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Inhibition of DNA Adduct Formation and Mutagenic Action of 3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole by Chlorophyllin-chitosan in *rpsL* Transgenic Mice

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We have studied the inhibitory effect of chlorophyllin-chitosan (Chl-Chi) complex, an insoluble form of chlorophyllin, on the DNA adduct formation and mutagenesis by a heterocyclic food mutagen-carcinogen, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), in mice carrying the *E. coli rpsL* gene as a mutagenesis reporter. Upon administration of a diet containing 0.002% or 0.01% Trp-P-2, DNA adducts were formed in various tissues in a dose-dependent manner, with the maximum level observed in the liver. Addition of 3% Chl-Chi to the diet reduced the Trp-P-2 adduct by up to 90%. The *rpsL* mutant frequencies increased significantly in both the liver and spleen upon administration of a 0.01% Trp-P-2 diet. Addition of Chl-Chi to the diet decreased these induced mutant frequencies to the background level. No harmful effect of Chl-Chi was detected during these experiments. The results show that Chl-Chi may be a candidate chemopreventive agent against the genotoxic action of Trp-P-2, and possibly also other aromatic carcinogens in the diet.

Key words: Chlorophyllin — Trp-P-2 — Somatic mutation — Chitosan — Chemoprevention

Food that humans eat every day contains mutagenic and carcinogenic compounds such as heterocyclic amines.[1)] Food also contains antimutagenic and anticarcinogenic agents.[2,3)] It is of great interest to seek edible, safe chemicals that could prevent the genotoxic actions of some components in food. Recently, a new anti-mutagenic compound, chlorophyllin-chitosan (Chl-Chi) was developed.[4)] This insoluble material is a complex of chlorophyllin and chitosan, both of which are used widely in food and medicine as safe materials. Chlorophyllin can inhibit binding of heterocyclic amines to DNA,[5–8)] and inhibits heterocyclic amine-induced mutagenesis[5,9–11)] and carcinogenesis.[12,13)] The basis of the inhibitory actions *in vivo* is supposed to be its high affinity for heterocyclic amines and subsequent efficient excretion from the body in a form of a chlorophyllin-carcinogen complex.[14)] For this purpose, the insoluble form of chlorophyllin, Chl-Chi, might be more suitable than chlorophyllin itself. This insoluble Chl-Chi complex has affinity for heterocyclic amines as strong as that of free chlorophyllin.[4)] Therefore, when Chl-Chi is present with heterocyclic amines in the diet, it can be expected that it will trap the carcinogens as efficiently as chlorophyllin. The insoluble complexes may then be readily excreted. The insoluble nature of Chl-Chi may also favor avoidance of hitherto unknown adverse effects, if any, of chlorophyllin. In the present study we have explored the inhibitory effects of Chl-Chi on DNA adduct formation and mutagenesis induced by a short-term administration of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]-indole (Trp-P-2), a typical heterocyclic amine, in various organs in mice. Orally given Trp-P-2 is known to induce hepatocellular carcinoma in CDF1 mice, with markedly higher activity in females.[15)] For these analyses we used transgenic mice, HITEC, which carry the readily retrievable *rpsL* gene as a mutagenesis marker.[16–18)]

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MATERIALS AND METHODS

Materials Chl-Chi was prepared according to a salting-out procedure reported earlier.[4)] From 6 g of chitosan-500 (Wako, Osaka) and 12 g of sodium copper chlorophyllin (Nacalai Tesque, Kyoto), 12–15 g of Chl-Chi was obtained as a deep green powder with an Na-Cu chlorophyllin content of approximately 60% (w/w). The pH dependence and capacity of the Chl-Chi to bind Trp-P-2 were similar to those previously reported[4)]: the saturation level of Trp-P-2 was 0.6 mmol per gram of Chl-Chi.

Transgenic mice and administration HITEC mice (SSW2-14P) used in this study were developed by Gondo and collaborators.[19)] The mice contained integrated plasmid pSSW[19)] carrying the *rpsL* gene. Seven-week-old female HITEC mice and sibling female mice without the pSSW insert born from the same mothers simultaneously were obtained from Kyudo Co., Ltd. (Tosu, Saga). The presence of transgene in the mice was determined by Southern blot analysis using plasmid pSSW as a probe. The mice that carried pSSW were used for the mutational

analysis, while the mice without the plasmid were used for the measurement of DNA adducts using ^{32}P-postlabeling analysis. The presence of pSSW in the chromosome was assumed not to affect DNA adduction by Trp-P-2. After having been fed normal solid mouse diet, CE-2 (Clea Japan, Tokyo), the mice were fed a specific diet in a ceramic container, which was changed daily. For the preparation of test diets, 3 g of pulverized CE-2 per mouse with or without 6% Chl-Chi by weight was mixed with an equal weight of water containing 0.004% or 0.02% Trp-P-2 acetate, or water only. Drinking water was given freely. The mice with and without the transgene were placed in separate cages side-by-side and administered the same diet for 4 weeks. Then, the mice without the transgene were killed by cervical dislocation, and the organs were removed immediately, washed with saline, frozen in liquid nitrogen, and stored at −80°C until the ^{32}P-postlabeling analysis. For the mutagenesis assay, the transgenic mice were sacrificed 2 weeks after the non-transgenic animals were killed. We expected that the mutations would have been fixed during this withholding period.

Analysis of adduct formation and mutagenesis Measurement of Trp-P-2 DNA-adduct was performed using the ^{32}P-postlabeling method[20] as modified by Fukutome *et al*.[21] Mutant frequencies were measured according to published methods[16] with slight modifications. From the extracted DNA, pSSW carrying *rpsL* and Km^r, kanamycin resistance, was cut out by *Bgl*I digestion, and circularized with T4 DNA ligase. For each sample, 5 μg of the ligated DNA was introduced into competent *E. coli* cells DH10B (Str^r) by electroporation. One-hundredth of the treated cells was plated onto kanamycin plates to estimate the total number of recovered pSSW. The rest of the cells were plated on agar plates containing kanamycin and streptomycin for scoring the number of the plasmids that carried mutations in the *rpsL* gene. The 5 μg of DNA gave approximately 50 000 total transformants and 0 to 100 transformants with mutant plasmids, depending upon the mutagenic response. Mutant frequencies were calculated from the ratio of the transformants with mutant plasmids to total transformants.

Statistical analysis was performed using Student's *t* test.

RESULTS

Administration of Trp-P-2 and Chl-Chi The mice were fed a diet containing 0.002% or 0.01% Trp-P-2 with or without Chl-Chi. At both doses, every meal provided in a ceramic container had been consumed by the animals almost completely as observed at the daily exchange of the container. The mice showed no apparent preference for the diet without Chl-Chi. We estimate that about 20 or 100 μmol Trp-P-2/day/kg body weight was administered in the experiments using 0.002% or 0.01% Trp-P-2,

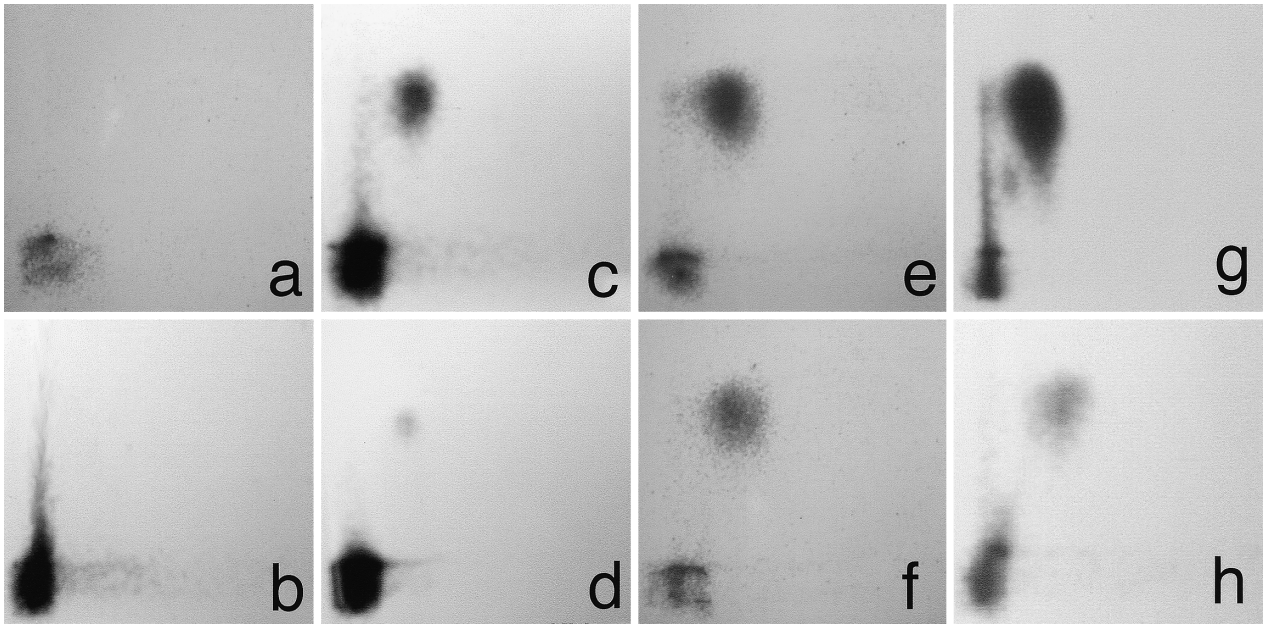

Fig. 1. Typical TLC patterns of ^{32}P-postlabeling analysis of Trp-P-2-DNA adducts. The patterns shown represent the analysis of DNA extracted from kidney (a to f), liver (g), and spleen (h): a control mouse (a), and mice treated with 3% Chl-Chi (b), 0.002% Trp-P-2 (c), 0.002% Trp-P-2 plus 3% Chl-Chi (d), 0.01% Trp-P-2 (e, g, h), and 0.01% Trp-P-2 plus 3% Chl-Chi (f).

respectively. At the doses employed here, Trp-P-2, Chl-Chi, and their combination each showed no apparent toxic effect. Increases in body weight during the experiments were similar among all groups (data not shown). No green material bound to the wall of the digestive tract was found at the time of analysis after administration of Chl-Chi. Throughout the period of Chl-Chi administration, the feces of the mice contained green, dispersed solid materials.

Adduct formation and its inhibition DNA samples were purified from various organs of the mice given test diets for 4 weeks and DNA adducts were analyzed by a ^{32}P-postlabeling method. Typical thin layer chromatograms are shown in Fig. 1. The DNA samples from mice fed Trp-P-2 gave a single migrating spot, which probably corresponds to the 5′-^{32}P-monophosphate of the Trp-P-2 adduct, (guanin-8-yl)Trp-P-2 deoxyriboside.[22] The adduct levels found in the experiments using 0.002% and 0.01% Trp-P-2 are summarized in Tables I and II, respectively. No Trp-P-2 DNA-adduct was detected in the control mice or in the mice treated with Chl-Chi only (panels a and b in Fig. 1, and Tables I and II). Although this spot was found in all the samples from Trp-P-2-treated animals, its amount varied by more than 10-fold among the organs analyzed (panels e, g, and h of Fig. 1, and Tables I and II). The order of the Trp-P-2 adduct level was liver>kidney>other organs in the experiment using 0.002% Trp-P-2 (Table I), and liver>heart>kidney>other organs for the experiment using 0.01% Trp-P-2 (Table II). The levels of the adduct in the liver, kidney, and large intestine in Table II (0.01% Trp-P-2) are one order of magnitude higher than those in Table I (0.002% Trp-P-2).

When Chl-Chi was added to the Trp-P-2 diet, the adduct levels were reduced markedly, most apparently in the liver and kidney (see panels c, d, e, and f in Fig. 1, and Tables I and II). In all cases where more than 1 adduct per 10^7 nucleotides was formed by treatment with Trp-P-2 alone, Chl-Chi efficiently reduced the adduct formation.

Table I. Formation of Trp-P-2 DNA-adduct in Organs of Mice Treated with 0.002% Trp-P-2 in Their Diet, and Inhibition of Adduct Formation by Chl-Chi

Organ	Number of adducts per 10^7 nucleotides				Inhibition (%)
	Control	Chl-Chi	Trp-P-2	Trp-P-2 +Chl-Chi	
Liver	<0.1	<0.1	12.2±2.2	5.2±1.2*	57.3
Lung	<0.1	<0.1	0.4±0.1	0.3±0.04	—
Kidney	<0.1	<0.1	1.7±0.7	0.4±0.3	76.5
Spleen	<0.1	<0.1	0.5±0.2	0.3±0.3	—
Forestomach	<0.1	<0.1	0.2±0.1	0.2±0.1	—
Large intestine	<0.1	<0.1	0.3±0.1	0.2±0.1	—
	(n=3)[a]	(n=3)	(n=4)	(n=3)	

* Significant difference vs. mice treated with Trp-P-2 alone (P<0.05).
a) *n*: number of mice used.

Table II. Formation of Trp-P-2 DNA-adduct in Organs of Mice Treated with 0.01% Trp-P-2 in Their Diet, and Inhibition of Adduct Formation by Chl-Chi

Organ	Number of adducts per 10^7 nucleotides			Inhibition (%)
	Control	Trp-P-2	Trp-P-2 +Chl-Chi	
Liver	<0.1	115.9±32.4	32.0±11.7*	72.4
Lung	<0.1	0.5±0.1	0.9±0.6	—
Kidney	<0.1	17.2±4.8	3.4±2.2*	80.2
Spleen	<0.1	1.8±0.2	0.8±0.4*	55.6
Forestomach	<0.1	0.4±0.2	0.1±0.02	—
Large intestine	<0.1	3.0±1.2	0.7±0.3	76.7
Heart	<0.1	29.6±10.0	7.7±3.3*	74.0
	(n=2)[a]	(n=3)	(n=4)	

* Significant difference vs. mice treated with Trp-P-2 alone (P<0.05).
a) *n*: number of mice used.

Table III. Induction of Mutagenesis in the *rpsL* Gene in Organs of Mice Fed 0.01% Trp-P-2 in Their Diet and Its Inhibition by Chl-Chi

Organ	Mutant frequency ($\times 10^{-5}$)		
	Control	Trp-P-2	Trp-P-2 +Chl-Chi
Liver	10.5±1.7	22.4±3.4*	9.4±3.9†
Lung	10.1±2.5	11.3±2.1	8.7±1.4
Kidney	11.6±4.9	19.0±4.5	7.3±6.2
Spleen	29.1±11.9	67.9±20.3*	23.1±11.4†
Large intestine	8.3±5.5	8.4±2.1	5.2±1.4
	(*n*=3)[a]	(*n*=3)	(*n*=3)

*Significant difference vs. control ($P<0.05$).
†Significant difference vs. mice treated with Trp-P-2 alone ($P<0.05$).
a) *n*: number of mice used.

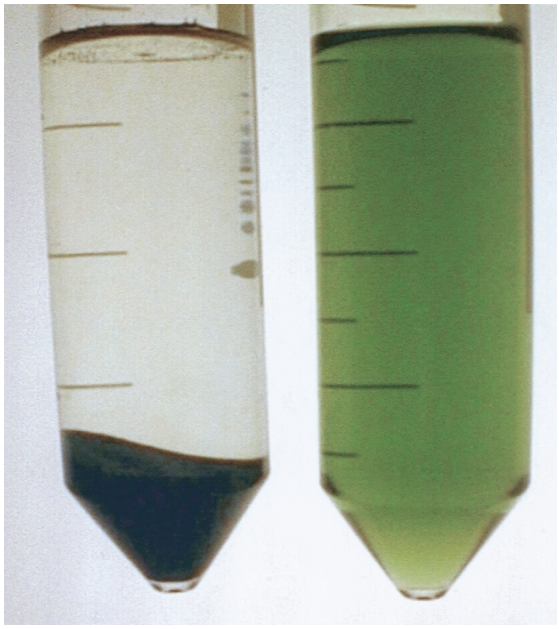

Fig. 2. Insoluble nature of chlorophyllin-chitosan (Chl-Chi). Chl-Chi (900 mg in 35 ml H_2O, left) and chlorophyllin (1.8 mg in 35 ml H_2O, right) in plastic tubes after centrifugation of homogeneous mixtures at 1500*g* for 15 min.

Mutagenic activity of Trp-P-2 and its inhibition by Chl-Chi In the mice fed the diet containing 0.01% Trp-P-2, the *rpsL* mutant frequency increased by 2.1-fold in the liver, and by 2.3-fold in the spleen (Table III). Induction of mutations in other organs was not apparent. Addition of Chl-Chi to the diet reduced the mutant frequencies in the liver and spleen, almost to the background level. Mutant frequencies for treatment with Chl-Chi alone were the same as the control (data not shown).

DISCUSSION

We have shown here that Chl-Chi, an insoluble form of chlorophyllin (Fig. 2), is an efficient inhibitor of adduct formation and mutagenesis induced by Trp-P-2 in mice. In the absence of Chl-Chi, Trp-P-2 added to the diet is expected to be absorbed through the intestine and activated to a direct-acting mutagen in the liver, where the highest adduct level was found (Tables I and II).

When an aqueous Trp-P-2 solution is mixed with a diet containing Chl-Chi for the preparation of the test diet, Trp-P-2 is expected to form an insoluble complex with Chl-Chi as a result of their high affinity.[4] This complex may be sufficiently stable during passage through the digestive tract, and may be excreted without absorption into the body. Indeed, Chl-Chi inhibited the genotoxicity of Trp-P-2 efficiently in almost all organs where genotoxicity was observed. In the liver and spleen, both adduct formation and gene mutation were reduced markedly by the addition of Chl-Chi to the diet containing 0.01% Trp-P-2 (Tables II and III).

Although the genotoxic effect of Trp-P-2 and antigenotoxic effect of Chl-Chi in these organs are clear, there seems to be no apparent quantitative correlation between the adduct formation and mutant frequencies. In the spleen, the adduct level was much lower than that in the liver, but increases in the mutation frequencies were similar, namely, about 2-fold over the control in both organs (Tables II and III). The lack of such a correlation has been reported for 2-amino-3,4-dimethylimidazo[4,5-*f*]-quinoline in Big Blue mice.[23] This phenomenon might reflect the higher cell proliferation rate in the spleen.

Chlorophyllin has been shown to be a potent inhibitor of mutagenesis and carcinogenesis.[5–13] However, it is very soluble and its complexes with heterocyclic amines retain their solubility. This property might result in uptake through the digestive organs, and might cause unexpected

adverse effects. This concern is based on the fact that weak co-mutagenic or tumor-promoting effects of chlorophyllin have been reported.[24, 25] In this respect, an insoluble complex of chlorophyllin, such as the one described here, may be preferable to the original soluble form. Chitosan is a suitable agent for such a purpose, because it is widely used as a food ingredient and has no known toxicity.[26] However, before application of Chl-Chi to human chemoprevention, further experiments may be required including the estimation of its effectiveness when Chl-Chi and the carcinogens are administered separately.

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