Use of Monoclonal Antibody Probes against Rat Hepatic Cytochromes P-450c and P-450d to Detect Immunochemically Related Isozymes in Liver Microsomes from Different Species

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Nine distinct monoclonal antibodies raised against purified rat liver cytochrome P-450c react with six different epitopes on the antigen, and one of these epitopes is shared by cytochrome P-450d. None of these monoclonal antibodies recognize seven other purified rat liver isozymes (cytochromes P-450a, b, and e–i) or other proteins in the cytochrome P-450 region of "Western blots" of liver microsomes. Each of the monoclonal antibodies was used to probe "Western blots" of liver microsomes from untreated, or 3-methylcholanthrene-, or isosafrole-treated animals to determine if laboratory animals other than rats possess isozymes immunochemically related to cytochromes P-450c and P-450d. Two protein-staining bands immunorelated to cytochromes P-450c and P-450d were observed in all animals treated with 3-methylcholanthrene (rabbit, hamster, guinea pig, and C57BL/6J mouse) except the DBA/2J mouse, where no polypeptide immunorelated to cytochrome P-450c was detected. The conservation of the number of rat cytochrome P-450c epitopes among these species varied from as few as two (guinea pig) to as many as five epitopes (C57BL/6J mouse and rabbit). The relative mobility in sodium dodecyl sulfate–gels of polypeptides immunorelated to cytochromes P-450c and P-450d was similar in all species examined except the guinea pig, where the polypeptide related to cytochrome P-450c had a smaller M_r than cytochrome P-450d. With the use of both monoclonal and polyclonal antibodies, we were able to establish that purified rabbit cytochromes P-450 LM_4 and P-450 LM_6 are immunorelated to rat cytochromes P-450d and P-450c, respectively.

Research over the past decade has demonstrated that there are marked species differences in the hepatic microsomal metabolism of drugs and xenobiotics. Kato (1) has reviewed a large amount of evidence that supports the concept that hepatic microsomal mixed-function oxidases are the most important enzymes controlling species differences in pharmacological and toxicological responses to xenobiotics. The unusually broad substrate capacity of this microsomal mixed function oxidase enzyme system is due, principally, to multiple isozymes of the terminal oxidase, cytochrome P-450.[2]

[2] The term cytochrome P-450 is used to refer to any or all forms of liver microsomal cytochrome P-450. We designate the rat hemoproteins in a nondescriptive manner in the order that they are purified, since a nomenclature for the various forms of cytochrome P-450 has not been established. Rat hepatic cytochromes P-450a–i are products of distinct mRNAs (2–7). Cytochrome P-450c is inducible in liver microsomes by as much as 50-fold when rats

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Much effort has been directed toward purifying and characterizing the cytochrome P-450 isozymes of rat and rabbit liver microsomes, and results from several laboratories have shown that at least 10 distinct isozymes exist in each of these species. The question has been asked whether any relationship exists among the cytochrome P-450 isozymes from different species. Recent results of amino acid and DNA sequence analyses have demonstrated substantial amino acid sequence homology (75%) between phenobarbital-inducible rat cytochrome P-450b (and P-450e) and rabbit cytochrome P-450LM$_2$ (2, 3, 12, 13). However, traditional methods of amino acid sequencing of purified isozymes or nucleotide sequencing of cDNA represent major time investments not likely to be quickly accomplished with all of the known isozymes. Additional approaches can be utilized to determine if structural homology exists among cytochrome P-450 isozymes in different species. Identification of structural homologs of cytochrome P-450c, the major MC[3]-inducible isozyme in rat liver (8), in other species could contribute significantly to our understanding of species differences in drug metabolism and induction, especially since species differences in response to induction by MC are known (15-18).

MAb[4] are characterized by high specificity for a single epitope (often a sequence of 4-7 amino acids) on a protein, and they can be highly effective as probes to detect the presence of that epitope on other proteins in the same species or other species (19-21). In addition, two isozymes that share common epitopes would be expected to share regions of similar amino acid sequences.

In this study we have used nine distinct MAb raised against rat liver microsomal cytochrome P-450c (14) to probe "Western blots" containing liver microsomes from four different species to determine if liver microsomes from these species possess an immunochemical homolog of rat cytochrome P-450c. We have shown previously (14) that these nine MAb are directed toward at least six epitopes on cytochrome P-450c.[5] One of these epitopes appears on another rat isozyme, cytochrome P-450d, which is recognized similarly by three of the nine MAb. Because of this immunochemical relationship, we were able to determine in the same experiments whether liver microsomes from other species contain immunochemical homologs of rat liver cytochrome P-450d as well as cytochrome P-450c.

EXPERIMENTAL PROCEDURES

Chemicals, enzymes, and antibodies. Horseradish peroxidase, MC, Tween 20 and 4-chloro-1-naphthol were purchased from Sigma. Nitrocellulose blotting paper (0.22 μM pore size) was obtained from Schleicher and Schuell (Keene, N. H.). Isosafrole (technical grade) and cyclohexane were purchased from Eastman-Kodak, and corn oil was obtained from Mallinckrodt Chemical Works. Agarose (Sea Kem ME) was obtained from Marine Colloids Division of FMC Corporation (Rockland, Maine). The

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are administered MC (8, 9) or certain other xenobiotics (10, 11). The induction of cytochrome P-450d is effected by the same xenobiotics which induce cytochrome P-450c, but to differing extents (10, 11).

[3] Abbreviations used: SDS, sodium dodecyl sulfate; MC, 3-methylcholanthrene; MAb, monoclonal antibody (antibodies); ELISA, enzyme-linked immunoabsorbant assay; B6, C57BL/6J mice; D2, DBA/2J mice; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

[4] Each MAb is designated with one or two letters and a unique number. The first letter, "C", refers to the antigen of immunization (i.e., rat hepatic cytochrome P-450c), while the presence of the letter "D" indicates antibody that cross-reacts with rat cytochrome P-450d (14). For simplicity, epitopes on cytochrome P-450c are designated with the same number used to designate the MAb. Where more than one MAb recognizes the same epitope, then the lowest number from the MAb is used. "Western

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blot" refers to the nitrocellulose replica of an SDS-polyacrylamide gel. Mixtures of proteins are first resolved in SDS-slab gels, and the proteins are subsequently transferred electrophoretically to the nitrocellulose, where the location of certain protein bands is achieved by specific antibody recognition via enzyme-catalyzed staining.

[5] Five of the epitopes are spatially distinct. The epitopes recognized by MAb C4 and C6 overlap but appear to be different based on several previous observations (14).

remaining chemicals were either reagent grade or have been described previously (10).

Rat liver cytochromes P-450c and P-450d were purified from Long-Evans rats as described (22, 23). Rabbit liver cytochromes P-450LM$_4$ and P-450LM$_6$ were purified from adult male New Zealand white rabbits as described (24, 25). Horseradish peroxidase-conjugated F(ab')$_2$ fragments of goat anti-mouse IgG (heavy- and light-chain specific) and horseradish peroxidase-conjugated F(ab')$_2$ fragments of goat anti-rabbit IgG (heavy- and light-chain specific) were purchased from Cappel Laboratories (West Chester, Pa.). The preparation of polyclonal antibodies, anti-P-450c and anti-P-450d, in rabbits, and the manner in which they were absorbed [anti-P-450c(-d) and anti-P-450d(-c)] have been described (26). The preparation and characterization of MAb against rat liver cytochrome P-450c (C1–C10) have also been described (14).

Xenobiotic treatment of animals and preparation of hepatic microsomes. Six male Hartley guinea pigs and nine male Golden Syrian (LaK:LVG) hamsters were obtained from Charles River Laboratories, (Wilmington, Mass.) at 6 weeks of age. Twelve male B6 (C57BL/6J) and 12 D2 (DBA/2J) mice were obtained from Jackson Laboratory (Bar Harbor, Maine) at 6 weeks of age. Three male New Zealand White PF/CF rabbits weighing 3.0 kg were obtained from HARE (Hewitt, N. J.). Animals were acclimated in our animal care facility for 1 week before xenobiotic treatments. Rabbits were fed Ralston Purina rabbit chow, and the other animals were fed Ralston Purina rodent chow 5001 *ad libitum*. Animals were divided into three groups, and one group (control) was given daily intraperitoneal injections of the vehicle (corn oil). The other two groups were given either isosafrole (150 mg/kg) or MC (25 mg/kg) intraperitoneally in corn oil daily for three consecutive days, and were killed by decapitation on the fourth day. The microsomal fraction of the livers was isolated and stored in small aliquots at $-76°C$ as described previously (10).

Other assay methods. Total microsomal cytochrome P-450 was determined from the CO-reduced difference spectrum (27) in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. Prior to the spectral determination of cytochrome P-450 in microsomes from isosafrole-treated animals, the metabolite complex of cytochrome P-450 formed *in vivo* was displaced by incubation of microsomes with cyclohexane (10 mM) for 20 min at 37°C, followed by 40 min at room temperature (23). Protein was determined by the method of Lowry *et al.* with bovine serum albumin as standard (28).

SDS-polyacrylamide gel electrophoresis was performed in 7.5% acrylamide gels by the method of Laemmli (29). The "Western blots" were performed (30, 31) using the apparatus and buffers exactly as described previously (14). Detection of bound antibody in "Western blots" was accomplished by the use of either the MAb directly coupled to horseradish peroxidase (32) or horseradish peroxidase-conjugated F(ab')$_2$ fragments of goat anti-mouse IgG. The location of the peroxidase activity on "Western blots" was detected by the oxidation of 4-chloro-1-naphthol with H$_2$O$_2$ as described (31).

Ouchterlony plates were made with 0.9% agarose in phosphate-buffered saline (137 mM NaCl, 2.6 mM KCl, 10 mM sodium-potassium phosphate buffer, pH 7.4, containing 0.2 mM EDTA) containing 0.1% sodium azide, and 3 ml was added to each Petri dish (50 × 9 mm). Wells were filled with antigens or antibodies, and diffusion was allowed to take place at room temperature in a humid atmosphere for at least 2 days. Photographs were taken with dark-field illumination on 10.2 × 12.7-cm Kodak Ektapan film (4162).

RESULTS

Preparation of "Western blots" involves the electrophoretic resolution of microsomal proteins on SDS-polyacrylamide gels, followed by the transfer of these proteins to a nitrocellulose sheet. Figure 1A shows an SDS-gel of liver microsomal proteins (stained for protein) from five mammalian species. The liver microsomal preparations from each species include a sample from corn oil-, isosafrole-, and MC-treated animals, respectively, from left to right. Each group of three microsomal samples is separated by a track containing a mixture of purified rat cytochromes P-450c ($M_r = 56K$) and P-450d ($M_r = 52K$). The same arrangement of standards and microsomal samples is used in Figs. 1–3. Comparisons of protein-staining patterns of liver microsomes from corn oil- and xenobiotic-treated animals reveal rather large differences in proteins in the M_r region of known cytochromes P-450 (45–60K) in some species (e.g., rabbit), but comparatively small changes are observed in others (e.g., guinea pig). Since some cytochrome P-450 isozymes have identical mobilities in SDS-gels (e.g., rat cytochromes P-450b and P-450d; cytochromes P-450f and P-450h), it is difficult to interpret the significance of changes in intensity of protein-stained bands.

MAb probes can be used to detect specifically a single epitope on a cytochrome

FIG. 1. Comparison of a protein-stained SDS–gel (A) and a "Western blot" of liver microsomes probed with C1 MAb (B) and detected with a second antibody. Parts A and B both have the same arrangement of groups of three liver microsomal preparations from the animals shown, with a mixture of purified rat liver cytochromes P-450c and P-450d separating each group. Each group of three samples includes liver microsomes from corn oil-, isosafrole-, and MC-treated animals, left to right, respectively. This same order of three microsomal samples, separated by rat cytochromes P-450c and P-450d, is repeated in Figs. 2 and 3. (A) The amount of protein was 10 μg for each microsomal sample and 0.3 μg each for both rat cytochromes P-450c and P-450d; (B) the amount of microsomal protein was 20, 5, and 5 μg, left to right, respectively, for each group of three microsomes. The amount of cytochromes P-450c and P-450d was 0.1 μg of each protein per well.
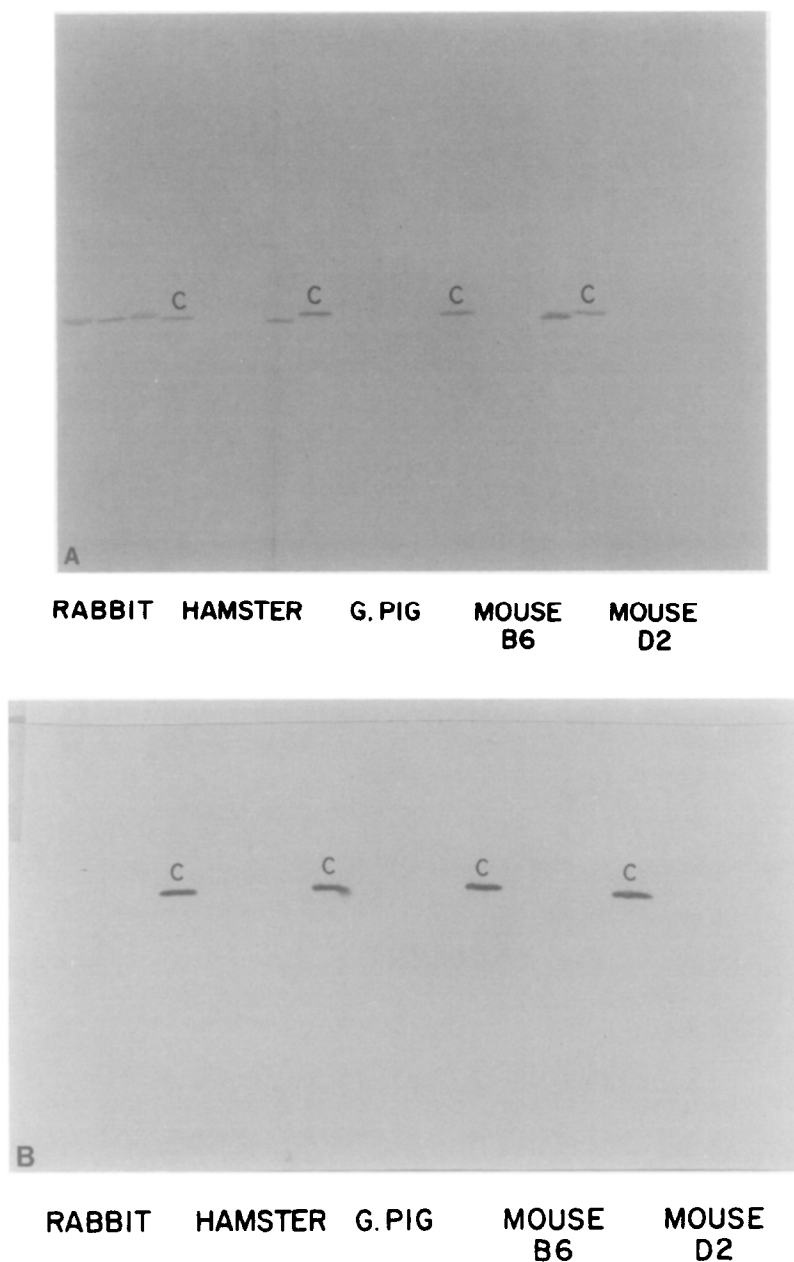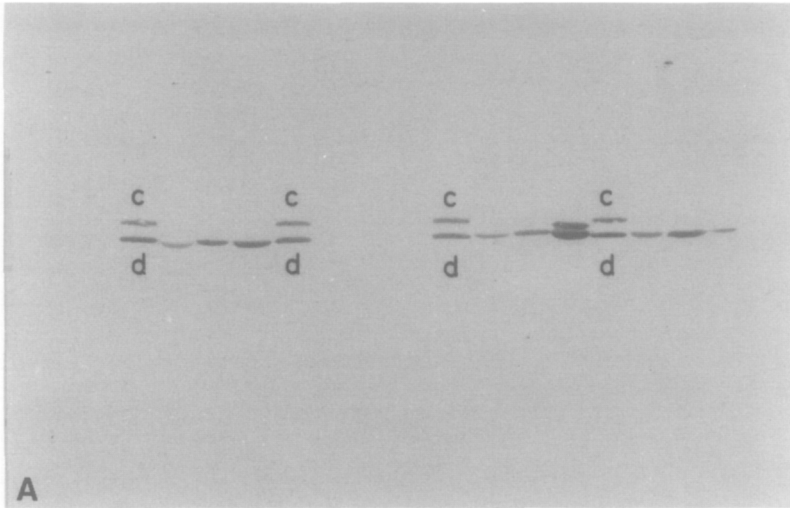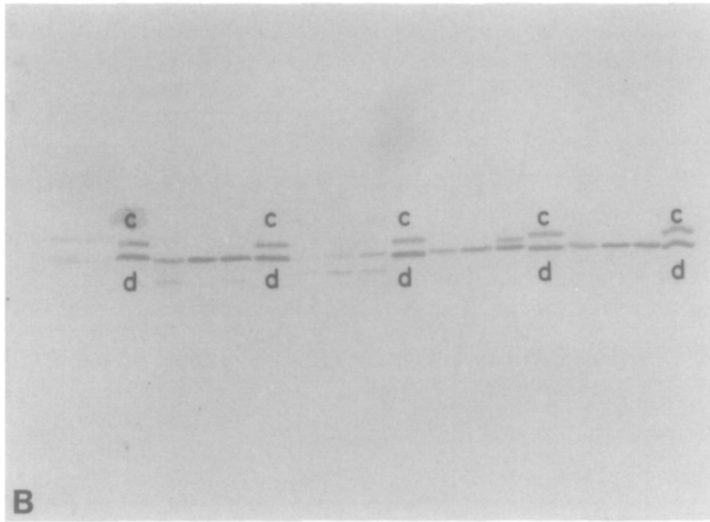
FIG. 2. "Western blots" of liver microsomes probed with either C4 (A) or C7 (B), and detected with a second antibody. The amount of microsomal protein was 20 μg (control), 5 μg (isosafrole), and 5 μg (MC), left to right, respectively, for each group of three microsomal samples. Rat liver cytochromes *P*-450c and *P*-450d were each used at 0.1 μg per well. Refer to Fig. 1 for additional details.

P-450 isozyme in the presence of a multitude of both related and unrelated proteins. This high specificity of MAb is clearly demonstrated by comparing the protein-stained gel in Fig. 1A with Fig. 1B, where C1 MAb was used to probe a

FIG. 3. "Western blots" of liver microsomes probed with CD5. The staining in (A) was achieved using horseradish peroxidase conjugated with goat anti-mouse IgG to detect CD5 binding. The staining in (B) was done using CD5 directly conjugated with horseradish peroxidase. The stained bands marked "c" and "d" refer to cytochromes P-450c and P-450d, respectively. The amount of microsomal protein for (A) was 20, 5, and 5 μg, left to right, respectively, for each group of three microsomal samples. The amount of microsomal protein in (B) was different for each group of three as follows (left to right): rabbit, 20 μg (control), 10 μg (isosafrole), and 10 μg (MC); hamster, 10, 2, and 2 μg; guinea pig, 20, 5, and 5 μg; B6 mouse, 10, 2, and 1 μg; and D2 mouse, 10, 2, and 10 μg. Refer to Fig. 1 for additional details.

"Western blot." Depending on the particular liver microsomal sample, either a single band or no band is detected with the C1 probe. Liver microsomal samples from control or xenobiotic-treated rabbits show a single peroxidase-positive band of a slightly larger apparent minimum M_r than rat cytochrome P-450c, while liver microsomal samples from guinea pigs show a single band with a substantially smaller apparent minimum M_r than rat cytochrome P-450c. Although rabbits, hamsters, guinea pigs, and B6 mice contain a microsomal polypeptide recognized by C1, in some species (hamster, B6 mouse) this polypeptide is detectable only when the animals are treated with MC. The absence of detectable reaction of C1 with the D2 mouse microsomes is the result of a mouse strain difference, since the MC-treated B6 mouse has C1 reactive protein. No staining by the C1 probe was detected in liver microsomes from hamsters or B6 mice treated with isosafrole or with corn oil.

It should be noted that, in Fig. 1B, 5 μg protein per track was used for all samples except microsomes from control animals, which were used at 20 μg protein per track to provide sufficient staining intensity. Consequently, the induction of cytochromes P-450 immunorelated to cytochromes P-450c and P-450d is greater than suggested by the similar staining intensity among some of the samples.

"Western blots" of liver microsomes probed with C4 and C7 MAb appear in Figs. 2A and B, respectively. The epitopes on cytochrome P-450c recognized by C4 and C7 have been shown to be spatially distinct from one another, and from the epitope recognized by C1 (14). The pattern of staining with C4 is very similar to the pattern of staining observed with C1 for all of the species tested except the guinea pig. Guinea pig liver microsomes contain a polypeptide detected with C1 (Fig. 1B) but no protein immunoreactive with C4 (Fig. 2A). All of the proteins that react with C4 have the same relative mobility compared to rat cytochrome P-450c as the proteins immunoreactive with C1 (Fig.

1B), suggesting that the same cytochrome P-450 is detected by both probes. The proteins recognized by C4 also react with C6 on "Western blots" of these liver microsomes (results not shown). We have previously provided evidence that the epitopes recognized by C4 and C6 are different; however, they appear to overlap sufficiently to allow only one MAb to bind to cytochrome P-450c at a time (14). As shown in Fig. 2B, no microsomal protein from rabbits, hamsters, guinea pigs, or mice is detected by C7. Analogous results were obtained when C10 was used to probe a "Western blot" of these same microsomal samples (data not shown). While C7 and C10 are distinct MAb, they do compete for binding to cytochrome P-450c; thus, their identical epitope specificity is not surprising.

Three MAb raised against rat cytochrome P-450c (CD2, CD3, CD5) are directed against an epitope which is shared by rat cytochrome P-450d (14). Consequently, these three MAb react equally well with rat cytochromes P-450c and P-450d in an ELISA or on "Western blots" (14). Figure 3 shows the results of probing "Western blots" of liver microsomes with CD5. CD5 reacts equally with both purified cytochromes P-450c and P-450d, as can be seen by the equivalent staining intensity of the bands marked "c" and "d," respectively, Figs. 3A and B. The CD5 probe reacts with two closely spaced proteins in the liver microsomes from MC-treated B6 mice, but only the lower protein is observed after isosafrole or corn oil treatment. The upper protein band in microsomes from MC-induced B6 mice was also recognized by the MAb specific for epitopes unique to cytochrome P-450c (Figs. 1B and 2A). The lower protein band is immunoreactive with the MAb to the epitope shared by cytochromes P-450c and P-450d. By deduction, the bands recognized by CD5 in all species can be assigned as immunochemically related to either cytochrome P-450c or P-450d. For example, the protein recognized by CD5 in hamster liver microsomes (Fig. 3A) is immunorelated to cytochrome P-450d

based on its mobility, which is distinct from the other hamster microsomal protein that is not recognized by CD5, but is recognized by C1 (Fig. 1B), C4 (Fig. 2A), and C6 (not shown).

Figure 3 also shows examples of two methods of staining the "Western blots" for detection of immunoreactive proteins. MAb binding was detected in Fig. 3A with horseradish peroxidase covalently coupled to anti-mouse IgG, i.e., a second antibody. In Fig. 3B the MAb, CD5, was coupled directly to horseradish peroxidase, making it unnecessary to use a second antibody. In both cases, the oxidation product of 4-chloro-1-naphthol with the cosubstrate H_2O_2 was used to stain for the presence of the peroxidase enzyme. Generally, the use of peroxidase-coupled second antibody gave lower background staining, but was less sensitive in detecting weak reactions such as those of guinea pig and rabbit cytochromes P-450 with CD5 (see Figs. 3A and B). With hamster liver microsomes, the single-antibody technique also detected a lower M_r protein. This could be due to a proteolytic degradation product of the cytochrome P-450 immunoreactive with CD5 or, alternatively, to another protein that is poorly recognized by this antibody. Since we did not want to miss weak reactions of any of the MAb with immunoreactive proteins in different species, we prepared "Western blots" using both staining procedures for each MAb.

Table I summarizes the reactivity of all nine MAb and polyclonal anti-P-450d(-c) on "Western blots" of mammalian liver microsomes from MC-treated animals, since the highest concentrations of immunoreactive proteins were always obtained with these animals. As can be seen from this table, a microsomal protein in rabbits and B6 mice has five epitopes in common with rat cytochrome P-450c, but the guinea pig immunoreactive protein has only two epitopes in common with the rat isozyme. Epitopes 2 and 7 are both recognized by more than one MAb; consequently, the number of MAb reacting with a protein does not always equal the number of common epitopes.

The notations "+" or "++" in Table I

serve to identify a positive staining reaction with directly conjugated MAb or second antibody, respectively. In no case did second antibody detect a protein that was not detected with the MAb conjugated directly to horseradish peroxidase, although the opposite did occur in several cases (e.g., C8 recognition of the rabbit protein). We found that the increased sensitivity of the directly conjugated MAb was due primarily to the shorter washing time of the blot, since the long incubation with second antibody was not performed. When directly conjugated MAb C8 or CD2 was used to detect a polypeptide in rabbit liver microsomes, the addition of a 90-min wash step abolished the staining seen if the wash step was limited to three 5-min washes. Consequently, a single "+" also indicates lower affinity of the MAb for a polypeptide compared to "++."

To determine if MAb C7 and C10 recognized immunorelated proteins in other species in the absence of SDS and β-mercaptoethanol, we tested antibody binding in an ELISA. The ELISA was performed in 96-well polystyrene microtest plates that were coated with liver microsomes from the four species, and MAb binding was detected with second antibody as previously described (14). In comparison to results with microsomes from MC-treated rats, C7 and C10 bound very weakly to microsomes from all other species treated with MC (approximately 2% compared to the rat), and no binding was detected with microsomes from MC-treated D2 mice. In contrast, C1 recognized a protein in all species on "Western blots," and also bound efficiently in the ELISA (20–90% compared to the rat) to liver microsomes from each species treated with MC except for the D2 mouse. The results from ELISA and "Western blots" are comparable for these MAb despite substantial differences in methodology. It would thus appear from the data in Table I that epitope 1 is highly conserved and epitope 7 is the least conserved among the five species.

It is clear from the results in Table I that no single MAb stains both immunoreactive proteins in every species; there-

TABLE I

SUMMARY OF ANTIBODY REACTIVITY WITH LIVER MICROSOMES FROM DIFFERENT SPECIES ON "WESTERN BLOTS"

Antibody probe		Liver microsomes from MC-treated animals					
MAb	Epitope[a]	Rat	Rabbit	Hamster	Guinea pig	B6 Mouse	D2 Mouse
Immunochemically related to rat *P*-450c							
C1	1	++	++	++	++	++	−
CD2 ⎫		++	+	−	+	++	−
CD3 ⎬	2	++	++	−	++	++	−
CD5 ⎭		++	±	−	+	++	−
C4	4	++	++	++	−	++	−
C6	6	++	++	+	−	++	−
C7 ⎫	7	++	−	−	−	−	
C10 ⎭		++	−	−	−	−	−
C8	8	++	+	−	−	++	−
Immunochemically related to rat *P*-450d							
CD2 ⎫		++	±	++	+	++	++
CD3 ⎬	2	++	−	++	++	++	++
CD5 ⎭		++	±	++	+	++	++
Polyclonal Anti-*P*-450d(-c)	Several Epitopes	++	++	++	++	++	++

Note. "Western blots" of liver microsomes from different species treated with MC were probed separately with nine MAb directed against cytochrome *P*-450c or polyclonal anti-*P*-450d(-c). Antibody binding to regions of the "Western blot" were detected as described under Experimental Procedures. Results are expressed with "+" denoting binding of antibody directly conjugated with horseradish peroxidase or "++" denoting antibody binding detected with a second antibody, i.e., anti-mouse IgG conjugated with horseradish peroxidase or anti-rabbit IgG conjugated with horseradish peroxidase (see text for significance). The symbol "±" denotes a variable and/or weak reaction with horseradish peroxidase-conjugated MAb, whereas "−" denotes no detectable binding of MAb.

[a] Epitopes are designated with the number corresponding to the MAb (14). MAb whose binding to cytochrome *P*-450c is blocked by another MAb are considered to bind to the same epitope, and this epitope is designated with the lowest number from among the MAb used. Although C4 and C6 block each others binding to cytochrome *P*-450c, other data suggest that they bind to different epitopes which overlap spatially (14).

fore, it is difficult to compare mobilities of protein bands appearing on different "Western blots." To facilitate comparisons of the relative mobilities of these proteins, a composite diagram was constructed (Fig. 4) based on the results obtained when each MAb was used to probe individual "Western blots." The relative mobility of each immunoreactive protein is compared with rat cytochromes *P*-450c and *P*-450d. Except for the guinea pig, the minimum M_r of the immunochemical homolog of cytochrome *P*-450c is larger than the immunochemical homolog of cytochrome *P*-450d.
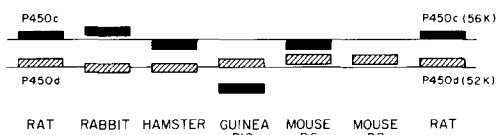

FIG. 4. Composite diagram comparing the mobility of immunoreactive proteins from several species using "Western blots" probed with MAb against rat liver cytochromes *P*-450c and *P*-450d. The diagram is drawn to scale, with the anode or tracking dye front at the bottom. Rat cytochromes *P*-450c and *P*-450d are placed on both sides, and are connected by horizontal lines as references for mobility differences among the species. The diagram depicts the results obtained when microsomes were isolated from animals treated with MC.

Although it is not possible to quantitate absolute levels of these immunoreactive proteins in different species, one can estimate fold increases within the same species by serially diluting the amount of microsomal protein from the livers of inducer-treated animals until the staining intensity on "Western blots" matches the control animals. With this method, we were able to estimate the approximate fold induction of the two proteins in all species after either isosafrole or MC treatment (Table II). MC treatment of animals resulted in elevated levels of both immunoreactive proteins in all animals except the D2 mouse, where only one protein recognized by CD2, CD3, and CD5 was induced. Cytochrome "P-450c"[6] was undetected in D2 mice regardless of the treatment. Isosafrole treatment induced cytochrome "P-450d" in all animals, but induced cytochrome "P-450c" in only the rat, rabbit, and hamster. Clearly the effectiveness of isosafrole or MC as inducers of either immunoreactive protein varies greatly among the species examined.

When homogeneous rabbit cytochromes P-450LM$_6$ and P-450LM$_4$ were electrophoresed in SDS–gels, and "Western blots" were probed with the nine MAb, the stained bands comigrated with the immunoreactive bands in rabbit liver microsomes (results not shown). Moreover, those MAb recognizing epitopes unique to cytochrome P-450c reacted only with cytochrome P-450LM$_6$, whereas MAb CD2 and CD5, as well as polyclonal anti-P-450d(-c), reacted with cytochrome P-450LM$_4$. As shown in Fig. 5A, cytochrome P-450LM$_6$, but not cytochrome P-450LM$_4$, reacts with C8 MAb in Ouchterlony immunodiffusion analysis, showing a line of identity with rat cytochrome P-450c. This monoclonal

[6] The terms cytochrome "P-450c" and cytochrome "P-450d" are used here to indicate proteins in species other than the rat that are immunochemically related to rat cytochromes P-450c and P-450d. The primary justification for identifying a given polypeptide in a "Western blot" as a cytochrome P-450 is because at least three distinct MAb react with the polypeptide. It is important to emphasize that immunochemical relatedness does *not* necessarily imply functional relatedness.

antibody (C8) has been previously shown to be a precipitating antibody (14). When polyclonal anti-P-450c is used in immunodiffusion analysis, cytochrome P-450LM$_6$ reacts with the antibody, giving a weak reaction of partial identity with rat cytochrome P-450c (Fig. 5B). When polyclonal anti-P-450d is allowed to diffuse against cytochromes P-450LM$_6$ and P-450LM$_4$, both proteins react with the antibody (Fig. 5C). The immunochemical relationships of the precipitin bands obtained, as well as the intensity of immunoprecipitin bands, indicate that rat cytochrome P-450d has more epitopes in common with rabbit cytochrome P-450LM$_4$ than with cytochrome P-450LM$_6$. When antibody specifically absorbed against cytochrome P-450c [anti-P-450d(-c)] is allowed to diffuse against cytochromes P-450LM$_6$ and P-450LM$_4$, only cytochrome P-450LM$_4$ is observed to react with this antibody, since the antibody directed against epitopes shared by rat cytochrome P-450c have been removed (Fig. 5D). Clearly, based on these results we can conclude that rabbit P-450LM$_4$ is immunochemically related to rat cytochrome P-450d, and that rabbit cytochrome P-450LM$_6$ is immunochemically related to rat cytochrome P-450c.

DISCUSSION

We have previously demonstrated the high specificity of nine MAb raised against rat hepatic microsomal cytochrome P-450c. Three of these MAb recognize the immunorelated protein, cytochrome P-450d, but none of these MAb recognize seven other purified rat cytochrome P-450 isozymes or any other proteins in the cytochrome P-450 region of "Western blots" of total microsomal protein (14). Because of their specificity, these MAb are well suited as probes to determine if other species possess proteins immunorelated to rat cytochromes P-450c and P-450d.

By using each MAb individually to probe "Western blots" containing the liver microsomes from four species, we found that each species possessed proteins immunochemically related to cytochromes P-450c

TABLE II

INDUCTIVE EFFECTS OF ISOSAFROLE AND MC ON MAMMALIAN HEPATIC ISOZYMES IMMUNORELATED
TO RAT CYTOCHROMES *P*-450c AND *P*-450d

Species	Treatment	Total *P*-450 (nmol/mg protein)	Induction of isozymes immunorelated to rat cytochrome	
			P-450c	*P*-450d
Rabbit	Control	1.5	++	++
	Isosafrole	2.5	Low	Low
	MC	2.9	Low	Low
Hamster	Control	1.4	ND	++
	Isosafrole	2.5	ND	Medium
	MC	2.6	Medium[a]	Medium
Guinea pig	Control	1.0	++	+
	Isosafrole	1.7	Low	Medium
	MC	1.5	Low	Medium
B6 Mouse	Control	0.8	ND	++
	Isosafrole	2.1	ND	Medium
	MC	2.3	High[a]	High
D2 Mouse	Control	1.0	ND	++
	Isosafrole	1.7	ND	Medium
	MC	0.9	ND	Low
Rat[b]	Control	0.9	+	++
	Isosafrole	2.3	Medium	Medium
	MC	1.8	High	Medium

Note. Estimates of the staining intensity of isozymes from control animals are indicated by "+" or "++" for weak and moderate staining intensity, respectively. The relative fold increase in specific isozymes was estimated by varying the amount of microsomal protein until staining intensity of proteins from control animals and inducer-treated animals were approximately the same. Fold increase is reported as low, medium, or high for 2–7, 8–20, or >21-fold increases, respectively. "ND" indicates that no protein was detected even when as much as 30 μg or more of microsomal protein was applied to the SDS-gel.

[a] Despite the absence of detectable staining in the control group, the lower limits to the fold induction can still be estimated by comparing the lowest amount of protein where staining is still detected in the microsomes from MC-treated animals versus the highest amount of protein in control microsomes where no staining is seen. This lower limit to the fold induction was at least 10- and 100-fold for hamster and B6 mice, respectively.

[b] For the rat, fold increases and absolute levels of cytochromes *P*-450c and *P*-450d have been determined more precisely by radial immunodiffusion (10).

and *P*-450d. The proteins immunorelated by cytochrome *P*-450c in liver microsomes from other species were recognized by as many as seven MAb (B6 mouse and rabbit) or as few as three MAb (hamster), as summarized in Table I. The proteins immunorelated to cytochrome *P*-450d in liver microsomes were stained by at least two of the three MAb directed against the epitope shared with cytochrome *P*-450d as well as by polyclonal anti-*P*-450d(-c). In addition to MAb recognition, there are other properties of these immunoreactive polypeptides which are consistent with their being cytochromes *P*-450 and, in particular, analogs of rat cytochromes *P*-450c or *P*-450d. These characteristics include (a) their minimum M_r is in the cytochrome *P*-450 range, i.e., $M_r \sim 60K$–45K; (b) they are found in the microsomal
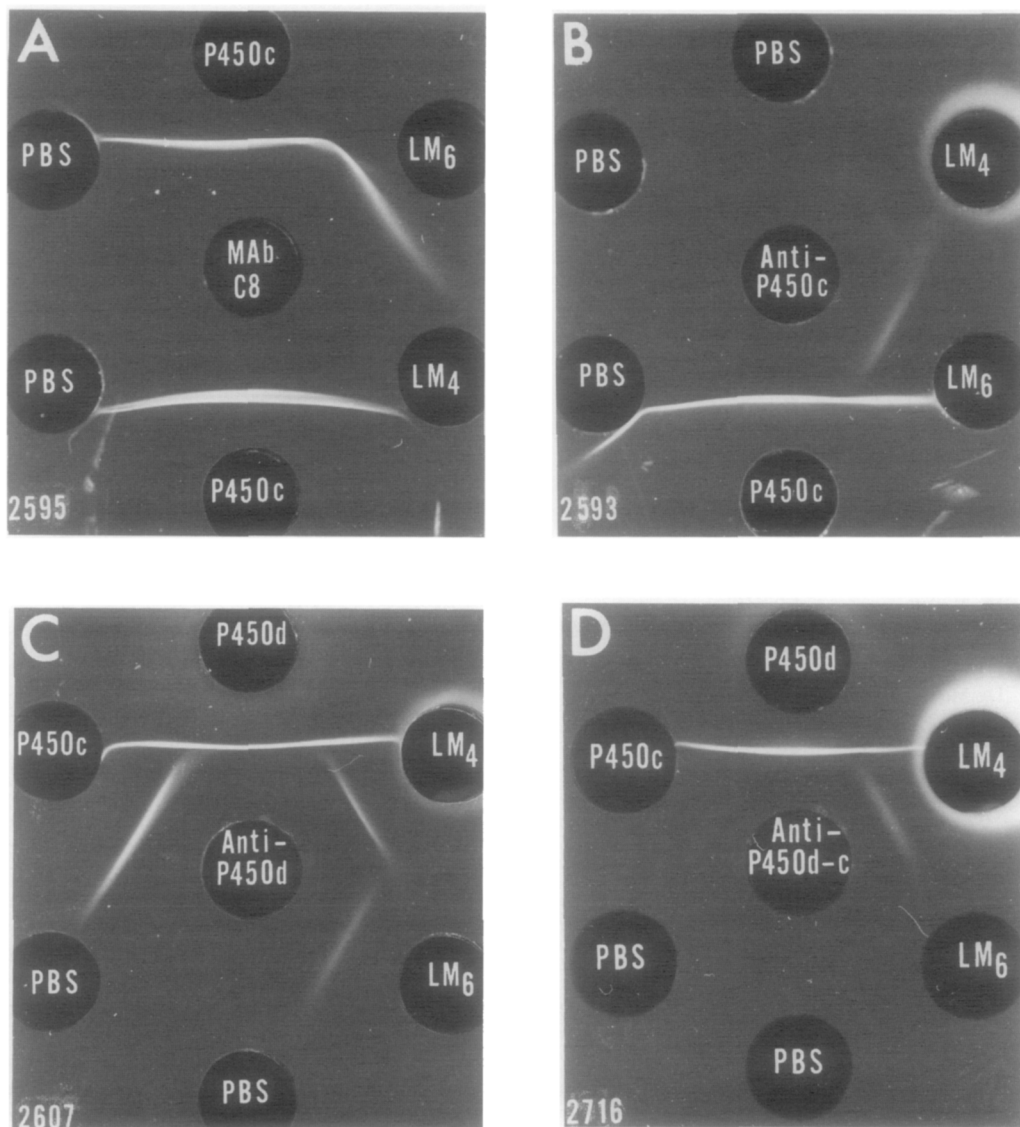
FIG. 5. Ouchterlony double-diffusion plates examining the reactivity of purified rabbit cytochromes P-450LM$_6$ and P-450LM$_4$ with antibodies prepared against rat cytochromes P-450c or P-450d. The central wells were filled (10 μl) with the indicated antibodies at 1, 25, 25, and 30 mg/ml for plates A–D, respectively. The wells surrounding the central well were filled (10 μl) with 10 μM of the indicated rat cytochromes P-450 (P-450c and P-450d) or with 10 μM rabbit cytochromes P-450 (LM$_4$ and LM$_6$) except in (D), where the rabbit cytochromes were used at 20 μM. The antibody used in (D) was back-absorbed against cytochrome P-450c (26). The antibody, termed anti-P-450d-c, is monospecific for cytochrome P-450d. Empty wells were filled with phosphate-buffered saline (PBS).

fraction of liver; (c) their levels in microsomes are elevated by administration of compounds characterized as inducers, i.e., isosafrole and/or MC (8–10); and (d) their reaction with MAb corresponds to the distribution of epitopes on the rat proteins, i.e., MAb only recognizing epitopes on cytochrome P-450c do not recognize cytochrome "P-450d" in other species. Conversely, putative cytochrome "P-450d"

polypeptides are recognized by polyclonal anti-*P*-450d(-c), but this antibody does not recognize putative cytochrome "*P*-450c" in the other species (Table I). The above considerations support the thesis that the two polypeptides recognized by the antibodies in "Western blots" in each species are immunochemical homologs of rat cytochromes *P*-450c or *P*-450d (see Footnote 6). It should be noted that assignment of immunochemical relatedness is possible only because this collection of MAb includes some that are specific for cytochrome *P*-450c, some that cross-react with cytochrome *P*-450d, and none that react with any other cytochromes *P*-450.

The relative mobility of the immunoreactive polypeptides on the "Western blots" of rabbit liver microsomes and their induction by MC and isosafrole indicates that these proteins are rabbit cytochromes P-450LM$_6$ and P-450LM$_4$. The reported characteristics of rabbit cytochromes P-450LM$_6$ and P-450LM$_4$ suggest homology with rat cytochromes *P*-450c and *P*-450d, respectively. Several properties distinguish cytochrome P-450c or P-450LM$_6$ from cytochrome P-450d or P-450LM$_4$, but show similarities for the putative rat and rabbit homologs, including (a) both cytochromes P-450d and P-450LM$_4$ are high-spin ferric hemoproteins whereas cytochromes P-450c and P-450LM$_6$ are low spin (22-25, 33, 34); (b) both cytochromes P-450d and P-450LM$_4$ have minimum M_r 52K-54K on SDS-gels, whereas the minimum M_r for cytochromes P-450c and P-450LM$_6$ is ~56K (22-25, 33, 34); (c) both cytochromes P-450d and P-450LM$_4$ elute from DEAE-cellulose at relatively low ionic strength, whereas cytochrome P-450c and P-450LM$_6$ elute with relatively high-ionic-strength buffers (22-25, 33, 34); (d) both cytochromes P-450d and P-450LM$_4$ have relatively high catalytic activity for *N*-hydroxylation of several substrates and low catalytic activity for metabolism of benzo[*a*]pyrene, whereas the relative substrate specificity of cytochromes P-450c and P-450LM$_6$ are reversed (22, 23, 33-36); and, (e) both cytochromes P-450c and P-450LM$_6$ are induced by MC and/or TCDD in the lung and

kidney, whereas cytochromes *P*-450d and P-450LM$_4$ are not induced in these tissues (37, 38). In 1975, we purified and directly compared rabbit cytochrome *P*448 (LM$_4$) and rat cytochrome *P*448 (*P*-450c). Both proteins had different substrate specificities, minimum M_r in SDS-gels, and immunological properties (34). These results are consistent with the data in the present study that rat cytochrome *P*-450c is more related to rabbit cytochrome P-450LM$_6$ than P-450LM$_4$. Recent partial sequence analysis of rabbit cytochrome P-450LM$_4$ indicates that it is about 70% homologous with rat cytochrome *P*-450d (39, 40). The use of purified rabbit cytochromes P-450LM$_6$ and P-450LM$_4$ in "Western blots" and Ouchterlony plates establishes their immunochemical homology with rat cytochromes *P*-450c and *P*-450d, respectively. Moreover, from the published reports referred to above, it appears that these immunochemically related isozymes have a number of similar biochemical properties.

No polypeptide in D2 mouse liver microsomes immunorelated to rat cytochrome *P*-450c was detected regardless of whether the mice were treated with MC or isosafrole. However, cytochrome "*P*-450c" was detected in B6 mice treated with MC but not in control or isosafrole-treated B6 mice. This difference agrees with the single autosomal dominant gene difference between these two mouse strains that controls *Ah*-responsiveness, i.e., AHH-inducibility or induction of benzo[*a*]pyrene hydroxylation (41). Therefore, it appears that the mouse cytochrome "*P*-450c" analog is cytochrome P_1-450 described by Negishi and Nebert (42).

Whether mouse cytochrome "*P*-450d" corresponds to mouse cytochrome *P*448 or P_2-450 cannot be determined from published data (42, 43). It is important to note that the regulation of mouse cytochrome "*P*-450d" is quite different from mouse cytochrome "*P*-450c," since mouse cytochrome "*P*-450d" is present in untreated D2 and B6 mice, and is induced in both strains by isosafrole and, to a lesser extent, MC (see Table II). However, the effectiveness of MC treatment for

induction of mouse cytochrome "P-450d" was much greater in the B6 mice compared to D2 mice. This differential effect of MC in the two strains could be related to the *Ah* locus, since it has been shown that the receptor has higher affinity for MC in B6 mice compared to D2 mice (44). It is interesting that, in rats, we have been unable to find an inducer of either cytochrome P-450c or P-450d which did not induce the other isozyme, although their induction is not under coordinate regulatory control. For example, isosafrole treatment of rats induced cytochrome P-450d to greater absolute levels than cytochrome P-450c, whereas MC-treatment of rats had the opposite effect. However, the mouse cytochrome "P-450c" can be induced in B6 mice by MC but not by isosafrole, whereas isosafrole treatment induces cytochrome "P-450d" in both B6 and D2 strains to similar extents despite the *Ah* receptor difference between these two strains. Clearly, factors other than the *Ah* receptor *per se* must be involved in the regulation of mouse cytochromes "P-450c" and "P-450d."

More limited information is available concerning hamster and guinea pig cytochromes P-450. Walz *et al.* (18), using two-dimensional gel electrophoresis of hamster liver microsomes followed by peptide mapping of separated proteins, concluded that this species has a cytochrome P-450 isozyme that corresponds to rat cytochrome P-450d. The hamster protein is induced by MC and shares ~40% homology with cytochrome P-450d. While Walz *et al.* (18) were not able to detect this protein in microsomes from untreated animals, the methodology used in the present study is more sensitive, and establishes that low levels of this protein are present in microsomes from control hamsters. Abe and Watanabe (45) purified an MC-inducible cytochrome P-450 from guinea pig liver microsomes that has a minimum M_r slightly greater than rat cytochrome P-450d (54K versus 52K). This guinea pig cytochrome P-450 is a high-spin ferric hemoprotein, exhibits an absorption maximum at 448 nm for the ferrous–carbonyl complex, and has low catalytic activity

toward benzo[*a*]pyrene relative to the purified rat isozyme. Our results indicate that this is the guinea pig homolog of rat cytochrome P-450d based on the "Western blots" presented here and the spectral and catalytic properties of the protein. Thus, our conclusions regarding immunological relatedness of guinea pig and hamster cytochromes P-450 with rat cytochromes P-450c and P-450d are consistent with the limited data available in the literature.

A few generalizations can be made about the magnitude of induction of the immunoreactive analogs of cytochromes "P-450c" and "P-450d" in the species studied other than the mouse. Cytochrome "P-450d" was easily detectable in untreated animals, and was increased in all species by both isosafrole and MC treatment, but to differing extents. Cytochrome "P-450c" was either low or not detectable in all of the omnivorous species and, except for the rat, these same omnivorous species showed no detectable induction of cytochrome "P-450c" by isosafrole. The control animals of the herbivorous species (rabbit and guinea pig) appeared to contain higher levels of cytochrome "P-450c" compared to the omnivorous animals, and relatively small increases in cytochrome "P-450c" were effected by either isosafrole or MC treatment. While additional species could be examined, our studies show that structural homologs of cytochromes P-450c and P-450d are present in hepatic microsomes from several species, and that the levels of these proteins in control animals and their magnitude of induction by MC and isosafrole differs among these species.

Note added in proof. Since the submission of this manuscript, Kimura *et al.* (46) published the total sequence of mouse hepatic microsomal P_3-450 from DNA-cloning techniques. This protein shows high sequence homology to rat hepatic microsomal cytochrome P-450d (40). Hence, the mouse protein immunorelated to rat cytochrome P-450d is probably cytochrome P_3-450.

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