0026-895X/88/060736-08$02.00/0

MOLECULAR PHARMACOLOGY, 34:736-743

Distribution and Induction Sites of Phenobarbital- and 3-Methylcholanthrene-Inducible Cytochromes P-450 in Murine Liver: Immunohistochemical Localization with Monoclonal Antibodies

P. G. FORKERT, P. MIREHOUSE-BROWN, S. S. PARK, and H. V. GELBOIN

Departments of Anatomy and Medicine, Queen's University, Kingston, Ontario, Canada (P.G.F., P.M.) and Laboratory of Molecular Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892 (S.S.P., H.V.G.)

Received May 10, 1988; Accepted September 8, 1988

SUMMARY

Monoclonal antibodies specific for cytochromes P-450 induced by 3-methylcholanthrene (Mab 1-7-1) and phenobarbital (Mab 2-66-3) have been used in an unlabeled peroxidase-antiperoxidase immunohistochemical procedure to investigate the intralobular distribution and induction sites of the hemoproteins within the livers of CD-1, C57BL/6, and DBA/2 mice. 3-Methylcholanthrene-specific cytochromes P-450 were localized predominantly in centrilobular hepatocytes of control mice from all strains and were present at higher levels in CD-1 and C57BL/6 mice than in DBA/2 mice. Treatment with either 3-methylcholanthrene or β-naphthoflavone produced striking increases of 3-methylcholanthrene-specific cytochromes P-450 in hepatocytes from all regions of the hepatic lobule in CD-1 and C57BL/6 mice, but not in DBA/2 mice. Phenobarbital-specific cytochromes P-450 were localized in hepatocytes throughout all segments of the lobule in control mice, with slightly greater hemoprotein content in centrilobular hepatocytes. Treatment with phenobarbital resulted in enhancement of cytochrome P-450 that was visualized in hepatocytes in all regions of the lobule. Strain-related differences were not observed for phenobarbital-specific cytochromes P-450. These results demonstrate that constitutive levels of 3-methylcholanthrene- and phenobarbital-specific cytochromes P-450 are localized predominantly in centrilobular hepatocytes of murine livers, and induction of the hemoproteins is manifested to the greatest extent in periportal hepatocytes, resulting in a more uniform distribution throughout the hepatic lobule.

Cytochromes P-450 are integral constituents of the mixed function oxidase system, which exist in multiple forms with overlapping stereo- and regioselectivities for a variety of substrates including drugs, chemical carcinogens, pesticides, steroids, and prostaglandins (1–4). The complexity of the cytochrome P-450 system, due to enzyme multiplicity, overlapping substrate specificities, and differing inducibilities, has limited specific characterization of the cytochrome P-450 phenotype in tissues as well as its role in biotransformation of xenobiotics. The development and availability of Mabs directed to epitopes belonging to individual cytochromes P-450 have provided a unique approach for determination and identification of isozymic profiles of various tissues from different species, at both basal and induced levels (5–8).

Immunochemical studies using Mabs in conjunction with techniques of enzyme inhibition, immunopurification, and radioimmunoassay have both qualitatively and quantitatively identified the dynamic contribution of individual cytochromes P-450 to the total hemoprotein pool within a tissue (9–11). Several of these studies have used Mab 1-7-1 and Mab 2-66-3 to investigate epitope-specific cytochromes P-450 in the tissues of experimental animals (9–12). Mab 1-7-1 defines 3-MC-induced cytochromes P-450, whereas Mab 2-66-3 defines PB-induced cytochromes P-450 (7, 8). Cytochromes P-450, as recognized by Mab 1-7-1, have been shown to be highly inducible in the livers of the responsive C57BL/6 strain of mice treated with 3-MC; in contrast, little increase is detected in the nonresponsive DBA/2 mice given the same treatment (9–11). In our previous studies using immunofluorescence and Mabs (Mab 2-66-3 and Mab 1-7-1), we have determined the presence of homologous cytochromes P-450 in pulmonary tissues of mice (13).

In the present study, we have used Mabs (Mab 1-7-1 and Mab 2-66-3) in conjunction with a highly sensitive immunohistochemical technique to investigate the hepatic distribution and cellular sites of induction of epitope-specific cytochromes P-450 in the responsive CD-1 and C57BL/6 and the nonre-

This research was supported by a grant from the Medical Research Council of Canada (P.G.F.). P.G.F. is a Career Scientist of the Ontario Ministry of Health, Health Research Personnel Development Program.

ABBREVIATIONS: Mab, monoclonal antibody; 3-MC, 3-methylcholanthrene; PB, phenobarbital; βNF, β-naphthoflavone.

sponsive DBA/2 strains of mice. We have also examined the responses of these three murine strains with regard to their inducibilities by PB and by the polycyclic aromatic hydrocarbons 3-MC and βNF.

Materials and Methods

Chemicals and reagents. Sodium phenobarbital was purchased from BDH Chemicals (Toronto, Ontario, Canada) and sodium pentobarbital (Somnotol) was supplied by MTC Pharmaceuticals (Hamilton, Ontario). Tissue Tek II O.C.T. compound was acquired from Lab-Tek Products (Division Miles Laboratories, Naperville, IL). The following chemicals were obtained from Sigma Chemical Co. (St. Louis, MO): 3-MC, βNF, paraformaldehyde, Tris hydrochloride, sodium borohydride, saponin, bovine serum albumin, 3,3′-diaminobenzidine tetrahydrochloride, and hydrogen peroxide. Soluble rabbit anti-mouse peroxidase-antiperoxidase complex (Dakopatts) was purchased from Dimension Laboratories (Mississauga, Ontario).

Treatment of animals. Male CD-1, C57BL/6, and DBA/2 mice (Charles River Canada, St. Constant, Québec), weighing 20–30 g, were maintained on a 12-hr light/dark cycle and kept in a sound-proofed and temperature (25 ± 1°)-controlled environment. Animals were housed over hardwood bedding (Beta Chip, Northeastern Products Corp., Warrensburg, NY) and were provided with food (Purina Rodent Chow) and water *ad libitum*. Mice were allowed to stabilize under laboratory conditions for at least 7 days before initiation of experimental procedures.

Groups of mice (3–6) from each strain were administered either 3-MC (80 mg/kg) or βNF (80 mg/kg) intraperitoneally in corn oil at 72 and 48 hr before death. The injection volume for 3-MC and βNF was 0.08 ml/10 g of animal weight. Additional groups of mice (3–6) were administered PB (25 mg/kg or 80 mg/kg) in saline for 4 consecutive days and killed 24 hr after the last dose. Phenobarbital was given by intraperitoneal injection in a volume of 0.05 ml/10 g of animal weight. Control animals received equivalent volumes of the respective vehicles using a similar protocol.

Tissue preparation. Animals were anesthetized by intraperitoneal administration of sodium pentobarbital (120 mg/kg). Livers were rapidly rinsed with physiological saline through the aorta to eliminate blood from the tissues; the perfusate was allowed to escape from an incision made in the right atrium. This step was followed by perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at a hydrostatic pressure of 20 cm; perfusion was maintained for approximately 7 min for all animals. Livers were then removed, sectioned into blocks, and immersed in the perfusion fixative for an additional 2 hr at room temperature. After the primary fixation, tissue sections were kept overnight in 2% paraformaldehyde containing 30% sucrose at 4°. Blocks of liver tissues were subsequently frozen in isopentane precooled in liquid nitrogen and stored at −70°.

Immunohistochemistry. Mab 1-7-1 and Mab 2-66-3 were each reacted with liver sections from CD-1, C57BL/6, and DBA/2 mice that were administered either corn oil or saline, the two vehicles used in this study, or PB, βNF, or 3-MC. Controls for Mab 1-7-1 comprised tissue sections from all strains of mice treated with the vehicles and PB, whereas controls for Mab 2-66-3 comprise tissues from mice treated with the vehicles, βNF, and 3-MC.

Immunohistochemical localization of cytochromes P-450 in hepatic tissues was achieved by application of the unlabeled antibody peroxidase-antiperoxidase immunostaining technique described by Sternberger, *et al.* (14). Tissue sections (5 μm) were cut on a cryostat at −20° and adhered to gelatin-coated slides. After rinsing in 0.05 M Tris-buffered saline, pH 7.6, sections were reacted for 10 min with 1% sodium borohydride in Tris buffer, to block free aldehyde groups generated by aldehyde fixation. After further washing in buffer, sections were treated for 5 min with 3% hydrogen peroxide in water to inhibit endogenous peroxidase. After incubation for 20 min with normal rabbit serum to block nonspecific binding of antibody, sections were exposed for 16 hr at 4° to mouse antisera to cytochromes P-450 (Mab 1-7-1 and Mab 2-66-3). Incubations were performed at dilutions of primary antibody ranging from 1:100 to 1:3200 to determine concentrations for optimal staining. Appropriate comparisons of various treatment protocols were accomplished in the same incubations so that conditions of staining were optimally controlled and standardized.

Control incubations were performed with HyHel 9, a Mab specific only to egg white lysozyme (15) (positive control), or with omission of the primary antibody (negative control). Tissue sections from the same livers were used for both specific and control incubations, which were conducted simultaneously. Sections were then exposed to rabbit anti-mouse IgG antiserum for 30 min and this was followed by treatment with soluble horseradish peroxidase-mouse antihorseradish peroxidase complex for 20 min at room temperature. Subsequently, the slides were incubated in the dark for 3 min in a solution of 3,3′-diaminobenzidine tetrahydrochloride (0.05%) containing hydrogen peroxide (0.015%). Slides were placed in two washes of Tris buffer between each incubation step. The first series of slides was prepared to depict the cellular sites of insoluble oxidized diaminobenzidine alone and were not counterstained, because additional staining may obscure the location of weak staining due to low levels of antigen. For reference to morphology, a second series of slides was initially reacted with diaminobenzidine and subsequently counterstained with Mayer's hematoxylin for 1 min. Sections were finally dehydrated in acetone, cleared in xylene, and mounted with Permount.

In view of the "Bigbee effect" (16), analyses of the distribution and intensity of labeling in different murine strains subjected to various treatment protocols were performed on tissue sections reacted with optimal rather than identical dilutions of primary antibody. This effect is a phenomenon in which a high concentration of target antigen and insufficient dilution of the first antibody results in steric hindrance to the formation of bonds by the second antibody. Comparisons of staining reactions were conducted on tissue sections that were stained with the peroxidase anti-peroxidase complex and were not counterstained. Patterns and degrees of labeling were assessed from black and white photomicrographs of the same magnification. Regional distribution of specific cytochromes P-450 were confirmed on sections that were counterstained. Sections were examined on a Zeiss Photomicroscope II and color photography was accomplished using Ektachrome 160 Tungsten film with a camera setting of ASA 160. Black and white photomicrographs were obtained from exposure of Kodak Technical Pan film at 6 V using a green filter.

Results

Immunohistochemistry. Our observations of the cellular sites of cytochromes P-450 are based on the structure of the classical hepatic lobule, which is usually polygonal or hexagonal in shape on cross-section. Plates of hepatocytes radiate from the central structure of the lobule, the central vein. The portal canals containing the portal triads (branches of the hepatic artery, portal vein, and bile duct) are generally situated at the angles of the lobule at the perimeter. We examined the localization of immunohistochemical staining for cytochromes P-450 within the hepatic lobule and focused on centrilobular and periportal regions.

The antigenic sites for cytochromes P-450 were visualized using the immunohistochemical staining technique of Sternberger, *et al.* (14). In this immunohistochemical staining procedure, the primary antibody is linked to the peroxidase-antiperoxidase complex by a secondary antibody directed against the first antibody. In the presence of its substrate (hydrogen peroxide) horseradish peroxidase forms a complex with hydrogen peroxide. Upon the addition of diaminobenzidine, which serves as an electron donor, a second complex forms that dissociates, leaving the insoluble oxidized diaminobenzidine as

a brown precipitate (14). The use of the peroxidase-antiperoxidase technique, in conjunction with fixation and preparatory protocols that included the use of a mild fixative, paraformaldehyde, and the exclusion of solvents and embedding medium before immunostaining, resulted in optimal preservation of the target antigen and permitted the visualization of even low levels of cytochromes P-450. Optimal dilutions of primary antibody were similar for Mab 1-7-1 and Mab 2-66-3. A dilution of 1:400 produced optimal staining in liver sections from control mice, whereas a dilution of 1:800 of the appropriate Mab was optimal for staining tissues of induced mice, due to the presence of greater concentrations of cytochromes P-450, the target antigens.

All control incubations produced negative immunolabeling. The specificity of the immune reactions was further validated by the lack of cross-reactivity between Mab 1-7-1 and Mab 2-66-3.

Distribution of Mab 1-7-1-reactive cytochromes P-450. Table 1 summarizes the distribution of 3-MC-specific cytochromes P-450 in the livers of CD-1, C57BL/6, and DBA/2 mice after various treatments. Fig. 1 (A–H) depicts representative patterns of immunohistochemical staining obtained by Mab 1-7-1. Mab 1-7-1 produced positive staining in tissues of all murine strains administered saline, corn oil, or PB (Table 1; Fig. 1, A–C). Staining was predominantly concentrated in the centrilobular region of the lobule but was not detectable in the periportal region (Fig. 1, A–C). There was a gradation of staining so that hepatocytes situated adjacent or close to the central vein demonstrated the most intense staining, whereas hepatocytes in the midzonal area were less intensely stained (Fig. 1C). Within the positively stained zones were hepatocytes with differing as well as negative reactivities for Mab 1-7-1, resulting in a heterogeneous cell population with respect to staining intensities. Although the sites and pattern of staining were similar for all three strains of mice examined, staining intensities were considerably reduced in the hepatocytes of DBA/2 mice as compared with those of CD-1 and C57BL/6 mice (Table 1; Fig. 1, A and B). Nevertheless, the staining in centrilobular hepatocytes was clearly delineated in the livers of the DBA/2 strain (Fig. 1B). Mab 1-7-1 produced enhancement of immunostaining in the livers of CD-1 and C57BL/6 mice pretreated with either βNF or 3-MC (Table 1; Fig. 1, E–H). The hepatic response to βNF treatment was indistinguishable from that to 3-MC treatment, resulting in a characteristic cellular distribution of the intense immunolabeling evoked by both polycyclic aromatic hydrocarbons. Centrilobular, midzonal, and periportal hepatocytes were stained to equivalent extents, so that intralobular regions could not be distinguished on the basis of staining properties as was possible in the case of control mice reacted with Mab 1-7-1. The number of hepatocytes with positive staining markedly increased, so that the majority exhibited labeling, which appeared highly variable in intensity within individual hepatocytes (Fig. 1, E and F).

Staining by Mab 1-7-1 was confined to the cytoplasmic compartment of the hepatocyte and was not observed in nuclear components. The reaction product was of a particulate nature and appeared as irregular aggregates that occupied discrete areas of the cytoplasmic matrix (Fig. 1, F and H). The aggregates were prominent within hepatocytes of βNF- or 3-MC-treated mice and were frequently confluent, thus imparting a characteristic "foamy" appearance to the cytoplasm

When positive controls were performed by incubating sections with HyHel 9 instead of Mab 1-7-1, staining was negative (Fig. 1D). Similar negative staining was also obtained when the primary antibody was omitted and the incubations were carried out with only the diluent for the antibody. The absence of staining was confirmed in liver sections from vehicle-treated mice and mice pretreated with PB, 3-MC, or βNF.

Although strain-related differences in Mab 1-7-1-specific immunoreactivity were apparent between the control responsive CD-1 and C57BL/6 mice and the nonresponsive DBA/2 mice, the differences were even more dramatic in mice that were pretreated with βNF or 3-MC. The inducibility of 3-MC-specific cytochromes P-450 demonstrated by CD-1 and C57BL/6 mice was not found in DBA/2 mice, and increases in staining were not evident in the latter strain. It is noteworthy, however, that a small number of hepatocytes in the centrilobular region exhibited an intensity of staining that was not seen in the hepatocytes of control DBA/2 mice.

Distribution of Mab 2-66-3-reactive cytochromes P-450. Fig. 2 (A–H) depicts representative patterns of immunostaining in the livers of CD-1, C57BL/6, and DBA/2 mice using Mab 2-66-3 as an antibody probe for PB-specific cytochromes P-450, and Table 1 summarizes the observations. Mab 2-66-3 produced positive immunolabeling in hepatic tissues; analogous staining reactions were obtained in tissues from all strains of mice treated with corn oil, saline, βNF, or 3-MC (Fig. 2, A–C), and thus no strain-related differences were detected in the control mice (Table 1). Mab 2-66-3 detected antigenic sites in hepatocytes situated throughout the hepatic lobule and staining was visible in centrilobular, midzonal, and periportal hepatocytes. Although the distribution of hepatocytes containing PB-specific cytochromes P-450 was relatively uniform throughout all intralobular regions, Mab 2-66-3 was more reactive with hepatocytes in the centrilobular region than with those in either the midzonal or periportal regions. The number of labeled hepatocytes as well as the intensity of staining were slightly greater in the centrilobular than in the periportal regions (Fig. 2B). Hepatocytes that were strongly reactive were interspersed with hepatocytes with reduced or negative labeling. However, the disparities in staining between centrilobular and periportal hepatocytes were never as pronounced with Mab 2-66-3 as those found with Mab 1-7-1 in control mice.

When immunohistochemistry was performed on liver sections from mice that had previously been administered PB (25 mg/kg, 80 mg/kg), the population of cells that was positively stained with Mab 2-66-3 increased significantly, and there was enhancement of immunolabeling in individual cells (Fig. 2, D–

TABLE 1

Distribution of 3-MC- and PB-specific cytochromes P-450 in livers of CD-1, C57BL/6, and DBA/2 mice

+–++++, increasing intensity of labeling obtained by peroxidase-antiperoxidase immunohistochemistry. PB (80 mg/kg), βNF (80 mg/kg), 3-MC (80 mg/kg), and their respective vehicles are administered according to protocols described in Materials and Methods.

Strain	Mab 1-7-1[a]			Mab 2-66-3[b]		
	Vehicle	PB	βNF/3-MC[c]	Vehicle	PB	βNF/3-MC[c]
CD-1	++	++	++++	+	+++	+
C57BL/6	++	++	++++	+	+++	+
DBA/2	+	+	+	+	+++	+

[a] Detects 3-MC-specific cytochromes P-450.
[b] Detects PB-specific cytochrome P-450.
[c] βNF and 3-MC produce similar labeling.

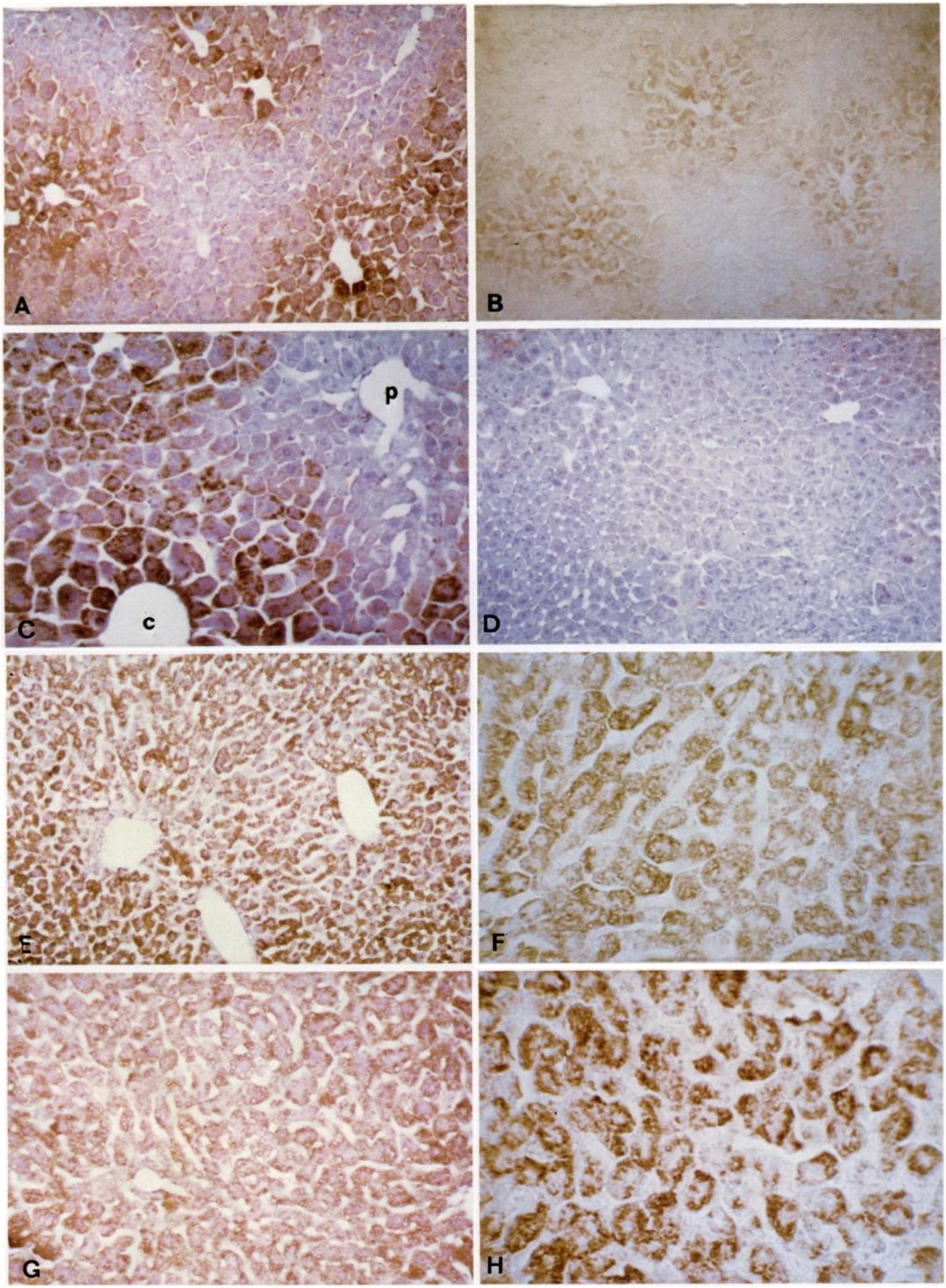

Fig. 1. Immunohistochemical localization of 3-MC-specific cytochromes P-450 within livers of CD-1, C57BL/6, and DBA/2 mice, as detected by Mab 1-7-1 and peroxidase-antiperoxidase immunostaining. A, C57BL/6 mouse treated with saline; staining predominates in centrilobular region; B, DBA/2 mouse treated with corn oil; low levels of staining in centrilobular region; C, CD-1 mouse treated with PB (80 mg/kg); gradation of staining from centrilobular to periportal region; D, CD-1 mouse treated with corn oil; negative staining in section reacted with HyHel 9 instead of Mab 1-7-1; E, C57BL/6 mouse treated with βNF (80 mg/kg); striking increases in staining throughout all regions of the hepatic lobule; F, C57BL/6 mouse treated with βNF (80 mg/kg); majority of hepatocytes within a lobule exhibit staining; G, CD-1 mouse treated with 3-MC (80 mg/kg); uniform distribution of intense staining as found with βNF treatment; H, C57BL/6 mouse treated with 3-MC (80 mg/kg); reactivity to cytoplasmic but not nuclear constituents. *c*, central vein; *p*, portal triad. Dilutions of Mab 1-7-1, 1:400 (A–C); 1:800 (E–H). Dilution of HyHel 9, 1:400 (D). Counterstained with Mayer's hematoxylin (A, C, D, E, and G). Original magnification: 100 × (A, B, D, and E); 160 × (G); 250 × (C, F, and H).

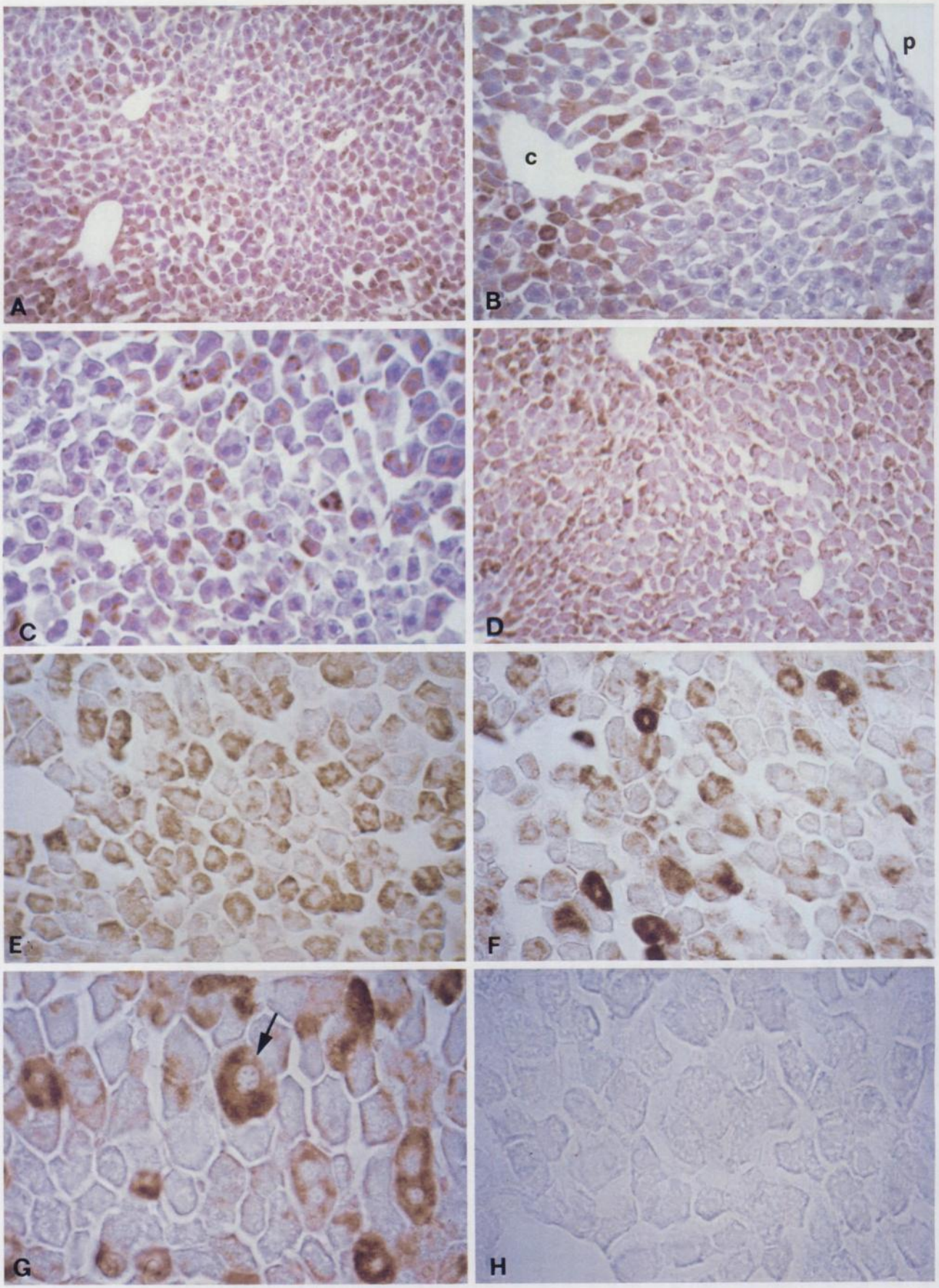

Fig. 2. Immunohistochemical localization of PB-specific cytochromes P-450 within livers of CD-1, C57BL/6, and DBA/2 mice, as detected by Mab 2-66-3 and peroxidase-antiperoxidase immunostaining. Strain-related differences in immunostaining were not detected for all three murine strains. A, C57BL/6 mouse treated with corn oil; staining in hepatocytes in all regions of the lobule; B, DBA/2 mouse treated with βNF (80 mg/kg); staining in centrilobular region slightly greater than in periportal region; C, C57BL/6 mouse treated with corn oil; variability of staining intensity in hepatocytes; D, CD-1 mouse treated with PB (80 mg/kg); increased staining within all regions of the lobule; E and F, C57BL/6 mice treated with 25 mg/kg (E) and 80 mg/kg (F) of PB; greater intensity of staining obtained with both dosages; G and H, C57BL/6 mouse treated with PB (80 mg/kg); positive labeling of nucleus by Mab 2-66-3 (G, *arrow*) and negative labeling by HyHel 9 (H). *c*, central vein; *p*, portal triad. Dilutions of Mab 2-66-3, 1:400 (A–C); 1:800 (D–H). Counterstained with Mayer's hematoxylin (A–D). Original magnification: 100 × (A and D); 160 × (B); 250 × (C, E, and F); 400 × (G and H).

F). The augmentation of staining evoked by the lower dosage of PB was only slightly less than that produced by the higher dosage (Fig. 2, E and F). However, the administration of either dosage of PB caused an increase in the number of hepatocytes containing PB-specific cytochromes P-450 throughout the hepatic lobule, resulting in a more even distribution. Hepatocytes that were intensely stained were scattered among cells that were less well stained, and among these were hepatocytes that demonstrated no staining for Mab 2-66-3. As found in vehicle-treated mice of the CD-1, C57BL/6, and DBA/2 strains, no observable differences in immunoreactivity were elicited by Mab 2-66-3 in mice of all strains pretreated with either 3-MC or βNF (Table 1).

Staining characteristics resulting from the use of Mab 2-66-3 differed from those using Mab 1-7-1. In contrast to the vacuolated appearance of cytoplasmic staining produced by Mab 1-7-1, Mab 2-66-3 yielded staining that was more uniformly distributed throughout the cytoplasmic matrix. However, variability exists with regard to the extent of Mab 2-66-3-specific immunolabeling within a lobule, and the most reactive hepatocytes possessed staining that virtually covered the entire cytoplasmic compartment. The lack of nuclear staining by Mab 1-7-1 contrasts with the staining obtained by Mab 2-66-3, which reacted positively with the nucleus of the hepatocyte, including the nuclear envelope (Fig. 2G). All control reactions, including that using HyHel 9 as primary antibody (Fig. 2H), produced negative staining. Thus, the staining properties demonstrated by Mab 1-7-1 and Mab 2-66-3 in the livers of mice appear to differ not only at the regional but at the cellular level as well.

Discussion

The observations presented in this study demonstrate the extraordinary sensitivity of the application of Mabs and immunohistochemistry for the detection and localization of epitope-specific forms of cytochrome P-450. Under optimal conditions of fixation and preparation of tissues for immunostaining, low levels of cytochromes P-450, such as were present in the livers of control C57BL/6 and DBA/2 mice, were visualized. The use of this methodology in conjunction with Mab 1-7-1 and Mab 2-66-3 have permitted precise detection and localization of epitope-specific forms of cytochrome P-450 within the hepatic lobule.

Our findings demonstrate a clear demarcation in the intralobular distribution of different cytochromes P-450 within the livers of control mice. These represent cytochromes P-450 that are presumably present at relatively low levels in a constitutive form. Phenobarbital-specific cytochromes P-450 are found in hepatocytes that are distributed throughout the liver lobule, and only slightly greater hemoprotein content is present in hepatocytes of the centrilobular region (Fig. 2, A–C). On the other hand, the 3-MC-specific cytochromes P-450 are predominantly localized in centrilobular hepatocytes and are not detectable in periportal hepatocytes (Fig. 1, A–C). A similar pattern of distribution has been shown for PB- and 3-MC-specific cytochromes P-450 in the livers of untreated rats (17). However, the intralobular sites of the two forms of cytochromes P-450 in the rat appear to be different from those exhibited by the control mice of this study. In the rat, PB-specific cytochromes P-450 are localized predominantly in centrilobular hepatocytes, whereas the 3-MC-specific cytochromes are distributed more uniformly throughout the hepatic lobule (17). Although the pattern of distribution of isozymic forms of cytochrome P-450 within the hepatic lobule is rather unique for the same tissues from different animal species, the data confirm the high concentration of cytochrome P-450 content within centrilobular hepatocytes of both mice and rats. This property may contribute to some of the factors underlying the frequent centrilobular manifestation of hepatotoxicities caused by numerous xenobiotics that undergo metabolic activation through the cytochrome P-450-dependent monooxygenase system (18, 19).

The intralobular sites of induction produced by PB, 3-MC, and βNF in mice are clearly defined. Pretreatment with PB elicits induction, a process that is manifested in increases in the content of PB-specific cytochromes P-450 in numerous hepatocytes in all segments of the hepatic lobule (Fig. 2D). Similar induction is also observed for 3-MC-specific cytochromes P-450 after pretreatment with either 3-MC or βNF (Fig. 1, E–H). The inductive response is reflected in enhancement of immunostaining within the hepatic lobule so that a more homogeneous distribution of positively stained hepatocytes is found throughout all regions. After treatment with 3-MC or βNF, the patterns of distribution obtained for 3-MC-specific cytochromes P-450 are indistinguishable, indicating that the same hemoproteins or hemoproteins bearing the same epitope are induced. This observation is in accord with the results of other studies that have shown that 3-MC and βNF identify and induce the same major isozymic forms of cytochrome P-450 (1, 17, 20, 21). However, regardless of the inducing agent used, inducibility within the liver is manifested in the recruitment of hepatocytes throughout the hepatic lobule, so that hepatocytes containing cytochromes P-450 are scattered throughout the lobule in a relatively more uniform fashion. Consequently, the degree and location of inducibility appear to be dependent primarily on the concentration and sites of particular forms of cytochromes P-450 that are present constitutively. Thus, in the present study, induction of 3-MC-specific cytochromes P-450 is maximal in the periportal hepatocytes, because the basal forms reside predominantly in the centrilobular regions. In the case of PB-specific cytochromes P-450, the basal forms are found in hepatocytes distributed throughout all regions of the hepatic lobule, and induction produces an accentuation of the existing pattern of distribution, resulting in a greater number of hepatocytes containing greater amounts of hemoprotein than found in the livers of control mice. Nevertheless, the final result of induction is a wider distribution of hepatocytes that demonstrate expression of specific cytochromes P-450 determined by the inducing agent.

The sites of PB- and 3-MC-specific cytochromes P-450 differ not only at the regional but also at the cellular level. Whereas the sites of reactivity to Mab 1-7-1 are primarily cytoplasmic and are negative in the nucleus (Fig. 1, F and H), Mab 2-66-3 produces both cytoplasmic as well as nuclear staining, including the nuclear membrane (Fig. 2, E–G). More precise nuclear localization requires the application of immunoelectron microscopy; these studies are currently in progress in this laboratory. The labeling to the nuclear membrane for PB-specific P-450 in the present study is in agreement with similar observations in rats in which the same P-450 species is found to be associated with the nuclear envelope (17). Other studies have used immunoelectron microscopy to demonstrate labeling of

PB-inducible cytochrome P-450 to the outer nuclear membrane (22).

As expected, no strain-related differences in immunoreactivity for PB-specific cytochromes P-450 are evident in all the mice examined in this study. Neither is there any difference in immunohistochemical staining for the 3-MC-specific cytochromes P-450 in the responsive CD-1 and C57BL/6 strains. It is only in the nonresponsive DBA/2 mice that differences in immunoreactivity for the 3-MC-specific cytochromes are apparent. We detected constitutive basal levels of 3-MC-specific cytochromes P-450 in the livers of DBA/2 mice, albeit at considerably lower concentrations than found in the livers of responsive mice (Fig. 1, A and B). Furthermore, the high inducibility evoked by 3-MC and βNF in the responsive mice is not observed in DBA/2 mice. These results are consistent with the findings of numerous investigations that have established the lack of inducibility of DBA/2 mice and the high inducibility of C57BL/6 mice by 3-MC (23–26). Inducibility, as found in the prototypic responsive C57BL/6 strain, is regulated by the *Ah* receptor, which is present in the cytosol in a high affinity form in genetically responsive strains of mice (27). The *Ah* receptor has been undetectable in the nonresponsive murine strain, and the lack of this receptor has been correlated with the noninducibility demonstrated by the DBA/2 strain of mice. However, using more refined techniques of isolation, Okey's laboratory has recently identified low concentrations of a low affinity *Ah* receptor in nonresponsive strains of mice (28, 29). Whether any correlation can be demonstrated between the low levels of cytochromes P-450 we observed in the livers of control and 3-MC-treated DBA/2 mice, as assessed by immunohistochemical staining, and the presence of low levels of a low affinity *Ah* receptor remains to be established.

Immunochemical studies have demonstrated that Mab 2-66-3 detects one form of cytochrome P-450 with M_r 54,000 (12, 30), and Mab 1-7-1 reacts with two forms of cytochromes P-450 in 3-MC-treated C57BL/6 mice and binds to two species of microsomal proteins with M_r 57,000 and 56,000 (11). However, in 3-MC-treated DBA/2 mice, Mab 1-7-1 binds to only one form of cytochrome P-450 with M_r 56,000 (11). Structural analyses has shown that the NH_2-terminal sequence of the M_r 57,000 polypeptide is the same as that for mouse P_1-450, as deduced from its cDNA nucleotide sequence, except that the 57,000 polypeptide possesses proline and serine as two additional residues (31–33). The amino terminal sequence for the M_r 56,000 polypeptide is, however, identical to that of the mouse P_3-450 (31, 33) as well as to that of cytochrome P-450d of Long-Evans rats (34). The data indicate that the M_r 57,000 and 56,000 polypeptides detected by Mab 1-7-1 correspond to mouse P_1-450 and P_3-450, respectively. Accordingly, the immunohistochemical staining observed in centrilobular hepatocytes of DBA/2 mice in the present study represents the M_r 56,000 cytochrome P-450, which corresponds to mouse P_3-450. In C57BL/6 mice, the immunohistochemical staining represents both the M_r 57,000 and 56,000 polypeptides, which correspond to the mouse P_1-450 and P_3-450.

In summary, the results of this study demonstrate that cytochromes P-450 specific for PB and 3-MC are present constitutively in the livers of CD-1, C57BL/6, and DBA/2 mice and are localized in hepatocytes that occupy well defined regions of the hepatic lobule. These cytochromes P-450 exhibit inducibility, a process that is reflected in the presence of high concentrations of hemoprotein that reside in hepatocytes throughout all regions of the hepatic lobule. These findings emphasize the significant diversity of the hepatocyte population, as well as its dynamic capacity for inducibility with respect to the cytochrome P-450 system.

Acknowledgments

We thank Dr. Allan Okey for valuable discussions and for making available to us manuscripts in press. We also thank Dr. G. V. Childs for helpful advise on immunohistochemical techniques.

References

1. Lu, A. Y. H., and S. West. Multiplicity of mammalian microsomal cytochromes P-450. *Pharmacol. Rev.* **31**:277–295 (1980).
2. Conney, A. H. Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev.* **19**:317–366 (1967).
3. Conney, A. H., and J. J. Burns. Metabolic interactions among environmental chemicals and drugs. *Science* (Wash. D. C.) **178**:576–586 (1972).
4. Gelboin, H. V. Benzo[*a*]pyrene metabolism, activation, and carcinogenesis: role and regulation of mixed-function oxidases and related enzymes. *Physiol. Rev.* **60**:1107–1166 (1980).
5. Park, S. S., A. V. Persson, M. J. Coon, and H. V. Gelboin. Monoclonal antibodies to rabbit liver cytochrome P450 LM2. *FEBS Lett.* **116**:231–235 (1980).
6. Park, S. S., S. J. Cha, H. Miller, A. V. Persson, M. J. Coon, and H. V. Gelboin. Monoclonal antibodies to rabbit liver cytochrome $P\text{-}450_{LM2}$ and cytochrome $P\text{-}450_{LM4}$. *Mol. Pharmacol.* **21**:248–258 (1981).
7. Park, S. S., T. Fujino, D. West, F. P. Guengerich, and H. V. Gelboin. Monoclonal antibodies that inhibit enzyme activity of 3-methylcholanthrene-induced cytochrome P-450. *Cancer Res.* **42**:1798–1808 (1982).
8. Park, S. S., T. Fujino, H. Miller, F. P. Guengerich, and H. V. Gelboin. Monoclonal antibodies to phenobarbital-induced rat liver cytochrome P-450. *Biochem Pharmacol.* **33**:2071–2081 (1984).
9. Song, B.-J., T. Fujino, S.-S. Park, F. K. Friedman, and H. V. Gelboin. Monoclonal antibody-directed radioimmunoassay of specific cytochromes P-450. *J. Biol. Chem.* **259**:1394–1397 (1984).
10. Fujino, T., D. West, S. S. Park, and H. V. Gelboin. Monoclonal antibody-directed phenotyping of cytochrome P-450-dependent aryl hydrocarbon hydroxylase and 7-ethoxycoumarin deethylase in mammalian tissues. *J. Biol. Chem.* **259**:9044–9050 (1984).
11. Cheng, K.-C., H. V. Gelboin, B.-J. Song, S.-S. Park, and F. K. Friedman. Detection and purification of cytochromes P-450 in animal tissues with monoclonal antibodies. *J. Biol. Chem.* **259**:12279–12284 (1984).
12. Friedman, F. K., R. C. Robinson, S.-S. Park, and H. V. Gelboin. Monoclonal antibody-directed immunopurification and identification of cytochromes P-450. *Biochem, Biophys. Res. Commun.* **116**:859–865 (1983).
13. Forkert, P. G., M. L. Vessey, J. S. Elce, S. S. Park, H. V. Gelboin, and S. P. C. Cole. Localization of phenobarbital- and 3-methylcholanthrene-inducible cytochromes P-450 in mouse lung with monoclonal antibodies. *Res. Commun. Chem. Pathol. Pharmacol.* **53**:147–157 (1986).
14. Sternberger, L. A., P. H. Hardy, J. J. Cuculis, and H. G. Meyer. The unlabeled antibody-enzyme method of immunohistochemistry: preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-anti-horseradish peroxidase) and its use in the identification of spirochetes. *J. Histochem. Cytochem.* **18**:315–333 (1970).
15. Smith-Gill, S. J., A. C. Wilson, M. Potter, E. M. Prager, R. J. Feldmann, and C. R. Mainhart. Mapping the antigenic epitope for a monoclonal antibody against lysozyme. *J. Immunol.* **128**:314–322 (1982).
16. Bigbee, J. W., J. C. Kosek, and L. F. Eng. Effects of primary antiserum dilution on staining of 'antigen-rich' tissues with the peroxidase anti-peroxidase technique. *J. Histochem. Cytochem.* **25**:443–447 (1977).
17. Baron, J., J. A. Redick, and F. P. Guengerich. An immunohistochemical study on the localizations and distributions of phenobarbital- and 3-methylcholanthrene-inducible cytochromes P-450 within the livers of untreated rats. *J. Biol. Chem.* **256**:5931–5937 (1981).
18. Brodie, B. B., W. D. Reid, A. K. Cho, D. Sipes, G. Krishna, and J. R. Gillette. Possible mechanism of liver necrosis caused by aromatic compounds. *Proc. Natl. Acad. Sci. USA* **68**:160–164 (1971).
19. Slater, T. F. Biochemical studies on liver injury: structural aspects, in *Biochemical Mechanisms of Liver Injury* (T. F. Slater, ed.). Academic Press, New York, 2–44 (1978).
20. Guengerich, F. P., and M. V. Martin. Purification of cytochrome P-450, NADPH-cytochrome P-450 reductase, and epoxide hydratase from a single preparation of rat liver microsomes. *Arch. Biochem. Biophys.* **205**:365–379 (1980).
21. Guengerich, F. P., P. Wang, P. S. Mason, and M. B. Mitchell. Immunological comparison of rat, rabbit and human microsomal cytochromes P-450. *Biochemistry* **20**:2370–2378 (1981).
22. Matsuura, S., Y. Fujii-Kuriyama, and Y. Tashiro. Immunoelectron microscope localization of cytochrome P-450 on microsomes and other membrane structures of rat hepatocytes. *J. Cell Biol.* **78**:503–519 (1978).
23. Anderson, L. M., J. M. Ward, S. S. Park, A. B. Jones, J. L. Junker, H. V.

Gelboin, and J. M. Rice. Immunohistochemical determination of inducibility phenotype with a monoclonal antibody to a methylcholanthrene-inducible isozyme of cytochrome P-450. *Cancer Res.* **47**:6079–6085 (1987).
24. Negishi, M., and D. W. Nebert. Structural gene products of the *Ah* complex: increases in large mRNA from mouse liver associated with cytochrome P_1-450 induction by 3-methylcholanthrene. *J. Biol. Chem.* **256**:3085–3091 (1981).
25. Haugen, D. A., M. J. Coon, and D. W. Nebert. Induction of multiple forms of mouse liver cytochrome P-450: evidence for genetically controlled *de novo* protein synthesis in response to treatment with β-naphthoflavone or phenobarbital. *J. Biol. Chem.* **251**:1817–1827 (1976).
26. Nebert, D. W. The genetic regulation of drug-metabolizing enzymes. *Drug. Metab. Disp.* **16**:1–8 (1986).
27. Eisen, H. J., R. R. Hannah, C. Legraverend, A. B. Okey, and D. W. Nebert. The *Ah* receptor: controlling factor in the induction of drug-metabolizing enzymes by certain chemical carcinogens and other environmental pollutants, in *Biochemical Actions of Hormones* (G. Litwack, ed.), Vol. 10. Academic Press, New York, 227–257 (1983).
28. Manchester, D. K., S. K. Gordon, C. L. Golas, E. A. Roberts, and A. B. Okey. *Ah* receptor in human placenta: stabilization by molybdate and characterization of binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 3-methylcholanthrene, and benzo(*a*)pyrene. *Cancer Res.* **47**:4861–4868 (1987).
29. Okey, A. B., M. S. Denison, P. A. Harper, and R. D. Prokipcak. The *Ah* receptor: first step in the mechanism of response to 3-methylcholanthrene-type chemicals in rodents and in man, in *Microsomes and Drug Oxidations* (J. Miners, D. J. Birkett, R. Drew, and M. McManus, eds.). Taylor and Francis, London, 31–38 (1988).
30. Hietanen, E., C. Malaveille, F. K. Friedman, S.-S. Park, J.-C. Béréziat, G. Brun, H. Bartsch, and H. V. Gelboin. Monoclonal antibody-directed analysis of cytochrome P-450-dependent monooxygenases and mutagen activation in the livers of DBA/2 and C57BL/6 mice. *Cancer Res.* **46**:524–531 (1986).
31. Cheng, K.-C., S. S. Park, H. C. Krutzsch, P. H. Grantham, H. V. Gelboin, and F. K. Friedman. Amino-terminal sequence and structure of monoclonal antibody immunopurified cytochromes P-450. *Biochemistry* **25**:2397–2402 (1986).
32. Kimura, S., F. J. Gonzalez, and D. W. Nebert. The murine *Ah* locus: comparison of the complete cytochrome P_1-450 and P_3-450 cDNA nucleotide and amino acid sequence. *J. Biol. Chem.* **259**:10705–10713 (1984).
33. Kimura, S., F. J. Gonzalez, and D. W. Nebert. Mouse cytochrome P_3-450: complete cDNA and amino acid sequence. *Nucleic Acids Res.* **12**:2917–2928 (1984).
34. Botelho, L. H., D. E. Ryan, P.-M. Yuan, R. Kutny, J. E. Shively, and W. Levin. Amino-terminal and carboxy-terminal sequence of hepatic microsomal cytochrome P-450d, a unique hemoprotein from rats treated with isosafrole. *Biochemistry* **21**:1152–1155 (1982).

Send reprint requests to: Dr. P. G. Forkert, Department of Anatomy, Queen's University, Kingston, Ontario, Canada K7L 3N6.