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PREPARATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST A FORM OF HAMSTER LIVER CYTOCHROME P-450 HIGHLY SPECIFIC TO AFLATOXIN B_1

Momoko Sunouchi[1,*] Yukio Ishimi[2], Keinosuke Mizokami[1], Morio Fukuhara[3], Akira Takanaka[1], Fumio Hanaoka[2] and Masa-atu Yamada[2]

[1]Division of Pharmacology, Biological Safety Research Center, National Institute of Hygienic Sciences, 1-18, Kamiyoga 1-Chome, Setagaya-ku, Tokyo 158, Japan

[2]Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

[3]Department of Pharmaceutical Sciences, The Institute of Public Health, Shirokanedai, Minato-ku, Tokyo 108, Japan

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SUMMARY: Monoclonal antibodies were prepared against a form of cytochrome P-450 (designated as cytochrome P-450-I) purified from 3-methylcholanthrene-treated hamster livers which is highly specific to aflatoxin B_1. The cytochrome P-450-I was detected in ELISA and Western blots in liver microsomes from 3-methylcholantherene-treated hamsters and also from non-treated and phenobarbital-treated hamsters in smaller amounts. However, none of the liver microsomes from 3-methylcholanthrene-treated rat, rabbit, guinea pig and Suncus murinus contained the cytochrome P-450-I. These results suggest that cytochrome P-450-I is specific to hamster and is induced mainly by 3-methylcholanthrene.

Cytochrome P-450s are known to play an important role in the metabolism of xenobiotics including drugs and environmental pollutants (1,2) and also endogenous substances including steroids and vitamins (3,4). A number of mutagens and carcinogens are transformed to active metabolites by cytochrome P-450s (2,5,6). However, multiplicity and broad and overlapping substrate specificity of cytochrome P-450s (5,6) make it difficult to study species differences in carcinogenesis.

Aflatoxin B_1, a potent hepatocarcinogenic and mutagenic mycotoxin produced by Aspergillus flavus (7), is transformed by cytochrome P-450s to metabolites with potent mutagenic activity

*To whom correspondence should be addressed.

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(8). Cytochrome P-450 species which are involved in aflatoxin B_1 activation and carcinogenesis have not been known yet. Recently, we have isolated a form of cytochrome P-450 from livers of hamsters treated with 3-methylcholanthrene(MC)(9). This isozyme designated as cytochrome P-450-I is about 50- to 250-fold greater than the other cytochrome P-450s purified from the livers of MC-treated and phenobarbital(PB)-treated rats in the activity to transform aflatoxin B_1 to mutagenic metabolites (9). To elucidate the differences in susceptibility of various animals to carcinogenicity of aflatoxin B_1 and to clarify the role of the cytochrome P-450-I in the hepatocarcinogenesis of aflatoxin B_1, it is important to quantify this cytochrome P-450-I in various organs or animals under various states, especially when animals are exposed to chemicals. With monoclonal antibodies specific to hamster cytochrome P-450-I, we could detect and quantify the hamster cytochrome P-450-I, which might reveal the correlation between hepatocarcinogenesis and aflatoxin B_1. We report here the preparation and characterization of monoclonal antibodies against the cytochrome P-450-I and describe their use in the detection of this isozyme in livers of the other experimental animals.

MATERIALS AND METHODS

Preparation of cytochrome P-450s and microsomal fractions. Hepatic cytochrome P-450s were purified from MC-treated hamsters and rats(MC 25mg/kg/day, ip., for 4 days) as described by Mizokami et al.(9). The specific contents of hamster cytochrome P-450-I (low spin state) and cytochrome P-450-II (high spin state) were 15.3 and 13.6 nmoles/mg protein, respectively. The specific contents of rat cytochrome P-448-L1 (low spin state), cytochrome P-448-L2 (low spin state) and cytochrome P-448-H (high spin state) were 13.6, 14.2 and 13.2 nmoles/mg protein, respectively. Liver microsomal fraction were prepared from non-treated, MC- or PB-treated hamster and MC-treated rat, rabbit, guinea pig and Suncus murinus as described previously(10).

Mouse immunization and cell hybridization Balb/c mice (Nippon Bio-Supp. Center, Japan) were immunized by subcutaneous injections of 25 μg of hamster cytochrome P-450-I in Freund's complete or incomplete adjuvant (Gibco) every two-weeks for four times. After the last injection, 15 μg of the antigen was injected intravenously. Four days after the final booster, spleen cells (1×10^8) prepared from the mouse were fused with NS-1 (2×10^7) or P3U1 (2×10^7) myeloma cells using 50% polyethylene glycol and then the obtained positive hybridoma cells were cloned by the limited dilution(11). The supernatants of culture medium were used for binding activity with the antigen. This binding activity for monoclonal antibody screening was tested by Dot-binding assay

using mouse peroxidase-anti peroxidase (mouse-PAP, Jackson Immuno Research, U.S.A.), 3,3'-diaminobenzidine and H_2O_2 according to method of Hanks et al.(12).

Characterization of established monoclonal antibodies Class and subclass of the monoclonal antibodies were determined by Ouchterlony double-diffusion plates (mouse monoclonal antibody typing kit, Miles, U.S.A.). ELISA for monoclonal antibodies to cytochrome P-450s or microsomal fractions was carried out by the method of Engvall et al.(13). Wells of polystylene microtiter plates (Sumitomo, MS-3596H, Japan) were coated with purified cytochrome P-450s(12.5 pmole/ml) or microsomal cytochrome P-450s(20 pmole/ml) by incubation at 4 ^{o}C, overnight.

Electrophoresis on a SDS-polyacrylamide gel (14) was done with cytochrome P-450s or microsomes. Western blots were carried out by the methods of Guengerich et al. (15). Proteins on SDS-polyacrylamide gel were stained by silver staining(16). Cytochrome P-450 contents were measured by the method of Omura and Sato(17). Protein contents were assayed by the method of Lowry et al.(18).

RESULTS

We have obtained five hybridoma cell lines that secreted monoclonal antibodies(named Mab.MIT601 to MIT605) against the hamster cytochrome P-450-I. Spent media collected from these hybridoma cell cultures were used in the following experiments. All monoclonal antibodies were of IgG type with kappa light chain. Mab.MIT601 and MIT602 had subclass 2b, while MIT603, MIT604 and MIT605 had subclass 1.

Reactivities of the monoclonal antibodies with purified cytochrome P-450s obtained from liver microsomes of MC-treated hamsters and rats were shown in Table 1. In solid phase ELISA, Mab.MIT601 and MIT602 highly reacted with the hamster cytochrome P-450-I and weakly reacted with hamster cytochrome P-450-II. The other monoclonal antibodies (Mab.MIT603, MIT604 and MIT605) were reactive with the hamster cytochrome P-450-I but not with hamster cytochrome P-450-II. None of these monoclonal antibodies cross-reacted with rat cytochrome P-448-L1, cytochrome P-448-L2 and cytochrome P-448-H.

Using these monoclonal antibodies, Western blots and immunochemical staining were done to know the reactivity with other forms of the purified cytochrome P450s. Fig.1A shows the electrophoretical mobilities of hamster cytochrome P-450-I, cytochrome P-450-II, rat cytochrome P-448-L1, cytochrome P-448-L2 and cytochrome P-448-H. In Fig. 1B are shown immunochemical

Table 1. Reactivity of the obtained monoclonal antibodies with cytochrome P-450s purified from 3-methylcholanthrene-treated hamsters and rats

P-450 species	Monoclonal antibodies				
	MIT601	MIT602	MIT603	MIT604	MIT605
Hamster P-450-I	1.36	1.34	0.19	0.62	0.85
Hamster P-450-II	0.13	0.13	0	0	0
Rat P-448-L1	0	0	0	0	0
Rat P-448-L2	0	0	0	0	0
Rat P-448-H	0	0	0	0	0

The reactivity of the Mabs with cytochrome P-450s was assayed by ELISA using anti-mouse IgG(gamma chain), mouse peroxidase anti-peroxidase and 3,3'-diaminobenzidine tetrahydrochloride. Wells were coated with cytochrome P-450s at 1 nmole/ml in 0.1M carbonate buffer(pH9.5). Data shown are absorbance at 492 nm.

staining, and Mab.MIT604 stained only 1 band that may correspond to hamster cytochrome P-450-I but not hamster cytochrome P-450-II, rat cytochrome P-448-L1, cytochrome P-448-L2 and cytochrome P-448-H. The profile of immunochemical staining by Mab.MIT604 was the same to the results obtained using Mab.MIT603 and MIT605(data not shown). Mab.MIT.601 and MIT602 reacted with 2 bands that might correspond to the hamster cytochrome P-450-I and cytochrome

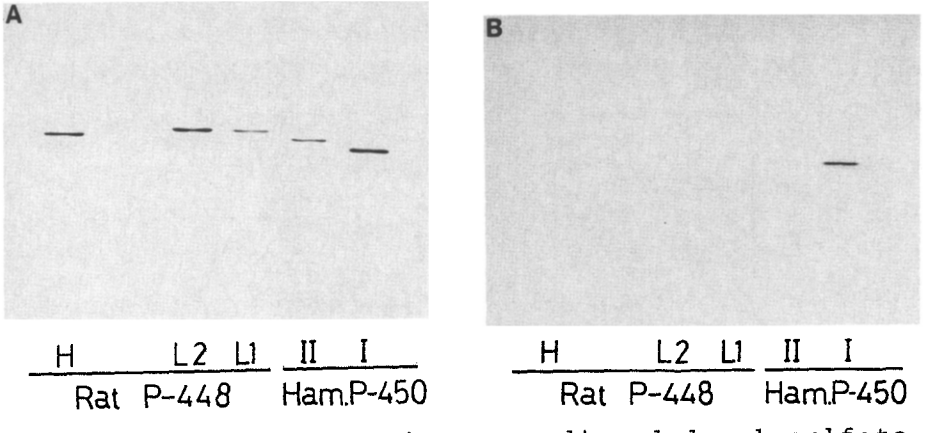

Fig. 1A. Electrophoresis on sodium dodecyl sulfate-polyacrylamide gel with 10% of acrylamide and silver staining of purified cytochrome P-450-I and cytochrome P-450-II from MC-treated hamsters and cytochrome P-448-L1, cytochrome P-448-L2 and cytochrome P-448-H from MC-treated rats(cytochrome P-450s, 0.1 to 0.2 μg/lane).

Fig. 1B. Western blots of Mab.MIT604 with purified cytochrome P-450s from MC-treated hamsters and rats.

Table 2. Reactivity of monoclonal antibody MIT604 with liver microsomes of various animals

Animals	Treatment	Mab.MIT604
Hamster	non-treated	0.04
Hamster	PB-treated	0.04
Hamster	MC-treated	0.49
Rat	MC-treated	0
Rabbit	MC-treated	0
Guinea pig	MC-treated	0
Suncus murinus	MC-treated	0

The reactivity of monoclonal antibody MIT604 with liver microsomes was assayed by ELISA. PB(60 mg/kg/day, i.p.) was administered to hamsters for 3 days. Data shown are absorbance at 492 nm.

P-450-II(data not shown). All monoclonal antibodies did not react with rat cytochrome P-448-L1, cytochrome P-448-L2 and cytochrome P-448-H. Thus the results from Western blots and immunochemical staining as well as those from ELISA have shown that Mab.MIT603, MIT604 and MIT605 were specific to the hamster cytochrome P-450-I.

In order to ascertain whether the hamster cytochrome P-450-I exists in liver microsomes of non-treated and PB-treated hamster, ELISA(Table 2) and Western blots(Fig. 2) were performed by using

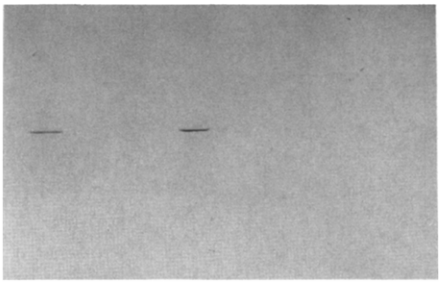

Fig. 2. Western blots of Mab.MIT604 with liver microsomes from non-treated(C), MC-treated(MC) and PB-treated(PB) hamsters. (right; 2 µg of microsomal protein / lane, left; 4 µg of microsomal protein / lane).

Fig. 3. Western blots of Mab.MIT604 with liver microsomes from MC-treated hamster, rat, rabbit, guinea pig and Suncus murinus (A; Suncus murinus, B; hamster, C; rat, D; rabbit, E; guinea pig, Protein used: 0.2 µg / lane for cytochrome P-450-I, 2 µg / lane for hamster, 20 µg / lane for the other animals).

Mab.MIT604. The antibody strongly reacted with liver microsomes of MC-treated hamsters and weakly reacted with liver microsomes of non-treated and PB-treated hamsters. We have then studied if this form of cytochrome P-450s exists in liver microsomes of the other experimental animals. None of liver microsomes of MC-treated rat, rabbit, guinea pig and Suncus murinus reacted with Mab.MIT604 in solid phase ELISA(Table 2) as well as Western blots(Fig. 3).

DISCUSSION

In the present study, we have obtained monoclonal antibodies specific to hamster liver cytochrome P-450-I, highly specific to aflatoxin B_1. The study on the identification of cytochrome P-450-I using these monoclonal antibodies with purified cytochrome P-450s or liver microsomes from various animal species demonstrated that hamster cytochrome P-450-I is specific to hamster and is specifically induced by MC-treatment.

Aflatoxin B_1 is the most potent hepatocarcinogen and is also activated by cytochrome P-450s(8,19). It has been reported that amounts of aflatoxin B_1-DNA adducts were more produced by hepatic cytochrome P-450 from PB-treated rats than by cytochrome P-448 from polychlorinated biphenyls(PCB)-treated rats (19). However, these cytochrome P-450s of rats were capable to activate not only aflatoxin B_1 but also other substrates such as benzo(a)pyrene and Trp-P-2(5). Thus it is difficult to elucidate the correlation of hepatocarcinogenesis induced by aflatoxin B_1 and these forms of cytochrome P-450s that have wide and overlapping substrate specificity. Substrate specificity of hamster cytochrome P-450-I to aflatoxin B_1 is much higher than that of the other cytochrome P-450s from rats(9). With the monoclonal antibodies we have prepared, a specific form of cytochrome P-450s could be quantified in small amounts, since immunochemical assay is highly sensitive compared with the spectrophotometric assay. These monoclonal antibodies are, therefore, useful to clarify (a) the role of cytochrome P-450-I in carcinogenesis induced by aflatoxin B_1, (b) the induction mechanism of cytochrome P-450-I after MC-treatment

by identification of mRNA translation products and (c) the quantification and localization of cytochrome P-450-I in various organs and tissues. In addition, these antibodies could be used for the simple and rapid purification of the cytochrome P-450-I by immunoaffinity column chromatography.

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