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Inhibition of 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline-mediated DNA-adduct Formation by Chlorophyllin in *Drosophila*

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The effect of chlorophyllin on 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx)-mediated DNA-adduct formation in *Drosophila* was studied. Third-instar larvae of *Drosophila* were fed MeIQx at 1 mg/6.5 g-feed/bottle, with or without chlorophyllin (100–300 mg). After a 6 h feeding exposure to MeIQx, the larvae were divided into 2 groups. The first group was examined for covalent DNA adducts by ^{32}P-postlabeling assay. The second group was assayed for DNA damage by allowing the larvae to develop to adults and measuring the male/female ratio (males, DNA repair-deficient; females, DNA repair-proficient). The ^{32}P-postlabeling results indicated a significant decrease in DNA adduct levels in larvae treated with MeIQx and 300 mg chlorophyllin (1.7 ± 0.7 adducts/10^7 nucleotides) as compared with MeIQx-treated larvae (6.5 ± 2.1 adducts/10^7 nucleotides). The results on male/female sex ratios also indicated a chlorophyllin-induced decrease in DNA damage by exposure to MeIQx. The suppressive effect of chlorophyllin on the genotoxic actions of a polycyclic mutagen, MeIQx, may be a result of complex formation between chlorophyllin and the mutagen.

Key words: MeIQx — Chlorophyllin — MeIQx-DNA adduct — ^{32}P-Postlabeling of *Drosophila* DNA

Chlorophyllin, a water-soluble derivative of chlorophyll, has been studied extensively as an inhibitor of the actions of mutagens.[1,2] For example, mutations inducible by heterocyclic amines in bacteria and in *Drosophila* can be suppressed by simultaneous administration of chlorophyllin.[3–5] A feature of this phenomenon is that the structures of the mutagens susceptible to the inhibition are mostly polycyclic, and it has been proposed that the inhibition by chlorophyllin arises from complex formation between the planar aromatic structure of chlorophyllin and these planar mutagens.[1,6] Recent reports on the inhibition by chlorophyllin of animal carcinogenesis induced by aflatoxin B_1,[7] PhIP,[8] and IQ[9] have demonstrated that chlorophyllin is indeed preventative against cancers induced by these polycyclics, and raised the possibility that it might suppress carcinogenesis involving a wide range of polycyclic compounds.

2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) is one of the major carcinogenic heterocyclic amines present in cooked food,[10] and there is evidence that humans are exposed continuously to it.[11] In the *Drosophila* DNA-repair test, feeding the larvae with MeIQx-containing diet resulted in selective killing of DNA repair-deficient males while repair-proficient females were not affected, and the presence of chlorophyllin in the feed inhibited this cytotoxic effect.[5] It is important to demonstrate more directly the protective effect of chlorophyllin against the DNA-damaging effect of MeIQx *in vivo*. We show in this report that MeIQx-adducts in *Drosophila* whole body DNA, as detected by the ^{32}P-postlabeling technique, are suppressed by chlorophyllin co-administered with MeIQx.

MeIQx used was a product of Wako (Tokyo), and sodium copper chlorophyllin was from Nacalai Tesque (Kyoto). The *Drosophila* strains used and the feeding protocols are described in our previous report.[5] A group of 3rd-instar larvae, which consisted of approximately equal numbers of DNA repair-deficient males (*mei-9*, *mei-41*) and -proficient females (*mei-9*$^+$, *mei-41*$^+$) ($\sim$100 each per culture bottle), was fed a diet containing MeIQx (1 mg/6.5 g diet/bottle), with or without chlorophyllin (dose, 0, 100, 200 or 300 mg), for a period of 6 h. The larvae from each dosing program were then divided into two groups. The first group was immediately frozen in liquid nitrogen and later subjected to ^{32}P-postlabeling analysis of DNA adducts, and the second group was further grown on the normal diet for 7 days to form adult flies. The numbers of males and females in this group of flies were then scored. The male/female ratio is an indicator of somatic cell DNA lesions that are repairable by the excision repair (*mei-9*) and post-replicational repair (*mei-41*) functions of the animal.[12]

DNA from the frozen larvae was isolated by phenol-chloroform extraction. Briefly, the frozen larvae were ground with a pestle that had been immersed in liquid nitrogen, then lysed with a sodium dodecyl sulfate-

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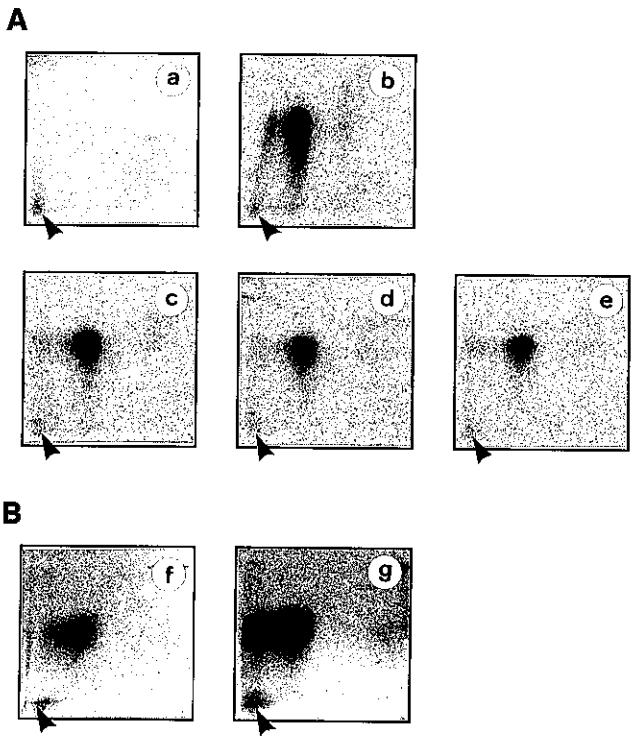

Fig. 1. Autoradiograms of MeIQx-DNA-adducts. Panel A shows adducts from *Drosophila* fed MeIQx with or without chlorophyllin. ^{32}P-Postlabeling analysis was done by nuclease P1-phosphodiesterase I treatment of intensively labeled nucleotide mixtures[13]: (a) no-MeIQx control, (b) MeIQx 1 mg only, (c) MeIQx + chlorophyllin 100 mg, (d) MeIQx + chlorophyllin 200 mg, (e) MeIQx + chlorophyllin 300 mg. This series of photos corresponds to Exp. 2 in Table I. Panel B shows the results of an experiment in which adducts from mouse (f) and *Drosophila* (g) fed MeIQx were compared. The origins of the chromatograms are indicated by arrows.

containing Tris-buffer at pH 8.0, treated with proteinase K, and subjected to phenol-chloroform extraction. The aqueous extract was treated with RNase A and RNase T1, and extracted with phenol-chloroform. The DNA was precipitated with ethanol and subjected to extensive dialysis to remove any remaining ribonucleotides. The DNA obtained by subsequent ethanol precipitation showed an A260/A280 value of 1.82; about 0.1 mg DNA was obtained from 1 g (~800) of larvae.

The DNA was then subjected to the modified adduct-intensification analysis.[13] Briefly, the DNA was digested with micrococcal nuclease and spleen phosphodiesterase (Worthington, Freehold, NJ), and the digest (10 μg DNA equivalent) was then labeled with ^{32}P under the adduct-intensification conditions[14] by use of T4 polynucleotide kinase (Takara, Kyoto) and [γ-^{32}P]ATP (800 Ci/mmol, 15 μM, ICN Biochemicals, Irvine, CA). The labeled nucleotide mixture was further digested with nuclease P1 (Yamasa, Choshi) and phosphodiesterase I, PDE I (Worthington), under the conditions described in the literature.[13] The digest was subjected to TLC on polyethyleneimine cellulose (Polygram, Cel 300 PEI, Machery-Nagel, Duren, Germany), and the plate was developed four times as described.[14] The radioactivities of adduct spots were analyzed with a Bio-Imaging Analyzer (BAS 2000, Fuji Photo Film, Tokyo) with an exposure time of 2 h. The adduct level was determined as the relative adduct labeling (RAL),[15] i.e., "RAL in the modified adduct-intensification method" = [(count rate in adduct nucleotides)/(count rate in total nucleotides)] $\times$ $(IF)^{-1}$, where IF (intensification factor) is the value obtained in an MeIQx-only experiment, i.e., IF = [RAL in the modified adduct-intensification method]/[RAL in the modified standard method[13]].

For detection of MeIQx-adducts in DNA of mice, male CDF1 mice at 7 weeks of age (Charles River Japan, Atsugi) were administered MeIQx at 50 mg/kg body weight by gavage once a day for 3 successive days, and killed 24 h after the last administration. The DNA was isolated from the liver by phenol extraction and analyzed by the ^{32}P-postlabeling method[13] using the intensification protocol described above.

The ^{32}P-postlabeling of *Drosophila* DNA after treatment with MeIQx with or without chlorophyllin gave a spot on TLC at a position where no spot was found for a no-MeIQx control (Fig. 1A). The position of the spot was identical with that of the adduct from the liver of mice fed MeIQx (Fig. 1B). The intensity of the spot from *Drosophila* was decreased by administration of chlorophyllin, and the decrease was greater with greater amounts of chlorophyllin (Fig. 1A). The single adduct we observed on the TLC plate was probably N^2-(deoxyguanosin-8-yl)-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline 5′-phosphate (dG-C8-MeIQx 5′-phosphate), since it is known that the major MeIQx adduction is between the 2-amino of MeIQx and position 8 of guanine[14] and that the nucleotide formed in the nuclease P1-PDE I procedure is nucleoside 5′-phosphate.[13] Turesky *et al.*[16] isolated a second adduct 5-(deoxyguanosin-N^2-yl)-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (dG-N2-MeIQx) from the *in vitro* treatment of DNA with MeIQx, and reported that the ratio of the C8 adduct to the N2 adduct was 8–10. The minor N2 adduct that could have been formed in the *Drosophila* larvae exposed to MeIQx was probably present in amounts too small to allow detection in our analysis.

Table I summarizes the results of three feeding DNA-analysis experiments. The decrease in adduct level by chlorophyllin was statistically significant at 300 mg chlorophyllin, and intermediate doses of chlorophyllin also tended to be effective in suppressing the adduct forma-

Table I. Inhibition by Chlorophyllin of MeIQx-adduct Formation in *Drosophila*[a)]

Chlorophyllin (mg)		Adduct (per 10^7 nucleotides)		DNA-repair test (male/female)	
		Found	Mean±SD	Found	Mean±SD
0	Exp. 1	8.9		0.13	
	Exp. 2	5.5	6.5±2.1	0.04	0.10±0.05
	Exp. 3	5.0		0.12	
100	Exp. 1	4.6		0.28	
	Exp. 2	2.7	3.4±1.0	0.16	0.21±0.06
	Exp. 3	3.0		0.20	
200	Exp. 1	5.6		0.30	
	Exp. 2	3.1	3.4±2.1	0.28	0.28±0.03**
	Exp. 3	1.5		0.25	
300	Exp. 1	1.5		0.32	
	Exp. 2	1.1	1.7±0.7*	0.34	0.31±0.04**
	Exp. 3	2.5		0.26	

a) Larvae of *Drosophila* were fed MeIQx 1 mg/6.5 g diet/bottle for 6 h together with chlorophyllin (0–300 mg). MeIQx-adducts were analyzed by the ^{32}P-postlabeling with a modified intensification procedure.[13)] The DNA repair test was performed as described previously.[5)] Three independent experiments were performed, and the results are given in this table. The adduct levels and the sex ratios are averages of duplicate determinations. * $P<0.05$; ** $P<0.01$. The significance of differences was evaluated with Student's t test.

tion. The general damage in somatic cell DNA detected by the repair test was high in the case of administration of MeIQx (0.10±0.05), but was lowered significantly by the presence of chlorophyllin (at both 200 mg and 300 mg). With the lowest dose of chlorophyllin (100 mg), there was an increase in the sex ratio, although it was statistically insignificant. Throughout the entire series of experiments, no significant cytotoxic effect against the females was detected, suggesting that most of the damage inflicted in the larval stage had been repaired during growth. In addition, 300 mg of chlorophyllin alone (with no MeIQx) was not DNA-damaging; the male/female ratio was 1.19.

These results clearly show that chlorophyllin protects somatic cell DNA in *Drosophila* against the adduction of MeIQx. Chlorophyllin can form complexes with heterocyclic amines in general, including MeIQx[17)]: it was shown that Sepharose-supported chlorophyllin can serve as a ligand to adsorb MeIQx. It is conceivable that, given the large dose (100–300 fold) of chlorophyllin co-administered with MeIQx, extensive association between MeIQx and chlorophyllin molecules took place in the feed and in the larval digestive tract, resulting in the inhibition of MeIQx absorption in *Drosophila*. Using radiolabeled IQ, Dashwood has shown that its absorption from the digestive tract in rats is suppressed by chlorophyllin and that IQ binding to DNA in the liver is inhibited.[18)] Our data here constitute evidence that the covalent linking of a heterocyclic amine to a DNA base is indeed inhibited by chlorophyllin.

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