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IMMUNOHISTOCHEMICAL LOCALIZATION OF CYTOCHROME P450b/e* IN HEPATIC AND EXTRAHEPATIC TISSUES OF THE RAT

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Abstract—The cellular distribution of cytochrome P450b/e has been studied in the liver and a number of extrahepatic tissues in the Wistar rat by immunocytochemistry, using two monoclonal antibodies, 10/1 and 1/4, raised against the major phenobarbitone-inducible form of cytochrome P450. The specificity of these antibodies was verified by a number of techniques. In Western blotting, both antibodies recognised a single band of M_r 52,000 in liver microsomes from rats pre-treated with phenobarbitone, acetone and isosafrole, which co-migrated with a purified preparation of cytochrome P450b. In untreated rats, a weak specific immunostain was visible across the whole of the liver lobule, with stronger staining in a few hepatocytes around the central vein. Immunoreactive cytochrome P450b/e was also found in the Clara cells of the lung and in the enterocytes of the small intestine, with maximal staining at the tips of the villi. No immunoreactive cytochrome P450b/e was detected in kidney, testis or pancreas. Phenobarbitone treatment resulted in a strong, specific immunostain of all hepatic centrilobular cells, antibody titration indicating that induction of cytochrome P450b/e had occurred. Marked induction was also found in the enterocytes of the small intestine, a strong immunostain being apparent in cells along the length of the villus, from crypt to tip. No induction was apparent in lung, kidney, testis or pancreas. Immunoquantification of cytochrome P450b/e, by densitometric scanning of dot blots probed with a monoclonal antibody, 10/1, confirmed these observations. Thus, there are very marked, specific inter- and intra-tissue differences in both the expression and inducibility of cytochrome P450b/e in the rat.

Cytochrome P450, a multi-isoenzyme family of haemoproteins, plays an important role in the elimination of a wide range of xenobiotics. Although extrahepatic tissues usually contribute little towards this process, they may be involved in the generation of reactive metabolites and would thus be targets for metabolically initiated cytotoxicity or genotoxicity. The extent to which this occurs will be dictated largely by the tissue-specific expression and inducibility of the isoenzymes of cytochrome P450. For example, in rabbit lung the constitutive phenobarbitone (PB)-inducible isoenzyme, form 2, is almost exclusively responsible for the bioactivation of 4-ipomeanol and several other toxic chemicals [1].

Rat hepatic cytochromes P450 b and e are co-induced by PB and several other unrelated compounds [2, 3]. These two proteins, which are members of the P450IIB sub-family, differ in only 13 of 491 amino acids and are immunochemically indistinguishable with polyclonal anti-P450b or anti-P450e [4] or with a monoclonal antibody (2-66-3) to P450b [5].

The hepatic distribution and inducibility of the major PB-inducible isoenzymes of cytochrome P450, as assessed immunohistochemically using polyclonal antibodies, is well established in some strains of rat [6, 7]. However, little information is available on their localization within extrahepatic organs which have been reported to be unresponsive to PB-treatment [8, 9]. Recently, the tissue-specific expression and regulation of mRNAs coding for P450b and P450e have been investigated [10, 11]. An attempt to quantify the proteins within extrahepatic tissues has also been made by Western blotting of subcellular fractions [5]. Together, these studies provide some indication for the presence of cytochrome P450b and/or P450e within kidney, lung, small intestine and testis, albeit at very low levels. The question of whether induction does occur in any of these tissues, and to which sub-populations of cells this is restricted, requires clarification. We have further investigated the constitutive expression and inducibility of the major PB-inducible form(s) (P450b/e) in rat tissues, using immunocytochemical techniques.

MATERIALS AND METHODS

Materials. Dichlorodifluoromethane (Arcton), synthetic mounting medium containing resin in xylene (Pertex), TissueTek II embedding medium, hydrogen peroxide, counterstains, 3-methylcholanthrene (MC) and acetone (ACT) were

* MAbs 10/1 and 1/4 recognise only a single band corresponding to cytochrome P450b (the product of the P450IIB1 gene) on Western blotting. However, these MAbs recognise both cytochrome P450b and P450e (the product of the P450IIB2 gene) in dot blotting. Therefore specific immunocytochemical staining in this manuscript has been referred to as with P450b/e.

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obtained from BDH Chemicals Ltd (Poole, U.K.); sodium phenobarbitone was from Macarthys (London, U.K.) and most of the reagents used for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE), 4-chloro-1-naphthol and nitrocellulose membrane were provided by Bio-Rad Laboratories Ltd (Watford, U.K.). Isosafrole (ISF) was from Eastman Kodak (Rochester, NY) and pregnenolone-16α-carbonitrile (PCN) and clofibrate (CL) were kind gifts from G D Searle (Skokie, IL) and from Pharmaceuticals Division, ICI plc (Macclesfield, U.K.), respectively. Rabbit anti-mouse (RAM) IgG conjugated to horseradish peroxidase was obtained from Miles Laboratories Ltd (Slough, U.K.) and goat anti-mouse (GAM) IgG conjugated to 5 nm colloidal gold and the silver enhancement kit were provided by Janssen Life Sciences Products (Wantage, U.K.). All other reagents were supplied by the Sigma Chemical Co. (Poole, U.K.). Cytochrome P450c was purified as previously described [12]. An apparently homogeneous preparation of cytochrome P450b was a generous gift of A Paine, St Bartholomew's Hospital Medical College, London.

Treatment of animals. Male Wistar rats (180–200 g, from OLAC, Bicester, U.K.) were housed in groups of three per cage with food (PRD diet; Labsure Animal Products, Poole, U.K.), until 16 hr prior to killing, and water provided *ad lib*. Groups of animals were left untreated or were treated daily with PB in saline (80 mg/kg body wt) for 4 days by intraperitoneal (i.p.) injection, MC in corn oil (80 mg/kg) as a single i.p. injection 48 hr prior to killing, CL in saline (200 mg/kg) daily for 3 days by i.p. injection, ISF in corn oil (160 mg/kg) by i.p. injection daily for 4 days, PCN in corn oil (100 mg/kg) daily for 3 days by i.p. injection or ACT as a 25% solution (5 ml/kg) intragastrically 22 hr prior to killing. For isoniazid (INH) treatment, rats received 0.1% w/v in their drinking water (adjusted to pH 7.4 with 100 mM HCl) for 11 days, followed by an i.p. injection of 50 mg/kg INH in saline 3 hr prior to killing. With the exception of MC, ACT and INH, animals were killed 24 hr after the last dose of the inducer.

Monoclonal antibodies. The production and purification of the monoclonal antibodies (MAbs) to cytochromes P450b/e, 1/4 and 10/1, used in these studies have been described previously [12]. These antibodies were derived from the fusion of the mouse myeloma cell line P3.NS1.1-Ag4-1 (NS1 cells) with splenic lymphocytes obtained from Balb/c mice immunized with a preparation of cytochrome P450b partially purified from the livers of rats treated with PB, to a specific content of 5 nmol/mg, as described previously [13]. Antibody was prepared in quantity by inoculation of mice with actively growing hybridoma cells as previously described [13]. The ascites fluid thus produced was subjected to ammonium sulphate precipitation and DEAE Affi-Gel Blue chromatography to produce purified IgG preparations of the MAbs. The specificity of the antibodies was confirmed by screening against a homogeneous preparation of cytochrome P450b and other isoenzymes [12].

Tissue preparation. Animals were killed by stunning and exsanguination and their livers were perfused *in situ* with ice-cold 0.9% NaCl and then removed. Tissue blocks, prepared from the medial lobes of the livers and also from extrahepatic organs (kidney, lung, duodenum, pancreas and testis), were rapidly frozen in melting Arcton (at −156°; precooled in liquid nitrogen) and stored at −80° until required for sectioning. Sections of approximately 7 μm thickness were cut on a cryostatic microtome (Slee, London, U.K.) at −20°, mounted onto poly-L-lysine coated slides [14], air dried at room temperature overnight and stored, with dessicant, at −80° until required for immunocytochemistry. Microsomal fractions were prepared from the remainder of the liver and extrahepatic organs as described previously, except that 1 mM EDTA was added to the buffers [15]. Heparin, 3 units/ml of homogenate, was added to homogenates of small intestine to aid "clean" sedimentation of the microsomal pellet.

Electrophoresis, Western and dot blotting. Microsomal proteins were separated by SDS–PAGE, using 9% polyacrylamide, and electrophoretically transferred to 0.45 μm nitrocellulose membranes as described previously [13]. Dot blotting was performed using a Bio-Dot microfiltration apparatus from Bio-Rad Laboratories (Watford, U.K.). Protein samples were diluted appropriately in phosphate buffered saline (PBS: 140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4), pH 7.4, and aliquoted directly onto the nitrocellulose membrane through the wells by applying suction with a vacuum pump.

Specific antibody–antigen interaction, following incubation of membranes with monoclonal antibody followed by RAM IgG conjugated to horseradish peroxidase, was detected by exposure to 1 mM 4-chloro-1-naphthol in PBS containing 0.01% H_2O_2 for 15 min.

Immunoquantification of the major PB-inducible isoenzyme(s) of cytochrome P450 was achieved by computerised integration of the peaks obtained by densitometric scanning of dot blots probed with MAb 10/1 [16]. There was a linear relationship between the amount of protein applied to the nitrocellulose sheets and the density of the dot, for P450b and for all of the microsomal preparations studied, over the range of concentrations used in the present investigation ($r \geq 0.94$). The gradients of the concentration–optical density lines were the same within a tissue before and after treatment of the animals with PB. However, there was a slight difference in the gradients amongst tissues, leading to a maximum discrepancy in calculated P450b/e content between tissues of two-fold. Thus, within-tissue comparisons of P450b/e content should be quantitative, and between-tissue comparisons, of less importance in the present study, should at worst be semi-quantitative.

Indirect immunoperoxidase staining of sections. Tissue sections, warmed to room temperature, were fixed for 15 min in cold acetone and then rehydrated in PBS, pH 7.4. Non-specific binding sites were blocked by incubation in PBS containing 1% bovine serum albumin for a minimum of 10 min. Sections were drained and exposed to varying dilutions (1:10–1:1000) of monoclonal antibody (stock protein = 1 mg/ml) overnight at 4° in a humidified chamber.

Sections were washed 3 × 5 min in PBS prior to incubation with the secondary antibody, RAM IgG conjugated to horseradish peroxidase diluted in PBS containing 5% normal rat serum (1:200 dilution), for 90 min at room temperature. Sections were washed as before and specific antigen–antibody complexes were then visualised by development in 0.025% 3,3-diaminobenzidine tetrahydrochloride in PBS containing 0.03% hydrogen peroxide for 20 min. Sections were counterstained with Cole's haematoxylin, dehydrated and mounted in Pertex.

Immunogold-silver staining (IGSS) of sections. Immunogold-silver staining is a highly sensitive technique particularly suitable for detection of extremely low levels of antigens [17]. The level of enhancement in sensitivity of this technique over immunoperoxidase detection was estimated in liver sections from rats pre-treated with PB before using this system to check those extrahepatic tissues in which levels of antigen appeared to be very low. The IGSS technique improved the limit of detection by 10–20-fold.

Staining was carried out as previously described [18] with exclusion of trypsin treatment of the sections. Slides were transferred to silver development solution [19] and incubated in the dark for 4–6 min. After washing in photographic fixer, diluted 1:4, for 1–2 min and running tap water for 10 min, sections were counterstained as above.

Tests of specificity of staining. Negative controls included replacement of the primary antibody with diluent alone, serum or MAb 107, which does not cross-react with rat cytochromes P450 [20]. This antibody is specific to rabbit cytochrome P450, form 4. In the IGSS technique, in addition, the second antibody, GAM IgG conjugated to 5 nm colloidal gold, was replaced by diluent to check that the silver enhancement solution did not itself react with the tissue.

Positive control incubations were also performed using 3/4/2, a monoclonal antibody specific, in the rat, to the polycyclic-hydrocarbon inducible isoenzyme of cytochrome P450, form c [13]. The staining characteristics of this antibody in rat tissues have been described previously [21].

The inducibility of cytochrome P450 in each tissue examined was assessed semi-quantitatively from the changes in the staining intensities of the sections when the monoclonal antibodies were used at varying dilutions, i.e. induction of cytochrome P450 in a tissue results in binding of the monoclonal antibody at lower concentrations (higher dilutions) than in the tissues of untreated animals. A minimum of five sections for each animal were examined, there being three animals per group. At no time were inter-individual differences in staining within a group greater than those seen between groups.

RESULTS

Specificity of monoclonal antibodies 10/1 *and* 1/4

The specificity of the purified MAbs was first determined by the enzyme linked immunoassay (e.l.i.s.a.) technique. Both antibodies reacted strongly with the homologous antigen, cytochrome P450b, and with the microsomal fraction from PB-treated rats (Table 1). Neither antibody reacted with P450c. Immunotitration revealed that 10/1 had a higher affinity (by ≈10-fold) than 1/4 for its antigen (data not shown). The reaction of the MAbs with microsomal proteins was further investigated by the technique of Western blotting. Both antibodies recognized a single protein band of approximate M_r 52,000 in microsomes prepared from rats pre-treated with PB, ACT and ISF, which corresponded to the band recognized in an apparently homogeneous preparation of P450b. No bands were detected in microsomes from untreated rats or from rats pre-treated with any of the other inducing agents or in hepatic microsomes from other mammalian species (Fig. 1).

Table 1. Reactivity of MAbs with their homologous antigen determined by e.l.i.s.a. assay

MAb	Antigen	A_{492} nm
1/4	P450b	0.851 ± 0.039
	P450c	0.030
	Microsomes from PB-treated rat liver	0.707 ± 0.018
10/1	P450b	0.980 ± 0.135
	P450c	0.040
	Microsomes from PB-treated rat liver	0.775 ± 0.075
	PBS	0.027 ± 0.011

Results are mean ± SD, where shown, of at least two determinations.

Liver

An optimal fixation protocol was established in preliminary experiments in which the reactivities of 1/4 and 10/1 in unfixed frozen tissue sections were compared to those in sections which had been fixed with methanol (5 min), acetone (15 min), methanol–acetone, 1:1 (v/v) (5 min) or 0.5% *p*-benzoquinone (5 min). Sections fixed in acetone showed the best retention of antigenicity, comparing favourably in staining characteristics to unfixed tissue. Methanol abolished all antigenicity, whilst *p*-benzoquinone markedly reduced the intensity of staining (data not shown). Acetone fixation was therefore used in all subsequent studies with these antibodies.

In liver from untreated animals, specific immunostaining was detected with both 1/4 and 10/1, but only when the antibodies were used at dilutions of less than 1:50 (Table 2). Immunohistochemical staining for cytochrome P450b/e was most intense in a few, single, randomly distributed hepatocytes radiating out from the central vein over a short distance, of not more than 3–4 cell rows (Fig. 2a). A very weak immunoreaction was detected in hepatocytes throughout the rest of the lobule. No staining of the bile duct epithelium was evident. The immunoreaction product was diffuse, appearing as a homogeneous stain uniformly dispersed across the hepatocytes. Control sections stained with 107 showed no specific staining (Fig. 2b).

Immunohistochemical staining for cytochrome P450b/e following pre-treatment of rats with PB was apparent at antibody dilutions as high as 1:1000 with 10/1 when using indirect immunoperoxidase

Fig. 1. Western blotting of microsomal fractions from rat liver with MAb 10/1. Microsomal proteins were separated by SDS–PAGE and then subjected to Western blotting as described in Materials and Methods. Hepatic microsomal fractions (20 μg protein per well) were as follows; lane 1, untreated rat; lane 2, PB-treated rat; lane 3, MC-treated rat; lane 4, ACT-treated rat; lane 5, PCN-treated rat; lane 6, INH-treated rat; lane 7, ISF-treated rat; lane 8, CL-treated rat; lane 9, human; lane 10, purified P450b, 10 pmoles; lane 11, untreated Balb/c mouse; lane 12, untreated New Zealand white rabbit; lane 13, untreated Duncan Hartley guinea-pig and lane 14, untreated Syrian hamster.

detection and greater than 1 : 10,000 with IGSS detection (Table 2). Thus, silver enhancement increased the detection limit of the indirect two stage technique by approximately 10–20-fold in frozen sections. In contrast to untreated rats, following treatment with PB all of the centrilobular cells were intensely stained and, in some areas, staining also extended marginally into midzonal regions (Fig. 3a). The distribution of staining was the same with the two methods of detection. However, whereas the immunoperoxidase stain appeared homogeneous, the degree of silver precipitation varied within individual cells, as appar-

Table 2. Immunotitration of the binding of MAbs, 1/4 and 10/1, to liver from untreated and xenobiotic-treated rats

		MAb Dilution				
Treatment	MAb	1:10	1:100	1:500	1:2000*	1:10,000*
None	1/4	++	+	−	−	−
	10/1	++	+	−	−	−
PB	1/4	+++	+++	++	++	++
	10/1	+++	+++	++	+++	+++
MC	1/4	+	−	−	nd	nd
	10/1	+	−	−	nd	nd

* Immunodetection by immunogold-silver staining (IGSS). Degree of staining: +++ intense; ++ moderate; + weak; − none. nd: not determined.

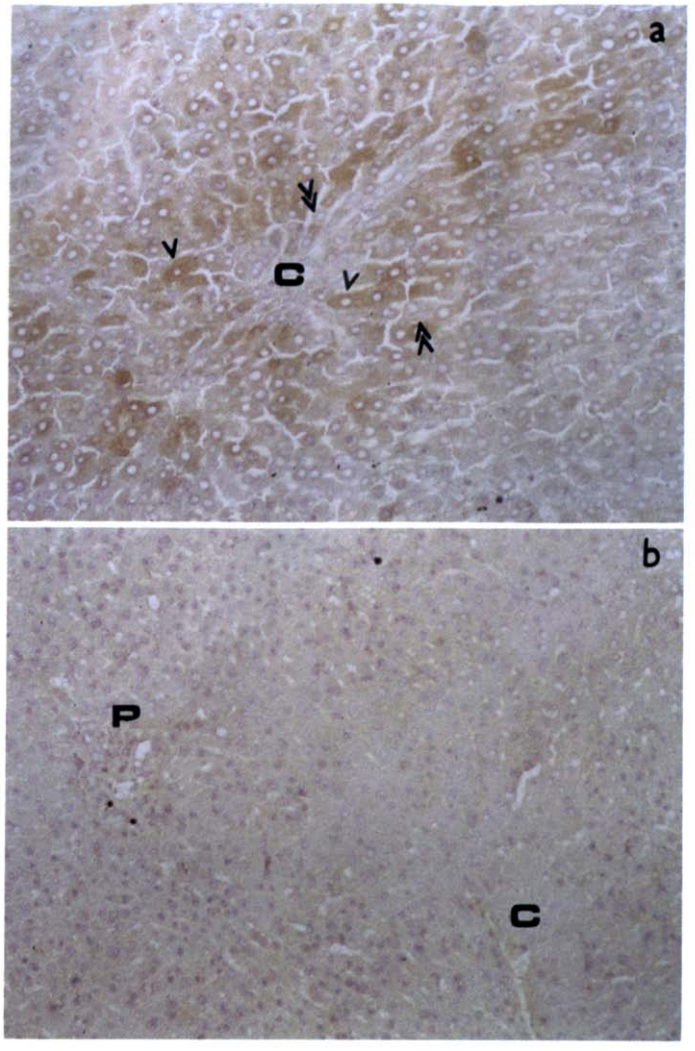


Fig. 2. Immunocytochemical localization of cytochrome P450b/e in livers from untreated rats. (a) Section incubated with MAb 1/4 at a dilution of 1:10. Note intense staining of some centrilobular (C) cells (>), whilst other cells are negative (>>). (b) Section incubated with control MAb 107 at the same dilution as MAb 1/4. No specific staining is evident. P—Periportal. Magnification: 135x.

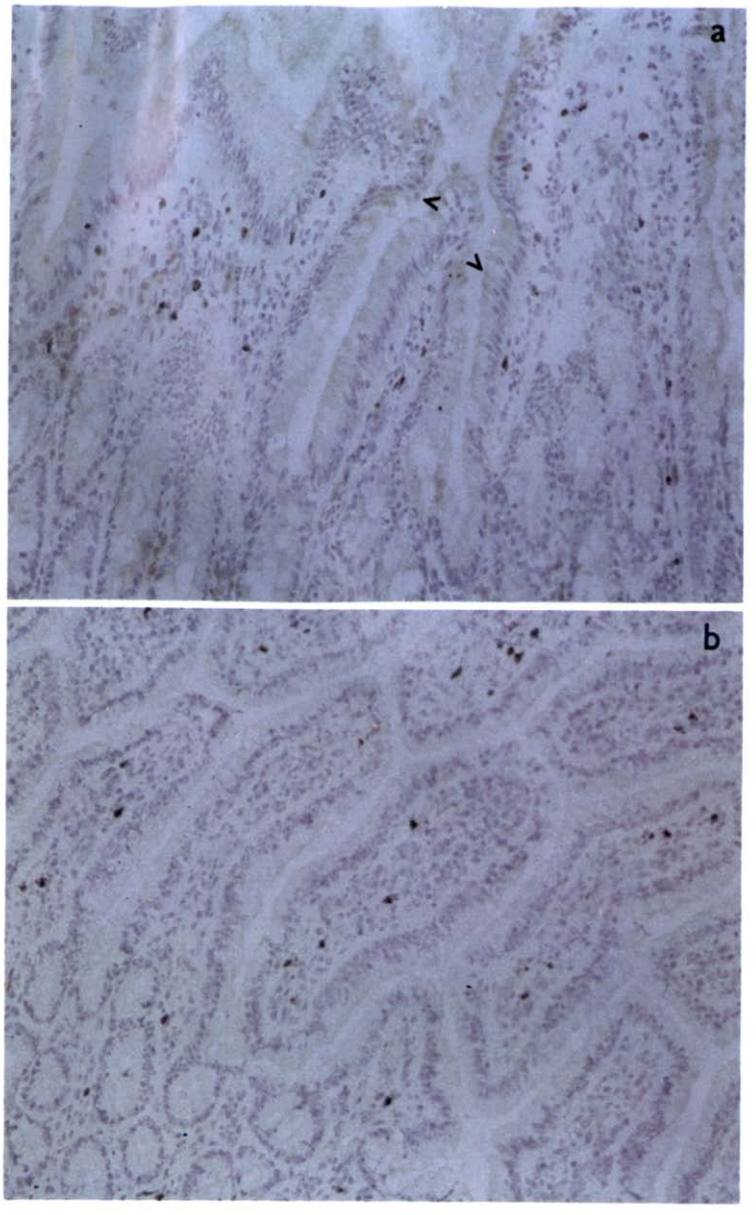


Fig. 6. Immunocytochemical localization of cytochrome P450b/e in the small intestine of untreated and PB-treated rats. Sections were incubated with either MAb 1/4 (a,c,e,g) or with control MAb 107 (b,d,f,h) at a dilution of 1:10. (a–d) Sections from untreated rats. Specific staining, (a,c) is stronger at the tips of the villi and appears to be more intense at the apical side of epithelial cells (>). (e–h) Sections from PB-treated rats. An intense immunoreaction (e,g) is apparent along the length of the villi (>), but this is slightly weaker in cells of the crypts of Lieberkuhn (> >).

MAb 107 produced negligible staining of sections from either untreated (b,d) or PB-treated (f,h) rats.

Non-specific staining of macrophages (m) is evident in sections incubated with either MAb 1/4 or MAb 107. Magnification: (a,b,e,f) 200×; (c,d,g,h) 500×.

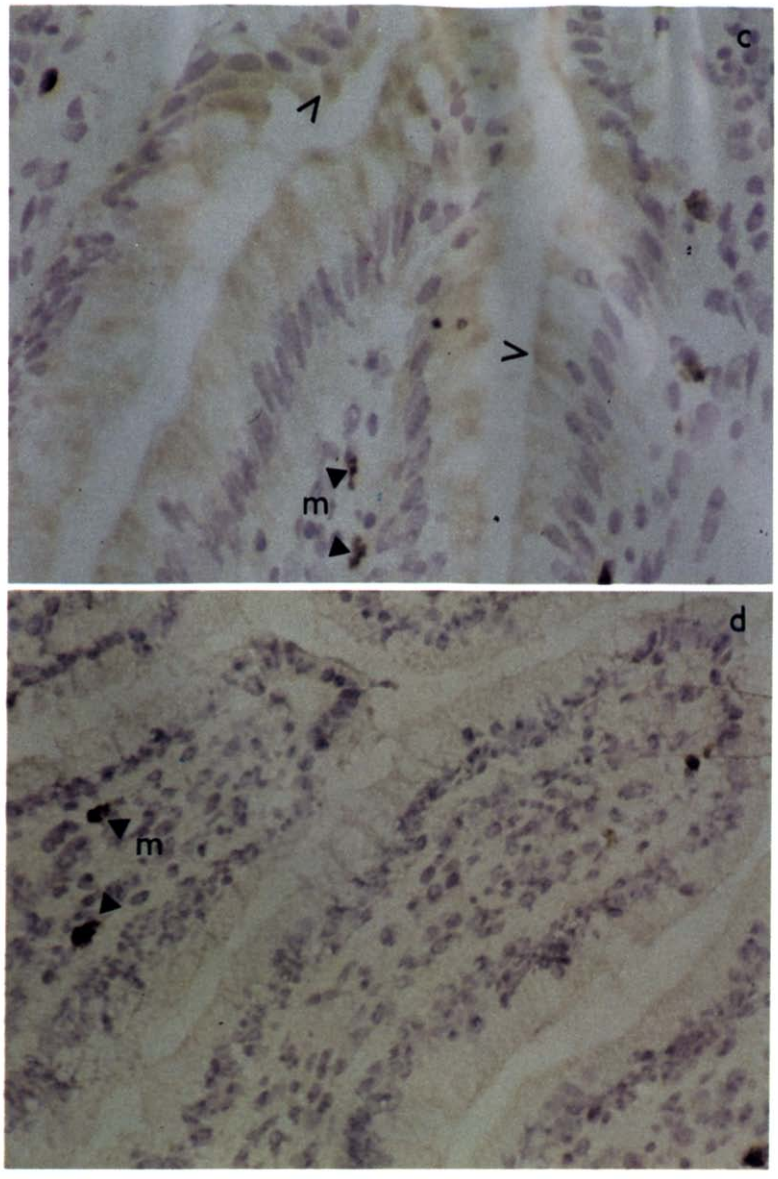
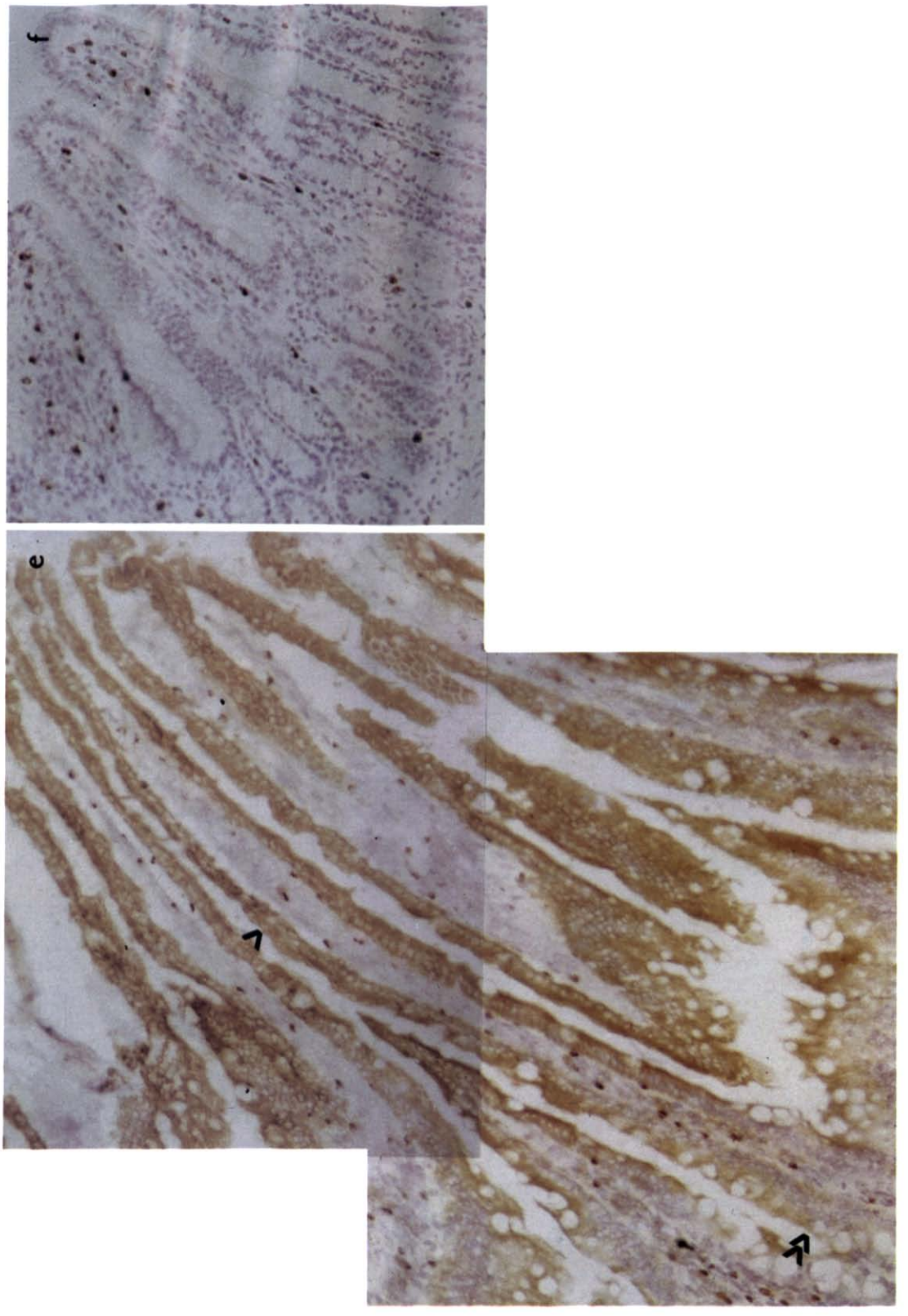


Fig. 6. Continued.



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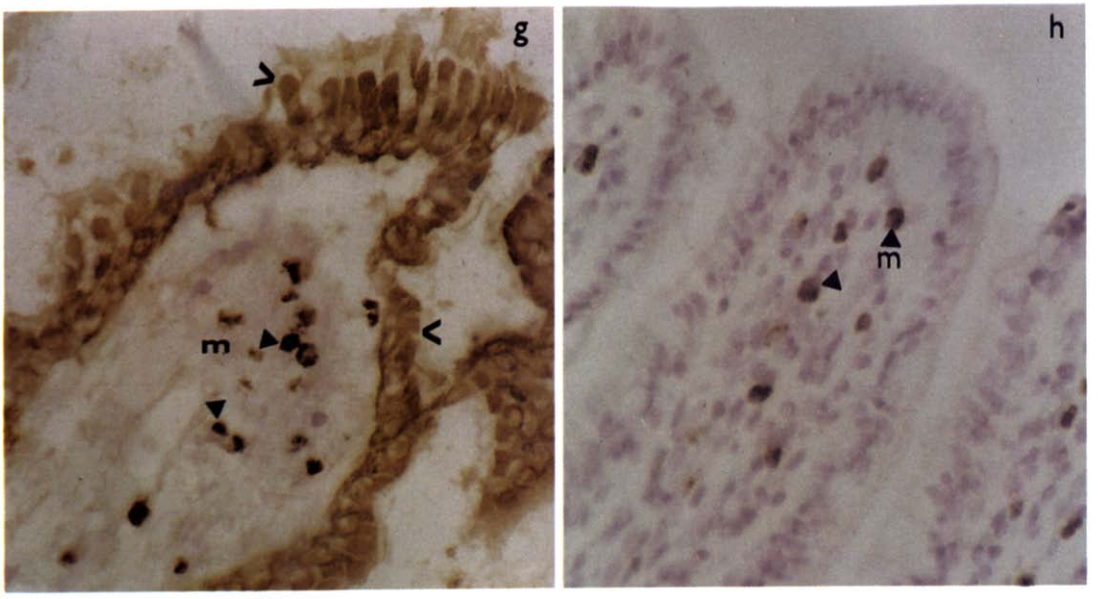

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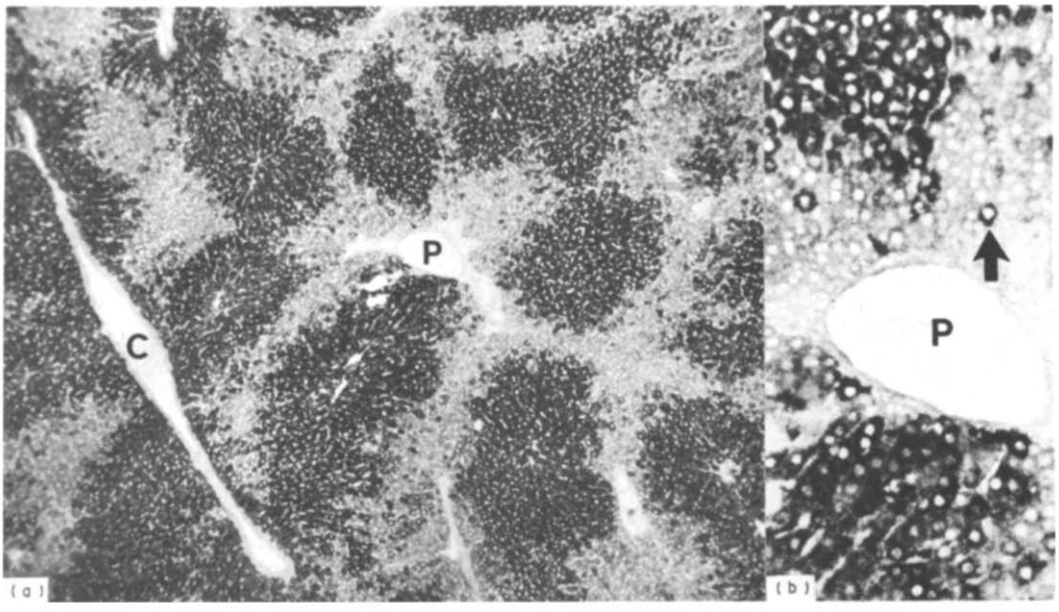

Fig. 3. Immunocytochemical localization of cytochrome P450 in liver of rats treated with PB using 1:10,000 dilution of MAb 10/1. Specific immunoreaction was detected using GAM conjugated to 5 nm colloidal gold as the secondary antibody and the signal enhanced with silver enhancement solution. (a) Specific staining extends radially from central veins (C), magnification 52×. (b) Some periportal (P) cells were positive (→), magnification 180×.

ent when viewed at higher magnification. The cytoplasm within a few cells showed a heavy even reaction, whereas in others the distribution of precipitate was irregular. The nucleus of some cells was surrounded by a halo of black silver deposit and some grains could also be seen sitting over the nucleus. At these higher magnifications, the presence of a specific immunoreaction was also apparent in scattered, single hepatocytes in the periportal region (Fig. 3b). Replacement of the anti-P450b/e antibodies with 107 resulted in a total lack of staining of the liver sections (data not shown).

Following treatment of rats with MC, a weak immunoreaction was apparent only at antibody dilutions of 1:10. Staining intensity was less than with sections from untreated rats (Table 2).

Extrahepatic tissues

Kidney sections from untreated and PB-treated rats exposed to either 1/4 or 10/1 showed no immunostaining at antibody dilutions as low as 1:10 (Table 3). The possibility that this was due to insensitivity of the second antibody was excluded by the use of IGSS which, in liver, had previously increased the limit of detection by 10–20-fold. Despite this increase in sensitivity, no immunoreactive product above background staining could be detected, even after PB treatment of the animals (Fig. 4a). Only with MAb 3/4/2, used as a positive control in these experiments, was staining apparent, which was similar to that previously described [21], with cytochrome P450 localized in cells of the S_3 portion of the proximal convoluted tubule (Fig. 4b).

In sections of lung from untreated rats immunoreactive cytochrome P450 was clearly evident in the bronchial epithelial cells and in the non-ciliated bronchiolar epithelial, or Clara, cells. These cells were readily identifiable by their bulbous, domed shape. Although the intensity of the immunoreaction product varied amongst Clara cells it was always greater in the apical than in the basal cytoplasm (Fig. 5a). No staining was apparent in adjacent ciliated cells, even at low dilutions of antibody. The distribution of cytochrome P450b/e in the lung was not changed by treatment of the animals with PB, nor was there any apparent induction, the reaction product being clearly visible in sections from both untreated and treated animals, at antibody dilutions greater than

Table 3. Immunotitration of binding of MAbs, 1/4, 10/1 and 3/4/2, to kidney from untreated and xenobiotic-treated rats

		MAb Dilution		
Treatment	MAb	1:10	1:50	1:100
None	1/4	–	–	–
	10/1	–	–	–
	3/4/2	++	+/–	–
PB	1/4	–	–	–
	10/1	–	–	–
	3/4/2	++	+/–	–
MC	1/4	–	–	–
	10/1	–	–	–
	3/4/2	+++	++	++

Degree of staining: +++ intense; ++ moderate; + weak; +/– some weakly positive areas; – none.

Table 4. Immunotitration of the binding of MAbs, 1/4, 10/1 and 3/4/2, to lung from untreated rats and rats treated with PB

Treatment	MAb	MAb Dilution 1:10	1:50	1:100	1:1000	1:10,000	1:50,000
None	1/4	+++	+++	+++	+++	++	++
	10/1	+++	+++	+++	+++	++	++
	3/4/2	++	++/+	+/−	−	nd	nd
PB	1/4	+++	+++	+++	+++/++	++	++
	10/1	+++	+++	+++	+++/++	++	++
	3/4/2	++	++/+	+/−	−	nd	nd

Degree of staining: +++ intense; ++ moderate; + weak; +/− some weakly positive areas; − none. nd: not determined.

1:10,000 (Table 4). Due to the limitations of the techniques employed, resolution of other specific pulmonary cell types, particularly in alveolar walls, was difficult. Furthermore, to preserve the integrity of the sections, endogenous peroxidase was not blocked by pre-incubation in H_2O_2. Consequently, there was a relatively high background staining, particularly in the connective tissues of blood vessels and alveolar walls. Hence, it was not possible in these experiments to determine the extent to which the antibodies may have preferentially bound to cell types within these regions. In contrast, 3/4/2 produced only a moderate immunoreaction in the same cell types at antibody dilutions <1:50 (Table 4). No specific staining was apparent in Clara or other cells when the MAbs were replaced with 107 as a negative control (Fig. 5b).

A very weak, specific immunoreaction was observed in enterocytes in sections cut from the duodenal area of the small intestine, with a gradient of staining from the crypts to the tips of the villi (Fig. 6a and c). This pattern of staining was maintained, but with a greatly intensified signal, when the rats were pre-treated with PB (Fig. 6e and g). Both the cells lining the length of the villi and those within the crypts of Lieberkuhn were positively stained. Whereas staining was apparent in sections from untreated animals with antibody dilutions only of 1:10, following PB treatment immunoreactive protein was detectable at MAb dilutions as high as 1:200, indicating considerable induction of this isoenzyme (Table 5). The Paneth cells did not appear to be stained in sections from either untreated or PB-treated animals, with either of the antibodies used. Appropriate control sections treated with MAb 107 showed no staining of any cells (Fig. 6b, d, f, h).

No specific staining was apparent in sections of pancreas (Fig. 7a) or testis (Fig. 7b) from rats either before or after treatment with PB.

Levels of cytochrome P450b/e in liver and extrahepatic tissues of untreated animals are very low or absent. Indeed, no band corresponding to P450b/e could be detected in Western blots with 10/1 or 1/4 of microsomes from kidney, lung or gut (data not shown). Therefore, in an attempt to quantify the immunocytochemical observations, the method of dot blotting, where unseparated proteins were applied directly to nitrocellulose membranes, was used. In agreement with the immunocytochemical data, constitutive levels of P450b/e in the liver were found to represent only a minor fraction (<6%) of the total spectrally determined cytochrome P450. These levels increased >40-fold upon PB treatment (Table 6). Further, no cytochrome P450b/e could be detected in microsomes from kidney of either untreated rats or rats pre-treated with PB (Table 6). Constitutive levels of this isoenzyme in lung microsomes were very low, although they did represent approximately 50% of the total cytochrome P450 pool, and were refractive to PB-treatment. In contrast, the barely detectable levels of immunoreactive protein in microsomes from small intestine of untreated rats were increased six-fold by PB-treatment (Table 6). This was reflected as only a doubling of the total cytochrome P450 pool.

DISCUSSION

The tissue distribution of cytochrome P450b/e has been determined by immunocytochemistry utilizing two monoclonal antibodies raised against the major PB-inducible isoenzyme(s) of cytochrome P450. It

Table 5. Immunotitration of the binding of MAbs, 1/4 and 10/1, to small intestine from untreated rats and rats treated with PB

Treatment	MAb	MAb Dilution 1:10	1:50	1:200
None	1/4	+/−	−	−
	10/1	+	−	−
PB	1/4	+++	++	++
	10/1	+++	+++	++

Degree of staining: +++ intense; ++ moderate; + weak; +/− some weakly positive areas; − none.

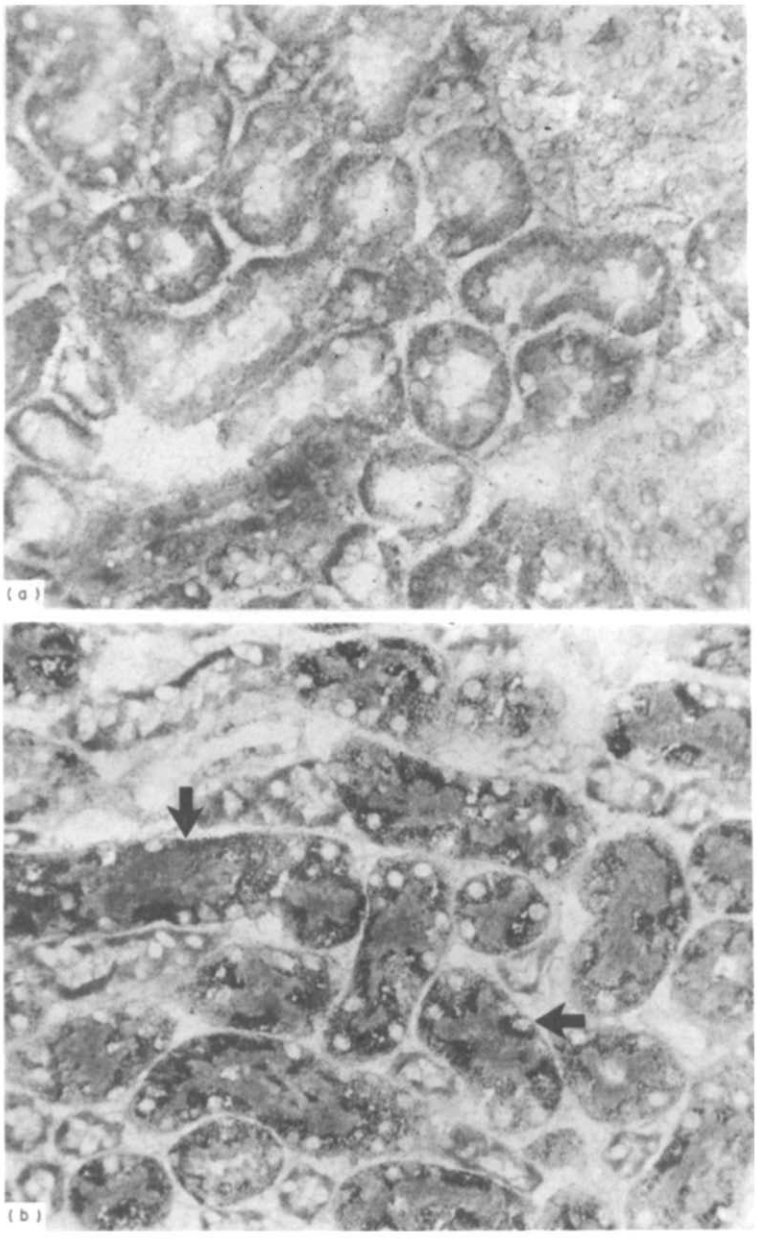

Fig. 4. (a) A kidney section from rat treated with PB, incubated with MAb 10/1 at low dilution (1:10). No immunoreactive P450b/e is apparent. (b) A section from the same kidney incubated with MAb 3/4/2 at a dilution of 1:10 shows specific immunoreaction in proximal tubule cells (→). Magnification 370×.

was shown by Western blotting (under conditions such that P450b is resolved from P450e) that these antibodies react with only a single protein band in hepatic microsomes from rats treated with PB, with the same molecular weight as that of purified cytochrome P450b. However, at this stage, we cannot exclude the possibility that these antibodies react with P450e, although such binding would have to be with markedly lower affinity than to P450b. Although a low affinity interaction may not be apparent in Western blots, it might be detected under conditions, such as those used here in immunocytochemistry, where the protein is subject to less severe denaturing treatment. In fact, screening various fixatives for their effect on the reactivities of 1/4 and 10/1 in immunocytochemistry revealed that the epitope to which these MAbs bind is particularly susceptible to relatively mild denaturing treatments. The procedures used in immunoblotting lead to unfolding of the protein and this may well reduce its antigenicity.

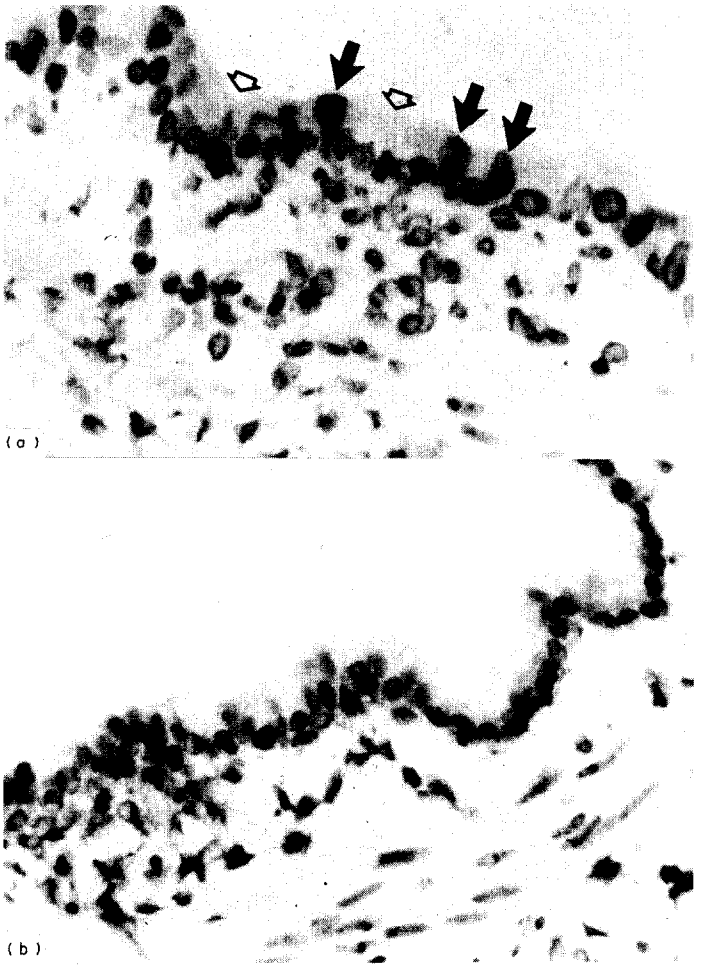

Fig. 5. (a) Immunocytochemical localization of cytochrome P450b/e in Clara cells (→) of the lung of a rat treated with PB, using MAb 1/4 at a dilution of 1:200. Staining is absent from ciliated cells (⇒). (b) A serial section of lung incubated with MAb 107. No staining is apparent with this antibody. Magnification 400×.

Table 6. Immunoquantification of P450b/e in liver and extrahepatic tissues of the rat by dot blotting as described in Materials and Methods

Tissue	Treatment	Total P450	Immunoreactive P450b/e
		pmol/mg protein	
Liver	None	755 ± 115 (6)	44 ± 6 (3)
	PB	1290 ± 258 (6)	1830* ± 316 (9)
Kidney	None	119 ± 5 (3)	bd (4)
	PB	102 ± 42 (3)	bd (4)
Lung	None	38 ± 7 (3)	24
	PB	40 ± 3 (3)	21
Gut	None	46 ± 14 (3)	8 ± 2 (3)
	PB	84 ± 17 (4)	42* ± 10 (8)

Results are means ± SD, where shown, from at least two separate microsomal preparations. Numbers in parentheses indicate the number of determinations. bd: below detection.

* Significantly different from untreated animals at $P < 0.001$.

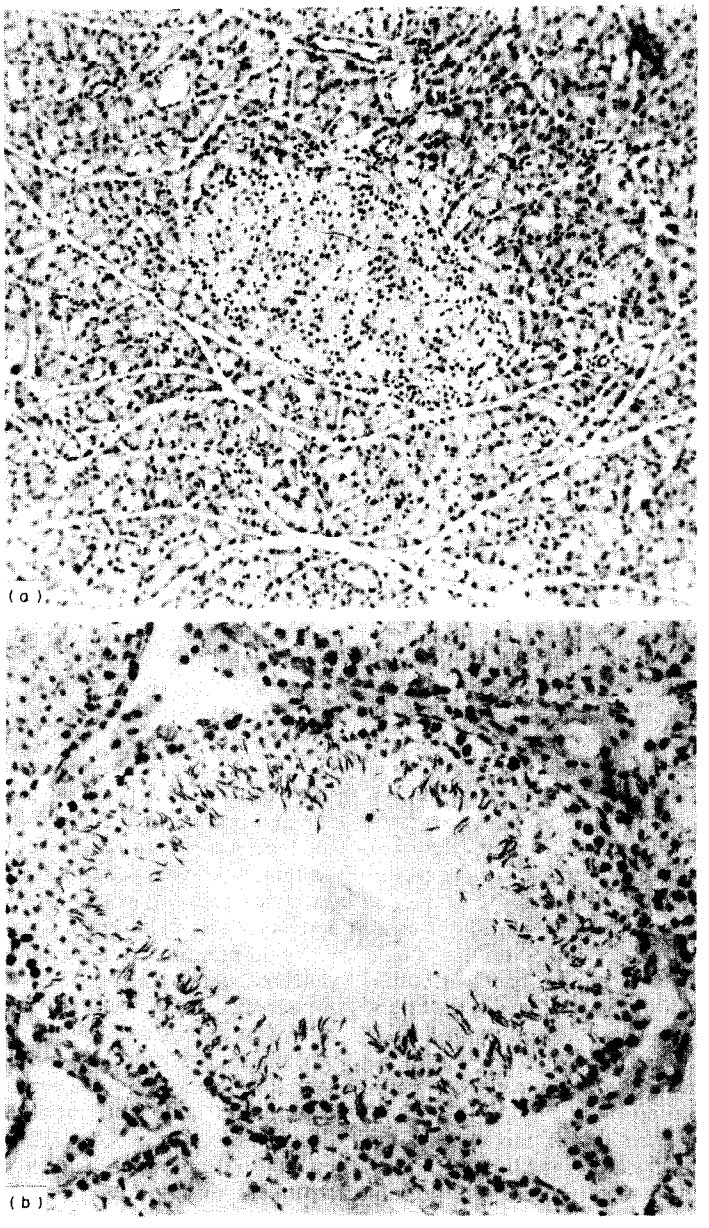

Fig. 7. Section of (a) pancreas and (b) testis of rat treated with PB exposed to MAb 10/1 at a dilution of 1:10. No specific immunoreaction was apparent in either tissue. Magnification 160× and 250×, respectively.

It is of interest that, to date, all antibodies (polyclonal or monoclonal) raised to individual PB-inducible holoenzymes, either P450b or P450e, cross-react with both forms and also in some instances, with variant forms of P450b, if not in Western blots, in reconstituted enzyme systems to produce inhibition of catalysis [22–25]. This has been attributed to the very high homology (>97%) between these isoenzymes in their amino acid sequences. However, a monoclonal antibody against P450b has been described that recognises P450e only under certain conditions, and even then with much reduced affinity [24]. It is possible that 10/1 and 1/4 also react with a modified epitope on P450e. Results of dot blotting with the pure proteins support this suggestion (unpublished data). For this reason, in the present study, specific immunocytochemical staining has not been assumed to reflect interaction of MAbs with only P450b but possibly also with its variant forms and P450e. Such binding has been referred to as with P450b/e. Recent studies have shown that in the liver of untreated rats the content of P450e greatly exceeds that of P450b, whereas P450b exceeds P450e in lung [5].

The staining of cytochrome P450b/e in livers from untreated rats is very weak, and is concentrated largely within a few cells scattered around the central vein. This finding is in strong contrast to previous reports. The extent of binding with a polyclonal antibody in hepatic sections from Sprague–Dawley rats, although greatest in the centrilobular region, was relatively moderate throughout the entire lobule [26]. In a similar study in Wistar rats, again with a polyclonal antibody, staining was evenly distributed, at a low level, throughout the liver, with no evidence of zonal localization [27]. Although these differences could be due, in part, to strain differences (Sprague–Dawley compared to Wistar) some other factor must also be involved. It is possible that differences in reactivity profile of the antibodies contribute to these findings.

Following treatment of rats with phenobarbitone, the centrilobular localization of P450b/e was similar to that described by others, also using Wistar rats [7, 27, 28]. These results show that not only does non-homogeneity of the hepatocyte population exist with respect to expression of P450b/e between zones, but also amongst individual cells within a zone, as emphasized by the staining pattern of hepatic sections from untreated rats. Furthermore, although not all cells within the centrilobular zone express cytochrome P450b/e at detectable levels, they all respond to PB-treatment of the rats with increased synthesis of P450b/e. In contrast, within the periportal region only the odd cell shows induction. Thus, the extent to which individual hepatocytes respond to induction varies. This suggests the presence of intercellular differences in the mechanisms for regulating both the expression and inducibility of P450b/e. Whether this is at the level of different isoenzyme variants or in the amount of a single isoenzyme has yet to be determined. The distribution of staining within individual hepatocytes is consistent with an endoplasmic reticular localization of P450b/e. However, the exact distribution of antigen will be detemined only when studies at the electron microscope level are performed.

Initial reports suggested that induction of P450 isoenzymes by PB is restricted to the liver [8]. However, a recent study [5], together with the present work, have demonstrated the differential inducibility of P450b/e by PB in extrahepatic tissues in a tissue-specific manner. Constitutive levels of cytochrome P450 in the small intestine are very low but detectable, with P450b/e increasing from the crypts to the tips of the villi. Following PB-treatment of rats, staining of P450b/e in the small intestine with 1/4 and 10/1 is considerably increased. These findings, to our knowledge the first time that the cellular localization of P450b/e has been demonstrated in the small intestine, corroborate previous work by Bonkovsky and co-workers, who quantified levels of cytochrome P450b/e in microsomes prepared from cells isolated from upper villus, lower villus and crypts, and found that levels increase in the cells as they mature [29]. This gradient of P450b/e is maintained following PB-treatment of the rats and is not related to the route of administration of the inducer, as no differences were found in the pattern or intensity of staining when PB was administered either intraperitoneally or orally at 0.1% in the drinking water for 7 days (data not shown). Interestingly, there have been reports of cytochrome P450-dependent monooxygenases in microsomes from small intestinal mucosa which are inducible by PB treatment and which, after PB-induction, are inhibited (>70%) by an anti-P450b/e monoclonal antibody, 2-66-3 [5, 30]. There is intense staining of macrophages in sections of intestine from both control and PB-treated animals. However, this is presumably due to high residual levels of peroxidase, as similar staining is observed with the control MAb 107 and when only the second antibody is used.

Cytochrome P450b/e is strongly expressed in the Clara cells of the lung of untreated rats, as indicated by the persistently strong immunoreaction, even at MAb dilutions lower than 1:10,000. This contrasts with the low expression of P450b/e in hepatocytes of untreated animals, where specific staining is lost at low MAb titres (<1:100). The specific content of constitutive cytochrome P450b/e in liver and lung is approximately the same but whereas this reflects expression in many hepatocytes, there are only a limited number of Clara cells. The localization of cytochrome P450b/e found in the lung is in agreement with that found by other groups [26, 31, 32]. However, the presence of this isoenzyme in additional pulmonary cells, such as type II pneumocytes, as has been reported for both rat and rabbit [31–33], cannot be excluded as it is notoriously difficult to differentiate between alveolar cell types at the light microscopic level. This is particularly true in the present study since the preservation of immunoreactivity depended upon the use of mild fixation (acetone), which was achieved at a reduction in resolution of cell types.

No immunoreactive cytochrome P450 could be detected with either 1/4 or 10/1 in kidney from Wistar rats, even after PB-treatment, a finding supported by recent data which show that renal monooxygenase activity of PB-treated rats could not be inhibited by a monoclonal antibody which recognises all of the PB-inducible isoenzymes [30]. In addition, no expression of mRNAs coding for P450b and P450e could be demonstrated in kidney, even after PB-treatment, using highly specific 18mer synthetic oligodeoxyribonucleotide hybridization probes [10]. In contradiction, however, Shepherd and co-workers have reported the presence of mRNA to cytochrome P450b in kidney [11]. Further, one laboratory has reported the presence of very low, but detectable levels of immunoreactive cytochrome P450b/e in kidney, but only three of six groups of Sprague–Dawley rats treated with PB were positive [5]. The inducibility of isoenzymes (b, b_2 and e) by PB in the liver differs amongst rat strains [25], and the same may be true for extrahepatic organs, which could account for the discrepancies existing amongst these reports. An additional factor that will tend to confound comparison between laboratories is the reactivity profile of the antibody used. In some studies, a polyclonal antibody that would react with all of the closely related PB-inducible isoenzymes, was used, whilst in others, including that described here, more specific monoclonal antibodies were employed.

Comparatively little is known about the expression

or inducibility of cytochrome P450b/e in pancreas or testis, although immunoreactive P450b/e has been demonstrated in acinar cells of the pancreas with a polyclonal antibody [26]. In contrast, using highly purified MAbs, no cytochrome P450b/e could be detected in either of these organs, either before or following PB treatment.

Using these highly specific monoclonal antibodies, which do not cross react with other proteins, marked intra- and inter-tissue differences in the expression and inducibility of the major PB-inducible form(s) of cytochrome P450 were demonstrated. Although the presence of immunoreactive cytochrome P450 cannot be directly equated with monooxygenase activity, these results may explain, at least in part, the tissue and cell-specific toxicity of compounds activated by this isoenzyme.

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