Inhibitory Effects of Beer on Mutation in the Ames Test and DNA Adduct Formation in Mouse Organs Induced by 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)

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An evaluation of the antigenotoxic potential of beer components against carcinogens contained in the human diet, namely heterocyclic amines (HCAs) including 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), was determined. The protective mechanism involved was also investigated. Beer samples were found to inhibit the mutagenicity of HCAs in the Ames test. Beer solution, consisting of a freeze-dried and dissolved sample, given as drink-water significantly reduced the formation of PhIP-DNA adducts in mouse colon and lung compared to control mice fed with PhIP in the absence of beer solution. Furthermore, beer solution added in the diet as a food additive mimic significantly reduced the amount of DNA adducts present in the liver and lung of mice fed with PhIP. In an effort to investigate the mechanism responsible for the observed protective effect, the effect of beer solutions on HCA metabolizing enzymes was investigated. Beer solutions inhibited the activity of CYP1A1 and CYP1A2, as determined from deethylation and demethylation assays using 7-ethoxy- and 7-methoxyresolufin, respectively. Considering the overall suppression of PhIP genotoxicity by beer, this study confirmed that beer components can interfere with the enzyme activity involved in the metabolism of HCAs and subsequently suppress the observed genotoxicity. The results of this study showed that beer components act in a protective capacity against the genotoxic effects of heterocyclic amines *in vivo*.

Key words 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP); beer; DNA adduct; *in vivo* effect; antigenotoxicity; antimutagenicity

Heterocyclic amines (HCAs) including 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) have been identified as potent mutagens and carcinogens in rodents and are produced in foods during the process of cooking.[1,2] Since humans are frequently exposed to HCAs, these compounds are suspected as being human carcinogens.[3] Epidemiological studies have established an apparent association between the consumption of well-cooked red meat and certain types of cancer. Ohgaki *et al.*[4] showed that mice fed with PhIP in the diet had a higher incidence of lymphoma and leukemia than control mice, and rats fed with PhIP developed colon and prostate carcinoma in males, while mammary gland carcinomas appeared in females. The antimutagenicity and anticarcinogenicity of dietary components is currently receiving a great deal of attention,[5,6] and protection against PhIP genotoxicity has been investigated.[7—10] We previously investigated the inhibitory effect of beer on the bacterial mutagenicity of preactivated heterocyclic amines including 3-hydroxyamino-1-methyl-5*H*-pyrido[4,3-*b*]indole, 2-hydroxyamino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole,[11] 2-chloro-4-methylthiobutanoic acid[12] and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.[13] Beer also prevented radiation-induced chromosome aberrations in human lymphocytes,[14] and suppressed PhIP genotoxicity in V79 cells as determined by the comet assay.[15]

In this study, the effects of beer on the mutagenicity of promutagenic PhIP and several other heterocyclic amines in the presence of metabolic enzymes were investigated using the Ames test. The *in vivo* effect of beer samples in relation to PhIP-induced DNA adduct formation under conditions relevant to human dietary habits was also evaluated. The protective effects were studied in the colon (the target organ associated with PhIP tumorigenesis) and other important organs (liver, lung and kidney) of mice fed with a beer-solution or by the addition of beer components to the diet, the latter being a food additive mimic. Since HCAs are promutagens and require cytochrome P450-mediated activation for mutagenicity, the effects of beer on metabolic activation were evaluated in an effort to determine the protective mechanism involved.

MATERIALS AND METHODS

General PhIP (CAS 105650-23-5), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) (CAS 76180-96-6), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) (CAS 77500-04-0) and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) (CAS 62450-07-1) were obtained from Wako Chemicals (Osaka, Japan). S9 was prepared from livers of Sprague–Dawley rats (6 weeks old) from Charles River Japan (Atsugi, Japan) induced by phenobarbital and β-naphthoflavone (Wako Chemicals). C57BL/6N mice (male, 6 weeks old) were also obtained from Charles River Japan. Four different beer samples (beers A, B, C, D) produced in Japan were examined. Beer-A was a stout beer, while beer-B, -C and -D were lager beers. All were purchased in local stores in Okayama. For the *in vivo* experiments, beer-A was freeze-dried to remove ethanol and the solid obtained was dissolved in water to one-fifth, one-half or an equal volume of the original sample volume. These samples were referred to as "beer solution (×5)", "beer solution (×2)" and "beer solution (×1)", respectively. For the enzyme assay, beer samples were freeze-dried and the residues were dissolved in water equal to the original sample volume. "μl eq." repre-

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sents the amount of beer equivalent to that in a corresponding volume of beer.

Mutagenicity Assays and Inhibition Experiments The preincubation method[16] was employed for the Salmonella mutagenicity assays.[17] The modifying effects of compounds on mutagenicity were examined as previously described.[11] The revertant colonies that resulted were counted manually. When the number per plate exceeded 2000, colonies in a certain square area were counted, and the total number on the plate was estimated from the average count derived from five such areas. Experiments were performed in duplicate and the averages were determined. Statistical analyses were performed using the one-way ANOVA. Results were considered significant at $p<0.05$. Mutagenic activity (%) given in the Figures was derived as follows:

$$100\times[(\text{His}^+ \text{ revertants in the presence of beer}) - (\text{spontaneous revertants})]/[(\text{His}^+ \text{ revertants in the absence of beer}) - (\text{spontaneous revertants})]$$

Detection of DNA Adducts in Mice C57BL/6N mice were housed as one or two mice in a cage with access to diet and water *ad libitum*. The diet for the mice was prepared by mixing 3 g of feed (MF powder, Oriental Yeast, Tokyo, Japan) with 3 ml of 0.005% PhIP solution or 3 ml of water (for the control diet), to yield a diet-paste (6 g). The calorie content of the diet was adjusted using maltose. In investigations concerning the influence of beer on PhIP-induced DNA adduct formation, experiment-1 consisted of continuously providing beer-A solution to mice in lieu of drinking water for 5 d. Diet-paste (6 g) carrying 0.005% PhIP was given for three subsequent days at 3 p.m. Four groups of mice received a diet mixed with PhIP (group 1), PhIP in the diet and beer-A solution (×1) in water (group 2), PhIP in the diet and beer-A solution (×2) in water (group 3), or a control diet (group 4). On day 6, mice were sacrificed by cervical dislocation, tissues were excised, washed with ice-cold 0.15 M KCl, frozen in liquid nitrogen, and then stored at −80 °C until use. Experiment-2 consisted of mice being fed with a paste that consisted of mixing beer solution-A and 0.005% PhIP with an equal weight of control diet (powder). For 2 d, mice received a control diet mixed with water (groups 1 and 4), a diet mixed with beer-A solution (×1) (group 2), or a diet mixed with beer-A solution (×5) (group 3). For the subsequent three days, mice were given a diet mixed with PhIP (group 1), beer-A solution (×1) and PhIP (group 2), beer-A solution (×5) and PhIP (group 3), or a control diet (group 4). On day 6, mice were sacrificed. Subsequent procedures were similar to those outlined for experiment-1.

The amount of PhIP-DNA adducts in treated mice tissue was determined by a modified adduct-intensification analysis of the ^{32}P-postlabeling method.[18] The level of adduct was estimated by measuring relative adduct labeling using a Bio-Imaging analyzer (BAS 2000, Fuji Film, Tokyo, Japan). Each detection and quantitative analysis of PhIP-adduct was carried out in triplicate and the reproducibility was confirmed. All experiments were carried out in accordance with the Guidelines for Animal Experiments at Okayama University Advanced Science Research Center, Japanese Government Animal Protection and Management Law (No. 105), and Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6). Statistical analyses were performed using the unpaired Student's *t* test. Results were considered significant at $p<0.05$.

Measurement of Enzyme Activity 7-Etoxyresorufin *O*-deethylase (EROD) and 7-methoxyresorufin *O*-demethylase (MROD) activities were measured by fluorophotometric quantification of each metabolite produced from CYP1A1-mediated *O*-deethylation and CYP1A2-mediated *O*-demethylation, respectively, as previously described.[19] The fluorescence emitted from the metabolite was not influenced by the presence of beer solution at the doses tested. Each experiment was carried out in triplicate and averages were determined. Experiments were repeated twice and the reproducibility was confirmed. Statistical analyses were performed using the one-way ANOVA. Results were considered significant at $p<0.05$.

RESULTS

The antimutagenicity of four samples of beer was examined using the Ames test. Three beer samples (beer-A, -B, -C) significantly inhibited PhIP mutagenicity (Fig. 1A). The amount of beer sample needed for 40% inhibition (ID_{40}) of PhIP mutagenicity was approximately 20, 100 and 100 μl/plate for beer-A, -B and -C, respectively. Beer-C was sig-

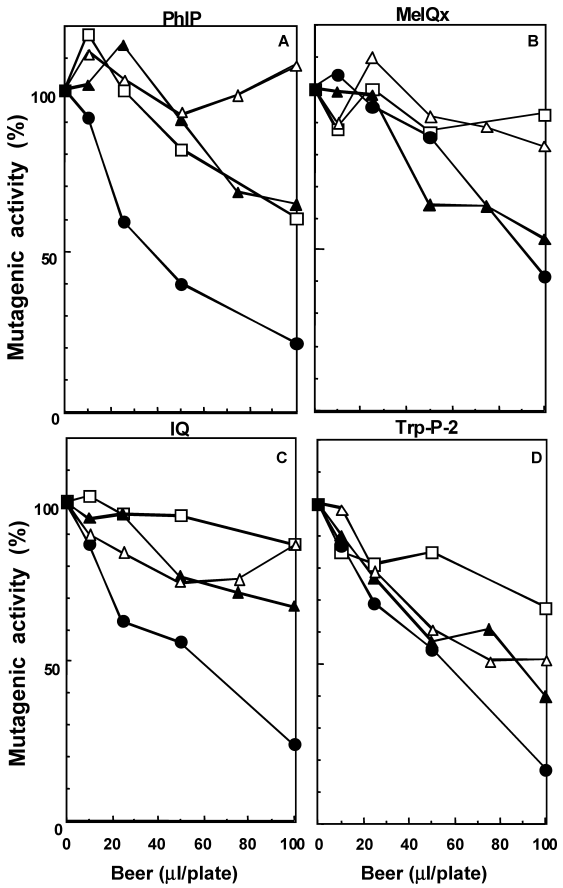

Fig. 1. Effect of Beer Samples on the Mutagenicity of PhIP (A), MeIQx (B), IQ (C) and Trp-P-2 (D): Beer-A (●), Beer-B (□), Beer-C (▲) and Beer-D (△)

The number of His$^+$ revertants from *S. typhimurium* TA98 per plate found in the absence of inhibitor was 814±20 for 2 nmol of PhIP, 3263±76 for 80 pmol of IQ, 3509±341 for 0.2 nmol MeIQx and 1895±206 for 0.1 nmol of Trp-P-2. The number of revertants spontaneously formed was 20—43 with TA98. The averages of the duplicate data are shown. Experiments were repeated and the reproducibility was confirmed.

nificantly effective in inhibiting the mutagenicity of MeIQx (Fig. 1B), while beer-A and -C were significantly effective in inhibiting the mutagenicity of IQ (Fig. 1C), and all beer samples examined significantly inhibited Trp-P-2 mutagenicity (Fig. 1D).

The *in vivo* effects of beer on the formation of DNA adducts in mice fed with PhIP were investigated. As the ID_{40} of beer-A against PhIP mutagenicity was one-fifth or less relative to the other three samples, the effect of beer-A on the formation of DNA adducts in mice was examined. The diet-paste given to mice was completely eaten before 9 a.m. the following day. PhIP-induced DNA adducts were formed in the colon, liver, lung and kidney of mice given 0.005% PhIP in the diet (Tables 1, 2). No adducts were observed in the tested organs of mice fed a control diet (group 4) (data not shown). The formation of DNA adducts in the colon and lung of mice given PhIP in the diet significantly decreased following the administration of drinking beer-A solution (×2) *ad libitum* compared with mice given PhIP without beer-A solution (Table 1). Beer-A solution (×1) and (×5) administered in the diet also significantly decreased the amount of DNA adducts in the liver and lung of mice given PhIP in the diet (Table 2).

To examine the effect of beer samples on the enzymes that metabolize heterocyclic amines, the relative enzyme activity in the presence of beer was determined and is shown in Fig. 2. MROD activity was significantly inhibited in the presence of beer solutions (beer-A, -B, -C, -D). The amount of beer sample needed for 50% inhibition (IC_{50}) of the demethylase activity was approximately 60, 200, 100 and 200 μl eq./ml for beer-A, -B, -C and -D, respectively (Fig. 2A). EROD activity was slightly enhanced in the presence of a small amount of beer solution, and then inhibited. The differences were significant at $p<0.05$. IC_{50} values of deethylase activity determined were 80, 140 and 200 μl eq./ml for beer-A, beer-C and beer-B, respectively (Fig. 2B).

Table 1. Effect of Beer Solution in the Drinking Water on DNA Adduct Formation in Mice Fed with 0.005% PhIP (Experiment-1)

Tissue group	Beer solution added in the drinking water	Adducts/10^8 nucleotide	No. of mice
Colon			
1	Water	38.6±28.2	7
2	Beer-A solution (×1)	44.8±40.8	8
3	Beer-A solution (×2)	15.6±16.8*	8
Liver			
1	Water	17.9±7.96	8
2	Beer-A solution (×1)	15.1±7.31	8
3	Beer-A solution (×2)	11.9±7.71	8
Lung			
1	Water	11.1±3.91	8
2	Beer-A solution (×1)	7.42±4.00	6
3	Beer-A solution (×2)	7.91±2.91*	7
Kidney			
1	Water	23.7±11.4	8
2	Beer-A solution (×1)	17.0±5.67	8
3	Beer-A solution (×2)	15.1±9.53	8

* Significantly different from group 1 at $p<0.05$. Experimental details as described in Materials and Methods.

Table 2. Effect of Beer-A Solution Mixed in the Diet on DNA Adduct Formation in Mice Fed with 0.005% PhIP (Experiment-2)

Tissue group	Beer solution added in the diet	Adducts/10^8 nucleotide	No. of mice
Colon			
1	Water	2.69±1.43	8
2	Beer-A solution (×1)	4.50±3.73	8
3	Beer-A solution (×5)	2.69±2.30	8
Liver			
1	Water	5.18±1.71	8
2	Beer-A solution (×1)	3.17±1.37*	8
3	Beer-A solution (×5)	2.44±2.02**	8
Lung			
1	Water	22.9±6.43	8
2	Beer-A solution (×1)	0.611±1.24***	8
3	Beer-A solution (×5)	1.60±2.23***	8
Kidney			
1	Water	1.92±1.23	8
2	Beer-A solution (×1)	3.94±6.99	8
3	Beer-A solution (×5)	1.10±1.00	8

Significantly different from group 1 at *$p<0.05$, **$p<0.01$ and ***$p<0.001$. Experimental details as described in Materials and Methods.

DISCUSSION

Results obtained from the Ames test showed that certain beer samples could inhibit the mutagenicity of heterocyclic amines (PhIP, MeIQx, IQ and Trp-P-2) that were present in cooked food. Considering the relevance of human dietary habits, the effects of beer solution in the form of drinking beverages were investigated. Beer-A solution in drinking

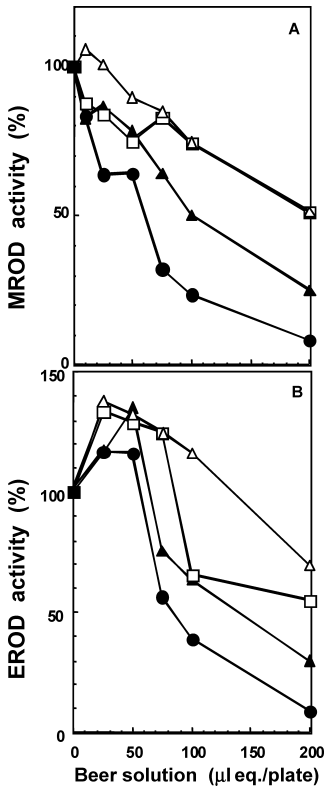

Fig. 2. Effects of Beer Solutions on 7-Methoxyresorufin *O*-Demethylase (MROD) (A) and 7-Ethylresorufin *O*-Deethylase (EROD) (B) Activities

Relative activities are shown in the presence of beer-A (●), beer-B (□), beer-C (▲) and beer-D (△) solutions, where 100% is defined as the activity of each enzyme in the absence of beer solution, which is 75.2 and 246 pmol/min/mg protein for MROD and EROD, respectively. "μl eq." represents the amount of beer equivalent to that in a corresponding volume of beer. Averages of the duplicate data are shown. Experiments were repeated and the reproducibility was confirmed.

water decreased the amount of DNA adducts formed in the colon and lung of mice fed with PhIP (Table 1). This result suggested that beer components in the drinking water could suppress the formation of PhIP-DNA adducts in target organs (colon) associated with carcinogenesis in male rats. The effects of beer solution mixed in the diet as a food mimic were then investigated. The addition of beer-A solution in the diet decreased the amount of DNA adducts formed in the liver and lung of mice given PhIP, however this effect was not observed in the colon (Table 2). The observed differences in the suppression of DNA-adduct formation in the liver and colon between experiment-1 and -2 could be accounted for by the feeding methods employed. It is known that enterohepatic circulation is important for PhIP metabolism. Watkins *et al.*[20] reported that feces was the major route of excretion of PhIP, and suggested biliary excretion of PhIP. Buonarati *et al.*[21] reported that urinary and fecal excretion over 24 h accounted for 16% and 42—56% of the dose, respectively, in mice administered [^{14}C]PhIP (i.p.). This suggested that colon cells had been exposed to PhIP for over 24 h following administration. Mice in experiment-1 were given beer-A solution throughout the experimental period as drinking water, while mice in experiment-2 received the diet mixed with beer-A solution from 3 p.m. to 9 a.m. at the following day but received no diet mixed with beer-A solution from 9 a.m. to 3 p.m. Continuous presence of beer components throughout the experimental period might be more effective in providing protection against PhIP-induced DNA damage in the colon. In contrast, administration of beer together with PhIP in the diet might be more effective in suppressing the activation of PhIP by CYP enzymes at the first pass through the liver of mice.

It was found that the amount of DNA adducts in the liver, lung and kidney of mice fed with MeIQx decreased significantly following the administration of beer-A solution mixed in drinking water as well as in the diet.[22] A common mechanism concerning the inhibitory effect of components in beer-A towards mutagenicity in the Ames test and DNA adduct formation induced by PhIP and MeIQx might be involved. The metabolism of mutagens represents one protection mechanism associated with the use of certain chemopreventive agents.[5,8,9] PhIP was metabolically activated by CYP1A1, CYP1A2 and CYP1B1 through a process involving *N*-hydroxylation.[23,24] The CYP1A2-catalyzed *N*-hydroxylation pathway was shown to account for 70% of the overall elimination of a PhIP dose ingested by human volunteers and 91% of ingested MeIQx.[24] The ID_{40} of beer solution against PhIP mutagenicity was beer-A<beer-B=beer-C<beer-D, and the IC_{50} of beer solution against the CYP1A1 and CYP1A2 activities were also beer-A<beer-C<beer-B≦beer-D. Suppression of CYP1A2 and CYP1A1 activities suggested that the antimutagenicity observed with the Ames test was related to the inhibition of metabolic activation. The inhibition of DNA adduct formation might also be linked with the inhibition of metabolic activation.

Miranda *et al.*[25] reported that 8-prenylnaringenin and xanthohumol from hops could inhibit the mutagenic activation of CYP1A2-mediated IQ. These components from hops could be responsible for the inhibition of CYP enzymes by beer. Although the antimutagenic effect towards heterocyclic amines remains to be determined, it is assumed that beer components have at least one target associated with the observed antimutagenicity, namely, the inhibition of CYP enzyme activity for PhIP. The formation of DNA adducts and associated genetic changes play critical roles in processes involving carcinogenesis.[26] The results of the present study has provided candidates that might act as potential modulators of heterocyclic amine-induced carcinogenesis.

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