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PHENOTYPING OF PHENYTOIN TREATED RAT LIVER CYTOCHROME P-450 WITH MONOCLONAL ANTIBODIES

Hak Song Jin, Sun Me Won, Myung Gull Lee, Sang Shin Park*, Harry V. Gelboin* and Nak Doo Kim

College of Pharmacy, Seoul National University, Seoul 151-742, Korea.
Laboratory of Molecular Carcinogenesis, National Cancer Institute,
National Institute of Health, Bethesda, MD 20892 U.S.A.*
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ABSTRACT: *The phenotyping of cytochrome P-450 in hepatic microsomes induced by phenytoin in the rats was carried out by using several monoclonal antibodies (MAbs) against specific P-450 isozymes. Phenytoin (180 mg/kg) was administered intrapritoneally for three consecutive days to the male Sprague-Dawley rats (100-120g). Solid phase radioimmunoassay showed higher binding affinity of MAb PB 2-66-3 and PCN 2-13-1 to the microsomes from phenytoin treated rats than those to from untreated rats, which was comparable to the level in phenobarbital induced rat hepatic microsomes. Immunoblot analysis exhibited selective binding of both PB 2-66-3 and PCN 2-13-1 in both phenoabarbital and phenytoin induced microsomes supporting that phenytoin induces phenobarbital inducible form. MAbs, PB 2-66-3 immunochemically inhibited hexobarbital hydroxylase activity in either phenobarbital or phenytoin induced microsomes. Hexobarbital sleeping time was significantly shortened in the PB and DPH-pretreated group. These results suggest that phenytoin may induce the phenobarbital inducible form of P-450.*
Key words: *Phenytoin, cytochrome P-450, monoclonal antibody*

INTRODUCTION

Phenytoin (diphenylhydantoin, DPH), anticonvulsant and antiarrythmic drugs, is a primary durg for most types of epilepsy. It is well known that phenytoin is metabolized via hydroxylation by microsomal enzymes (Kett *et al.*, 1970) and it also has been shown that metabolism of other drugs was increased by phenytoin. That includes 6-β-hydroxycortisol to 17-hydroxycorticosteroid, dexamethasone, DTT, and possibly vitamin D_3 (Conney *et al.*, 1968, Werk *et al.*, 1971, Jubiz *et al.*, 1970 and Dent *et al.*, 1970). However it is not identified which form(s) of

P-450 isozyme is (are) inducible by the treatment of phenytoin. Present study was aimed at the identification of the phenotype of isozymes induced by phenytoin. In the study, the phenotype of cytochrome P-450 induced by phenytoin in rats was investigated by using several MAbs against specific P-450.

MATERIALS AND METHODS

Materials and Animals

Phenobarbital (PB), 3-methylchlanthrene(3-MC), digoxin, phenytoin (DPH), propranolol, reserpine, NADPH and hexobarbital were purchased frm Sigma Chemical Co.. Sodium dodecyl sulfate, acrylamide, N,N'-methylene-bis-acrylamide, trizma base, ammonium persulfate, TEMED, ang glycine were obtained for electrophoresis from BioRad Lab.. Prestained protein molecular weight standards (range 14.3-200 kDa) were purchased from Bethesda Research Lab. and goat serum, phosphatase labeled affinity purified goat antibody to mouse IgG (γ) and BCIP/NBT, the substrate of phosphatase were purchased from Kirkegaard and Perry Lab.. Nitrocellulose membrane was purchased from Promega Biotec. Nonfat milk was obtained from Difco Co., and β-mercaptoethanol was obtained from Merck Co.. All inorganic chemicals were reagent grade. Sprague-Dawley male rats (100-120 g) were chosen in this experiment because cytochrome P-450 isozymes in rats have very similar homology with those in human, and intraspecies differences in rats were not detected (Billings *et al.*, 1984, Kim *et al.*, 1986). Sprague-Dawley rats were supplied from the Experimental Animal Breeding Center of Seoul National University.

Preparation of Liver Microsomes Treated with Phenytoin and Other Drugs

Young male rats were given intraperitoneally for 3 consecutive days by phenytoin (180 mg/kg), propranolol (10 mg/kg), digoxin (10 mg/kg) and reserpine (5 mg/kg) in physiological saline solution, phenobarbital Na (100 mg/kg) in saline and 3-methylcholanthrene (25 mg/kg) in corn oil as reference drugs, and physiological saline solution was used as control. The rats were sacrificed by cervical dislocation following 18 hrs fasting from the last dose. Liver was perfused with ice cold 0.15 M NaCl, excised, weighed and minced. Liver homogenate was prepared by usng teflon-glass homogenizer in 0.1 M phosphate buffer and centrifuged at 1,000 g for 10 min. Supernatant was centrifuged at 12,000 g for 10 min (Ultracentrifuge, MSE, Europa. Rotor number 75.13). Supernatant was centrifuged again at 113,000 g for 1 hr. Supernatant was discarded and pellet was resuspended with 0.15 M KCl and centrifuged at 113,000 g again for 1 hr. Liver microsome was obtained from the pellet by resuspension with 0.1 M phosphate buffer. All these procedures were performed in 0-4℃ and the microsome obtained and pellet was stored at −70℃ (Eriksson *et al.*, 1978). Protein content of liver microsome was assayed by the method of Lowry *et al.* (Lowry *et al.*, 1951).

Monoclonal Antibodies

Monoclonal antibodies were prepared according to the general hybridoma method of Koehler and Milstein (Koehler *et al.*, 1975). Briefly, female BALB/c mice were immunized with purified cytochrome P-450 prepared from 3-methylcholanthrene (Park *et al.*, 1982a), phenobarbital (Park *et al.*, 1982b, Park *et al.*, 1984), pregnenolone 16-α cabonitrile (Park *et al.*, 1986) and ethanol (Ko *et al.*, 1987)-induced rat liver microsomes. After repeated immunizations, the spleens were removed and fused with myeloma cells (RGNS-1). Hybrid cells were selected after several passages and cloned. Hybridomas producing monoclonal antibodies to cytochrome P-450 were identified by a solid phase radioimmunoassay. The hybridoma cells were subcloned two additional times and those that remained positive by radioimmunoassay were grown further and inoculated intraperiotoneally into BALB/c mice primed with Pristane. The ascites fluid containing the specific monoclonal antibodies was collected and used as the antibody preparation in this study. Hybridoma cells made from normal spleen cells of nonimmunized BALB/c mouse fused with myeloma cells were inoculated into mice and the ascites fluid was used as a control (NBS).

Characterization of Cytochrome P-450 Isozymes Induced by Phenytoin and Other Drugs with Several Specific Monoclonal Antibodies

Solid-phase Radioimmunoassay

The radioimmunoassay (RIA) was carred out according to the method of Part *et al.* (Park *et al.*, 1984), using 35-S labeled monoclonal antibodies and 96-well microtiter plates (rigid polystyrene, Dynatech). The wells were precoated by incubation with 100 μg of microsome (3 mg/m*l* in phosphate buffered saline, PBS) for 2 hrs at 37℃. The remaining nonspecific sites were blocked with bovine serum albumin by replacing the microsome solution with PBS containing 3% bovine serum albumin and 2% sodium azide and incubating for 30 min. The wells were washed 3 times with PBS and incubated for 2 hrs with MAbs. The MAbs bound to enzyme then incubated with 35-S labelled anti-mouse IgG(k) overnight and washed 5 times with PBS. The individual wells were placed in scintillation vials containing 8 m*l* of Aquazol and the radioactivity was measured by liquid scintillation counter (LKB, Biochro. Engl.).

Immunoblotting: SDS-polyacrylamide gel electrophoresis

Microsomes were solublized by boiling at 100℃ for 2 min in a reaction mixture containing 5% β-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2.5% SDS and 0.5% bromophenol blue. Proteins were separated by the 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (1970) (Friedman *et al.*, 1983). The samples were electrophoresed at 25 mA/plate until tracking dye, bromophenol blue, reached the bottom of the stacker and continued at 35 mA/plate for 4-5 hrs.

Western blotting and immunodetection

After SDS-PAGE, the gels were electrophoretically transferred onto a nitrocellulose filter paper in a buffer containing 25 mM Tris, 192 mM glycine and 20% (*v/v*) methanol at 4℃ overnight. Strips of the transfer were first incubated with 3% nonfat milk in PBS for 2 hrs at room temperature, then exposed to MAb solution in PBS at 1:200 dilution at room temperature overnight. After washing with 0.05% tween 20/PBS twice and PBs once for 15 min respectively strips were incubated with goat anti-muse IgG conjugated with alkaline phosphatase at 1:1, 000 dilution in 5% goat serum in PBs for 2 hrs at room temperature. Following further washing with PBS containing 0.05% Tween 20 three times and PBs once respectively, Strips were developed using the BCIP/NBT phosphatase substrate system.

Hexobarbital hydroxylase activity

Hexobarbital hydroxylase activity was determined with HPLC by measuring the amount of hexobarbital remained after reaction. Reaction mixture contained 0.6 m*l* of microsomal protein (500 μg) and 0.4 m*l* of mixture containing 2 μ*l* of hexobarbital sodium (7 mg/m*l*), 30 μ*l* of 0.1 M $MgCl_2$, 100 μ*l* of NADPH (5 mg/m*l*) and 270 μ*l* of distilled water. After incubation for 15 min at 37℃, the reaction was stopped by adding 1.5 m*l* of acetonitrile and centrifuged for 2 min at 4,000 rpm. 20 μ*l* of supernatant was injected onto a reversed phase C18 column (5 μm, 15×0.46 cm, NOVAPACK, Waters) with a flow rate of 0.6 m*l*/min at room temperature. The mobile phase was 3.5 mM NaH_2PO_4 in water-acetonitrile (67: 33), with the pH adjusted to 2.7 with phosphoric acid. The effluent was monitored spectrophotometrically with a variable wavelength detector (Hitachi, Japan) set at 238 nm. For analysis of antibody inhibition in hexobarbital hydroxylase activity, 500 μg of microsomal protein in 350 μ*l* of phosphate buffer (pH 7.4) was incubated with 250 μ*l* of antibody fluids (1.5 mg MAb in PBS) for 30 min in ice with gentle agitation and the mixture was assayed for hexobarbital hydroxylase activity as described above.

Measurement of Hexobarbital Sleeping Time in Rats Treated with Phenytoin

Phenytoin (180 mg/kg), propranolol (10 mg/kg), phenobarbital (100 mg/kg), and 3-methylcholanthrene (25 mg/kg) were administered intraperitoneally to 5 rats for 3 consecutive days respectively. After 18 hrs fasting from the last dose of each drugs, hexobarbital sodium (100 mg/kg) was given i.p. to the rats and measured the sleeping time. Sleeping time was measured based on the duration of loss of righting reflex. The statistical analysis was performed by student t-test.

RESULTS

Characterization of Cytochrome P-450 Isozymes by Using Several Monoclonal Antibodies

Identification of phenytoin and othr drugs-induced P-450 by radioimmunoassay

The binding of MAbs to the microsomes obtained from treated with phenytoin and other drugs was examined by MAb-directed immunoassay. The epitope specific-cytochrome P-450 in each microsome was detected quantitatively by the binding of specific MAb to each microsome. The MAb binding to specific epitope of certain cytochrome P-450 was detected by second Ab, 35-S methionine rat anti-mouse IgG(k) 1-87-1 (34,000 cpm/well). Table 1 shows the data of the RIA to phenytoin, reserpine, propranolol, digoxin, phenobarbital and 3-methylcholanthrene treated microsomes. MAb NBS 1-48-5 binds nonspecifically about 3000 cpm in microsomes from rats treated with all drugs used in the study. Both MAbs, PB 2-66-3 and PCN 2-13-1 showed higher binding affinity to the microsomes from phenytoin treated rats, which was comparable with binding to the microsomes from the phenobarbital treated rats. Whereas binding of MC 1-7-1 and MC 1-36-1 to the microsomes from ethanol, digoxin, reserpine, and propranolol treated rats was similar to that observed in nonspecific MAb NBS 1-48-5. The results suggest that the cytochrome P-450 induced by phenytoin has the epitope for the binding of PB 2-66-3 and PCN 2-13-1 and the P-450 induced by phenobarbital has the epitope for the binding of PCN 2-13-1.

Table 1. Radioimmunoassay for microsomes with MAbs

MAbs	Binding to P-450 (RIA; cpm)						
	Saline	PB	DPH	3-MC	Reserpine	Propranolol	Digoxin
NBS 1-48-5	3969	3858	3985	3436	3463	3432	3332
MC 1-7-1	3295	3791	3940	4995	3326	3659	3171
MC 1-36-1	3130	3659	3486	5070	3520	3656	3406
PB 2-66-3	2967	5069	5057	3099	2982	2736	2842
PCN 2-13-1	3637	5021	4801	4208	4034	4095	3612
EtOH 1-981	2514	2904	2823	3200	2846	2883	2629

Each microsome (3 mg/m*l*) was precoated on 96-microtiter wells at 4℃ overnight and incubated with MAbs. The bound antibodies were detected with 35-S labeled rat anti-mouse IgG(k).
*Drugs denoted on the table indicate the microsome obtained from rats treated with that drug. PB: phenobarbital, DPH: phenytoin, 3-MC: 3-methylcholanthrene.

Identification of Phenytoin and Other Drugs-induced P-450 by Immunoblotting

The characterization of cytochrome P-450 in microsomes was further examined qualitativey in western blotting and immunodetection. Liver microsomes of rats

treated with saline, 3-methylcholanthrene, phenobarbital, phenyltoin, digoxin, propranolol and reserpine were used. Fig. 1 and 2 show that selective binding of MC 1-7-1 and MC 1-36-1 to the microsome 3-MC treated rats, but no binding was observed between MC 1-7-1 and MC 1-36-1 and microsomes from rats treated with phenobarbital, phenytoin, digoxin, propranolol and reserpine. Selective binding to PB 2-66-3 and PCN 2-13-1 to the cytochrome P-450 from phenobarbital and phenytoin treated rats was recognized on the NC sheets (Fig. 3 and 4), whereas weaker binding between PCN 2-13-1 and microsomes from rats treated with digoxin, propranolol, reserpine, and 3-MC was observed. EtOH 1-98-1 and nonspecific MAb, NBS 1-48-5 did not yield visible bands with any of the microsomal proteins. These results demonstrate that the cytochrome P-450 induced by PB and DPH has the epitope for the binding of PB 2-66-3 and PCN 2-13-1. That is, the cytochrome P-450 induced by phenytoin has the antigenic determinant group which is contained in the cytochrome P-450 induced by phenobarbital.

Hexobarbital Hydroxylase Activity

The specificity of MAbs for the inhibition of specific enzyme activity has been demonstrated (Park *et al.*, 1984, 1986, 1982a, 1982b, Ko *et al.*, 1987).

PB 2-66-3, PCN 2-13-1 and NBS 1-48-5 were used to detect the reaction phenotype of the microsomes treated with phenytoin. Hexobarbital hydroxylase activity in the microsomes from PB and DPH treated rats was increased significantly in the control in the absence of MAb and the increased enzyme activities were not inhibited in the presence of NBS 1-48-5. Whereas, the increased

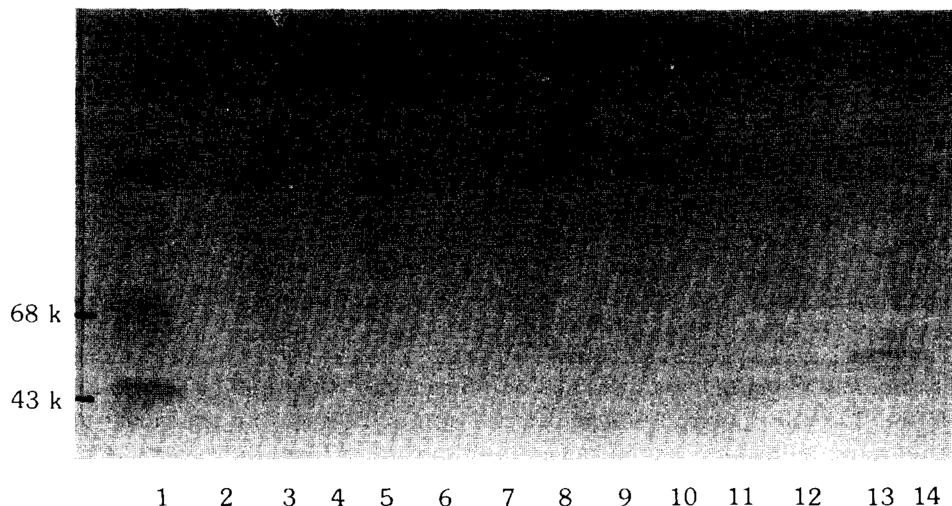

Fig. 1. Western blotting and immunodetection of microsomes with MAb, MC 1-7-1.
Lane 1 contains molecular weight standards. Lane 2 to 14 contains 20 μg of microsomes obtained from rats treated with saline (2), PB (3,4). DPH (5, 6), reserpine (7, 8), digoxin (9, 10), propranolol (11, 12), and 3-MC (13, 14) respectively.

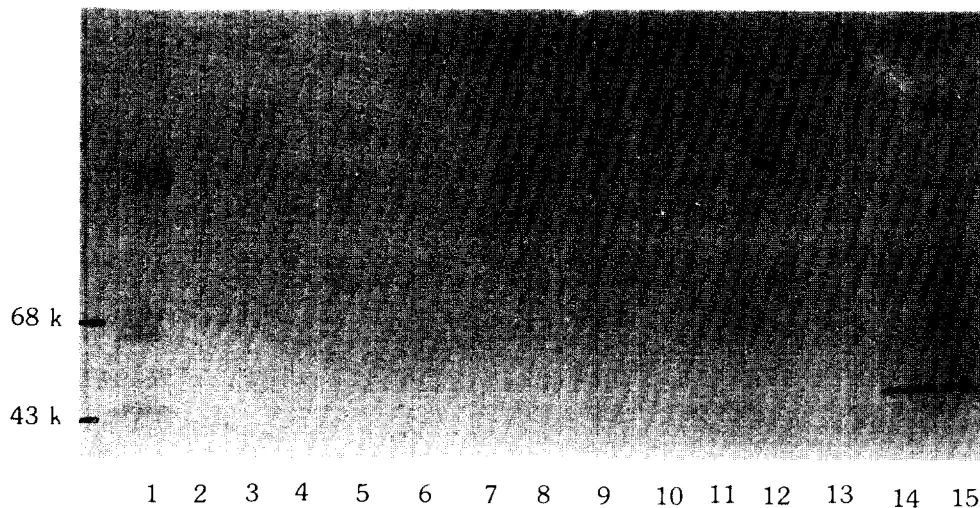

Fig. 2. Western blotting and immunodetection of microsomes with MAb, MC 1-36-1.
Lane 1 contains molecular weight standards. Lane 2 to 14 contains 20 μg of microsomes obtained from rats treated with saline (2), PB (3, 4), DPH (5, 6), reserpine (7, 8), digoxin (9, 10), propranolol (11, 12), and 3-MC (13, 14) respectively.

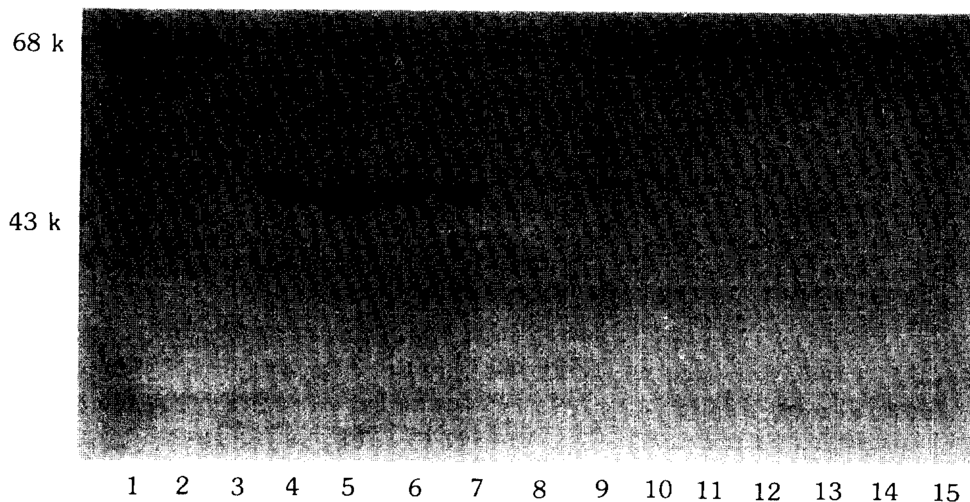

Fig. 3. Western blotting and immunodetection of microsomes with MAb, PB 2-66-3.
Lane 1 contains molecular weight standards. Lane 2 to 14 contains 20 μg of microsomes obtained from rats treated with saline (2), PB (3, 4), DPH (5, 6), reserpine (7, 8), digoxin (9, 10), propranolol (11, 12), and 3-MC (13, 14) respectively.

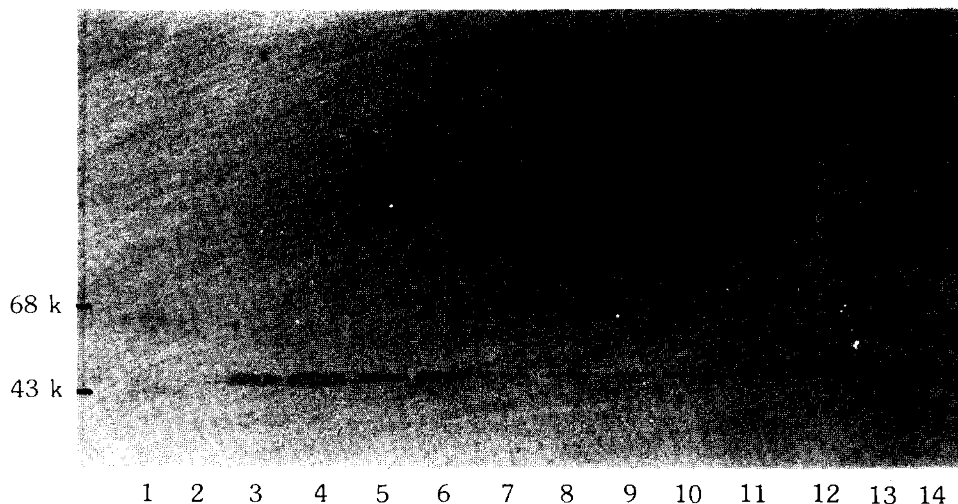

Fig. 4. Western blotting and immunodetection of microsomes with MAb, PCN 2-13-1. Lane 1 contains molecular weight standards. Lane 2 to 14 contains 20 μg of microsomes obtained from rats treated with saline (2), PB (3, 4), DPH (5, 6), reserpine (7, 8) digoxin (9, 10), propranolol (11, 12), and 3-MC (13, 14) respectively.

Table 2. Hexobarbital Hydroxylase Activity and Inhibition by MAbs

Treatment	Hexobarbital Hydroxylase Activity (nmole/mg protein/min)					
	Control	MAbs				
		NBS 1-48-5	PB 2-66-3	MC 1-7-1	PCN 2-13-1	EtOH 1-98-1-1-2
Saline	<0	<0	<0	150	<0	<0
PB	1570	1869	<0	1668	1823	1074
3-MC	217	148	107	<0	341	315
DPH	1771	2354	<0	1657	1781	1652

PB: phenobarbital, 3-MC: 3-methylcholanthrene, DPH: phenytoin

hydroxylase activities induced by phenobarbital or phenytoin were inhibited in the presence of PB 2-66-3 (Table 2). The inhibited hydroxylase activity in the presence of PB 2-66-3 was as low as to that observed in the control group.

Effect of Phenytoin on Hexobarbital Sleeping Time

Twenty five S.D. rats were divided into 5 groups and phenytoin (180 mg/kg), propranolol (10 mg/kg), phenobarbital (100 mg/kg), 3-MC (25 mg/kg) and saline were administered intraperitoneally to rats for 3 consecutive days. After 18 hrs fasting, hexobarbital sodium (100 mg/kg) was administered and duration of loss of righting reflex was measured. In the control group, the duration of loss of

Table 3. Hexobarbital Sleeping Time

Pretreated Group	Hexobarbital Sleeping Time (min)
Control	30.0±4.9
Phenobarbital	4.1±1.8***
Phenytoin	6.1±1.6**
3-Methylcholanthrene	37.7±5.1
Propranolol	45.3±1.0

These values are means±S.E.
**Significantly different from control, $p < 0.01$
***Significantly different from control, $p < 0.001$
Rats were pretreated with drugs for 3 consecutive days intraperitoneally and the sleeping time was tested by measuring the duration of loss of righting reflex after injection of hexobarbital sodium 100 mg/kg intraperitoneally.

righting reflex was 30.0 min. In the DPH treated group, sleeping time was 6.1 min and in the PB treated group, 4.1 min. Sleeping time in the PB and DPH treated rats was shortened by 86 and 80% respectively. Whereas, in the rats treated with 3-MC and propranolol, the sleeping time was similar to that in the control.

DISCUSSION

A large multiplicity of cytochrome P-450 has been reported in rats, rabbits, and humans. (Lu *et al.*, 1980, Guengerich *et al.*, 1982, Wang *et al.*, 1983, Dutta *et al.*, 1983 and Shoun *et al.*, 1983). Certain forms of cytochrome P-450 are inducible by a variety of inducers and the level of specific inducers or to a variety of physiological conditions, *e.g.* fasting (Tu *et al.*, 1982). These phenotypings of cytochrome P-450 isozymes are important to understand the role of cytochrome P-450 for individual differences in drug metabolism and in the activation of chemical carcinogens. It has been reported that phenytoin induced microsomal enzyme and increased the metabolism of corticosteroids (Werk *et al.*, 1971 and Jubiz *et al.*, 1970), insecticides (Conney *et al.*, 1968), and possibly vitamin D_3 (Dent *et al.*, 1970). Previous studies showed that phenytoin half-life was decreased by coadministration of phenobarbital, suggesting enzyme induction is involved in the clearance of phenyltoin (Cucinell *et al.*, 1963, Kutt *et al.*, 1969 and Morselli *et al.*, 1971). This implies that the PB-treated microsomal enzyme may have similar isoforms with the DPH-treated microsomal enzyme. The multiplicity, however, makes it difficult to understand which specific form of cytochrome P-450 is responsible for the induction by phenytoin. Thus the characterization of specific form of cytochrome P-450 induced by phenytoin is important step for the evaluation of the types of cytochrome P-450 isozymes and for understanding drug interaction of phenytoin with other antiepileptic drugs and individual differences in the drug metabolism. MAbs are ideal tools of the isolation and elucidation of

the multiplicity of cytochrome P-450 since they are precise probes for specific antigenic determinants and they can recognize even small difference in P-450s. In this study, the phenotyping of cytochrome P-450 induced by phenytoin was determined by using some of MAbs.

Cytochrome P-450 induced by DPH was not recognized by the specific MAbs against P-450 isozymes induced by 3-MC and ethanol but the MAbs against P-450s induced by PB and PCN in RIA and immunoblotting. These results suggested that possibility that the forms(s) of cytochrome P-450 induced by DPH might be identical with that inducible by either PB and PCN. It was reported that chronic and extensive consumption of alcohol caused an increase in the disappearance of phenytoin from the serum of alcoholics as compared to non-alcoholic humans by the induction of microsomal enzyme (Kater *et al.*, 1976). However, the phenotype of P-450 induced by ethanol may be different from that of P-450 induced by DPH, that is, the increased clearance of DPH by chronic use of ethanol is occurred by another mechanism (Chung *et al.*, 1976). There was 3-4 fold increase in hexobarbital hydroxylase activity in microsomes from rats treated with DPH and PB compared to saline-treated microsomes, and the enzyme activity was inhibited by PB 2-66-3 as much as the activity of saline-treated microsomes. P-450s induced by PB and DPH have the epitope for binding of PB 2-66-3. This result indicates that the reaction phenotype of P-450 induced by DPH may be identical with that of P-450 induced by PB. The measurement of enzyme inhibition by PB 2-66-3 and PCN 2-13-1 confirmed that there existed MAb detectable homologies between PCN 2-13-1 and PB 2-66-3 (Park *et al.*, 1986 and Reubi *et al.*, 1984) although there was little overall sequence homology between both of P-450 forms. PB 2-66-3 inhibited the hexobarbital hydroxylase activity more significantly than PCN 2-13-1. This may imply that PB 2-66-3 is directed to the active site of the P-450 responsible for hexobarbital hydroxylase activity more significantly than PCN 2-13-1. We also found that a decrease of hexobarbital sleeping time by DPH was comparable to the level in the rats pretreated with PB.

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