BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **250,** 150–153 (1998)
ARTICLE NO. RC989202

In Vivo Detection of Mutations Induced by Aflatoxin B_1 Using Human CYP3A7/HITEC Hybrid Mice

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Received July 8, 1998

CYP3A7-M10 mouse is a transgenic mouse carrying human CYP3A7 cDNA, in which CYP3A7 is expressed in the small intestine but not in the kidney. HITEC mouse is a transgenic mouse developed to detect mutagenic potency of various chemicals *in vivo.* The M10/HITEC mouse was established by crossmating of these two strains of mice. When a 9,000 × g supernatant fraction prepared from the small intestine was added to an incubation mixture for Ames test with *Salmonella typhimurium* TA98 strain to examine the mutagen-producing activity from aflatoxin B_1 (AFB_1), the mutagen-producing activities of the 9,000 × g supernatant fraction from the small intestine was found to be 1.7-fold higher in the M10/HITEC mice than in HITEC mice. Such a difference in the capacity to activate AFB_1 was not seen with the 9,000 × g supernatant fraction from the kidney from both strains of mice. Male M10/HITEC mice of 8 weeks old were treated with a single i.p. injection of AFB_1 (8 mg/kg body weight). The mutation of the introduced *rpsL* gene in the genomic DNA from the small intestine and the kidney was analyzed. The mutation frequency in the small intestine of M10/HITEC mice was significantly higher ($p<0.05$) than that of HITEC mice, while the mutation frequency in both strains was similar in the kidney. These results provide the first evidence for the toxicological function of CYP3A7 *in vivo.*

Key Words: **CYP3A7; human fetus; HITEC mouse; AFB_1.**

The P450 monooxygenase system plays central roles in the oxidation of various compounds, including exogenous agents such as drugs and chemical carcinogens, as well as endogenous steroids and fatty acids (1, 2). In general, the oxidative reactions catalyzed by P450 lead to detoxication. However, some of the oxidative reactions result in the formation of reactive intermediates. The reactive intermediates thus formed bind to endogenous molecules such as DNA, RNA and proteins to induce the toxicity in tissues.

CYP3A7 belongs to the CYP3A subfamily, and is the main P450 isoform expressed in the human fetal liver specifically (3). The amounts of CYP3A7 expressed in the human fetal liver account for about 30% of total P450 in the human fetal liver. CYP3A7 was first purified by Kitada *et al.* (4, 5) and cloned by Komori *et al.* (6). To clarify the catalytic functions of CYP3A7, several approaches have been performed *in vitro* using the purified enzyme (7) and cells transformed with CYP3A7 cDNA (8, 9, 10). These studies demonstrated that CYP3A7 was capable of activating some promutagens including AFB_1 and heterocyclic amines (11, 12, 13, 14), suggesting the possibility that CYP3A7 plays important toxicological roles in human fetuses.

To further clarify the toxicological roles of CYP3A7 *in vivo,* CYP3A7 transgenic mouse lines, M2 and M10, were established by Li *et al.* and the nature of these lines of mouse was evaluated (15, 16, 17). In the M10 line of mouse, CYP3A7 mRNA is expressed in the small intestine but not in the kidney (15).

HITEC mice are of another line of mouse which carries the *rpsL* gene of *E. coli* as a monitor gene for mutations. This transgenic mouse was developed by Gondo *et al.* (18, 19) to detect the mutations caused by chemicals *in vivo.*

The purpose of this study was to clarify the toxicological significance of CYP3A7 *in vivo,* particularly on the roles for genotoxicity via the activation of promutagens. Then we crossmated CYP3A7-M10 mice with HITEC

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Abbreviations used: P450 or CYP, cytochrome P450; AFB_1, aflatoxin B_1; HITEC, hyper-sensitive *in vivo* test of carcinogenicity; *E. coli, Escherichia coli;* DMSO, dimethyl sulfoxide; KM, kanamycin; SM, streptomycin.

0006-291X/98 $25.00

mice to establish a new line of mice, namely M10/HITEC mice. We expected to detect the metabolic activation of AFB_1 by CYP3A7 expressed in the small intestine as a mutation occurring in the *rpsL* gene. To confirm the catalytic activity of CYP3A7 protein expressed in the M10/HITEC mice *in vitro*, we first performed the Ames test using the *Salmonella typhimurium* TA98 strain in the presence of a 9,000 × g supernatant fraction prepared from the small intestine and the kidney using AFB_1 as a promutagen. The M10/HITEC mice were treated with AFB_1, and the mutation frequency which occurred in the *rpsL* transgene in the small intestine and the kidney was analyzed.

MATERIALS AND METHODS

Chemicals and reagents. AFB_1 was purchased from Wako Pure Chemical Industries (Tokyo, Japan). Other chemicals and reagents were of the highest quality commercially available.

Ames test. The mutagenicity test using the *Salmonella typhimurium* TA98 strain was carried out according to the method of Maron *et al.* (20). Briefly, the 9,000 × g supernatant fraction was prepared from the small intestine and the kidney of both M10/HITEC mice and HITEC mice. Each 9,000 × g supernatant fraction added to an incubation contained the same amount of protein, 0.825 mg/ assay. AFB_1 was dissolved in DMSO and diluted with distilled water to give a final concentration of 0, 16.7, 83.5, 167 and 835 nM, respectively. The reaction mixture contained the 9,000 × g supernatant fraction and 0.1 ml of AFB_1 in DMSO in a final volume of 0.6 ml. Preincubation was started immediately after the addition of 0.1 ml of TA98 suspended in a nutrient broth, and carried out at 37°C for 20 minutes. After the preincubation, the reaction mixture was immediately poured onto a minimal glucose plate with a top agar. Assays were carried out in triplicates at each dose.

Animals and treatment. The CYP3A7-M10 mice were crossmated with HITEC mice (C57BL/6J strain, *rpsL* hemizygote) to establish a mouse line, M10/HITEC mice, carrying both the homozygous *CYP3A7* gene and hemizygous *rpsL* gene. Male M10/HITEC and HITEC mice of eight weeks old were used. Both strains of mice were divided into two groups. The first group of mice for each strain was treated with AFB_1 at a dose of 8 mg/kg body weight by a single i.p. injection. Another group of mice for each strain was treated with DMSO as a vehicle of AFB_1. Two weeks after treatment, mice were sacrificed, and organs were collected for analysis.

Analysis of mutation frequency. Analysis of mutation was carried out according to the method described by Gondo *et al.* (19). Briefly, genomic DNA was prepared from the small intestine and the kidney from both M10/HITEC mice and HITEC mice. A shuttle plasmid pML4 carrying the *rpsL* gene was recovered from the genomic DNA by *Ban*II digestion. The DNA thus recovered was self-ligated by T4 DNA ligase. The plasmid pML4 was introduced into the host *E. Coli* RR1 cells by electroporation at 25 μFD, 200 =4 and 1.8 kV. When RR1 cells were transfected with pML4 carrying a wild type of *rpsL* gene, they were resistant to KM, and formed colonies on the plates containing KM alone. On the other hand, when RR1 cells were transfected with pML4 carrying the *rpsL* gene with a mutation(s), they were resistant to both KM and SM, and formed colonies on the plates containing both KM and SM. Mutation frequency was calculated as follows: Mutation frequency = colony number on the plate containing KM and SM/colony number on the plate containing KM alone.

RESULTS AND DISCUSSION

To confirm the expression of catalytically active CYP3A7 protein in the small intestine of M10/HITEC mice, we performed the Ames test using the 9,000 × g supernatant fraction prepared from the small intestine of M10/HITEC mice and determined the mutagen-producing capacity using AFB_1 as a promutagen. The results of the mutation assays using the 9,000 × g supernatant fraction from the small intestine and the kidney are shown in Fig.1. When the 9,000 × g supernatant fraction prepared from the small intestine was used, the mutagen-producing activity of the 9,000 × g supernatant fraction was higher in M10/HITEC mice than HITEC mice, as indicated by 32.4 (induced revertants/nmol) and 19.0 (induced revertants/nmol), respectively. Thus, CYP3A7 expressed in the small intestine of M10/HITEC mice was assumed to be catalytically active, and the difference between the values from M10/HITEC and HITEC mice was thought to be due to CYP3A7 expressed in the small intestine of M10/HITEC mice. When the 9,000 × g supernatant fraction prepared from the kidney was used, the mutagen-producing activity of the 9,000 × g supernatant fraction from the kidney was almost the same between M10/HITEC mice and HITEC mice, as indicated by 45.2 (induced revertants/nmol) and 40.0 (induced revertants/nmol), respectively.

To further examine the *in vivo* toxicological significance of CYP3A7, we analyzed the mutation which occurred in the *rpsL* gene. The M10/HITEC and the HITEC mice were treated with AFB_1 at a dose of 8 mg/kg body weight, and the mutations in the *rpsL* gene in the small intestine and the kidney were analyzed. The results of the experiment to analyze the mutations in the small intestine are shown in Fig. 2(a). The mutation frequencies in the small intestine from mice treated with AFB_1 were 7.15 ± 2.67 ($\times 10^{-5}$) and 3.74 ± 1.30 ($\times 10^{-5}$) for M10/HITEC and HITEC mice, respectively, while the mutation frequencies in the small intestine from both transgenic mice treated with DMSO as a control were 3.11 ± 1.04 ($\times 10^{-5}$) and 4.50 ± 1.78 ($\times 10^{-5}$) for M10/HITEC and HITEC mice, respectively. Thus, the mutation frequency of M10/HITEC mice was about 1.9-fold higher than that of HITEC mice; the difference was statistically significant ($p<0.05$). The difference in the mutation frequency between M10/HITEC and HITEC mice was similar to that of the mutagen-producing activity seen in the Ames test. Thus, the increased mutation frequency in M10/HITEC mice may be caused by CYP3A7 expressed in the small intestine of M10/HITEC mice.

AFB_1 is metabolized to AFB_1-8,9-epoxide, the reactive metabolite. CYP3A and CYP1A2 are reported to catalyze this metabolic activation (21). Regarding the metabolic activation of AFB_1 in the small intestine of mice, it appears that CYP3A11 (22) and CYP1A2 (23) in addition to CYP3A7 are also responsible for the metabolic activation. This may be true, while the level of the mutation frequency seen in HITEC mice treated with AFB_1 was not higher than the levels seen in the

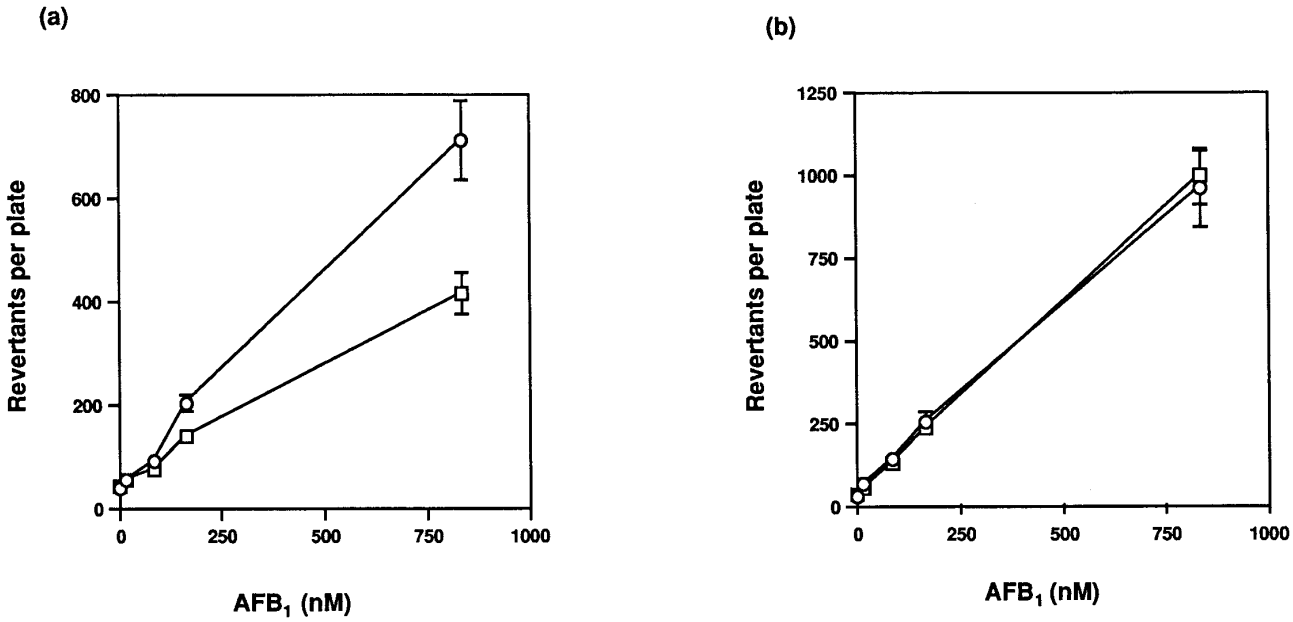

FIG. 1. Examination for the capacity of human CYP3A7 expressed in the small intestine of M10/HITEC mice to activate AFB_1 using *Salmonella typhimurium* TA98. The 9,000 × g supernatant fraction prepared from the small intestine (a), or kidney (b) was added to incubations. Each mixture contained the 9,000 × g supernatant fraction from a tissue pooled from 3 or 4 mice. The mutagenicity test was carried out as detailed under Materials and Methods. Open square, HITEC mice; open circle, M10/HITEC mice.

same strain of mice treated with DMSO. This apparent discrepancy may be accounted for by assuming that once the metabolic activation by these mouse enzymes occur, the active metabolite is detoxified by glutathione *S*-transferase present in abundant amounts as reported previously (21), and that there are relatively low levels of enzymes involved in the metabolic activation in the mouse small intestine (22, 23).

The results obtained using the kidney from M10/HITEC and HITEC mice, in which CYP3A7 is not expressed, are shown in Fig. 2(b). The mutation frequencies in mice treated with AFB_1 were 2.83 ± 2.22 ($\times 10^{-5}$) and 3.56 ± 1.55 ($\times 10^{-5}$) for M10/HITEC and HITEC mice, respectively. In the same strains of mice treated with DMSO, the mutation frequencies were 1.78 ± 1.01 ($\times 10^{-5}$) and 2.02 ± 1.01 ($\times 10^{-5}$) for M10/HITEC and HITEC mice, respectively. It has been reported that CYP3A is expressed in the mouse kidney at levels below detection limit (24), while CYP1A2 is expressed at a low level (25). Since a portion of AFB_1 is distributed

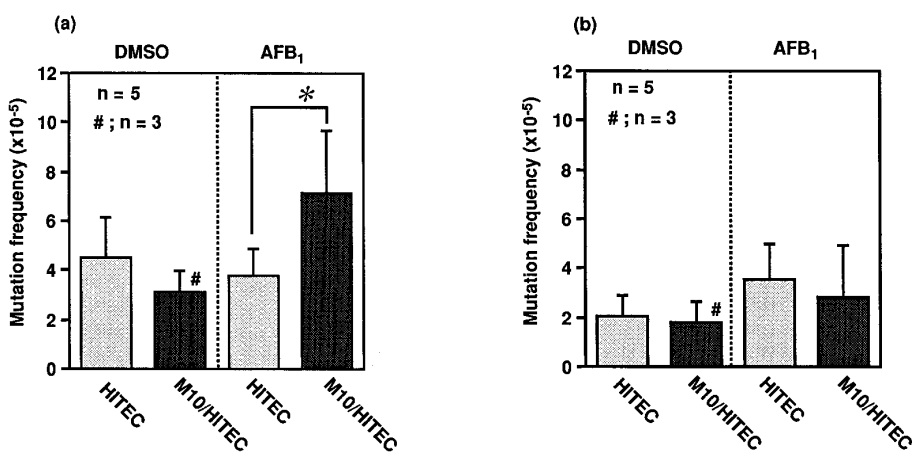

FIG. 2. The mutation frequency in the *rpsL* gene contained in the genomic DNA from the small intestine (a), or the kidney (b) of M10/HITEC mice and HITEC mice. The mutation frequency was analyzed as detailed under Materials and Methods. The mutation frequency is shown as mean ± SD (n = 5, except for the case indicated as # in which n = 3). * Indicates significantly different from the AFB_1-treated HITEC mice at $p<0.05$ (unpaired Student's *t* test).

in the kidney (26), it may be possible that AFB_1 is activated by the CYP1A2 present in the kidney. Despite this hypothesis, we found no significant increase in the mutation frequency by AFB_1, suggesting that the level of metabolic activation in the mouse kidney is negligible.

The half life of AFB_1-8,9-epoxide has been reported to be about 1 second (27). This is in accordance with our observation that no apparent increase of the mutation frequency in the kidney was seen. If the metabolite of AFB_1 formed in the liver is stable enough, then the metabolite is expected to be transferred to the kidney to cause mutations. Lending support to this idea, Li *et al.* reported that the level of AFB_1- *N*7- guanine adduct in the kidney of the CYP3A7-M10 mice was similar to that of non-transgenic mice (17).

To clarify the ability of CYP3A7 to activate AFB_1 *in vivo*, we established a hybrid mouse strain, M10/HITEC. The results indicate that CYP3A7 activates AFB_1 to induce mutations in the *rpsL* gene in the small intestine of the hybrid mouse, where CYP3A7 is expressed, but not in the kidney where CYP3A7 is not expressed. This *in vivo* study provides direct evidence that CYP3A7 plays an important toxicological role *in vivo.*

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid from The Ministry of Education, Science, Sports and Culture of Japan, and also by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Drug ADR Relief, R&D Promotion and Product Review of Japan, and also by research funds from the Japanese Society of Alternatives to Animal Experiments.

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