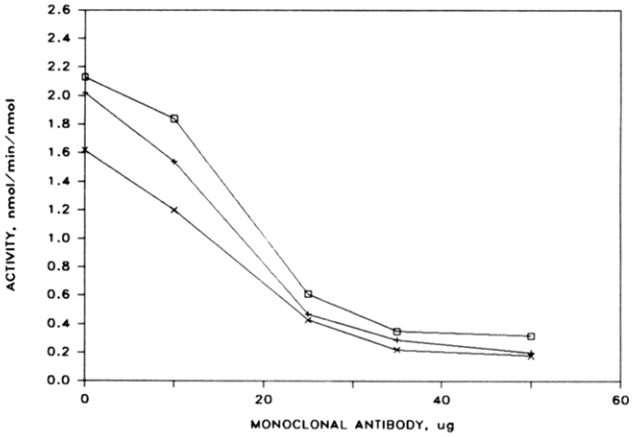

FIGURE 1: Inhibition by the monoclonal antibody of 6β- and 16α-hydroxylation of progesterone as catalyzed by reconstituted preparations of P-450 3b. The indicated amounts of antibody were incubated at 25 °C with 0.1 nmol of P-450 3b prepared from either IIIVO/J or NZW rabbits reconstituted with purified P-450 reductase and dilauroyl-L-α-lecithin. After 30 min, 10 nmol of [^{14}C]progesterone and 1 μmol of NADPH were added to initiate the reaction. The reaction was terminated after 5 min, and the substrate and products were extracted and analyzed as described (Dieter & Johnson, 1982). The rate determined in each case is shown for 6β- (□) and 16α-hydroxylation (+) catalyzed by the NZW P-450 3b and 16α-hydroxylation (×) catalyzed by 6β-hydroxylase-deficient P-450 3b prepared from strain IIIVO/J.

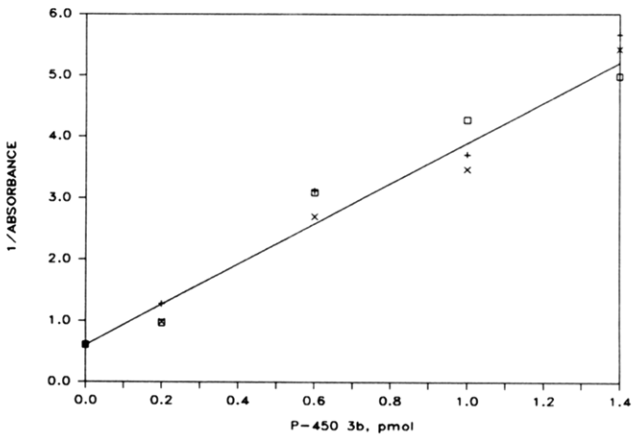

FIGURE 2: Solid-phase competitive immunoassay: 96-well microtiter plates were coated with 200 μL of the monoclonal antibody 8-27 in 0.1 M $NaHCO_3$, pH 9.6, at a concentration of 0.05 mg/mL and incubated for 3 h at 37 °C. Additional binding sites were reacted with 3% BSA for 1 h at 37 °C. Between each incubation the plates were washed once with 3% BSA and twice with PBS. A constant amount of the P-450–alkaline phosphatase conjugate was combined with increasing amounts of purified cytochrome in a final volume of 200 μL/well. The buffer used for sample incubations consisted of 50 mM potassium phosphate, pH 7.4, containing 20% glycerol, 0.1% Nonidet P-40, and 0.5% BSA. To determine the amount of bound P-450–alkaline phosphatase conjugate, 200 μL of *p*-nitrophenyl phosphate solution at a concentration of 1 mg/mL in 1 M diethanolamine, pH 9.8, and 0.5 mM $MgCl_2$ was added to each well and allowed to react for 20 min in the dark. The absorbance at 405 nM was read on a Titertek multiscan. A purified preparation of P-450 3b prepared from strain IIIVO/J is denoted by the symbol (+) whereas the other two preparations (×) and (□) were prepared from NZW rabbits.

case background values were obtained.

The effect of adding unconjugated purified preparations of P-450 3b on the binding of the conjugate is shown in Figure 2. The P-450–phosphatase conjugate was used in excess of the binding capacity of the wells, and the addition of increasing amounts of the unlabeled P-450 3b causes a proportional decrease in the binding of the conjugate to the well. A simple dilution curve is observed over a greater than 10-fold range

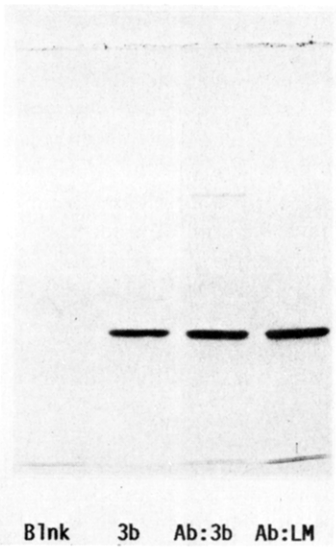

FIGURE 3: Analysis of the peptides bound by immunoadsorbent using SDS–polyacrylamide gel electrophoresis. A 25-μL sample of 8-27-Sepharose was incubated with an appropriate amount of antigen, in 1.5-mL microfuge tubes for 2 h at 4 °C on a rotating mixer. The suspension was then centrifuged and the antibody resin washed once with buffer (10 mM Tris–acetate, pH 7.4, 20% glycerol, 0.1 mM $EDTA(Na)_2$, 1% NP-40, and 0.2% sodium cholate) and then twice with H_2O to remove detergent. After washing, the bound antigen was eluted with 0.125 mL (5 × resin volume) of solubilization buffer (62.5 mM Tris, pH 6.8, 10% glycerol, and 2% SDS) without either DTE or bromophenol blue. The latter were added prior to sample preparation and electrophoresis as described by Laemmli (1970) using 7.5% acrylamide gels. The gel was fixed and then stained with Coomassie Blue. A total of 10–50 μL of each eluent was applied to each lane (nominal dimensions of 8 mm × 1.5 mm × 90 mm). The lanes contain the following: Blnk, elution of the resin alone; 3b, a purified preparation of P-450 3b (1 μg); Ab:3b and Ab:LM, peptides bound and eluted from the antibody resin after reaction with a purified preparation of P-450 3b and solubilized liver microsomes, respectively. The high molecular weight band visible in the lanes containing purified P-450 3b and immunopurified P-450 3b has been attributed to dimers formed by cross-linking (Koop & Coon, 1979).

for the dilution of the conjugate by preparations of P-450 3b from either IIIVO/J or NZW rabbits. Substitution of P-450 3b with 100-fold excess amounts of highly purified preparations of either P-450 2 or P-450 4, the major forms induced by phenobarbital and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, did not inhibit the binding of the 3b conjugate to the adsorbed antibody.

Specificity for the 3b Electrophoretic Class of P-450. Although solid-state binding assays indicated that the antibody did not recognize other purified forms of P-450, we could not exclude the possibility that other immunoreactive cytochromes are present in microsomes. We therefore sought to immunopurify antigenic proteins from rabbit liver microsomes solubilized using the detergents sodium cholate and Nonidet P-40 and to analyze the isolated antigens by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. As would be expected, this monoclonal antibody does not precipitate the antigen directly over a range of concentrations as indicated by double immunodiffusion experiments. In order to preclude the interference of the heavy chain of the immunoglobulin in the electrophoretic analysis and to reliably sequester the immune complexes, the antibody was covalently linked to Sepharose prior to incubation with the microsomal proteins. When the proteins were eluted from the immunoadsorbent and analyzed by SDS–PAGE, a single protein band with the same electrophoretic mobility as P-450 3b is observed (Figure 3).

The antibody linked to Sepharose was also used to assess

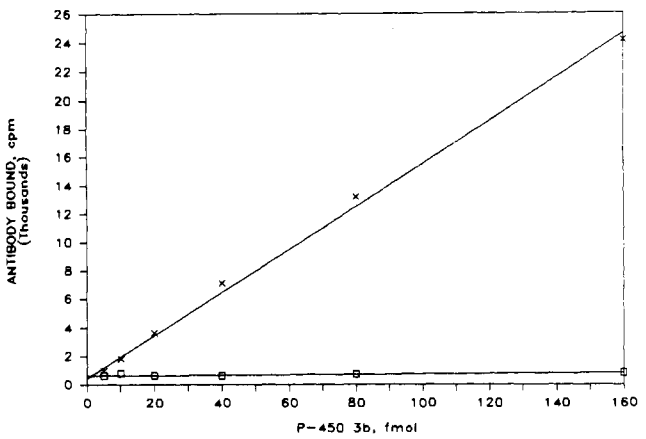

FIGURE 4: Two-site immunoradiometric assay. The wells of a 96-well polyvinyl chloride plate were coated by incubating 50 μL/well of a 50 μg/mL solution of the primary antibody in 0.1 M Na_2CO_3, pH 9.5, for 2 h at 37 °C. Residual binding sites were blocked by subsequently incubating 200 μL of 3% BSA for 1 h at 37 °C. After washing, the indicated amount of antigen, diluted in a total volume of 100 μL of 3% BSA, was allowed to react overnight at 4 °C. Following the removal of unbound antigen and extensive washing with PBS, 100 μL of a solution containing ^{125}I-labeled 8-27 antibody in 3% BSA was added to each well and left at 37 °C for 3 h. The plate was then thoroughly washed and air-dried, and the ^{125}I bound in each well was counted. The points designated (×) were obtained when the plate was coated with a second antibody 10-171 that recognizes an epitope shared by both P-450 1 and P-450 3b whereas the values obtained when unlabeled 8-27 antibody was used to coat the plate are designated (□). The amount and specific activity of the ^{125}I-8-27 antibody was 333 fmol/1.86 × 10^5 cpm per 100 μL/well.

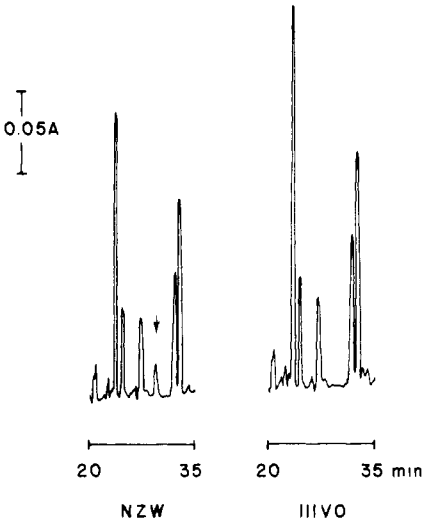

FIGURE 5: Tryptic peptide mapping. Liver microsomes from NZW and IIIVO/J rabbits containing a total of 400 nmol of P-450 were solubilized as described under Experimental Procedures and reacted with 0.75 mL of 8-27–Sepharose for 2 h at 4 °C on a rotating apparatus. The Sepharose was washed repeatedly before bound protein was eluted with 3 M NaSCN and dialyzed against 0.1 M NH_4HCO_3, pH 8.1. The tryptic peptides obtained from approximately 4 nmol of each preparation were separated by reverse-phase HPLC using a linear gradient of acetonitrile (0–70%) in 0.1% (v/v) phosphoric acid (Dieter & Johnson, 1982). Peptide elution was monitored at 210 nM. Examples of a portion of the chromatograms obtained with microsomes from either NZW or IIIVO/J animals are shown. The arrow designates the peptide associated with preparations of P-450 3b exhibiting the high efficiency, 6β-hydroxylase activity.

whether the antibody recognized all of the cytochrome in purified preparations of P-450 3b. When 100 μL of immunoadsorbent was incubated with 2 nmol of P-450 3b containing both variant forms of P-450 3b in a total volume of 1.2 mL, more than 97% of the cytochrome was bound by the resin as judged spectrophotometrically. These results indicate that the antibody recognizes essentially all of the P-450 in preparations of P-450 3b. Taken together with the capacity of the antibody to inhibit the 16α-hydroxylase activity exhibited by both enzymic variants of P-450 3b, these results suggest that the monoclonal antibody described here recognizes an epitope shared by all of the enzymic subforms of P-450 3b.

Recognition of a Single Nonrepeated Epitope. The behavior of the antibody in a two-site immunoradiometric assay was examined to determine whether it recognizes a single epitope. In this assay, the wells of a microtiter plate are coated with a monoclonal antibody. Antigen is then incubated in each well and sequestered by the adsorbed antibody. Subsequent incubation with a monoclonal antibody labeled with ^{125}I will lead to the formation of a hetero complex of labeled secondary antibody, antigen, and primary antibody (coating) when the two antibodies recognize distinct, nonoverlapping sites of the same antigen. As shown in Figure 4, when a heterologous monoclonal antibody, 10-171, is used to sequester P-450 3b, ^{125}I-8-27 is bound in amounts that are dependent on the amount of bound antigen. In contrast, negligible levels of binding are seen when 8-27 serves as both the primary and secondary antibodies. These results are independent of whether the P-450 3b is prepared from IIIVO/J or NZW rabbits and indicate that this antibody recognizes a single epitope on P-450 3b that is shared by both enzymic subforms.

Peptide Mapping of the Immunopurified P-450 3b. Previous work indicated that the P-450 3b prepared from strain IIIVO/J can be distinguished from that isolated from most NZW rabbits by tryptic peptide mapping (Dieter & Johnson, 1982). We therefore determined whether this difference was preserved when the proteins were isolated from solubilized microsomes by immunoaffinity chromatography. For this purpose, the proteins were eluted from the antibody–Sepharose in 3 M NaSCN and subsequently dialyzed against 0.1 M ammonium bicarbonate, pH 8.1. The protein was then subjected to the proteolytic action of trypsin, and the resulting peptides were analyzed by using reverse-phase high-performance liquid chromatography. The characteristic difference noted previously was duplicated when the proteins isolated by the immunoaffinity technique were mapped. The pertinent region of the chromatograph is reproduced in Figure 5. As shown in this figure, the characteristic peptide which is exclusively associated with preparations of P-450 3b exhibiting the low K_m high efficiency 6β-hydroxylase activity is present in the chromatogram of the immunopurified protein isolated from NZW liver microsomes but not from the IIIVO/J source.

Inhibition of Microsomal Activity. The kinetic properties of reconstituted purified P-450 3b prepared from most NZW rabbits suggest that this cytochrome should make a large contribution to the microsomal progesterone hydroxylase activity in NZW rabbits whereas the absence of the high efficiency 6β-hydroxylase for the cytochrome P-450 3b expressed in strain IIIVO/J suggests that P-450 3b in this strain should not contribute substantially to the microsomal metabolism of progesterone via the 6β-hydroxylation reaction. This was tested by using the inhibitory capacity of the monoclonal antibody. When the antibody was included in the reaction mixture, substantial inhibition of both the 6β-hydroxylation and 16α-hydroxylation of progesterone occurs for most microsomes isolated from NZW rabbits as shown in Table I. In contrast, only the 16α-hydroxylase reaction is significantly affected when microsomes prepared from strain IIIVO/J are assayed (Table II). As illustrated in Table I, the extent of inhibition observed was similar whether the microsomes from NZW rabbits exhibited a relatively high or low 6β-hydroxylase

Table I: Inhibition by a Monoclonal Antibody to P-450 3b of the Microsomal 6β- and 16α-Hydroxylation of Progesterone As Catalyzed by Microsomes Prepared from NZW Rabbits[a]

microsomes	control	AB	difference	% inhibition
		6β-Hydroxylation		
NZW-1	2.37	0.56	1.81	76
NZW-2	3.00	1.70	1.30	43
NZW-3	0.86	0.22	0.65	75
NZW-4	0.43	0.45	−0.02	−4
		16α-Hydroxylation		
NZW-1	1.14	0.28	0.86	75
NZW-2	1.12	0.46	0.64	58
NZW-3	0.42	0.14	0.28	66
NZW-4	0.66	0.29	0.37	56

[a] Rates are expressed as nanomoles of product formed per minute per milligram of liver microsomal protein. The rate obtained in the presence of the antibody is displayed in the column denoted AB. The column headed "difference" contains the results of subtracting the rate obtained in the presence of the antibody from that determined in its absence, "control". The column headed "% inhibition" displays the ratios of the difference in activity to the activity obtained for the control incubation. Experiments were conducted as described in the legend to Figure 1 using 30 μg of microsomal protein and 100 μg of antibody.

Table II: Differential Inhibition of 6β- and 16α-Hydroxylase Activity As Catalyzed by Microsomes Prepared from Strain IIIVO/J[a]

microsomes	control	AB	difference	% inhibition
		6β-Hydroxylation		
IIIVO-1	1.58	1.62	−0.04	−3
IIIVO-1 with pregnanediol	0.97	0.98	−0.01	−1
IIIVO-2	0.66	0.62	0.04	6
IIIVO-2 with pregnanediol	0.37	0.34	0.02	7
		16α-Hydroxylation		
IIIVO-1	0.60	0.37	0.23	38
IIIVO-1 with pregnanediol	1.00	0.32	0.68	68
IIIVO-2	0.44	0.23	0.21	48
IIIVO-2 with pregnanediol	0.74	0.19	0.56	75

[a] Additional Details are described in footnote *a* of Table I.

activity. This indicates that the difference in the hydroxylase activity noted between these preparations of microsomes may reflect either differences in the microsomal concentration of P-450 3b or of the catalytically distinct, polymorphic forms of P-450 3b.

When the amount of each enzyme activity attributed to P-450 3b is compared, a relatively constant ratio of 6β-hydroxylase to 16α-hydroxylase activity is found. At the substrate concentration utilized here (ca. 10 μM), the 16α-hydroxylase activity of the purified, reconstituted variant forms of P-450 3b is similar (Dieter & Johnson, 1982). Thus, the inhibitable 16α-hydroxylase activity should be relatively insensitive to the presence of variant forms of P-450 3b, and the differences in inhibitable 16α-hydroxylase activity (0.2–0.9 nmol min^{-1} mg^{-1}) are suggestive of differences in the microsomal concentration of P-450 3b. The relatively constant ratio of inhibitable 6β-hydroxylase/16α-hydroxylase activity suggests that the polymorphism of P-450 3b does not contribute often to the variations observed among microsomal preparations from the outbred NZW rabbits. An exception has been included in Table I that mimics the IIIVO/J phenotype in that the ratio of antibody-inhibitable 6β-hydroxylase/16α-hydroxylase activity is 0. This putative phenotype is rarely encountered among the NZW rabbits that we have examined (ca. 5%).

The difference that can be seen for the uninhibitable 6β-hydroxylase activity of the microsomes isolated from strain IIIVO/J indicates that differences in the occurrence of other forms of P-450 can contribute to variations in 6β-hydroxylase activity in this partially inbred strain and most likely in the outbred NZW rabbits. We have observed that the extent of inhibition by the antibody of the 6β-hydroxylase activity for the microsomes prepared from different NZW rabbits ranges from 40% to 80%. This variation in the extent of inhibition as well as the inability of the antibody to inhibit the microsomal activity to the same extent that is observed for the reconstituted cytochrome (Figure 1) is consistent with the contribution of other enzymes to this activity.

We also examined the effect of 5β-pregnane-3β,20α-diol on the 16α-hydroxylation of progesterone by microsomes prepared from strain IIIVO/J. This compound is an allosteric effector of the P-450 3b isolated from this strain, and as shown in Table II, this derivative of progesterone stimulates the rate of 16α-hydroxylation of progesterone catalyzed by liver microsomes prepared from IIIVO/J rabbits. The monoclonal antibody to P-450 3b inhibits this stimulated activity to the same residual level of activity that is observed in the absence of the pregnanediol. This result suggests that the effect of the pregnanediol on microsomal 16α-hydroxylation is mediated by P-450 3b. Further, the magnitude of the increase in antibody-inhibitable microsomal activity (3-fold) seen with this compound is similar to the degree of stimulation of the reconstituted cytochrome P-450 3b purified from strain IIIVO/J (Johnson et al., 1983). It can also be seen that the pregnanediol partially inhibits the 6β-hydroxylase activity of microsomes from IIIVO/J rabbits. This is further evidence for a distinct 6β-hydroxylase because this compound does not inhibit the catalytic activity of reconstituted purified P-450 3b.

Discussion

The results of this study indicate that the "high efficiency" progesterone 6β-hydroxylase shares an antigenic determinant with other functionally distinct enzymic subforms comprising P-450 3b. This determinant is also expressed in rabbit strain IIIVO/J although the high efficiency 6β-hydroxylase is not expressed in this genetically defined strain. Taken together with previous work (Dieter & Johnson, 1982), this suggests that the functional polymorphism exhibited by P-450 3b arises from microheterogeneity.

The monoclonal antibody described in this study exhibits a high degree of specificity for the 3b electrophoretic type of P-450 as demonstrated by the electrophoretic analysis of the antigens eluted from the immunoadsorbent. This specificity was also evident in the competitive immunoassay where a 100-fold molar excess of either P-450 2 or P-450 4 was unable to inhibit the binding of P-450 3b–phosphatase conjugate with this antibody.

The capacity of the antibody to selectively and extensively inhibit both 16α-hydroxylation and 6β-hydroxylation of progesterone indicates that P-450 3b can play a major role in these metabolic pathways. Although the extent of inhibition is large, it varies from 40 to 80%, suggesting that other forms of P-450 participate in 6β-hydroxylation. This is evident in strain IIIVO/J in which a form of P-450 3b is expressed that does not exhibit appreciable 6β-hydroxylase activity. The lack of inhibition by the monoclonal antibody of the 6β-hydroxylation of progesterone catalyzed by liver microsomes from strain IIIVO/J is consistent with the properties of P-450 3b isolated from this strain. The variable extent of inhibition observed with outbred rabbits appears to reflect differences in the expression of the low K_m, high efficiency 6β-hydroxylase (P-450 3b) superimposed against a variable background of low efficiency, high capacity activity such as that observed in strain

IIIVO/J. In contrast, the antibody was observed to inhibit 16α-hydroxylation in strain IIIVO/J as well as the stimulation of this activity by 5β-pregnane-3β,20α-diol. This is also consistent with the properties of P-450 3b isolated from strain IIIVO/J which exhibits the attributes of allosteric control of the 16α-hydroxylation of progesterone (Johnson et al., 1983). Taken together with other results reported here, the antibody clearly provides a link between the catalytic properties of the purified cytochrome and the catalytic properties observed in microsomal preparations. These results emphasize the contribution that the functional diversity of individual forms of P-450 make to microsomal metabolism.

The present study has relied on the unique properties of monoclonal antibodies that exhibit distinct, well-defined specificity. The monoclonal origin of this antibody was utilized to establish the presence of the epitope it recognizes on the two variant forms of P-450 3b since this precludes the possibility that this reactivity resides in distinctly separate antibody components. This monoclonal antibody was used in turn to demonstrate that the characteristic differences in tryptic peptide composition and catalytic properties are maintained by the P-450 3b antigen immunopurified from NZW and IIIVO/J rabbit liver microsomes. In addition, the cloning of this hybridoma allowed us to select an antibody that was both specific for the 3b electrophoretic class of P-450 and inhibitory of its catalytic activity. In other work from this laboratory, we have reported on monoclonal antibodies that recognize distinct epitopes shared among electrophoretically distinct forms of P-450 (Reubi et al., 1984). At this time, we have three monoclonal antibodies that exhibit distinct specificities and that recognize at least two nonoverlapping epitopes that are shared by both P-450 3b and P-450 1, the hepatic microsomal progesterone 21-hydroxylase (Dieter et al., 1982). This work emphasizes the advantages of monoclonal antibody technology for the production of immunological reagents to characterize a multigene family of enzymes such as the cytochrome P-450 system.

Monoclonal antibodies have additional advantages over serum-derived antibodies as specific inhibitors of the P-450 enzymes. The purity of these reagents greatly exceeds that of serum-derived antibodies in that their specificity is highly defined. This ensures uniformity of action and precludes potential competition for overlapping binding sites between inhibitory and noninhibitory components of antisera which might limit the extent of inhibition. Since a monoclonal antibody was used in this study, it is unlikely that the variable extent of inhibition encountered with microsomes arises from incomplete action of the antibody on P-450 3b. Inhibition experiments using purified reconstituted P450 3b do not show the same range of variability, and the extent of inhibition for the reconstituted cytochrome is much greater than with miocrosomes, suggesting that the variability reflects the contribution of other P-450 enzymes in microsomal metabolism. The pattern of inhibition observed for microsomes prepared from inbred and outbred strains suggests that the ratio of inhibitable 6β-hydroxylase/16α-hydroxylase activity may serve to type microsomes in order to detect those exhibiting the two phenotypes. This represents a considerable savings in cost and labor over the isolation and characterization of P-450 3b by conventional means.

Other workers have developed monoclonal antibodies to rabbit P-450 2 and 4 (Boobis et al., 1981; Park et al., 1980, 1982a,b), and we have described additional antibodies recognizing distinct epitopes that are distributed among constitutive forms of rabbit liver P-450 (Reubi et al., 1984). Monoclonal antibodies can be useful reagents with well-defined specificities for the characterization of P-450 multiplicity, and these reagents should help in unraveling the complexities of regulation and multifunctionality exhibited by this enzyme system.

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Registry No. Cytochrome P-450, 9035-51-2; progesterone, 57-83-0.

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