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A New Form of Cytochrome P-450 Responsible for Mutagenic Activation of 2-Amino-3-methylimidazo[4,5-*f*]quinoline in Human Livers[1]

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ABSTRACT

Antibodies to P-450IA2 strongly inhibited the mutagenic activation of 2-amino-3-methylimidazo [4,5-*f*]quinoline (IQ) and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole acetate but not aflatoxin B_1 in human liver microsomes. The anti-rat P-450IA2 antibodies were capable of recognizing two proteins which show different mobilities on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of human liver microsomes. A new form of cytochrome P-450 (designated P-450-HM4) cross-reactive with anti-rat P-450IA2 antibodies showing that the smaller molecular weight was purified from human liver microsomes by means of the fast-performance liquid chromatography system. The molecular weight of P-450-HM4 was estimated to be 49,000, which was apparently different from that of P-450PA (human P-450IA2). The antibodies to P-450-HM4 did not cross-react with P-450PA (human P-450IA2) but inhibited to various extents the mutagenic activation of IQ in microsomes from human livers. In addition, P-450-HM4 showed significant mutagen-producing activity from IQ in a reconstituted system. Together with these and other results reported previously, it is concluded that at least two forms of cytochrome P-450 [P-450-HM4 and P-450PA (human P-450IA2)] are involved in the mutagenic activation of IQ in human liver.

INTRODUCTION

Cytochrome P-450 has been known for many years to be capable of oxidizing a wide variety of endogenous and exogenous compounds including carcinogens (1, 2). Multiple forms of cytochrome P-450 with distinct but overlapping substrate specificities have been purified from liver microsomes of various animal species including human (3, 4). In addition, it has been generally accepted that the various forms of cytochrome P-450 show different rates of activation as well as detoxication of chemical carcinogens, and that the metabolic activation of promutagens by cytochrome P-450 occurs as the first step in the initiation of chemical carcinogenesis (5–7).

Sugimura (8) and Sugimura and Sato (9) have succeeded in isolating numerous forms of promutagens from food pyrolysates, some of which have proven to be carcinogens. Among these, IQ[3] is one of the promutagens exerting very high mutagenicity in the Ames test upon incubation with cytochrome P-450-containing reconstituted system, indicating that cytochrome P-450 is capable of activating IQ to a mutagen(s). Regarding the molecular forms of cytochrome P-450 involved in the activation of IQ, we found that rat P-450IA2 possesses a higher capacity to activate IQ as compared to other purified forms of the cytochrome (10). Following these studies, the involvement of cytochrome P-450 in the activation of IQ and other promutagens produced by protein and amino acid pyrolysis was investigated by using liver microsomes and purified forms of human liver cytochrome P-450 (11–13). The results showed that a form(s) of human liver cytochrome P-450 cross-reactive with antibodies to rat P-450IA2 catalyzes the mutagen production at a fast rate. Later, a purified preparation of cytochrome P-450 human livers, namely P-450PA (human P-450IA2), which was cross-reactive with antibodies to rat P-450IA2, was proven to be responsible for the activation of IQ to a mutagen(s) (14). On the other hand, although P_1–450 (human P-450IA1) has not yet been purified from human liver microsomes, it has been shown that the amino acid sequence of P_1–450 (human P-450IA1) is highly similar to those of P-450PA (human P-450IA2) and rat P-450IA1 (15), but P_1–450 (human P-450IA1) exhibits different mutagen-producing activities from those of P-450 PA (human P-450IA2) and rat P-450IA1 (16).

We have previously purified cytochrome P-450 purified from human liver microsomes, namely P-450-HM1 and P-450-HM2, which probably correspond to P-450 NF (P-450IIIA4) and P-450MP (P-450IIC10), respectively, as judged by their mobilities on SDS-PAGE and catalytic activities (17). During the course of our studies on human liver cytochrome P-450, we have found that there are at least two forms of cytochrome P-450 which can be recognized as two protein bands on Western blot analysis with the use of rat P-450IA2 antibodies. Thus, in the present study, we purified a form of cytochrome P-450 (designated P-450-HM4) which is distinct from the P-450PA (human P-450IA2) obtained from microsomes of human livers. The P-450-HM4 activated IQ.

MATERIALS AND METHODS

Materials. $NADP^+$, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast, Tokyo, Japan. IQ, Glu-P-1 and Trp-P-2 were from Wako Pure Chemicals, Tokyo, Japan. Aflatoxin B_1 was obtained from Makor Chemicals. Protein A Sepharose and Separose 4B were from Pharmacia, Uppsala, Sweden. DLPC was from Sendary Research Laboratory, Ontario, Canada. *Salmonella typhimurium* TA1535/pSK1002 and Emulgen 911 were kindly provided by Dr. T. Shimada, Osaka Prefectural Institute of Public Health and Kao-Atlas, Tokyo, Japan, respectively. Liver autopsy samples were obtained within 12 h after clinical or accidental death and stored at −80°C until use. Microsomes were prepared as described previously (18).

Assay for Mutagen-producing Activity. The mutagen-producing activity was measured according to the methods described previously (19–21). The induction of *umu* gene expression induced by the metabolic activation of promutagens was measured by using the tester strain *S. typhimurium* TA1535/pSK1002 carrying the plasmid, pSK1002, which contains a *umuC'-lacZ*-fused gene that produces a hybrid protein with β-galactosidase activity. A typical reaction mixture for the microsomal system consisted of 50 mM potassium phosphate buffer (pH 7.4), a NADPH-generating system (0.25 mM $NADP^+$, 5 mM glucose 6-phosphate, 0.5 unit of glucose-6-phosphate dehydrogenase, and 3.3 mM $MgCl_2$), 0.1 mM EDTA, liver microsomes (20 to 40 pmol of cytochrome P-450), 10 μM promutagens, and bacteria in a final volume of 1.0 ml.

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[3] The abbreviations used are: IQ, 2-amino-3-methylimidazo [4,5-*f*]quinoline; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole acetate; Glu-P-1, 2-amino-6-methyldipyrido(1,2*a*:3′,2′-*d*)imidazole acetate; AFB_1, aflatoxin B_1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DLPC, dilauroyl-*L*-3-phosphatidylcholine; P-450-HM1 and P-450-HM2 (17) are probably identical with P-450NF (39) and P-450MP (40), respectively.

Promutagens were dissolved in dimethyl sulfoxide at a concentration of 4 mM and 2.5 μl of the solution were added to the reaction mixture. A typical reaction mixture for a reconstituted system consisted of the same components as in the case of the microsomal system except that 5 to 10 nM cytochrome P-450, 0.05 unit of NADPH-cytochrome P-450 reductase, and 25 μg of DLPC were added in place of liver microsomes. The mixture was incubated aerobically at 37°C for 120 min with shaking. The activity of β-galactosidase was measured spectrophotometrically by using *o*-nitrophenyl-β-D-galactopyranoside as the substrate. The unit of the enzyme activity was calculated according to the method of Miller (22).

Purification of Cytochrome P-450 (P-450-HM4) from Human Liver Microsomes. Liver microsomes (3 g of protein) solubilized with sodium cholate were applied to an aminooctyl Sepharose 4B column (6.2 x 60 cm) which had been equilibrated with 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.75% sodium cholate (100 mM buffer A) as reported previously (23). After the column was washed with 10 mM buffer A, cytochrome P-450 was eluted by washing the column with 200 mM potassium phosphate (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5% sodium cholate, and 0.5% Emulgen 911. The mutagen-producing activity was measured in each fraction before the fractions were pooled. The fractions which exhibited mutagen-producing activity in the presence of NADPH-cytochrome P-450 reductase and DLPC were pooled. The pooled fraction was diluted 100-fold with 20% glycerol and then concentrated by ultrafiltration on a UK-50 membrane (Toyo, Tokyo, Japan). The concentrated fraction was applied to the fast performance liquid chromatography system (Pharmacia) equipped with a preparative DEAE-5PW column (Toso, Tokyo, Japan) previously equilibrated with 20 mM Tris-acetate (pH 7.5) containing 20% glycerol and 0.4% Emulgen 911 (Buffer A) as described elsewhere (24). Cytochrome P-450 was eluted into two peak fractions by washing the column with a linear gradient of sodium acetate, concentration 0 to 500 mM. The second peak fraction eluted by washing with Buffer A containing 300 to 400 mM sodium acetate was pooled, and then was diluted 50-fold with 20% glycerol. After the diluted sample was concentrated by ultrafiltration on a UK-50 membrane, the concentrated sample was applied to a preparative hydroxylapatite column (Koken, Tokyo, Japan). Cytochrome P-450 was eluted with a gradient of sodium phosphate, concentration 0 to 350 mM, pH 7.5, containing 20% glycerol and 0.4% Emulgen 911.

Other Methods. Protein concentration was determined by the method of Lowry *et al.* (25). The content of cytochrome P-450 and the activity of NADPH-cytochrome *c* (P-450) reductase were measured according to the methods of Omura and Sato (26), and Phillips and Langdon (27), respectively. P-450-HM1 (P-450IIIA4), P-450-HM2 (P-450IIC10) and rat P-450IA2 were purified as described elsewhere (28, 29). NADPH-cytochrome *c* (P-450) reductase was purified from liver microsomes of phenobarbital-treated rats according to the method of Yasukochi and Masters (30). One unit of NADPH-cytochrome *c* (P-450) reductase was defined as the amount of enzyme which catalyzed the reduction of 1 μmol cytochrome *c*/min. SDS-PAGE and Western blot analysis were carried out by the methods described elsewhere (31, 32). Antibodies to the purified preparations of cytochrome P-450 were raised to rabbits and IgG was prepared as described previously (33).

RESULTS

Table 1 shows the effects of antibodies to rat P-450IA2, P-450-HM1 (P-450IIIA4), and P-450-HM2 (P-450IIC10) on the metabolic activation of promutagens in human liver microsomes. The mutagen production from AFB_1 by human liver microsomes was inhibited by anti-P-450-HM1 antibodies but not significantly inhibited by antibodies to rat P-450IA2 and P-450-HM2 (P-450IIC10). In contrast, the metabolic activation of IQ and Trp-P-2 by human liver microsomes was inhibited by anti-rat P-450IA2 antibodies but not by antibodies to P-450-HM1 (P-450IIIA4) and P-450-HM2 (P-450IIC10), indicating that cytochrome P-450 related to rat P-450IA2 predominantly contributes to the mutagenic activation of pyrolysates such as IQ and Trp-P-2 in human liver microsomes. These observations are in agreement with the findings reported by other laboratories (11–14).

Fig. 1 shows the result of Western blot analysis of human liver microsomes with anti-rat P-450IA2 antibodies. Two protein bands with different mobilities on SDS-PAGE cross-reacted with the antibodies. A form of cytochrome P-450 immunochemically related to rat P-450IA2 has been purified from human liver microsomes and designated P-450PA (human P-450IA2) by Distlerath *et al.* (15). As shown in the figure, the mobility of the protein with higher molecular weight was apparently identical with that of P-450PA (human P-450IA2) on SDS-PAGE. In the present study, to clarify whether the cytochrome P-450 with lower molecular weight catalyzes the mutagenic activation of IQ in human liver microsomes, we purified the cytochrome from human liver microsomes with the guidance of cross-reactivity with anti-rat P-450IA2 antibodies and mutagen-producing activity from IQ.

Fig. 2 shows typical elution profiles of cytochrome P-450 and mutagen-producing activity from a DEAE-5PW column. Most of the cytochrome P-450 applied to a DEAE-5PW column was recovered as unadsorbed fraction. The cytochrome P-450 which remained on the column was eluted by washing the column with a linear gradient of sodium acetate concentration (300 to 400 mM). The mutagen-producing activity from IQ per nmol of cytochrome P-450 was higher in adsorbed fraction than in unadsorbed fraction. Cytochrome P-450 cross-reactive with anti-rat P-450IA2 antibodies was found in both adsorbed and unadsorbed fractions. Cytochrome P-450 that cross-reacted with antibodies to rat P-450IA2 and gave an apparently similar molecular weight to P-450PA (human P-450IA2), was eluted into the unadsorbed fraction. Then, cytochrome P-450 eluted by washing with Buffer A containing 300 to 400 mM sodium acetate was further purified by high-performance liquid chromatography equipped with hydroxylapatite column.

As shown in Fig. 3, the purified cytochrome P-450 (designated as P-450-HM4) gave a single protein band on SDS-PAGE and showed a cross-reactivity with anti-rat P-450IA2 antibodies. When compared with standard proteins (rabbit muscle phosphorylase *b*, M_r 94,000; bovine serum albumin, M_r 67,000; egg white ovalbumin, M_r 43,000), an apparent molecular weight of P-450-HM4 was estimated to be 49,000. The anti-rat P-450IA2 antibodies recognized both P-450-HM4 and P-450PA (human P-450IA2) in accordance with the results shown in Fig. 1, whereas anti-P-450-HM4 antibody did not cross-react with P-450PA (human P-450IA2) as well as P-450-

Table 1 *Inhibition by antibodies to rat P-450IA2, human P-450-HM1, and P-450-HM2 of genotoxic product formation from promutagens in human liver microsomes*

The mutagenic activation was measured as described in "Materials and Methods." P-45-HM1 and P-450-HM2 correspond to P-450IIIA4 and P-450IIC10, respectively. The amount of IgG was 2 mg/mg of microsomal protein. The activities to express *umu* expression by IQ, Trp-P-2, and AFB1 with human liver microsomes in the absence of antibodies were 24.0, 4.1, and 18.8 units/min/mg of protein, respectively.

	Antibodies (% of inhibition)			
Promutagens	P-450IA2	P-450-HM1	P-450-HM2	Major forms responsible
IQ	85	10	—[a]	P-450IA2
Trp-P-2	90	—[a]	—[a]	P-450IA2
AFB1	—[a]	90	—[a]	P-450IIIA

[a] —, no apparent effects were detected.

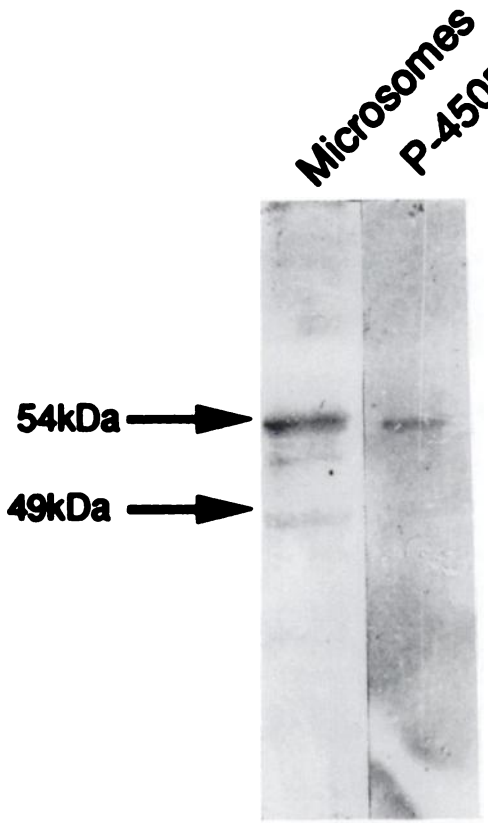

Fig. 1. Western blot analysis of human liver microsomes and purified P-450PA with anti-rat P-450IA2 antibodies. Western blot analysis was carried out as described in "Materials and Methods." Each lane contained 20 μg of microsomal protein or 2 μg of P-450PA (human P-450IA2).

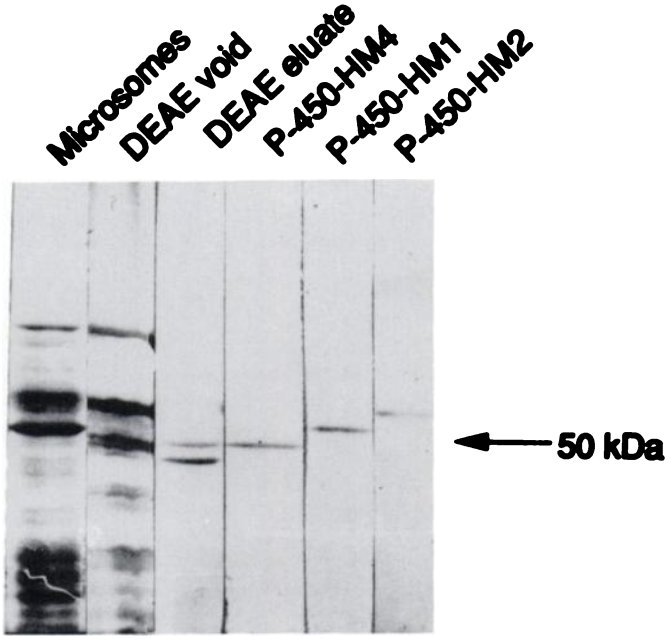

Fig. 3. SDS-PAGE of purified cytochrome P-450. The protein concentration of microsomes, DEAE-void fraction and DEAE-eluate fraction applied were 20, 20, and 10 μg, respectively. The amounts of purified P-450s applied were 1.2 pmol.

HM1 and P-450-HM2 (Fig. 4, *A* and *D*). In addition, the antibodies to P-450PA (human P-450IA2), P-450-HM1 (P-450IIIA4), and P-450-HM2 (P-450IIC10) did not cross-react with P-450-HM4 (Fig. 4, *B*, *C*, and *E*).

P-450-HM4 catalyzed the mutagenic activation of IQ in a reconstituted system (Table 2). The activity of P-450-HM4 to produce mutagen from IQ was much higher than those of P-450-HM1 (P-450IIIA4) and P-450-HM2 (P-450IIC10). Fig. 5 shows the effects of anti-P-450-HM4 antibodies on the mutagenic activation of IQ and AFB_1 in human liver microsomes. Antibodies to P-450-HM4 inhibited the activation of IQ in human liver microsomes, whereas the antibodies did not inhibit the mutagenic activation of AFB_1 at a concentration of 2 mg/mg of microsomal protein. These results indicate that since anti-P-450-HM4 antibodies did not cross-react with P-450PA (human P-450IA2), P-450-HM4 is also, in part, involved in the

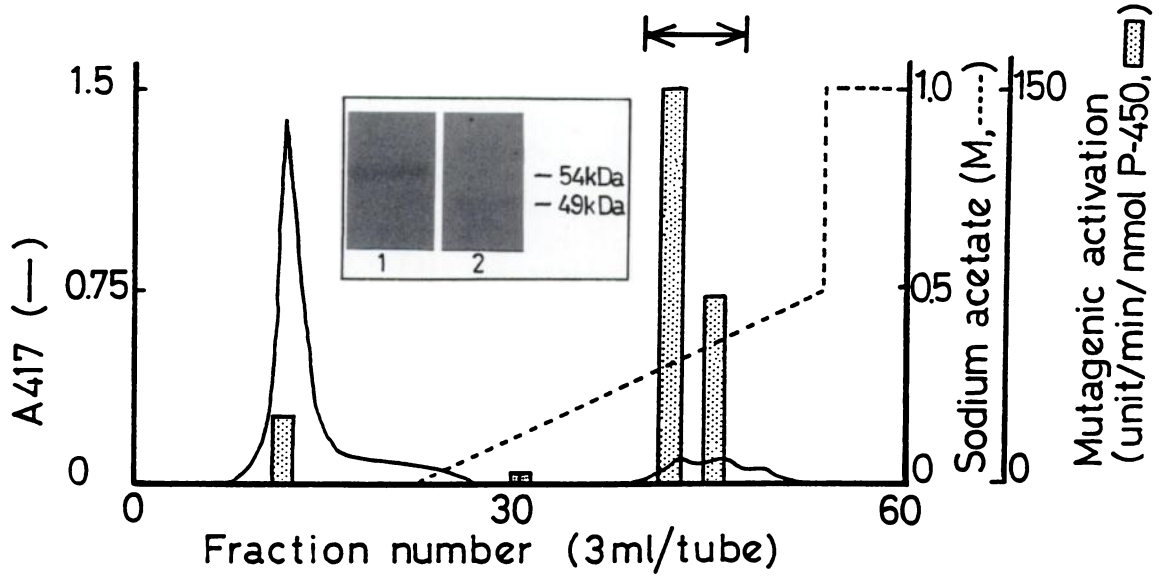

Fig. 2. Elution profiles from a DEAE-5PW column of cytochrome P-450 and mutagen-producing activity from IQ. Fractions eluted from an aminooctyl Sepharose 4B column, containing cytochrome P-450 which was cross-reactive with anti-rat P-450IA2 antibodies and exhibited mutagenic activation of IQ upon reconstitution with NADPH-cytochrome P-450 reductase and other necessary components, were pooled and applied to a DEAE-5PW column. The mutagen-producing activities from IQ were measured after every three fractions were pooled and concentrated by ultrafiltration on a UK-50 membrane, except that the activity with void fraction was measured after all of the unadsorbed fractions were pooled and concentrated. *Inset*: Western blot of the eluate with antibodies to rat P-450IA2. *Lanes 1* and *2* contain fraction 12 and 42, respectively. The *arrow* in the figure represents the fractions pooled, which were subjected for further purification.

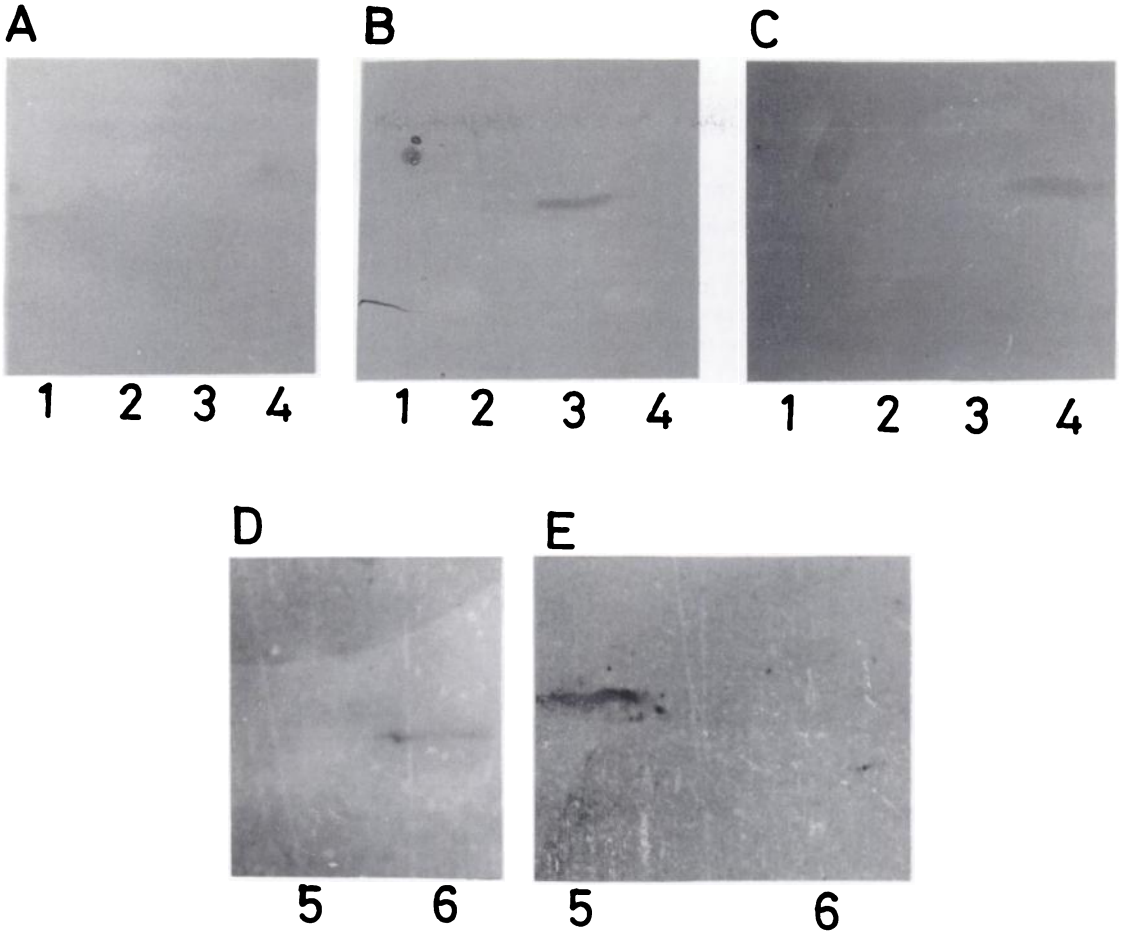

Fig. 4. Western blot analysis of purified cytochrome P-450. Western blot of purified P-450s with antibodies to P-450-HM4 (*A* and *D*), P-450-HM1 (*B*), P-450-HM2 (*C*), and P-450PA (*E*) were carried out as described in "Materials and Methods." *Lanes 1* to *4* contain purified P-450-HM4, P-450PA (human P-450IA2), P-450-HM1 (P-450IIIA4), and P-450-HM2 (P-450IIC10), respectively. *Lanes 5* and *6* contain P-450PA (human P-450IA2) and P-450-HM4, respectively. The amounts of P-450-HM4, P-450PA, P-450-HM1, and P-450HM2 were 0.5, 1, 2, and 2 μg/lane, respectively.

Table 2 *Genotoxic product formation from IQ in a reconstituted system*

Mutagen-producing activity was measured as described in "Materials and Methods." Each value represents the mean of duplicate determinations.

Cytochrome P-450	*umu* gene expression (units/min/nmol P-450)
P-450-HM4	414
P-450-HM1 (P-450IIIA4)	15
P-450-HM2 (P-450IIC10)	ND[a]

[a] ND not detectable.

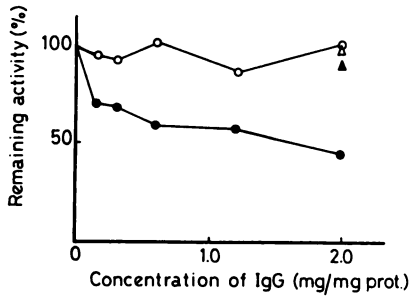

Fig. 5. Immunoinhibition of genotoxic product formation from promutagens in human liver microsomes. Mutagenic activation of IQ was measured in the presence of anti-P-450-HM4 IgG (●) or preimmune IgG (○) as described in "Materials and Methods." Mutagenic activation of AFB_1 was assayed in the presence of anti-P-450-HM4 (▲) or preimmune IgG (△), respectively. *Points*, percentage of control. The control values were 30 units/min/nmol P-450 for IQ and 32 units/min/nmol P-450 for AFB_1, respectively.

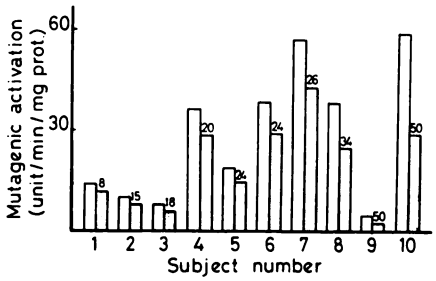

Fig. 6. Contribution of P-450-HM4 to the mutagenic activation of IQ in human liver microsomes. Mutagenic activation of IQ was measured in the presence of anti-P-450-HM4 antibodies (□) or preimmune IgG (□). The amount of IgG added was 2 mg/mg of microsomal protein. *Numbers*, percentage of inhibition.

mutagenic activation of IQ in human livers. The inhibitory effects of the anti-P-450-HM4 antibodies on the genotoxic product formation from IQ was varied among the liver microsomes used (Fig. 6). However, the relationship between the extents of inhibition and age, sex, or pathological conditions is unclear at present.

DISCUSSION

It has been demonstrated that several forms of cytochrome P-450 play important roles in the mutagenic activation of promutagens in liver microsomes of various animal species

(1, 2). The detailed knowledge of the enzymes responsible for the mutagenic activation is necessary to assess individual risks against promutagens. Polychlorinated biphenyl- and 3-methylcholanthrene-inducible forms of cytochrome P-450 (P-450IA1 and P-450IA2) have been shown to be responsible for the metabolic activation of pyrolysates such as IQ and Glu-P-1 to mutagens (34). To date, P_1-450 (human P-450IA1) has not been purified from human liver microsomes, which probably reflects its low level of expression in human livers. In contrast, P-450PA (human P-450IA2), which is involved in phenacetin *O*-deethylation (15), has been isolated from human liver microsomes (14). P-450PA (human P-450IA2) has been demonstrated to be a major form of cytochrome P-450 involved in genotoxic product formation from IQ in human liver microsomes (14). In the present study, we purified a form of cytochrome P-450 (P-450-HM4) which can catalyze the mutagenic activation of IQ, from human liver microsomes. P-450-HM4 was distinguishable from P-450PA (human P-450IA2) in their molecular weights, chromatographic behaviors, and immunochemical cross-reactivity. Although relative contribution of P-450-HM4 and P-450PA (human P-450IA2) to the activation of IQ in human liver microsomes is not unknown at present, antibodies to P-450-HM4 inhibited the mutagenic activation of IQ in human liver microsomes, indicating that P-450-HM4, at least in part, contributes to the mutagenic activation of IQ in human liver microsomes. From the results that the extents of inhibition of the mutagenic activation by anti-P-450-HM4 antibodies were varied among individuals studied, it is suggested that there appear to be individual differences in the expression of P-450-HM4 in human livers. This indicates that such factors might be one of the important determinants in individual susceptibility to genotoxicity of pyrolysates such as IQ. However, since we used autopsy samples of livers, the possibility that some factors other than an intrinsic or genetic factor(s) might affect the content of P-450-HM4 in liver microsomes cannot be excluded.

More recently, we purified a human fetal cytochrome P-450, P-450 HFLb, which was cross-reactive with antibodies to rat P-450IA2 and was also active for genotoxic product formation from IQ (35, 36). P-450-HM4 and P-450 HFLb were indistinguishable in their molecular weights and immunochemical properties. However, it remains to be investigated at present whether P-450-HM4 is identical with or very closely related to P-450 HFLb as shown in the case of P-450NF (P-450-HM1, 450IIIA4) and P-450HFLa (P-450IIIA7) (37, 38).

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