Establishment of a Salmonella Tester Strain Highly Sensitive to Mutagenic Heterocyclic Amines

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ABSTRACT

Heterocyclic amines (HCAs) that are present in cooked foods require metabolic activation to exert their genotoxicity. They undergo activation via N-hydroxylation by cytochrome P450 1A2 (CYP1A2), followed by O-esterification by O-acetyltransferase (OAT). To develop a Salmonella tester strain that is highly sensitive to mutagenic HCAs, we introduced a coexpression plasmid (pIA2OR) carrying human CYP1A2 and NADPH-CYP reductase cDNAs and an expression plasmid (pOAT) carrying Salmonella OAT to Salmonella typhimurium TA1538 to yield a TA1538/ARO coexpression plasmid (plA2OR). We also introduced an OAT gene expression system into TA1538/ARO strain and proved to express the enzymes, as indicated by high activities of 7-ethoxyresorufin O-deethylase and isoniazid N-acetylase.

The TA1538/ARO strain exhibited very high sensitivity to mutagenic HCAs 2-amino-3,4-dimethylimidazo[4,5-f]quinoline, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), and 2-amino-3,8-dimethylimidazo[4,5-f]quinoline and a somewhat higher sensitivity to 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine compared with the parent Ames tester strain TA1538. The plasmid pUC9/IA2 (23) was used as the source of cDNA for human CYP1A2. The NH2 terminus of CYP1A2 was modified as reported by Beverley, MA, respectively.

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The abbreviations used are: HCA, heterocyclic amine; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoline; CYP, cytochrome P450; NAT, N-acetyltransferase; OAT, O-acetyltransferase; OR, NADPH-CYP oxidoreductase; MeIQx, 2-amino-3,4-dimethylimidazo[4,5-f]quinoline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; MeAcC, 2-amino-3-methyl-9H-pyrido[2,3-b]indole; G-6-P, glucose-6-phosphate; EROD, 7-ethoxyresorufin O-deethylase.

For extrapolation of in vitro data to humans, it seems important to develop a test system that can detect the mutagenicity of HCAs at ordinary exposure levels. We have reported that genetically engineered mammalian cultured cells stably expressing CYP are sensitive in detecting the mutagenicities of promutagens, such as aflatoxin B1, (14, 18) and N,N-dimethylnitrosamine (19). Recent studies have shown that the expression yields of CYP in bacteria are higher than those in mammalian cultured cells (20). Therefore, the cDNA-mediated CYP expression system in the bacterial mutagenicity tester strain is expected to provide a useful tool for detecting picomolar levels of HCAs. Although the bacterial enzyme NADPH-flavodoxin reductase can substitute for the OR (21), the level of the activity is not efficient enough to support the full activity of CYP. Thus, the expression of OR seems to be required for the sufficient catalytic activity of CYPs. In fact, we have shown that simultaneous expression of monkey CYP1A1 and guinea pig OR enhanced the function of CYP1A1 (22) despite the mammalian cells that expressed endogenous OR. Thus, we attempted to establish a bacterial mutagenicity tester strain harboring human CYP1A2 and OR. We also introduced an OAT gene expression plasmid into this established strain to enhance the sensitivity to mutagenic HCAs.

MATERIALS AND METHODS

Chemicals. IQ, MeIQx, MeIQ, PhIP, and MeAcC were purchased from Wako Pure Chemicals Industries (Osaka, Japan). G-6-P, G-6-P dehydrogenase, NADP+, and liver homogenates (S9) from phenobarbital- and β-naphthoflavone-induced rats were obtained from Oriental Yeast Co. (Tokyo, Japan). α-Naphthoflavone was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Nutrient broth was obtained from Difco Laboratories (Detroit, MI). Plasmids pSE420 and pACYC184, used for a recombinant protein expression, were obtained from Invitrogen (Carlsbad, CA) and New England Biolabs (Beverly, MA), respectively.

Construction of Plasmids Carrying Human CYP1A2, OR, and OAT cDNAs. The plasmid pUC9/IA2 (23) was used as the source of cDNA for human CYP1A2. The NH2 terminus of CYP1A2 was modified as reported by
Fisher et al. (24). The CYP1A2 expression plasmid (pCW/1A2) was constructed according to the method reported by Sandhu et al. (25). Human OR cDNA from a human liver cDNA library prepared by the reverse transcriptase-PCR amplification was cloned by dividing it into two parts. The NH₂-terminal 1086 nucleotides were amplified using two specific primers. The sense primer corresponds to nucleotides −9 to −10 of OR (where nucleotide 1 corresponds to the first nucleotide of the start codon ATG; 5′-ATGATCA/CATGGA/GAGACT-3′). The Ncol site was introduced by site-directed mutagenesis at the start codon (underlined nucleotide). The antisense primer corresponds to nucleotides 1050–1077 (5′-TGCTTTGTTGGACTCC-3′). The COOH-terminal portion of OR cDNA was cloned using a sense primer that corresponds to nucleotides 964–984 (5′-GTGTACCCAGCCAACGACTCT-3′) and an antisense primer that corresponds to nucleotides 2109–2129 (5′-GGCAAAACA/CACCAGGAGAT-3′). The two amplified PCR fragments were ligated using an internal Ncol site at nucleotide 994. Subsequently, OR cDNA was introduced into the pSE420 expression plasmid (pSE/hOR). To obtain an OR cDNA fragment containing promoter and terminator regions, the pSE/hOR plasmid was digested with Sspl restriction enzyme. This fragment was introduced into nucleotides 131 bp upstream to 41 bp downstream of an OAT coding region. This fragment was inserted into the EcoRV site of the tetracycline resistance gene in pACYC184 plasmid (pOA/T; Fig. 1B). S. typhimurium TA1538 was transformed with the plA2OR plasmid, which was modified by S. typhimurium LB5000 (R± M+; Fig. 1A). The transformation of other plasmids (pOAT, pCW, and pACYC184) was carried out in the same manner as mentioned above. Bacterial strains and plasmids that were established or used in this study are summarized in Table 1.

Culture Conditions for Expression of CYP1A2, OR, and OAT. Twenty μl of bacterial stock solution were inoculated in 10 ml of nutrient broth supplemented with ampicillin (25 μg/ml) and chloramphenicol (10 μg/ml). Cultures were grown with shaking at 37°C for 8 h. Two μl of the culture were inoculated into 200 ml of modified Terrific Broth (25) and grown with shaking at 25°C for 8 h prior to induction with 1 mM isopropyl β-D-thiogalactopyranoside. The expression of recombinant proteins was achieved by a further incubation for 12 h at 25°C with shaking.

Assay of Catalytic Activities. Bacterial membrane and cytosol fractions were prepared as reported by Sandhu et al. (25). The content of CYP in whole bacterial cells and in the membrane fraction was determined by the method of Omura and Sato (28). EROD activity of the membrane fraction was determined essentially according to the methods of Lake (29), except for the components of an NADPH-generating system, in which the components of the NADPH-generating system in a reaction mixture were 5 mM MgCl₂, 5 mM G-6-P, 0.5 mM NADP⁺, and 1 unit/ml G-6-P dehydrogenase. The OR activity of the membrane fraction was determined with cytochrome c as an electron acceptor by measuring the absorbance change at 550 nm at 20°C according to the method of Phillips and Langdon (30). Watanabe et al. (26) reported that the transcript of Salmonella OAT gene had both NAT and OAT activities. Hence, we measured NAT activity with isoniazid as a substrate (26). The protein concentrations were determined by the method of Lowry et al. (31) using BSA as a standard.

Mutagenicity Assay. The number of viable cells was adjusted to give 1–2 × 10⁷ cells/ml by dilution with a nutrient broth prior to assays. The assay was carried out according to the method of Maron et al. (32). On the basis of a result of preliminary experiments, an NADPH-generating system was not added to a reaction mixture, except for an experiment in which bacterial membranes were added to the reaction mixture.

When IQ was metabolically activated outside of the cells, an appropriate amount of bacterial membrane or cytosol fraction from TA1538/ARO cells was added to a reaction mixture containing TA1538/OR or TA1538/C cells to give the same concentration of CYP and OAT. The NADPH-generating system and 1 mM acetyl CoA were also added to the reaction mixture.

Assays were carried out at least twice, with triplicate plates at each dose. The results were judged as positive when the number of colonies increased in a dose-dependent manner and reached a level twice as high as that obtained with vehicle alone as a control.

RESULTS

Expression of CYP1A2 in Salmonella. Fe²⁺-CO versus Fe²⁺ difference spectra of the genetically engineered Salmonella are shown in Fig. 2. The contents of CYP in whole cells of TA1538/ARO and TA1538/AR strains were approximately 1.1 × 10⁻⁷ and 7.7 × 10⁻⁸ pmol/cell, respectively. The contents of CYP in bacterial membranes and the catalytic activities of CYP and OAT are shown in Table 2. TA1538/ARO and TA1538/AR strains showed high EROD activity, whereas the EROD activity in TA1538/OR and TA1538/C strains was not detectable. TA1538/ARO and TA1538/C strains showed isoniazid NAT activities about 6–7 times as high as those of TA1538/AR and TA1538/C strains.

Effect of Preincubation on the Induction of Revertants. To achieve the maximum efficiency of metabolic activation of CYP1A2, we examined the effect of preincubation on the induction of revertants using IQ, MelIQ, and MeAaC. TA1538/ARO cells were preincubated with HCA for 0–60 min. As shown in Fig. 3, the number of revertants was the highest when cells were not preincubated. In particular, when the cells were treated with IQ, a decrease in the number of revertants was observed in all doses examined. Therefore, we did not preincubate in the following mutagenicity assays.

Efficiency of Genetically Engineered Salmonella in the Mutagenic Activation of HCAs. To evaluate the sensitivity of the established strains to HCAs, mutagenicities of MelIQ, IQ, MelQx, and PhiP...
were assayed. The TA1538/ARO and TA1538/AR strains showed positive responses to all HCAs examined. Among the four strains, the TA1538/ARO strain showed the highest sensitivity to HCAs (Fig. 4). The mutagenic potencies of HCAs in the TA1538/ARO strain were the highest with MeIQ and decreased in the order: MeIQ > IQ > MelIQ > PhIP (Table 3). Minimum concentrations of MeIQ, IQ, MelIQ, and PhIP giving positive results, judged as discussed in “Materials and Methods,” in the TA1538/ARO cells were 0.3, 3, 30, and 1000 pm, respectively. These values of MeIQ, IQ, and MelIQ, found in the TA1538/ARO cells, were approximately 10–100 times lower than those found in the TA1538/AR cells. When cells were treated with PhIP, the number of revertants at each dose in TA1538/ARO was slightly higher than that in TA1538/AR (Fig. 4D). However, mutagenic potencies of PhIP in TA1538/ARO (650 revertants/nmol) and in TA1538/AR (550 revertants/nmol) cells were nearly the same. Moreover, the minimum concentration of PhIP giving positive results in TA1538/AR was as same as that in TA1538/ARO. Four HCAs did not show mutagenicity in the TA1538/O cells that overexpress the OAT gene alone.

Inhibition of Mutagenic Activation in Genetically Engineered Salmonella Cells by α-Naphthoflavone. α-Naphthoflavone, one of the representative inhibitors of CYP1A2 (33), was added to a mixture containing the bacteria and a mutagen IQ. Plates were incubated at 37°C for 2 days. As shown in Fig. 5, α-naphthoflavone strongly inhibited the mutagenic activation of IQ in the engineered cells. Inhibitory effect was almost saturated at concentrations of α-naphthoflavone higher than 330 nm. This result indicates that CYP1A2 expressed in the cells is catalytically active and that OR can functionally associate with the cytochrome to exhibit its activity.

Effect of Intracellular Metabolic Activation on the Sensitivity of TA1538 Cells. Reactive intermediates are, in general, believed to be chemically unstable and reactive with cell components. Thus, if the reactive intermediates are formed outside of the cells, then the reactive metabolites are assumed to bind to cell components or the surface of the cell membranes. In our newly established cells expressing enzymes necessary to activate HCAs, it was expected that the reactive intermediates formed inside of the cells can bind efficiently with DNA to cause mutation of the Salmonella. To examine the effect of intracellular metabolic activation on the sensitivity of the established strain, the membrane and/or cytosol fractions of TA1538/ARO were added to the incubations containing TA1538/O and TA1538/C to compare the efficiency of intracellular and extracellular activations, respectively, using IQ as a promutagen (Fig. 6). The results showed that IQ induced a much higher mutation frequency in TA1538/ARO than it did in TA1538/O and TA1538/C, fortified externally with appropriate amounts of CYP1A2, OR, and OAT, indicating that intracellular activation of IQ is one of the important factors to raise the sensitivity of the cells to the promutagen.

DISCUSSION

In past years, several genetically engineered Salmonella tester strains have been reported (12, 15, 34, 35). For example, Grant et al. (13) and Wild et al. (15) reported on the establishment of strains

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### Table 2 CYP contents and catalytic activities in genetically engineered Salmonella cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>CYP activity (nmol/min/mg protein)</th>
<th>OR activity (nmol of cytochrome c reduced/min/mg of protein)</th>
<th>EROD activity (nmol/min/nmol of CYP)</th>
<th>Isozyme NAT activity (nmol/min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA1538/ARO</td>
<td>0.259 ± 0.016</td>
<td>123.8 ± 24.1</td>
<td>11.4 ± 0.18</td>
<td>135.9 ± 10.1</td>
</tr>
<tr>
<td>TA1538/AR</td>
<td>0.236 ± 0.012</td>
<td>91.3 ± 6.2</td>
<td>6.3 ± 0.09</td>
<td>19.3 ± 6.2</td>
</tr>
<tr>
<td>TA1538/O</td>
<td>&lt;0.002</td>
<td>1.4 ± 0.1</td>
<td>&lt;0.004</td>
<td>142.7 ± 25.7</td>
</tr>
<tr>
<td>TA1538/C</td>
<td>&lt;0.002</td>
<td>1.6 ± 0.1</td>
<td>&lt;0.004</td>
<td>22.0 ± 15.4</td>
</tr>
</tbody>
</table>

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Fig. 3. Effect of preincubation on the induction of revertants. TA1538/ARO cells were treated with IQ (A), MelQ (B), or MeAorC (C) at the concentrations indicated in the figure for 0–60 min. Immediately after the preincubation, the reaction mixture was poured onto a minimal glucose plate with a top agar. The number of revertants was counted after the 2 days of incubation at 37°C. Data points, mean (n = 3); bars, SD.

Fig. 4. Sensitivity of genetically engineered Salmonella to HCAs. Salmonella cells were treated with MelQ (A), IQ (B), MelQx (C), or PhIP (D). ○, TA1538/ARO; ●, TA1538/AR; ▼, TA1538/G; ▼, TA1538/C.

expressing human NAT1 or NAT2. Josephy et al. (35) reported on a strain expressing human CYP1A2. However, the sensitivities to HCAs in the Salmonella were almost equal or even lower than that of the parental Ames tester strain. Although the strain expressing human NAT2 as a phase II enzyme (12, 15) seems to be useful for assessing HCAs to human risks, rat S9 must still be added to N-hydroxylate HCAs. The established strain of cells expressing CYP1A2 (35) did not express OR. Therefore, the cells did not show sufficient monoxygenase activity of CYP1A2. Here, our genetically engineered strain TA1538/ARO showed a high sensitivity to HCAs without addition of rat S9. Thus, it may be possible that the simultaneous expression of OR is one of the important factors in raising the function of CYP1A2 in the bacteria.

We constructed a coexpression plasmid possessing two promoters to express both CYP1A2 and OR separately. This may be another reason for the success in obtaining the high expression yields of both enzymes in bacteria. Consequently, CYP1A2 in TA1538/ARO and TA1538/AR cells showed high functional monoxygenase activity (Table 2). Recently, Blake et al. (36) reported the coexpression system for human CYP3A4 and human OR in Escherichia coli carrying a promoter for expression of each enzyme. The independent two-promoter system may be effective for the simultaneous expression of CYP and OR in bacteria.

In mutagenicity assays, the preincubation of bacteria with an appropriate enzyme system such as rat S9 has been known to be effective, especially for the activation of promutagens during prein-
cubation (37). However, the preincubation was not needed for our established strain TA1538/ARO (Fig. 3). As shown in Fig. 3, the number of revertants decreased when the cells were preincubated with IQ. It has been reported that some HCAs generate oxygen free radicals by direct interaction with OR and that the amount of oxygen free radicals generated by IQ was relatively high, whereas the amount generated by MeAaC was low (38). The radical generation is considered to be an important factor in the causation of the cytotoxicities of certain anticancer agents (39, 40). In the TA1538/ARO cells, which express OR intracellularly, it seems possible that HCAs interact with OR to generate oxygen free radicals. Thus, the decrease in the number of revertants might be caused by the reduction of the numbers of viable cells resulting from the toxicity of oxygen free radicals. Further investigations are required for the explanation of this phenomenon.

MeIQx is one of the most abundant HCAs present in cooked foods. In contrast, IQ and MelQx are present in much lower quantities (3, 4). As shown in Table 3, the mutagenic potencies (induced revertants/nmol) of IQ and MeIQx were almost the same in the Ames tester strain when these HCAs were metabolically activated by externally added rat S9. Similar results were reported by Wild et al. (15). Calculating on the basis of both the quantities and the mutagenic potencies of HCAs in the Ames test, MelQx, IQ, and MeIQ are assumed to account for >60,6, and 4% of the total mutagenicity of fried beef, respectively (3). On the other hand, in this study with TA1538/ARO cells, the mutagenic potency of IQ was approximately 10 times as high as that of MeIQx (Table 3). These results suggest that the contribution of IQ in total mutagenicity of cooked foods has been underestimated in previous studies (3).

Although the same amounts of CYP1A2 and OAT were added to the reaction mixture, the TA1538/ARO strain showed the highest sensitivity among the established strains. Moreover, the TA1538/O strain showed higher sensitivity than the TA1538/C strain although approximately the same amount of OAT was added to the reaction mixture containing the TA1538/C cells (Fig. 6). The O-acetylated derivatives of N-hydroxy HCAs are highly reactive to bind to DNA and proteins. Hence, the intracellular expression of OAT also seems to be advantageous for these intermediates to interact with chromosomal DNA efficiently. These results suggest that the internal expression of CYP1A2 and OR also contributes to sensitization of the established strain. Although the response of TA1538/ARO toward PhIP was slightly different from that of TA1538/AR, values related to the mutagenicity of PhIP were nearly the same. Wild et al. (41) reported that the difference of OAT activity in Salmonella cells does not affect the mutagenicity of PhIP. Therefore, the difference in the mutagenicity of PhIP between TA1538/AR0 and TA1538 fortified with rat S9 may be reflected by the efficiency of CYP1A2 and other unknown factors on the metabolic activation of PhIP. As shown in Table 3, we found large differences between the efficiency of CYP1A2 expressed in TA1538/ARO and the efficiency of enzymes present in rat S9 to activate HCAs. Although the CYP1A2 enzymes in these systems came from different species, i.e., human and rat, it has been reported that there are no differences in the catalytic activities of CYP1A2 between the two species (42). Therefore, we postulate that the differences in the efficiency to activate HCAs might be caused by the difference in intracellular and extracellular activations. These results lend support to the idea that CYP1A2 inside of the cells could metabolically activate HCAs efficiently.

Murray et al. (43) reported that the concentrations of MeIQx in their urine collected for 12 h after meals were 10.6–17.8 pg/ml, when six male volunteers consumed about 300 g of cooked beef (approximately 290–850 ng of MeIQx ingested). Moreover, when cynomolgus monkeys were administered 2.2 μmol of IQ per body weight (in kg), the content of IQ in their blood at 1 h after dosing was estimated to be approximately 20 pmol/ml (44). These results indicate that only 1–2 ml of urine or 0.1 ml of blood is needed to detect mutagenic HCAs, if we analyze by using the TA1538/ARO cells. In fact, in preliminary experiments using the TA1538/ARO cells, we found that MeIQx could be detected with 0.1 ml of sera, which were collected from rats given 0.6 μmol of MeIQx per body weight (in kg).

Thus, the bacteria will be a valuable tool for assessing human risks of HCA mutagens and carcinogens.

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