Biochemical Pharmacology, Vol. 37, No. 24, pp. 4735–4741, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

MONOCLONAL ANTIBODY DIRECTED DETECTION OF CYTOCHROME P-450 (PCN) IN HUMAN FETAL LIVER

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(*Received* 24 *February* 1988; *accepted* 19 *July* 1988)

Abstract—Monoclonal antibodies (MAbs) raised to rat liver cytochrome P-450s induced by phenobarbital, 3-methylcholanthrene, and pregnenolone-16α-carbonitrile were used to detect these epitope specific P-450s in human abortion fetuses 14–24 weeks of age. This was performed using a Western blot technique. In parallel, ECOD was determined in the same tissue specimens.

Of seven different MAbs used MAb PCN 2-13-1/C2 was the only one that immunodetected a cytochrome P-450 band with Western blot analyses of human fetal liver microsomes. This band was consistently detected in all fetal liver specimens studied although the intensity varied among samples. No bands were detected in microsomal preparations from adrenal and renal tissues obtained from the same fetuses. The human adult liver microsomal specimens also contained a MAb PCN 2-13-1/C2 identified cytochrome P-450 band.

ECOD activity was detected in all but one of the human fetal livers and varied between 0.22 and 47.5 pmol min^{-1} mg $protein^{-1}$, as compared to 113 to 489 pmol min^{-1} mg $protein^{-1}$ in human adult livers. In all of the fetuses except one the adrenal ECOD activity (0.63–37.0 pmol min^{-1} mg $protein^{-1}$) exceeded that in the liver. The renal ECOD activities were, however, low. The hepatic and adrenal ECOD activities correlated with each other ($r = 0.95$). Although the ECOD activity is a function of several different P-450s there was also a correlation ($r = 0.78$) between the ECOD activity and the MAb immunodetected protein band intensity in Western blots of human fetal liver microsomes.

The presence of a MAb PCN 2-13-1/C2 identified band in fetal liver microsomes may be indicative of a steroid-dependent effect in fetal life.

The ontogenic development of various drug oxidative pathways has been studied in the human fetal [1, 2] and adult liver [3, 4]. Only limited characterization of the responsible fetal cytochrome P-450 species has been made, partly because of the restricted supply of tissue material. The large family of cytochrome P-450s now encompasses more than 20 species in the rat [5, 6]. In human adult liver an increasing number of P-450 species have been described (see Guengerich *et al.* [7]) and in fetal liver, a cytochrome P-450 form (P-450 HFLa) has recently been purified and characterized by Kitada *et al.* [8].

Knowledge of the metabolic specificity and contribution of human cytochrome P-450s will help us to understand how the "chemical environment" including pharmacological treatment affects the metabolism, efficacy and toxicity of various drugs and xenobiotics. Watkins *et al.* [4] recently described a cytochrome P-450 species (HLp) in human liver microsomes that was recognized in "Western" blots by a monoclonal antibody (MAb) against rat liver cytochrome P-450 induced by the antiglucocorticoid pregnenolone-16-alpha-carbonitrile (P-450 PCN).

Enzyme inhibitory MAbs are a powerful tool for "reaction phenotyping", i.e. for determining the contribution of individual cytochrome P-450s to specific drug metabolic reactions [9]. The MAbs may also possess the capacity to bind to unique cytochrome P-450 epitopes and thus be used to detect cytochrome P-450s in "Western" blots of microsomal proteins from various tissues and species. This cross-reactivity among species does not necessarily imply that identical or high structural homology exists. It indicates a common epitope and thus makes it possible, however, to study the human enzymes with MAbs that were raised against rat cytochrome P-450 [3, 10, 11, 12] or rabbit enzymes [4].

With the use of a library of MAbs towards different rat liver cytochrome P-450s we have investigated the existence of recognizable epitope specific cytochrome P-450s in microsomes from human fetal liver, kidney and adrenal as well as human adult liver specimens. In parallel we measured the catalytic activity of the microsomal preparations with ethoxycoumarin (EC) as substrate.

In this study we found that the MAb against PCN induced rat liver cytochrome P-450 recognizes a human liver protein in all fetal liver specimens investigated. This protein band was also detected in

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§ Abbreviations used: EC, 7-ethoxycoumarin; ECOD, 7-ethoxycoumarin-*O*-deethylase activity; DTT, dithiothreitol; kDa, kilodalton; MAb, monoclonal antibody; PCN, pregnenolone-16α-carbonitrile; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; HFLM, human fetal liver microsomes; HFAM, human fetal adrenal microsomes; HFRM, human fetal renal microsomes; HALM, human adult liver microsomes.

microsomes from human adult liver specimens. A number of monoclonal antibodies to other cytochrome P-450 species failed to detect their epitope specific P-450s.

MATERIALS AND METHODS

Chemicals. All reagents used for electrophoresis, transfer and immunodetection were purchased from Bio-Rad company, Vector Laboratories or Sigma (St. Louis, MO). Ethoxycoumarin and 7-hydroxycoumarin were purchased from Sigma (St. Louis, MO), beta-naphthoflavone from Sigma and phenobarbital from the hospital pharmacy. All other chemicals were of the highest grade available.

Preparation of monoclonal antibodies. Monoclonal antibodies (MAbs) were prepared by the hybridoma technique of Köhler and Milstein [13]. The details of their preparation and characterization have been described elsewhere [14–17].

MAbs towards the phenobarbital induced form of rat liver cytochrome P-450 were from the clones 4-29-5, 2-66-3 [15] and those raised against 3-methylcholanthrene induced rat liver cytochrome P-450 from clones 1-7-1 and 1-36-1 [14]. Two MAbs against pregnenolone-16-α-carbonitrile induced rat liver cytochrome P-450 were from clones 2-13-1 and 2-3-2 [17]. Finally, MAbs towards ethanol or acetone induced rat liver cytochrome P-450s were from clones 1-98-1 [16].

Biological material. Male Sprague–Dawley rats of about 200 g were induced as follows: phenobarbital 80 mg/kg/day for 3 days, betanaphthoflavone 40 mg/kg/day for 3 days or acetone 20% in the drinking water for 3 days. The rats were killed by cervical dislocation and the livers excised for microsomal preparation. Purified pregnenolone-16-α-carbonitrile induced rat liver cytochrome P-450 was a kind gift from Drs. J. W. de Pierre and A. Åström (Stockholm). This form is immunodetected by MAb PCN 2-13-1 and MAb PCN 2-3-2 as a rat isoenzyme form called PCN/PB.

Human fetal tissue specimens from 11 fetuses between 14 and 24 weeks of gestation were obtained at legal abortions made for socio-medical reasons. The abortions were performed by prostaglandin induction. The fetal tissues (liver, adrenals, kidneys) were excised usually within 30 min of the abortion and frozen at −70° until assay.

Human adult liver biopsy specimens (about 200 mg) were obtained at cholecystectomy after informed consent of the patients. In one case the liver was resected due to cholangiocarcinoma. The liver samples were frozen at −70° until assay.

Our study was approved by the Ethics Committee of the University Hospital.

Microsomal preparation. Microsomes from human and rat tissues were prepared by ultracentrifugation as described [18]. The microsomal pellets were resuspended in 50 mM Tris–HCl, pH 7.4, 0.25 M sucrose buffer, and stored at −70° until assay. Protein was measured according to Lowry *et al.* [19] using bovine serum albumin as a standard. The tissues were disrupted with an ultra-turrax Disintegrator T 18/10 KiKa 20000 rpm/min for 20 sec followed by sonication by Soniprep 150 for 30 sec twice (16 μm).

Enzyme assays. Ethoxycoumarin *O*-deethylase (ECOD) was measured according to Greenlee and Poland [20].

Immunoinhibition of ECOD was assayed as follows: microsomes were preincubated with the MAb for 30 min at room temperature. Subsequently the substrate and cofactors were added to the reaction mixture at 37°. The concentration of the MAb was 125 μg protein/ml incubate corresponding to an ascites protein: microsomal protein ratio of 1:2 according to previous experience [21]. Inhibition data are given as percentage of the control values without MAb or with non-specific MAbs present at the same concentration.

Electrophoresis and immunoblot techniques. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed with a discontinuous buffer according to Laemmli [22]. Ten per cent of polyacrylamide in 0.75 mm gels were used in a Miniprotean II dual slab cell Bio-Rad equipment. Protein samples were treated with a mixture containing 10 mM dithiothreitol (DTT), 1.8% sodium dodecyl sulphate (SDS), 45 mM Tris–HCl pH 8.8, 24% glycerol, 0.08 μg/ml bromophenol blue and boiled for 5–10 min. Alpha-iodoacetamide—an alkylating agent—was then added and allowed to stand for 25–30 min in order to complete the cleavage reaction of disulphide bridges. Ten microlitre samples containing 10 or 20 μg protein were applied to each well.

Transfer to nitrocellulose membranes was carried out over 4 hr at 4° in Mini-Trans-Blot cell (Bio-Rad equipment), according to Towbin *et al.* [23]. After transfer the nitrocellulose blots were placed in plastic bags with phosphate–saline-buffer pH 7.5 (PBS) with 10% newborn calf serum overnight. The following day immunodetection was carried out as follows: after two washes with PBS the nitrocellulose sheet was incubated 1.5 hr with the MAb in PBS containing 10% serum. Upon several washes with PBS the blots were then incubated 2 hr with the second antibody (goat anti-mouse–alkaline phosphatase conjugated antibody) from Bio-Rad. After several washes the colour was developed by 5-bromo-4-chloroindoxyl phosphate substrate and nitro-blue tetrazolium according to Blake *et al.* [24].

Gels and Western blots were scanned with an LKB laser light ultrascan XL equipment and the protein amount was estimated using the purified P-450 (PCN) as control. Previous experience showed a good correlation between the density values of the immunoblots and the protein amount. Molecular weights of the bands shown on SDS-PAGE were estimated by linear regression of the log standard molecular weight as a dependent variable versus the log migration rate according to Poduslo and Rodbard [25].

RESULTS

Human fetal tissues

Microsomal specimens of liver and adrenals and kidneys from eleven human fetuses were examined for ECOD activity and SDS-gel electrophoretic patterns.

The SDS-PAGE of total microsomal protein revealed two bands (M_r 52.7, 50.6) that were present

in all liver specimens. They are depicted together with the purified PCN-induced rat liver cytochrome P-450 (P-450-PCN) (M_r = 51.3) in Fig. 1a and b. There were at least 4–5 bands in the 48–57 kDa region in the liver microsomes. The adrenal microsomes did not contain any protein with the same migration characteristics (data not shown). The renal microsomes seemed to contain a weak band (M_r 52) at the position of P-450-PCN (M_r 51.3) (data not shown).

The microsomal proteins were analyzed with a panel of six monoclonal antibodies. Of these MAb PCN 2-13-1/C2 [17] bound to a human fetal liver protein species in all investigated microsomal preparations (Fig. 1, below). In contrast to the liver specimens, adrenal and renal microsomes did not contain any band that was recognized in Western blots by the MAb PCN 2-13-1/C2. The MAb PCN 2-3-2 recognizes a different epitope than PCN 2-13-1/C2 since it also has an affinity for the phenobarbital inducible cytochrome P-450 PB-4. This MAb did not bind to any band in Western blot analyses of fetal hepatic microsomes except for a single preparation of fetal liver microsomes (no. 1). The MAb PCN 2-3-2 was also unable to bind to any protein in blots of adrenal or renal microsomes.

The MAbs raised against phenobarbital (4-29-5, 2-66-3) or 3-methylcholanthrene (1-7-1, 1-36-1) induced rat cytochrome P-450 did not bind to any protein band in any of the fetal microsomal preparations.

All liver specimens (except no. 11) catalyzed the ECOD reaction at different rates (Table 1). The activity varied between 0.22 and 47.5 pmol $\times$ min^{-1} $\times$ mg protein^{-1}. The ECOD activities were compared with the densitometer scans of the MAb recognized bands in the Western immunoblots. There was a linear relation with a correlation coefficient of 0.78.

The adrenal microsomes from each fetus catalyzed the ECOD reaction at a considerably higher rate than the corresponding liver (0.6 to 37 pmol $\times$ min^{-1} $\times$ mg protein^{-1}) except in fetus no. 1. ECOD activities were detected in all renal microsomal preparations tested. The activity varied between 0.5 and 2.8 pmol $\times$ min^{-1} $\times$ mg protein^{-1}.

There was a correlation between the hepatic and adrenal enzyme activities. With the exclusion of fetus no. 1 (see above) the correlation coefficient was 0.95 (Fig. 2). No correlation existed between the liver and kidney enzyme activities.

Human adult tissues

Human adult liver specimens contained 5 bands in the 48–58 kDa region. One of these bands (M_r 51.2) co-migrated with the purified rat liver PCN cytochrome P-450 isoenzyme (M_r 52.2). This band was immunodetected by MAb PCN 2-13-1 in Western blots (Fig. 3). MAb PCN 2-3-2 and the other MAbs did not identify any protein band in the Western blot analyses.

The ECOD (Table 1) of adult liver was higher than that of fetal liver and varied between 112 to 489 pmol $\times$ min^{-1} $\times$ mg protein^{-1}.

Control MAb 1-48-5 and MAb Pb 2-66-3 had no significant inhibitory effect on ECOD of adult or fetal liver (Table 2). The MAb MC 1-7-1 however, inhibited the ECOD reaction by 30–40% in the fetal

Table 1. Ethoxycoumarin *O*-deethylase activity (ECOD) in microsomal preparations from human fetal and adult tissues*

Human specimen (No.)	Age (weeks of gest. or years)	Liver		Adrenal		Kidney	
Fetal samples							
Fetus no. 1	14 w	47.5 ± 0.57	(3)	9.4	(2)	0.5 ± 0.1	(3)
Fetus no. 2	18 w	18.0 ± 0	(3)	26.9 ± 1.6	(3)	1.13 ± 0	(3)
Fetus no. 3	19 w	16.2	(2)	37.0	(2)	1.14 ± 0	(3)
Fetus no. 4	18 w	12.8	(2)	26.7	(2)	2.8 ± 0.08	(3)
Fetus no. 5	24 w	3.85	(2)	10.5 ± 0.38	(3)	1.0 ± 0.2	(3)
Fetus no. 6	20 w	4.02	(2)	11.0	(2)	0.63 ± 0.1	(3)
Fetus no. 7	15 w	3.00	(2)	4.2 ± 0.13	(3)	—	
Fetus no. 8	15 w	1.30 ± 0	(3)	5.6 ± 0.13	(3)	—	
Fetus no. 9	18 w	0.58 ± 0	(3)	0.72 ± 0	(3)	—	
Fetus no. 10	21 w	0.22	(2)	0.63 ± 0.12	(3)	—	
Fetus no. 11	17 w	unmeasurable	(2)	1.9 ± 0	(3)	—	
Adult samples							
Patient no. 1a	59	489 ± 12.3	(4)				
Patient no. 1b	59	258 ± 8.5	(3)				
Patient no. 2	57	137.5	(2)				
Patient no. 3	43	163.3	(2)				
Patient no. 4	42	112.6 ± 4.1	(3)				
Patient no. 5	40	249.1	(2)				

* The incubate concentrations of the substrate and of NADPH were 0.5 and 1.0 mM, respectively. The incubation time was 45 min for human fetal microsomes and 30 min for human adult liver microsomes. The concentration of microsomal protein in the incubates varied from 0.2 to 1 mg with fetal microsomes and from 0.2 to 0.5 mg with human adult liver microsomes. The assay was performed under conditions of linearity with time and microsomal protein concentration. Enzyme activities were expressed as pmol $\times$ min^{-1} $\times$ mg protein^{-1} (metabolite formation) and the values represent the mean of duplicates or triplicate assays ± SEM. Numbers in parentheses indicate number of assays.

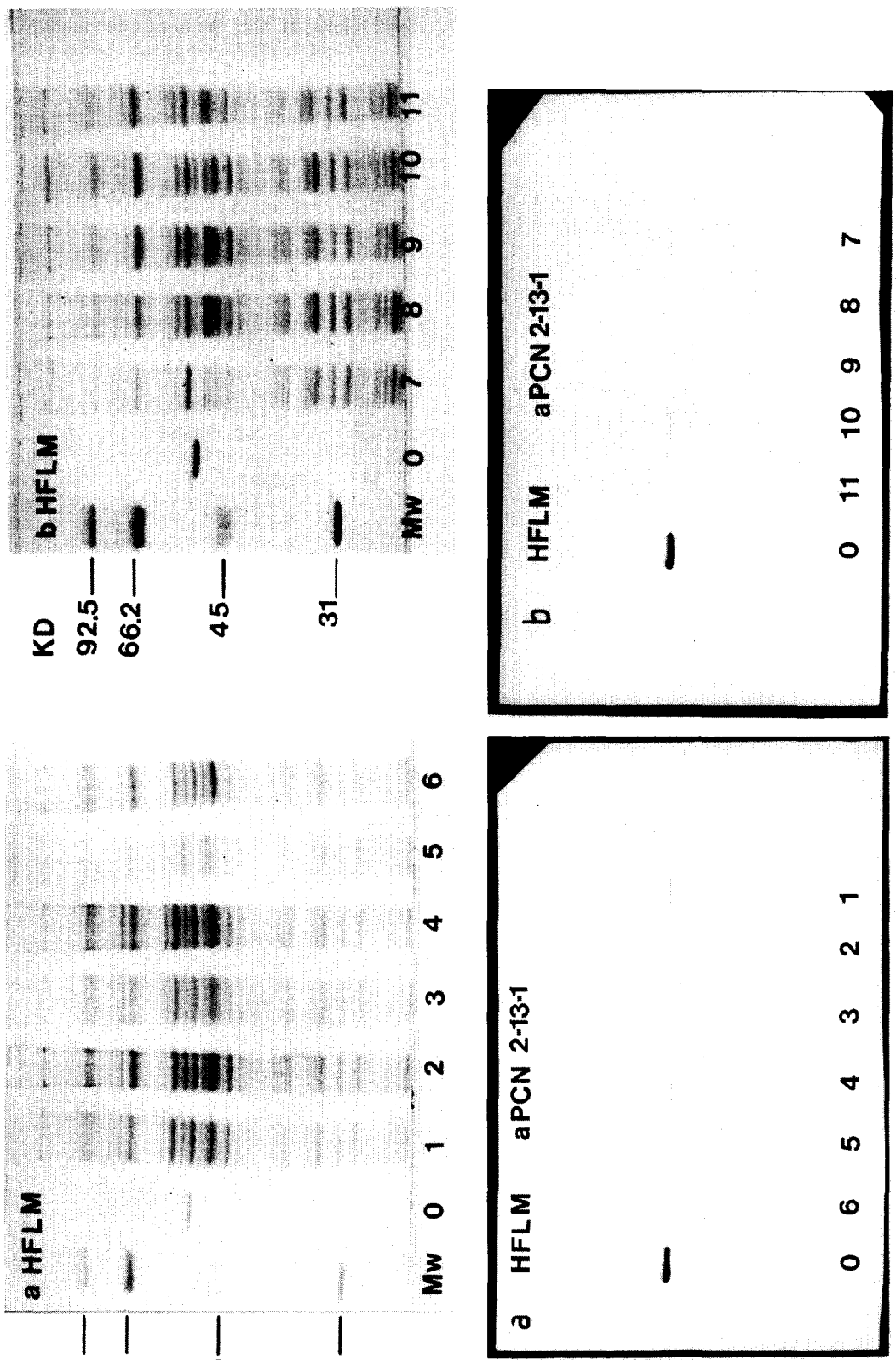

Fig. 1. SDS-gel electrophoresis patterns (above) with human fetal liver microsomal preparations—HFLM (a and b) from different fetuses (no. 1–11) and the corresponding immunoblots (below) with mouse anti-rat cytochrome P-450 (PCN) monoclonal antibody (MAb PCN 2-13-1). Liver microsomes contained a distinct protein band which migrates closely to the purified reference cytochrome P-450 from pregnenolone-16-alpha-carbonitrile induced rats (PCN, lane 0). Lane MW contains molecular weight standards.

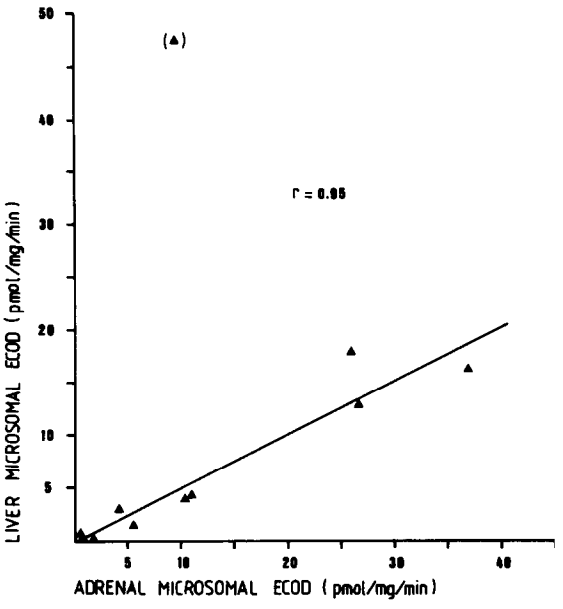

Fig. 2. Relation between ethoxycoumarin *O*-deethylase activity (ECOD) in liver and adrenal microsomes from 11 human fetuses. If fetus no. 1 is excluded (high extreme) there is a correlation of $r = 0.95$ between the ECOD activities in liver and adrenal microsomes.

liver specimens but had no inhibitory effect in adult liver. This is consistent with previous reports [21, 26]. MAb PCN 2-13-1 was not assayed because it is not inhibitory to ECOD in PCN induced rats [17].

DISCUSSION

Human fetal cytochrome P-450s have not been immunochemically characterized with the use of a library of MAbs. The reaction phenotyping we performed was with heterologous MAbs with in many cases known cross-reactivity with human enzymes and thus may give valuable information about the functional role of the identified P-450 species [3].

Table 2. Immunoinhibition of ethoxycoumarin *O*-deethylase (ECOD) in human fetal and adult liver microsomes by monoclonal antibodies (MAb) against phenobarbital or methylcholanthrene induced rat liver cytochromes P-450 (Pb 2-66-3), (MC 1-7-1), and unspecific monoclonal (1-48-5). The values are expressed as per cent of control (i.e. without MAb)

Specimen	NBS 1-48-5	Pb 2-66-3	MC 1-7-1
Human liver 1a	100 (2)	105 (2)	93 (2)
Fetus 1	88 (2)	72 (2)	61 (2)
Fetus 2	95 (2)	85 (2)	65 (2)
Fetus 3	98 (2)	95 (2)	59 (2)

Assay conditions: control ECOD activity was measured (in triplicates) with 250 μg microsomal protein per ml; 125 μg per ml of MAb ascites corresponding to 1:2 ascites/microsomal protein were used. The MAb incubations were duplicates with appropriate blanks. Fetus 1, 2, 3 had sufficient ECOD activity to perform inhibition test. Other details are given in Materials and Methods and the actual ECOD activities are shown in Table 1.

Of seven different MAbs raised against phenobarbital, 3-methylcholanthrene, ethanol or PCN induced rat liver cytochromes only the MAb PCN 2-13-1 [17] identified a protein with Western blot analyses with an SDS-PAGE migration similar to that of the purified rat liver cytochrome P-450 (PCN). This finding was consistently observed in all investigated fetal liver microsomes although the band intensity varied (Fig. 1). A similar or identical enzyme has previously been described by Watkins *et al.* [4] in human adult liver with the use of MAb against PCN induced rat liver cytochrome P-450 ("P-450p") or its homologue in the rabbit ("LM3c"). Their human enzyme was termed "HLp" and also found by us to be present in human adult livers. This P-450 thus seems to develop at an early gestational age in the human fetal liver. Its fetal presence may signify a glucocorticoid type of induction mechanism even though it was shown that pharmacological doses of glucocorticoids were required to induce HLp [4]. The fetal environment with high levels of steroid hormones [27] may be initiating the early development of this particular form of cytochrome P-450. The HLp cytochrome may also be induced by macrolide antibiotics and anticonvulsants [4]. Such drugs were, however, not used by the aborting women.

Another MAb against the PCN induced cytochrome P-450 (PCN 2-3-2) did not identify any fetal cytochrome species in the liver or adrenals or in human adult liver microsomes.

None of the MAbs against phenobarbital (MAb 4-29-5 and 2-66-3) or 3-methylcholanthrene (MAb 1-7-1 and 1-36-1) induced cytochrome P-450 was immunoreactive in the Western blots with either fetal or adult human liver. These results do not exclude the presence of such a cytochrome P-450 in the liver microsomes since recognizable epitopes or the requirements for adequate binding may be just lacking. The MAb 1-7-1 is, however, immunoinhibitory to AHH and ECOD. It inhibits AHH in microsomes of placentas from smoking women [10] and from several tissues in the rat [28]. Yet it does not bind in Western blots to the placental antigen.

The densitometer scans of the protein bands identified in Western blots were poorly related to the ECOD activity in the liver microsomal specimens (correlation coefficient $r = 0.78$). Other major cytochromes may be co-regulated with the cytochrome P-450 (HLp). The fact that MAb (Pb 2-66-3) did not inhibit and MAb (MC 1-7-1) only slightly inhibited the ECOD in these specimens as well as the poor correlation ($r = 0.78$) between the immunoblot scans and ECOD indicates that none of these isoenzymes is a major contributor to the ECOD reaction. We are currently studying the metabolism profile in PCN-induced rat liver. In the adrenal microsomes the ECOD activity was higher than in the liver. Yet, the MAb (PCN 2-13-1) did not identify any protein in the Western blots indicating that another cytochrome P-450 may be responsible for the ECOD activity in the adrenals.

In summary, our study has revealed the early fetal development in man of a cytochrome P-450 species

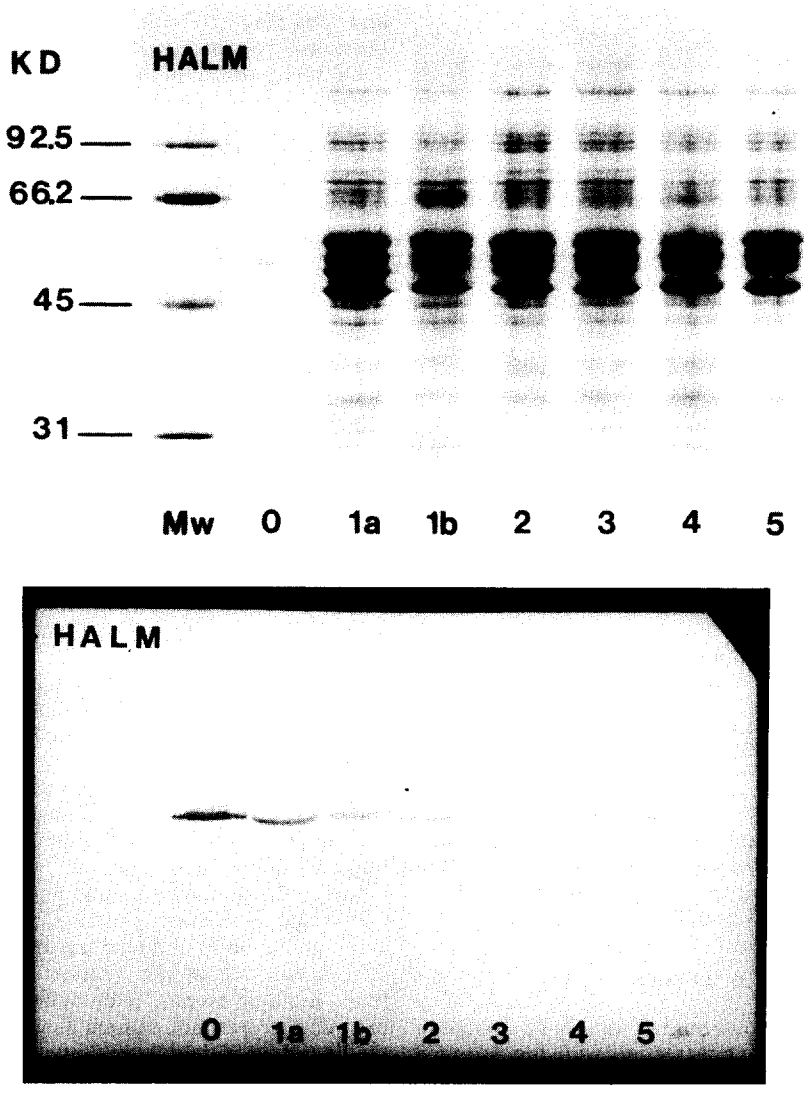

Fig. 3. SDS-gel electrophoresis (above) patterns and their corresponding immunoblots (below) with mouse anti-rat cytochrome P-450 (PCN) monoclonal antibody (MAb PCN 2-13-1) in human adult liver microsomes (-HALM). All specimens contain 4–5 bands with MW of 49 to 58 kDa. There is a band of 51.4 kDa which migrates very closely to the rat PCN purified isoenzyme (lane 0) (52.3 kDa) and probably is the band detected on immunoblot. Lanes 1a to 5 contain 20 μg of liver microsomal protein per well and lane MW contains molecular weight standards.

that is identified by a MAb against the PCN induced rat liver enzyme. These findings are suggestive of a fetal steroid induction process. Further studies are necessary to investigate what endo- or xenobiotics may enhance or inhibit this early natural development of the cytochrome P-450 HLp.

Acknowledgements—Mrs Birgitta Ask is acknowledged for expert technical assistance and Professor Birger Pettersson (Dept of Cell Biology, University of Uppsala) and Dr Mats Westman (Dept of Surgery, Samariter Hospital, Uppsala) for their kind help in the collection of tissue specimens. We also thank Drs J. W. de Pierre and A. Åstrom (Dept of Biochemistry, University of Stockholm) for kind supply of purified PCN induced rat liver cytochrome P-450. We also thank Ms Annika Lundin for excellent secretarial assistance.

This work was supported by grants from the Swedish Medical Research Council (14X-04496), the Expressen Prenatal Research Foundation and the National Board of Laboratory Animals (Dept of Agriculture, Stockholm).

Margarita G. Ladona was supported by the Swedish Institute and FISS (Ministerio de Sanidad y Consumo, Spain).

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