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Fecal Excretion of Dioxin in Mice Enhanced by Intake of Dietary Fiber Bearing Chlorophyllin

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Enhancing the fecal excretion of dioxins (PCDDs PCDFs and Co-PCBs), extremely toxic environmental contaminants that accumulates in the body, has been examined as a means of reducing the body burden. Previously, the effect of ingesting a substance capable of adsorbing dioxin such as activated charcoal was examined (Manara et al. 1984). More recently, it was reported that crude dietary fiber extracted from vegetables stimulated the fecal excretion of dioxins in rats (Morita et al. 1997a). The aim of this study was to develop a more effective substance with which to remove dioxin from the body. Six dietary fibers (locust bean gum, pectin, alginic acid, guar gum, chitin and cellulose) effective in binding dioxin isomers were selected from among 16 fibers based on previous experiments *in vitro* (Aozasa et al. 2001). The intake of these fibers except for alginic acid enhanced the fecal excretion of the dioxin isomer (HxCDD) in mice. Comparing the ability to bind HxCDD *in vitro* as well as to promote fecal excretion in mice, we concluded that the water-insoluble fibers were more effective for fecal excretion in mice. Cu-chlorophyllin has also been examined for its ability to promote the fecal excretion of dioxin by decreasing the intestinal absorption of carcinogen, and appeared to be more effective than the crude dietary fiber extracted from vegetables (Morita et al. 1997). Cu-chlorophyllin is water-soluble and we expected to enhance its effect by transforming it to a water-insoluble complex to bind with water-insoluble dietary fibers. Therefore, dietary fiber combined with chlorophyllin was prepared and its effect on the fecal excretion of dioxin was assessed. The most effective chlorophyllin and dietary fiber were selected to prepare a dietary fiber bearing chlorophyllin. The ability of each fiber to remove dioxin from the body was assessed based on the reduction in intestinal absorption as well as the promotion of excretion of dioxin.

MATERIALS AND METHODS

Six groups (4 or 5 mice) of 10-week-old C57BL/6 female mice were used in this experiment. A basal fiber-free diet and three diets containing 10% chitin, water-insoluble high molecular weight chitosan, or water-soluble low

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molecular weight chitosan were given to four groups of mice for 7 days. The composition of the basal diet was described elsewhere (Aozasa et al. 2001). The mineral and vitamin mixture was identical to AIN-76 in the basal diet. The low molecular weight chitosan was prepared by hydrolysis of the high molecular weight form. Another two groups were fed diets containing 1.0% of either chitosan. All six groups were given a single oral administration of HxCDD dissolved in ethanol: Tween 20: distilled water (1:10:89) at a dose of 10 μg/kg body weight on day 4. Feces collected after exposure for 3 days were analyzed for HxCDD using a gas chromatograph-mass spectrometer. The analytical conditions were reported previously (Aozasa et al. 2001).

Three groups (4 or 5 mice) of 10-week-old C57BL/6 female mice were used in this experiment. All three groups were given the basal fiber-free diet for 4 days, then treated with a single oral administration of dissolved HxCDD at a dose of 10 μg/kg body weight. The basal fiber-free diet was continuously given to all groups for 3 days, and feces containing HxCDD, which was not absorbed in the intestine, were collected. Thereafter, two groups were maintained on diets containing 10% water-insoluble and water-soluble chitosan for a period of 15 days. Another group (control) was further raised on the basal fiber-free diet. Feces were collected every 3 days for 15 days and analyzed for HxCDD using a gas chromatograph-mass spectrometer. Feces excreted from the beginning of day 3 after the administration are considered to contain the excreted form of HxCDD, which is absorbed and accumulated in the tissue.

Mice were fed the basal fiber-free diet and diets containing 1% porphyrins *ad lib.* for 7 days: the porphyrins were metal-free chlorophyllin iron-chlorophyllin, copper-chlorophyllin and copper-phthalocyanine. These mice were given HxCDD orally on day 4. The amount of HxCDD excreted in the feces after the oral administration was measured for 3 days as in experiment 1-1.

Dietary fiber bearing a chlorophyllin was prepared as follows; first, the dietary fiber (1.0 g) and chlorophyllin (1.3 g) were dissolved in 200ml of a methanol/0.5% hydrochloric acid solution (1:1) then dicyclohexylcarbodiimide (0.41 g) and 4-aminopyridine (0.06 g) were added This mixture was reacted at room temperature for 4 days. The reacted solution was washed with ethanol to dissolve the dietary fiber and remove the free chlorophyllin. The washed solution was filtered, and the residue dried and powdered in a mortar. The dietary fiber bearing chlorophyllin was mixed with the diet at 1%. The mixed diet was given to the mice, and the amount of HxCDD in the feces was measured as in experiment 1-1.

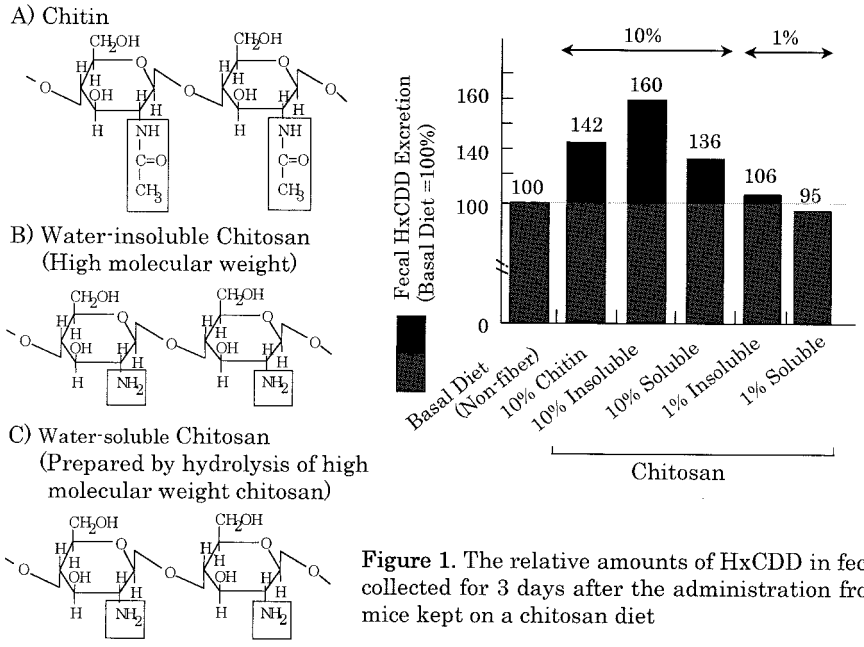


Figure 1. The relative amounts of HxCDD in feces collected for 3 days after the administration from mice kept on a chitosan diet

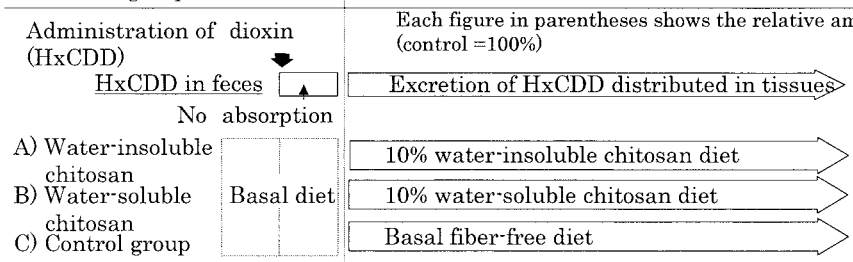
RESULTS AND DISCUSSION

We have previously reported that the intake of five dietary fibers increased the amount of dioxin isomer (HxCDD) in the feces of mice (Aozasa et al. 2001). These dietary fibers showed a high rate of adsorption of HxCDD in *in vitro* experiments and were selected from among 16 dietary fibers. They can be classified into water-soluble and insoluble fibers. In excreting the dioxin isomer, the water-insoluble fibers were more effective than the water-soluble fibers. Among the insoluble fibers, chitin was considered the most effective, having an acetyl amino group substituted for the hydroxyl of cellulose. Additionally, mice raised on a diet containing 10% chitosan were treated orally with the dioxin isomer (HxCDD) in this study. Fig.1 shows the relative amount of HxCDD in feces collected for 3 days after the administration of HxCDD in the mice on the chitosan diet. The 10% chitosan diet increased the relative amount to 160% of the control value (fiber-free). The increase (160%) in the amount excreted by chitosan was greater than by chitin (142%). However, the increase was markedly reduced by changing water-insoluble high molecular weight chitosan (160%) to water-soluble low molecular weight chitosan (136%). Water-insoluble high molecular weight chitosan was therefore considered to have promoted the elimination of dioxin from the body more efficiently than water-soluble low molecular weight chitosan. This finding supports the hypothesis made in our previous study *in vitro* and *in vivo* using dietary fiber (Aozasa et al. 2001). However, no increase in the fecal excretion of dioxin was

Table 1. The cumulative amount of HxCDD excreted in feces every 3 days in the 15-day experimental period when water-insoluble or water-soluble chitosan was given to mice beginning 3 days after oral administration of HxCDD

Treatment group	Cumulative fecal excretion of HxCDD (% of dose)				
	1-3 day	1-6 day	1-9 day	1-12 day	1-15 day
A) Water-insoluble Chitosan (High molecular weight)	0.54 (150)	0.81 (125)	1.2 (120)	1.7 (121)	2.1 (123)
B) Water-soluble Chitosan (Prepared by hydrolyzation of high molecular weight chitosan)	0.42 (117)	0.62 (95)	1.0 (100)	1.4 (100)	1.7 (100)
C) Fiber-free basal diet (control group)	0.36 (100)	0.65 (100)	1.0 (100)	1.4 (100)	1.7 (100)

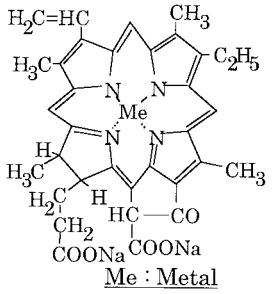
Each figure in parentheses shows the relative amount (control =100%)



observed upon the intake of soluble or insoluble chitosan when the content of chitosan in diet was reduced to 1.0% from 10%. The results indicate that as much as 10% fiber in the diet is needed to prevent the intestinal absorption of dioxin. A previous study in rats using crude dietary fiber extracted from vegetables also employed a mix of 10% (Morita et al. 1997).

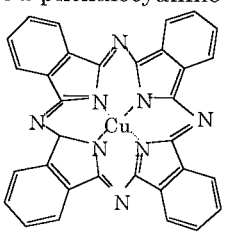
Besides preventing intestinal absorption, promoting the excretion of dioxin is important in reducing the burden. Table 1 shows the cumulative amounts of HxCDD excreted in feces every 3 days over 15-day experimental period when water-insoluble or water-soluble chitosan was given to mice beginning 3 days after the oral administration of HxCDD. Both chitosans were mixed with a non-fiber basal diet at 10%. In the control group given the non-fiber diet, HxCDD was excreted in feces at a rate of 0.36% of dose for the first 3 days of the experiment. The cumulative amount in the control group rose slightly throughout the experimental period, eventually increasing 1.7 percentage points. In contrast, the water-insoluble chitosan diet caused an increase in fecal excretion over 15 days of 2.1 percentage points equivalent to 123% relative to that in the control group (1.7%). However, in the mice given the water-soluble chitosan diet, no change in HxCDD excretion was observed. From these results, the insoluble chitosan stimulated the excretion of HxCDD more effectively than the water-soluble chitosan. In addition, the insoluble chitosan reduced the intestinal absorption of HxCDD. It seems that dietary fiber prevents the intestinal adsorption as effectively as it promotes the excretion of HxCDD.

A) Chlorophyllins



H : Metal-free Chlorophyllin
Fe : Fe-Chlorophyllin
Cu : Cu-Chlorophyllin

B) Cu-phthalocyanine



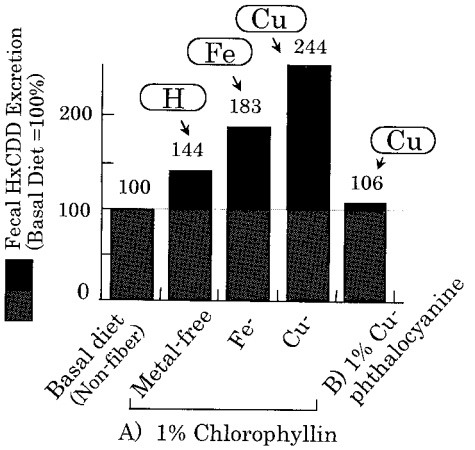


Figure 2. Effects of the four porphyrins on fecal excretion for 3 days in mice treated with HxCDD

Therefore, the efficiency in reducing the body burden of dioxin can be assessed based only on the reduction in intestinal adsorption.

Figure 2 shows the effects of the four porphyrins on fecal excretion for 3 days in mice treated with HxCDD orally. The four porphyrins were cupper-phthalocyanine, metal-free chlorphyllin, iron (Fe)-chlorphyllin, and cupper (Cu)-chlorphyllin. Each was added to the diet at 1.0%. At 1.0% there was no effect using the dietary fiber. The mice fed the diet containing porphyrins were administered HxCDD as a dioxin isomer. The excretion of HxCDD increased to 144% when metal-free chlorophyllin was present in the diet as compared to that in mice fed the fiber-free basal diets. Fe-chlorophyllin in the diet also caused an increase to 183%, but the maximum level (244%) of fecal excretion was observed in the mice given the Cu-chlorophyllin diet. It appeared that the effect on fecal excretion of the chlorophyllin derivative was dependent on the metal in the porphyrin ring, with copper having the greatest effect.

In rats fed 0.2% Cu-chlorophyllin, the fecal excretion of four dioxin isomers was determined after administration of a diet containing rice-bran oil contaminated with PeCDD, two HxCDDs and HpCDD (Morita et al. 1997). Consequently, in rats as well as in mice, the intake of Cu-chlorophyllin stimulates the fecal excretion of HxCDD. Chloropyllin is known to bind to planar compounds such as heterocyclic amines (Dashood 1992). Moreover, in a

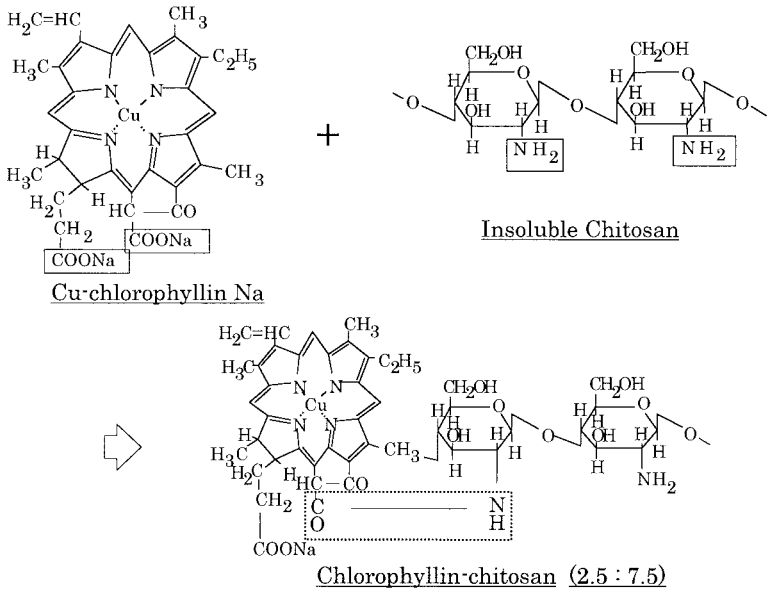


Figure 3. Proposal structure of water-insoluble high molecular weight chitosan bearing chlorophyllin (Chlorophyllin-chitosan)

Table 2. Intake of diet, weight of feces and fecal HxCDD excretion for 3 days in treated mice

Experimental diet	Intake of diet (g / mouse)	Weight of feces (g/ mouse)	Fecal HxCDD excretion (Non-fiber =100%)
1.0 % Chitosan bound Cu-chlorophyllin	10.4	0.53	137
0.7% chitosan + 0.3% Cu-chlorophyllin (Non-bound)	12.0	0.53	101
0.7% chitosan	10.4	0.45	99
0.3% Cu-chlorophyllin	10.1	0.46	93
Basal Diet (Non-fiber)	10.6	0.46	100

previous study using rats, it was suggested that the binding of chlorophyllin to dioxins as planar compounds resulted in a reduced intestinal absorption of the complex (Morita et al. 1997). The Fe derivative is more unstable in acid than Cu chlorophyllin (Buchler 1975), and its degradation in the stomach may reduce its ability to promote the fecal excretion of HxCDD. However, in the case of Cu-phthalocyanine, although copper is present in the porphyrin ring, no

increase (106%) in the amount of HxCDD in feces was observed. This finding indicated the effect of copper-phthalocyanine may be reduced by different functional groups. In other words, the excretion of dioxin is expected to be enhanced by changing the functional group bound to the porphyrin ring. Based on the results of the experiments with chloropyllin and dietary fiber, a complex was prepared by combining the porphyrin ring of Cu-chloropyllin with water-insoluble chitosan, a dietary fiber of high molecular weight, in an attempt to reinforce the effect to promote the fecal excretion of dioxin.

A water-insoluble high molecular weight chitosan bearing Cu-chlorophyllin was prepared by reacting the carboxyl group of water-soluble copper-chlorophyllin, which had the highest potency of the porphyrins tested, with the high molecular weight chitosan amino group. The preparation contained 25% Cu-chlorophyllin and 75% chitosan as shown in Figure 3, and was mixed with the non-fiber basal diet at 1.0%. The mice given the diet containing the specially prepared chitosan were administered HxCDD. Table 2 shows the intake of diet, weight of feces and HxCDD excretion for 3 days in the treated mice. There was no remarkable effect on intake of diet or weight of feces by the chitosan compared with the control. However, the preparation increased the 3-day excretion of HxCDD to 137% that mice fed the fiber-free diets. In contrast, the diet containing Cu-chlorophyllin and chitosan (2.5:7.5) not bound together produced no increase in excretion (101%). These findings demonstrate that the binding of Cu-chlorophyllin and chitosan enhanced the effect on the fecal excretion of dioxin. It is concluded that intestinal adsorption is further prevented by binding chlorophyllin to dietary fiber to produce a water-insoluble high molecular weight complex.

In this study, we compared water-soluble fiber, water-insoluble fiber, metal free, Fe- or Cu-chlorophyllin, Cu-phtalocyenine and chlorophillin-chitosan synthetic agent for fecal mediated dioxin removal in mice. The water-insoluble fibers were more effective than the water-soluble fibers in preventing intestinal absorption of dioxin. In addition, the water-insoluble fibers promote the excretion of dioxin. We suggested that water-insoluble substances are effective for removing dioxin from body. Actually, intestinal adsorption of dioxin is further prevented by binding water-soluble chlorophyllin to water-insoluble fiber to produce a water-insoluble complex.

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