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AFLATOXIN B_1-SPECIFIC CYTOCHROME P-450 ISOZYME (P-450-AFB) INDUCIBLE BY 3-METHYLCHOLANTHRENE IN GOLDEN HAMSTERS

MORIO FUKUHARA,* KEINOSUKE MIZOKAMI,† MASAHIRO SAKAGUCHI,‡ YASUKO NIIMURA, KAZUE KATO, SAKAE INOUYE‡ and AKIRA TAKANAKA†

Department of Pharmaceutical Sciences and ‡ Department of Microbiology, The Institute of Public Health, Shirokanedai, Tokyo 108; and † Division of Pharmacology, Biological Safety Research Center, National Institute of Hygienic Sciences, Kamiyoga, Tokyo 158, Japan

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Abstract—Hepatic microsomes of polychlorinated biphenyl (PCB)-treated Syrian Golden hamsters possessed a higher potency toward aflatoxin B_1 activation, based on the Ames test, than other animal species. This activity was induced in hamsters preferentially by treatment with 3-methylcholanthrene rather than phenobarbital. The contribution of an isozyme of cytochrome P-450 (P-450-AFB) to the activity of hamster livers for aflatoxin B_1 was studied. P-450-AFB, purified from 3-methylcholanthrene-treated hamster livers, was shown to possess the highest activation of aflatoxin B_1 in the Ames test. The quantification of this isozyme by a fluorometric sandwich enzyme-linked immunosorbent assay (ELISA) demonstrated that P-450-AFB was induced mainly in Syrian Golden hamsters but not in Chinese hamsters, or in other species. This isozyme constitutes approximately 40% of the total cytochrome P-450 of the hepatic microsomes from 3-methylcholanthrene-treated Golden hamsters but only 1% in the microsomes of phenobarbital-treated hamsters. Thus, we conclude that the high activity of Golden hamster livers towards aflatoxin B_1 activation was due presumably to this distinct and unique cytochrome P-450 isozyme which was induced mainly by 3-methylcholanthrene in Golden hamsters.

Aflatoxin B_1 (AFB_1‖) is known to be the most potent hepatocarcinogen [1] and has been found in a wide variety of foodstuffs [2]. The carcinogenicity of AFB_1 has been studied using various animal species [3]. However, it is not certain whether the results obtained with experimental animals are valid in evaluating the potential carcinogenicity of AFB_1 in humans. The carcinogenicity of AFB_1 depends upon its conversion to a genotoxic metabolite catalyzed by the cytochrome P-450 (P-450) dependent mixed-function oxidase system. Differences in the activity of this enzyme system in various animals may lead to different susceptibilities to AFB_1-induced tumorigenicity. Recently, a number of studies have demonstrated the presence of multiple forms of P-450 in the livers of various species of laboratory animals, and these are classified into several gene subfamilies according to their structure [4]. The characterization of isolated P-450s with respect to their substrate specificity would contribute to the elucidation of the mechanism of species and strain differences in the toxicity of certain chemicals including carcinogens. Although several forms of P-450s are known to have relatively high specificity to certain carcinogens, a few of them are not only highly specific to certain carcinogens but also to certain animal species and strains, thus elucidating the mechanism of species or strain specific chemical toxicity.

Several workers have isolated P-450 isozymes from animals [5–10] and humans [11] that are capable of activating AFB_1. Recently, we also purified an isozyme of P-450 highly active for AFB_1 from livers of 3-methylcholanthrene-treated Golden hamsters [12] and prepared monoclonal antibodies against this isozyme [13]. In the present report, we characterize this isozyme, designated P-450-AFB, with respect to species and strain specificity by comparing the abilities of liver fractions and purified P-450s to activate AFB_1 and by comparing the levels of this isozyme, by use of an immunoquantification assay.

MATERIALS AND METHODS

Materials. Amino-*n*-Sepharose 4B, diethylaminoethylcellulose (DE-52) and hydroxyapatite were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), Whatman Ltd (Maidstone, U.K.) and Bio-Rad Laboratories (Richmond, CA) respectively. AFB_1, dilauroyl-L-α-phosphatidylcholine, streptavidin-β-D-galactosidase and 4-methylumbelliferyl-β-D-galactoside were purchased from the Sigma Chemical Co. (St Louis, MO). β-D-Galactosidase was obtained from Zymed Laboratories (San Francisco, CA). NADPH, NADH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Boehringer-Mannheim Co. Ltd (Mannheim, F.R.G.). Other reagents were obtained from the Wako Chemical Co. (Osaka, Japan).

Preparation of liver fractions. Male hamsters

* Correspondence should be addressed to: Dr Morio Fukuhara, The Institute of Public Health, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan.

‖ Abbreviations: AFB_1, aflatoxin B_1; P-450, cytochrome P-450; P-450-AFB, the P-450 isozyme from Golden hamster livers, highly active toward aflatoxin B_1; PCB, polychlorinated biphenyls; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay; and PBS, phosphate-buffered saline.

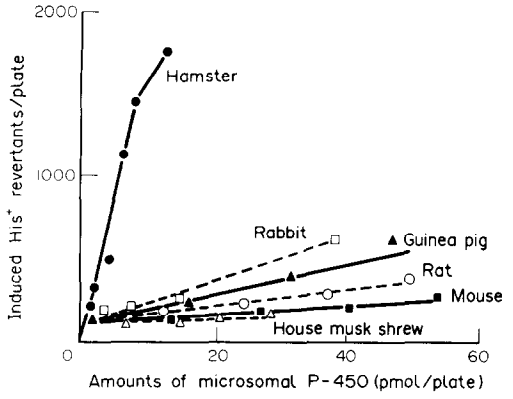

Fig. 1. Comparison of the abilities of hepatic microsomes from PCB-treated animals of different species to activate AFB_1. A mutagenicity test was done in the presence of 0.2 μg AFB_1 and various amounts of hepatic microsomes prepared from hamsters, rabbits, guinea pigs, rats, mice and house musk shrew, using *Salmonella typhimurium* TA100. Each value is the mean of two to three determinations.

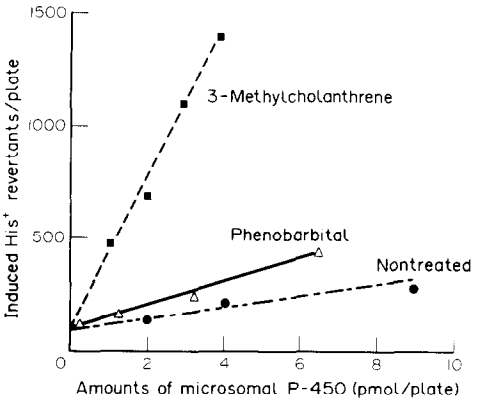

Fig. 2. Effects of microsomal enzyme inducers on the activation of AFB_1 by hepatic microsomes of Golden hamsters. Microsomes were prepared from non-treated hamsters or hamsters pretreated with 3-methylcholanthrene or phenobarbital. AFB_1 activity was determined by a mutagenicity test as described in the legend of Fig. 1.

(Syrian Golden), guinea pigs (Hartley), rats (Wistar) and mice (ddY) were obtained from Shizuoka Laboratory Animals Co. Ltd (Hamamatsu, Japan). Chinese hamsters were obtained from Sankyo Serv. (Tokyo, Japan) and rabbits (Japanese White) were from Nippon Ikagaku Dobutsu Co. Ltd (Tokyo, Japan). House musk shrew, *Suncus murinus*, was supplied by the Central Institute for Experimental Animals (Kawasaki, Japan). The animals were treated with a single i.p. injection of polychlorinated biphenyls (PCB; Kanechlor 500 diluted in olive oil) at a dose of 500 mg/kg except for the rabbits who received an injection of PCB at a dose of 250 mg/kg. After 5 days, the animals were killed and livers were removed and homogenized in 2 vol. of 0.154 M KCl. S9 fractions of the liver were prepared by centrifugation of the homogenates at 9,000 *g* for 15 min. Microsomes were obtained by centrifugation of the 9,000 *g* supernatant fractions at 105,000 *g* for 60 min. Hepatic microsomes were also prepared from Golden hamsters that were nontreated or treated with either 3-methylcholanthrene (25 mg/kg, i.p., for 4 days) or phenobarbital (60 mg/kg, i.p., for 4 days). These fractions were stored at −80° until used.

Purification of P-450s. P-450-AFB was purified, as described in our previous paper, from hepatic microsomes prepared from Golden hamsters treated with 3-methylcholanthrene [12]. Briefly, solubilized microsomes were applied on an amino-*n*-octyl Sepharose 4B column, from which P-450s were eluted into two regions, first by eluting with solution A consisting of glycerol, cholic acid, EDTA, dithiothreitol, phenylmethylsulfonyl fluoride, and Emulgen E-911 in a phosphate buffer (10 mM, pH 7.4) and second with solution A in a phosphate buffer (200 mM, pH 7.4). The first region was collected and subjected to chromatography on a DE column and on a hydroxyapatite column for further purification of P-450-AFB.

For comparative study, major components of P-450 from two other species of animals, rats and house musk shrew, were isolated. P-450s from livers of rats pretreated with 3-methylcholanthrene (25 mg/kg/day, i.p., for 4 days) or phenobarbital (0.1% in drinking water for 7 days) were isolated according to the methods of Kamataki *et al.* [14] and Waxman and Walsh [15] respectively. P-450s from house musk shrew pretreated with 3-methylcholanthrene (25 mg/kg/day, i.p., for 3 days) were isolated as described in our previous study [16].

Characterization of the purified P-450s. Physicochemical properties of the isolated P-450s were determined with respect to spectral properties [17] and relative molecular weights by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [18] with 10% acrylamide.

The catalytic activity toward AFB_1 was determined by a mutagenicity assay which was carried out principally by a modification of the Ames test [19]. The assay was performed using *Salmonella typhimurium* TA100 in the presence of different proportions of S9, microsomes or purified P-450s and AFB_1 (0.2 μg dissolved in dimethyl sulfoxide) in the preincubation mixture. Activity of the purified P-450s was studied by P-450-reconstituted system as described in our previous paper [16].

Immunochemical analysis. Antibody was raised against the purified P-450-AFB using female rabbits, and immunoglobulin G (IgG) fraction was prepared as described [20].

Western blot analysis was performed using the antibody on hepatic microsomes [21] after SDS–PAGE [18].

The quantification of P-450-AFB was done on solubilized hepatic microsomes by an immunoquantification assay which was developed on the basis of a fluorometric sandwich enzyme-linked immunoabsorbent assay (ELISA). The wells of microtiter plates were precoated with antibody to P-450-AFB by incubating at 37° for 3 hr with anti-P-450-IgG (5 μg protein/mL) in a carbonate buffer

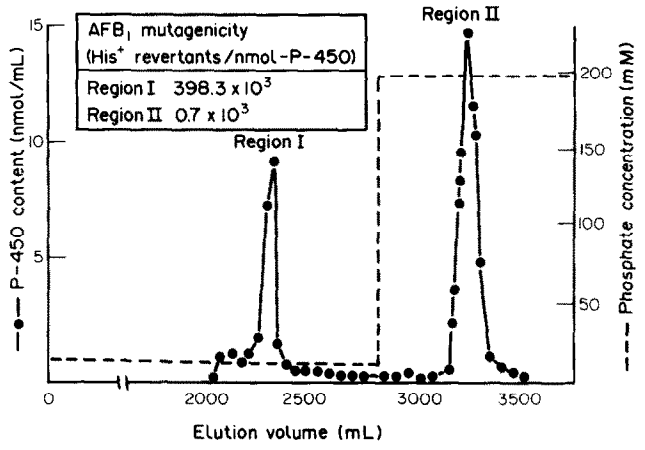

Fig. 3. Elution profile of P-450 from an amino-*n*-octyl Sepharose 4B column. Two fractions containing P-450, regions I and II, were obtained by eluting the column in a step-wise manner with solution A in 10 and 200 mM phosphate buffer respectively. The inset shows the activity of each fraction in AFB_1 activation which was determined using the reconstituted P-450-system, except that dialyzed fractions of regions I and II were used instead of purified P-450s.

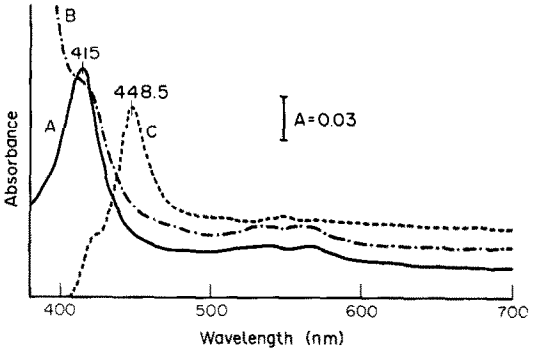

Fig. 4. Absolute spectra of purified P-450-AFB. The concentration of P-450-AFB was 17 nmol/mg in 0.1 M potassium phosphate buffer (pH 7.25) containing 20% glycerol, 0.1 mM EDTA and 0.1 mM DTT. Key: (A) oxidized, (B) dithionite-reduced, and (C) reduced CO-complex.

(50 mM, pH 9.3). The wells were incubated for 1 hr with bovine serum albumin in phosphate-buffered saline (PBS), and then were washed three times with PBS containing Tween 20 (0.05%). The wells were incubated overnight at room temperature with microsomes to be tested and then were washed. Biotinylated anti-P-450-AFB IgG (5 μg/mL) which was prepared as described previously [22], and then streptavidin-conjugated-β-D-galactosidase in PBS containing fetal calf serum (10%) and Tween 20 (0.05%) were added, and the wells were agitated for 1 hr. After washing, 4-methylumbelliferon-β-D-galactoside (0.1 mM) in PBS containing $MgCl_2$ (1 mM) and bovine serum albumin (1%) was added. The wells were incubated for 2 hr at 37°, and the enzyme reaction was stopped with glycine-NaOH (100 mM, pH 10.3). The fluorescence intensity was measured by a microplate fluorescence reader (Fluoroskan Flow Laboratories, McLean, VA, U.S.A.). Each microsomal sample was analyzed at several concentrations in the range where the linearity was obtained and quantified with a standard curve for the purified P-450-AFB.

RESULTS

Species differences in the mutagenic activation of AFB_1. The activities of hepatic microsomes from PCB-treated animals were compared in the activation of AFB_1, based on the mutagenicity test. As shown in Fig. 1, differences were observed from species to species, and the activity of Golden hamster liver microsomes was far greater than that of microsomes from other animals. Its potency was 25 times greater than that of rat liver microsomes.

Induction of the activity for AFB_1 in hamsters. The effects of microsomal enzyme inducers on the activity for AFB_1 mutagenicity were studied. Hepatic microsomes from 3-methylcholanthrene-treated hamsters were more potent than those from phenobarbital-treated and nontreated hamsters (Fig. 2). This finding indicates that P-450-AFB, which may be responsible for the metabolic activation of AFB_1, was more inducible by 3-methylcholanthrene than by phenobarbital.

Purification and characterization of P-450s. To compare P-450-AFB of Golden hamsters with other forms of P-450s from other animal species, major forms of P-450s were also purified from hepatic microsomes of rats and house musk shrew which showed medium and lowest activity in AFB_1 activation (Fig. 1). As for the purification of hamster P-450-AFB, P-450s of hepatic microsomes were eluted from an amino-*n*-octyl Sepharose 4B column into two regions. The first region was more active than the second region in the activation of AFB_1 mutagenicity (Fig. 3). P-450-AFB was purified from the first region to a specific content of 17.0 nmol/mg protein with 2.5% recovery of the original microsomal P-450. P-450-AFB showed an apparent molecular weight of

Table 1. Comparison of the abilities of various forms of P-450 to activate aflatoxin B_1

Animal species	P-450 isozyme	Inducer	CO-complex λ_{max} (nm)	Aflatoxin B_1 mutagenicity ($His^+ \times 10^{-3}$/nmol P-450)
Hamster	P-450-AFB	3-MC	448.5	720
Rat	P-450-L	PB	450	6.6
	P-448-H	3-MC	446.5	20.4
	P-448-L	3-MC	446.0	18.7
House musk shrew	P-448-H	3-MC	448.0	8.0
	P-448-L	3-MC	448.5	2.3
Rat*	P-450-male	Nontreated	451.0	7.67
	P-450Ic	PCB	449.5	0.29
	P-450Id	PCB	450.5	1.09
	P-450-H	PCB	447.0	8.17
	P-450-L	PCB	447.0	1.37
Rat†	UT-1	Nontreated		26.3
	UT-2		451	70.7
	UT-3		451	79.5
	UT-4		452	18.0
	UT-5		449	8.7
	UT-6		449	6.4
	UT-7			7.1
	UT-8			35.4
	UT-female			72.2
	PB-1	PB	449	9.7
	PB-2	PB	450	19.4
	PB-4	PB	450	16.8
	PB-5	PB	451	7.4
	MC-1	3-MC	447	22.8
	MC-5	3-MC	447	18.9

The abilities of the P-450s purified in the present study to activate aflatoxin B_1 were compared by the mutagenicity test. In addition, the results are also compared with P-450s previously reported, from which only the data comparable to the present results are presented. Abbreviations: 3-MC, 3-methylcholanthrene; PB, phenobarbital; H, high-spin state; and L, low-spin state.

* Data from Ishii *et al.* [9].

† Data from Shimada *et al.* [10].

56,000 and an absorption maximum at 448.5 nm in CO-reduced form with its heme iron in the low-spin state (Fig. 4).

From the hepatic microsomes of phenobarbital-treated rats, a major form of P-450 (P-450-L) was obtained with a specific content of 11.8 nmol/mg and an apparent molecular weight of 51,500. Two major forms (P-448-H and P-448-L) were obtained from 3-methylcholanthrene-treated rats, with specific contents of 17.0 and 13.6 nmol/mg and apparent molecular weights of 51,000 and 53,500 respectively.

From house musk shrew treated with 3-mehtylcholanthrene, two major forms (P-448-H and P-448-L) were obtained as described in our previous paper [16].

Catalytic activity toward AFB_1. The catalytic ability in the mutagenicity toward AFB_1 of P-450-AFB from hamsters was compared with those of rats and house musk shrew (Table 1). The activity of P-450-AFB was far greater in the induction of AFB_1 mutagenicity than that of the other P-450s. The comparison of the potency of P-450-AFB with those previously reported [9, 10] in the capability to activate AFB_1 by the Ames test disclosed that P-450-AFB was the highest of these P-450s.

Strain differences of hamsters in AFB_1 activation. The activities of S9 obtained from two different strains of hamsters were compared in the mutagenic activation of AFB_1 (Fig. 5). Golden hamsters exhibited higher activity than Chinese hamsters. Both of the hamsters were more capable of activating AFB_1 than Wistar rats.

Immunoquantification of P-450-AFB. The obtained antibody raised against the purified P-450-AFB (anti-P-450-AFB) recognized, by Western blot analysis, the purified P-450-AFB protein and only one band in the microsomes from 3-methylcholanthrene-treated hamsters at the same apparent molecular weight to P-450-AFB (Fig. 6). This strongly suggests that anti-P-450-AFB was highly specific to P-450-AFB, which allows immunoquantification by ELISA.

The immunoquantification assay developed in the present study could detect P-450-AFB at levels of less than 3 fmol. The amounts of P-450-AFB in hepatic microsomes were determined by this method in five different species of animals and two different strains of hamsters pretreated with PCB. As shown in Table 2, P-450-AFB was induced mainly in hepatic microsomes of Golden hamsters; the amounts in the other

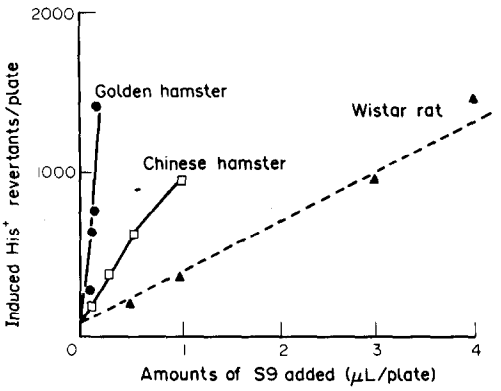

Fig. 5. Strain differences of hamsters in the activation of AFB_1 as determined by a mutagenicity test. S9-liver fractions were prepared from PCB-treated Golden and Chinese hamsters and Wistar rats and their activities for AFB_1 were compared by the mutagenicity test as described in the legend of Fig. 1. One microliter of S9 was prepared from an equivalent of 0.5 mg of wet liver tissue.

observed in the study using monoclonal antibodies [13]. In Chinese hamsters treated with PCB, P-450-AFB was observed in relatively low amounts compared with Golden hamsters.

As for the inducibility of this isozyme in Golden hamsters, P-450-AFB was induced in large amounts in 3-methylcholanthrene-treated hamsters, constituting approximately 40% of the total amount of P-450, while relatively smaller or negligible amounts were observed in phenobarbital- and non-treated hamsters (less than 1 and 0.5% of the total P-450 respectively).

DISCUSSION

The present study demonstrated that hepatic microsomes of Golden hamsters possess a remarkably high activity for AFB_1, which may be attributed to the endogenous level of a major 3-methylcholanthrene-inducible P-450 isozyme designated P-450-AFB. This isozyme was shown to be more highly active toward AFB_1 than any other major P-450 isozymes from rats and house musk shrew (Table 1). Several P-450 isozymes which possess activity for AFB_1 have been purified from rabbits [5], nontreated rats or rats treated with PCB, phenobarbital, or

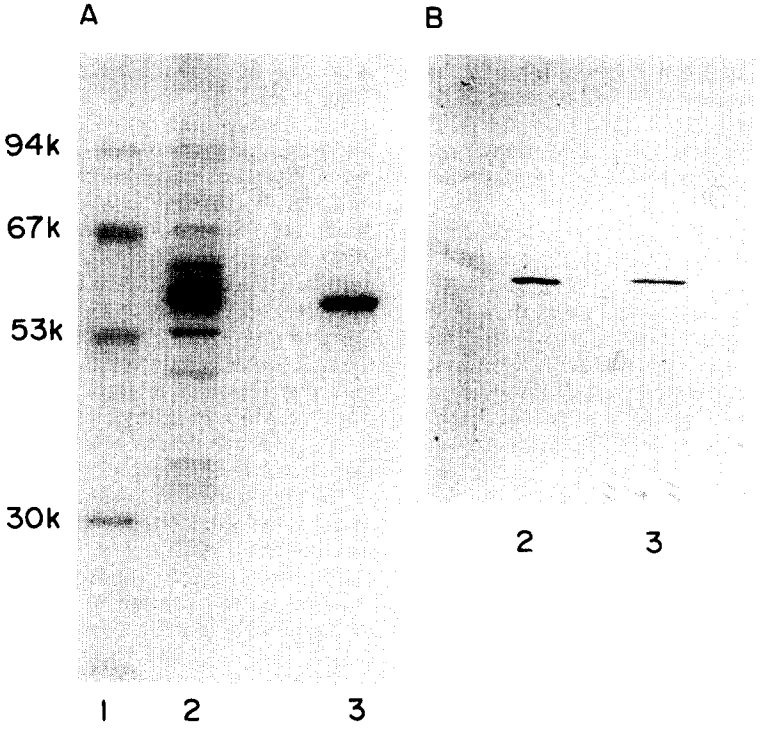

Fig. 6. SDS–PAGE (A) and immunoblot analysis (B) of purified P-450 and hepatic mircosomes of 3-methylcholanthrene-treated Golden hamsters. Panel A: molecular weight standard mixture (lane 1), solubilized hepatic microsomes from 3-methylcholanthrene-treated hamsters (lane 2, 1 μg protein) and purified P-450-AFB (lane 3, 0.2 μg) were electrophoresed in 10% acrylamide gels and stained with silver. Panel B: hepatic microsomes from 3-methylcholanthrene-treated hamsters (lane 2, 2 μg) and purified P-450-AFB (lane 3, 0.2 μg) were submitted to SDS–PAGE, transferred to nitrocellulose, and stained using the antibody against P-450-AFB and peroxidase-diamino-tetrahydrochloride.

species were negligible (less than 0.05 nmol/g liver). Whether P-450s quantified by this method have the same molecular weight to that of P-450-AFB could not be determined since the amounts in hepatic microsomes from these animals were too low to be detected by Western blot analysis, a factor also

Table 2. Immunoquantification of P-450-AFB in hepatic microsomes of various animal species and different strains of hamsters

Animal (strain)	Inducers	Total P-450 content (nmol/g liver)	P-450-AFB	
			Content (nmol/g liver)	Percent of total P-450
Hamster				
(Golden)	PCB	51.3 ± 5.8	4.54 ± 1.69	8.47
(Chinese)	PCB	28.6 ± 2.3	0.056 ± 0.001	0.19
Rat (Wistar)	PCB	33.8 ± 3.1	0.018 ± 0.001	0.06
Mouse (ddY)	PCB	21.4 ± 2.9	0.046 ± 0.002	0.20
Guinea pig (Hartley)	PCB	34.7 ± 3.8	0.016 ± 0.001	0.045
House musk shrew	PCB	22.9 ± 7.1	0.007 ± 0.003	0.057
Hamster (Golden)	3-MC	54.3 ± 2.4	21.1 ± 1.1	39.0
	PB	76.2 ± 0.8	0.81 ± 0.05	1.06
	Nontreated	30.2 ± 1.1	0.11 ± 0.01	0.38

Hepatic microsomes were obtained from various animals treated with PCB and also from Golden hamsters treated with 3-methylcholanthrene (3-MC) or phenobarbital (PB). Total amounts of P-450 were determined spectrophotometrically [17], and the contents of P-450-AFB were determined immunochemically by a sandwich ELISA. Values are the means ± SE of four animals.

naphthoflavone [5–7, 9, 10], rainbow trout [8] and also humans [11]. Although some of these [8, 11] demonstrate relatively high activity toward AFB_1, most of the P-450s catalyze AFB_1 activation at relatively low turnover rates and seem to be less potent than P-450-AFB.

The contribution of P-450-AFB to the high activity for AFB_1 activation by hamster livers is indicated by the fact that the estimated amounts of P-450-AFB were much higher in Golden hamsters than in the other species of animals pretreated with PCB (Table 2). Further, the amounts of P-450-AFB determined immunochemically correlated well with the activity for AFB_1 by the Ames test (Fig. 2) in the hepatic fractions of hamsters treated with microsomal enzyme inducers. The amounts in 3-methylcholanthrene-treated Golden hamsters also correlated well with the results of the purification experiments in which approximately half of the P-450s were collected in the first region eluted from the amino-*n*-octyl Sepharose 4B column that contained high activity toward AFB_1 (Fig. 3). All these facts support the idea that the high activity toward AFB_1 of hepatic microsomes of Golden hamsters is due presumably to P-450-AFB.

It should also be noted that P-450-AFB was inducible mainly in Golden hamsters and was not inducible in significant amounts in Chinese hamsters (Table 2). However, Chinese hamsters possessed relatively high activity toward AFB_1 in the mutagenicity test compared with rats (Fig. 5), indicating the presence of P-450 isozymes other than P-450-AFB that have relatively potent activity toward AFB_1.

Another feature of P-450-AFB is that this isozyme is inducible by 3-methylcholanthrene in hamster livers (Table 2). In contrast, the isozymes of rat livers responsible for the activation of AFB_1 are more inducible by phenobarbital than by 3-methylcholanthrene [6, 7, 23, 24]. It should be noted that the chromatographic behavior of P-450-AFB in the purification steps was rather similar to that of the major forms of phenobarbital-inducible P-450s of rats, for P-450-AFB was easily eluted from the amino-octyl Sepharose 4B column by 10 mM phosphate buffer (Fig. 3), which is the case with phenobarbital-inducible P-450s of rats. This fact and the catalytic properties of P-450-AFB [12] suggest that this isozyme is not similar to P-450c or P-450d of the IA gene subfamily, major 3-methylcholanthrene-inducible isozymes in rats [4]. In fact, our recent work* in which the complete sequence of cDNA for P-450-AFB was determined has shown high homology of its structure to the P-450IIA gene subfamily.

The high activity of P-450-AFB in AFB_1 activation may be attributed to the increased amounts of active metabolites of AFB_1 or to the formation of metabolites more active than those produced by P-450s of rats and other animals. Species differences in the metabolism of AFB_1 in other animals have been reported [25–28], and Hsieh *et al.* [29] observed a similarity in the metabolic profile of AFB_1 between normal hamsters and humans. However, we have no evidence as to what types of active metabolites are produced by hamster P-450-AFB.

The significance of the presence of P-450-AFB in Golden hamsters in AFB_1 hepatocarcinogenesis is not clear at present, since this species of animal is known to be relatively more resistant to AFB_1 carcinogenesis [30] and acute toxicity [3]. However, in these studies, nontreated hamsters in which the amounts of P-450-AFB were negligible (Table 2) were used. In this respect, whether or not P-450-AFB is induced in chronically AFB_1-treated hamsters should be determined to elucidate the role of this isozyme in the evolution of AFB_1 toxicity in hamsters. On the other hand, the activities of drug-metabolizing enzymes are more or less induced in the human body by the ingestion of a variety of

* Manuscript submitted for publication.

chemicals so that the role of induced enzymes may be important in the evolution of the toxicity of chemicals that could be activated by induced enzymes.

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