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Formation and Removal of DNA Adducts in the Liver of Rats Chronically Fed the Food-borne Carcinogen, 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline

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Effects of chronic administration of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) at 0.4, 8 and 400 ppm in the diet on DNA adduct formation and removal in the rat liver were examined by the ^{32}P-postlabeling method. The 0.4 and 8 ppm doses for 40 weeks resulted in time-dependent increases in MeIQx-DNA adduct levels until 16 and 8 weeks, respectively, with constant values being maintained thereafter. In the case of a carcinogenic dose (400 ppm) of MeIQx, the adduct levels reached a maximum at week 12, and then gradually decreased. Alteration of metabolism of MeIQx during liver carcinogenesis might be related to this decrease in DNA adduct levels. When MeIQx administration was stopped at week 20, 60–90% of the MeIQx-DNA adducts formed with the three doses (0.4, 8 and 400 ppm) of MeIQx were removed in a biphasic manner after return to a basal diet, with initial rapid removal followed by a slow change. No difference in the pattern of MeIQx-DNA adducts was detected on thin layer chromatography at any dose at any time point. Thus, it is suggested that there may be at least two types of damaged DNA, susceptible and resistant to removal of MeIQx-DNA adducts, after chronic administration of MeIQx.

Key words: Heterocyclic amine — MeIQx — DNA adduct formation — DNA adduct removal

Food contains a variety of mutagenic and carcinogenic compounds, including heterocyclic amines (HCAs).[1,2] 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), an HCA, induces mutations not only in bacteria but also in cultured mammalian cells,[1,2] and is carcinogenic in mice and rats, inducing mainly liver tumors in these animals.[3,4] MeIQx is present in various cooked foods[2,5–7] and furthermore, has been detected in all urine samples from healthy volunteers on a normal diet, but not in samples from in-patients receiving parenteral alimentation.[8] Thus, human eating a normal diet are continuously exposed to mutagenic and carcinogenic food-borne MeIQx in everyday life and human daily exposure is estimated to be in the range of 0.2 to 2.6 μg/person.[5]

MeIQx is metabolically activated by cytochrome P450IA2 to yield its *N*-hydroxyamino derivative. The *N*-hydroxylamine is further activated through esterification reactions and the resulting ultimate forms react with DNA to form MeIQx-DNA adducts.[9–13] ^{32}P-Postlabeling analysis under nuclease P1 conditions has revealed several adduct spots in the liver DNA of rats fed MeIQx. Two of them were identified as MeIQx-guanine adducts and the structure of one major spot was determined to be N^2-(deoxyguanosin-8-yl)-MeIQx 3′,5′-diphosphate (3′,5′-pdGp-C8-MeIQx).[14]

We previously demonstrated that dietary administration of MeIQx to rats in the dose range of 0.4 to 400 ppm, of which the highest dose induced hepatocellular carcinomas, resulted in a linear relationship between the dose of MeIQx and the level of MeIQx-DNA adducts in the liver at both 1 and 12 weeks.[15] This finding suggests that even at doses as low as human exposure levels, MeIQx could form DNA adducts in human organs.

It is very important to examine the dynamics of formation and removal of MeIQx-DNA adducts during chronic administration of various doses of MeIQx, to understand the role of such DNA adducts in carcinogenesis. In the present study, we therefore examined the time course of MeIQx-DNA adduct formation in rat liver during continuous administration of 0.4, 8 and 400 ppm MeIQx diet for 40 weeks. In addition, we studied the kinetics of removal of MeIQx-DNA adducts, when administration was stopped after 20-week continuous feeding. It has been reported that metabolic activation of carcinogens and detoxifying capacity are changed in preneoplastic and/or tumorous lesions in the liver of rodents.[16–20] Therefore, changes in histopathological parameters and formation of glutathione S-transferase placental form (GST-P)-positive foci, considered to be preneoplastic lesions, were also examined.

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MATERIALS AND METHODS

Materials MeIQx was obtained from the Nard Institute (Osaka). Polyethyleneimine (PEI)-cellulose thin layer chromatographic sheets (POLYGRAM CEL 300 PEI) were purchased from Macherey-Nagel (Düren, Germany). Micrococcal nuclease and spleen phosphodiesterase were from Worthington Biochemical Co. (Freehold, NJ, USA). T4 polynucleotide kinase and nuclease P1 were purchased from Takara Shuzo Co. (Kyoto) and Yamasa Shoyu Co. (Choshi), respectively. [γ-^{32}P]ATP (>7000 Ci/mmol) was obtained from ICN Biomedicals (Irvine, CA, USA). Potato apyrase was from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and treatments Male F344 rats, 5 weeks old, were purchased from Charles River Japan (Atsugi). MeIQx was added at a concentration of 0.4, 8 or 400 ppm to CE-2 basal powder diet (CLEA Japan, Tokyo) and each mixture was pelleted. Rats were housed four per wire cage and maintained in an air-conditioned animal room. After acclimatization for 1 week, they were given diets containing one of the three doses of MeIQx for 20 weeks, then divided into two groups. One group received a further 20-week administration of MeIQx (group I). The remaining group of rats was instead placed on CE-2 basal diet for the same period (group II). Control rats received CE-2 diet for the entire 40 weeks. MeIQx contents of diets were analyzed by HPLC after extraction with 50% methanol, and confirmed to be more than 90% of that added.[21] Body weights and diet intakes for each group were measured every 4 weeks.

Two rats of each group were killed every 4 weeks, with the exception of two rats of group II only at week 22, and the organs were examined and excised. After resection of the liver, a 2–3 mm thick slice was prepared from the left lateral lobe, fixed, embedded and cut into 5 μm sections for hematoxylin and eosin staining and GST-P immunohistochemistry. The remaining parts of the liver were frozen, stored at −80°C and used for analysis of DNA adducts. In addition, all visible tumorous lesions were taken and histological examinations of hematoxylin and eosin-stained sections were carried out.

Analysis of MeIQx-DNA adducts by the ^{32}P-postlabeling method DNA, isolated from rat liver by phenol extraction, was digested with micrococcal nuclease and spleen phosphodiesterase at 37°C for 3 h, and ^{32}P-labeled under nuclease P1 conditions.[22] MeIQx-DNA adducts were separated on PEI-cellulose sheets with the same development solvent systems as previously reported[14] and detected by autoradiography on Kodak XAR-5 film with intensifying screens at −80°C. Relative adduct labeling was estimated from the radioactivity of each adduct spot and of the total nucleotides, by the method of Gupta.[23] The levels of total adducts for each group were calculated as averages of four assays, two each on two rats. The variation of the total adduct levels in four assays was within 35%.

Analysis of GST-P-positive foci Immunohistochemical examination of GST-P binding was performed by using the same procedure as reported previously,[21] using GST-P rabbit antibody (MBL Co., Ltd., Nagoya). Numbers and areas of GST-P-positive foci consisting of more than 10 cells in transection were manually measured with the aid of a color video image processor (SP-500, Olympus Co., Tokyo) in the entire sections of the slices from the left lateral lobe of each rat. The data are presented as average values.

RESULTS

Body weights of rats in each group increased time-dependently. The average body weights of rats fed diets containing 0.4 and 8 ppm for 20 and 40 weeks did not differ from those of control rats, whereas values for rats given 400 ppm MeIQx demonstrated a 20% reduction throughout the experimental period. In the case of group II rats fed the diet containing 400 ppm MeIQx for 20 weeks and basal diet for the subsequent 20 weeks, the body weights gradually recovered after cessation of MeIQx administration, being 10% reduced after 4 weeks and only 2% after 12 weeks. The average diet intakes in the experimental and control groups were essentially the same, and the average intakes of MeIQx in rats given 0.4, 8 and 400 ppm were 6.6, 129 and 6320 μg per day per rat, respectively.

Fig. 1 shows autoradiograms of DNA adducts in the livers of rats given 0.4, 8 and 400 ppm MeIQx for 12 weeks. By the nuclease P1 method, four MeIQx-DNA adduct spots were observed in the samples from rats fed 400 ppm of MeIQx. The same DNA adduct pattern was also detected in the samples from rats fed 0.4 and 8 ppm of MeIQx. Moreover, the same adduct patterns were detected at all time points examined in both groups I and II. None of these adducts was detected at any time point in the control samples.

Fig. 2A shows the time course of MeIQx-DNA adduct levels with continuous feeding of 0.4 ppm MeIQx. When MeIQx was given to rats for 40 weeks (group I), the levels of MeIQx-DNA adducts gradually increased with increase of feeding period until 16 weeks and the value then stayed relatively constant for the remaining 24 weeks of the experiment. The levels of total adducts at 16 and 40 weeks in group I were 0.23 and 0.22 per 10^7 nucleotides, respectively. When MeIQx feeding was stopped at week 20 (group II), MeIQx-DNA adduct levels decreased to 90% over the first 4 weeks, and then rapidly dropped to only 20% of the peak value over the

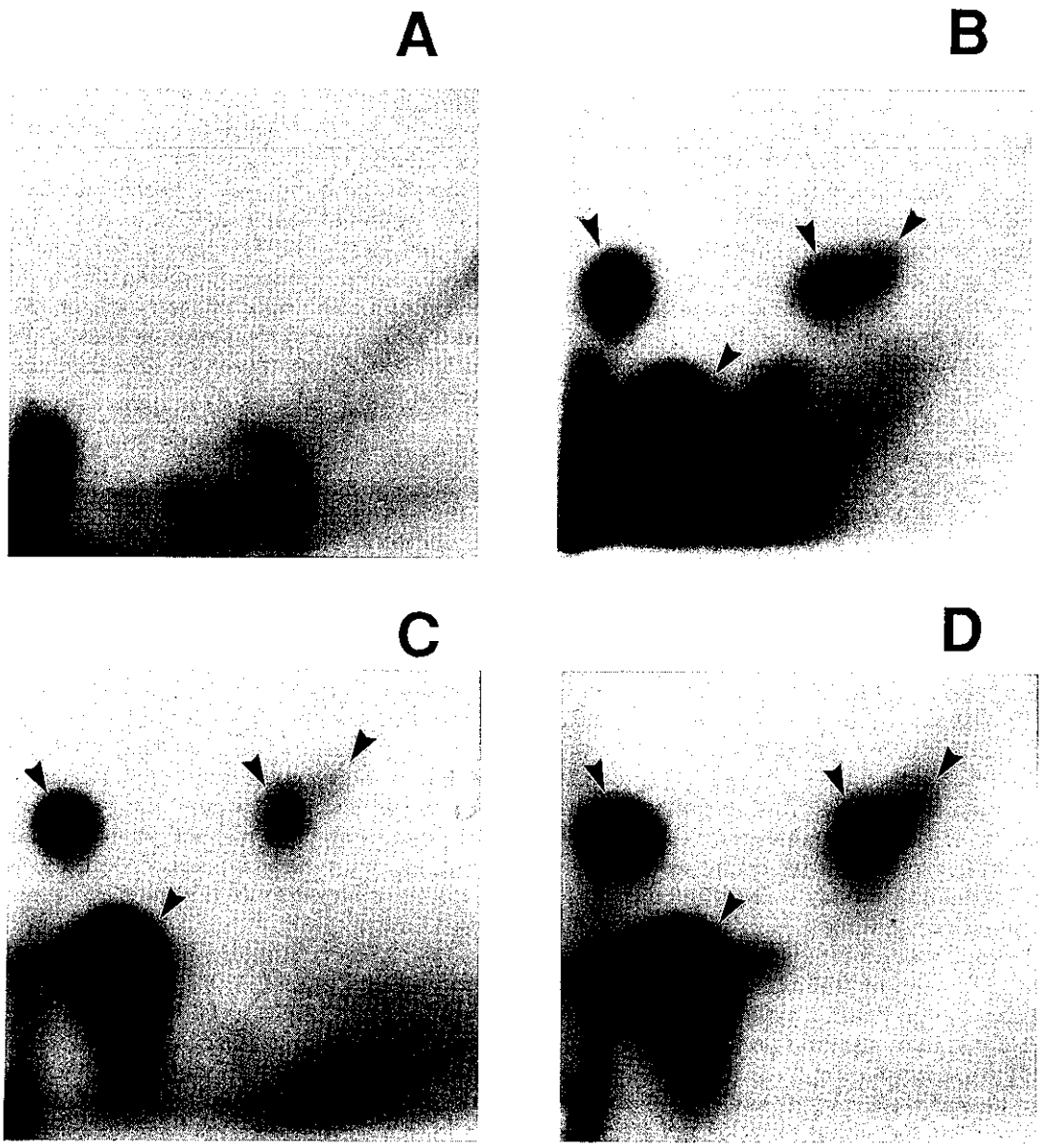

Fig. 1. Autoradiograms of MeIQx-DNA adducts detected by the nuclease P1 method. DNA was prepared from the livers of rats fed control (A), 0.4 ppm MeIQx (B), 8 ppm MeIQx (C) and 400 ppm MeIQx (D) diets for 12 weeks. The X-ray film exposure times were 16 h for (A), 14 h for (B) and 3 h for (C) at −80°C and 3 h for (D) at room temperature. MeIQx-DNA adduct spots are indicated by arrowheads.

next 4 weeks, with a gradual further reduction thereafter (Fig. 2B).

Fig. 3A shows changes of DNA adduct levels in the livers of rats administered 8 ppm MeIQx. The group I values increased for the first 8 weeks and then remained almost constant. After cessation of MeIQx feeding (group II), the peak adduct level of 3.0 per 10^7 nucleotides was reduced to 80 and 60% after 2 and 4 weeks, respectively, and then gradually decreased to 1.2 per 10^7 nucleotides (to 40%) at week 40 (Fig. 3B).

In the groups receiving 0.4 and 8 ppm MeIQx treatment for 20 or 40 weeks, neither induction of GST-P-positive foci nor histological changes were observed in the liver.

With chronic administration of the carcinogenic dose (400 ppm) of MeIQx for 40 weeks (group I), the adduct

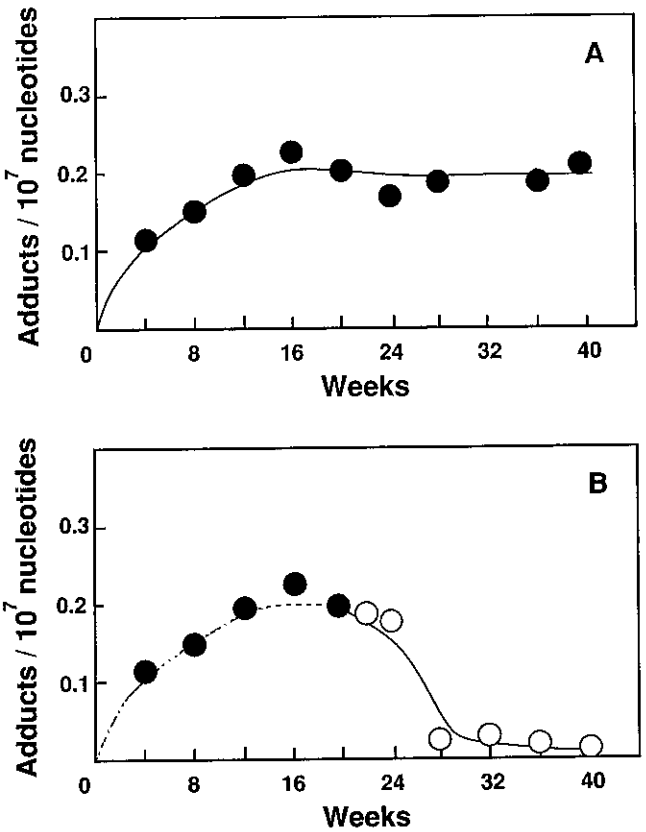

Fig. 2. Time course of formation of MeIQx-DNA adducts and their removal in the liver of rats fed 0.4 ppm MeIQx. Rats of group I (A) were chronically fed diet containing MeIQx for 40 weeks. Rats of group II (B) were fed diet containing MeIQx for 20 weeks and then basal diet for the subsequent 20 weeks.

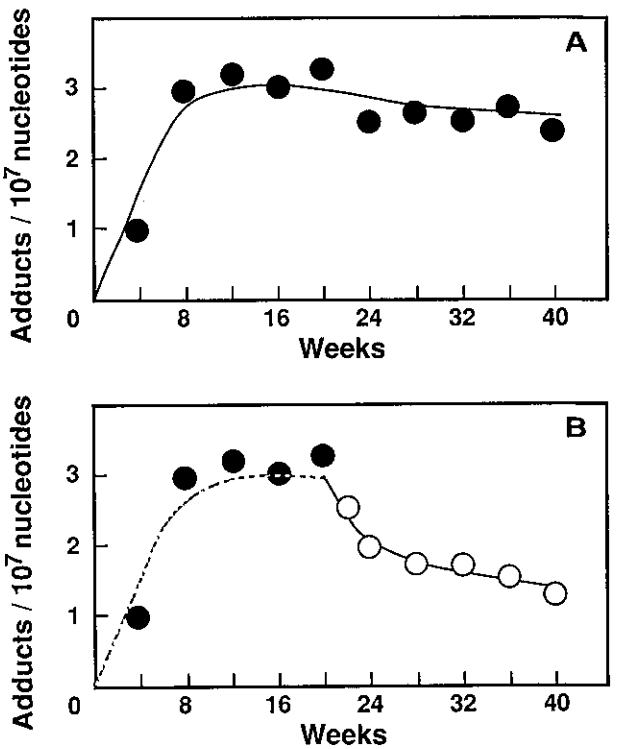

Fig. 3. Change of DNA adduct levels in the livers of rats fed 8 ppm MeIQx. The treatment periods with MeIQx for groups I (A) and II (B) were the same as described in Fig. 2.

levels reached a maximum of 120 per 10^7 nucleotides at week 12, and then gradually dropped to 54 per 10^7 nucleotides at week 40 (Fig. 4A). When MeIQx feeding was stopped at week 20 (group II), the adduct levels decreased to 90% in the first 2 weeks and to 35% in the next 2 weeks, and then gradually declined to 25% in the remaining 16 weeks (Fig. 4B).

In the livers of rats treated with 400 ppm MeIQx for 40 weeks (group I) enzyme-altered foci expressing GST-P were first observed after 12 weeks, the values being 32/cm^2 for number and 2.9 mm^2/cm^2 for area. The maximum number of 122/cm^2 was reached at week 24, and then there was a gradual decrease to 91/cm^2 at week 32 and 76/cm^2 at week 40. Areas of GST-P-positive foci increased throughout the treatment period, being 34, 50 and 63 mm^2/cm^2 at weeks 24, 32 and 40, respectively. In the case of group II, the numbers of GST-P-positive foci observed were 105, 107 and 90/cm^2 at weeks 24, 32 and 40, respectively. The areas gradually decreased, being 29, 24 and 22 mm^2/cm^2 at weeks 24, 32 and 40, respectively.

In the rats receiving 400 ppm MeIQx treatment for 40 weeks (group I), histological examination of the livers revealed centrilobular degeneration and necrosis at week 8 and slight irregularity of the trabecular structure at week 12. Small numbers of altered cell foci were detected at week 12, and the numbers then gradually increased with increase in treatment period. Development of an adenoma was first found in a liver sample at week 28. Hepatocellular carcinomas showing a trabecular pattern were observed in a rat at week 40. Histological changes in the livers of rats in group II were almost the same as in group I: namely, an adenoma and a hepatocellular carcinoma were first observed at weeks 28 and 40, respectively.

DISCUSSION

The present study showed that the levels of MeIQx-DNA adducts in the liver first increased time-dependently, and then became constant when 0.4 or 8 ppm MeIQx was given in the diet for 40 weeks. In contrast,

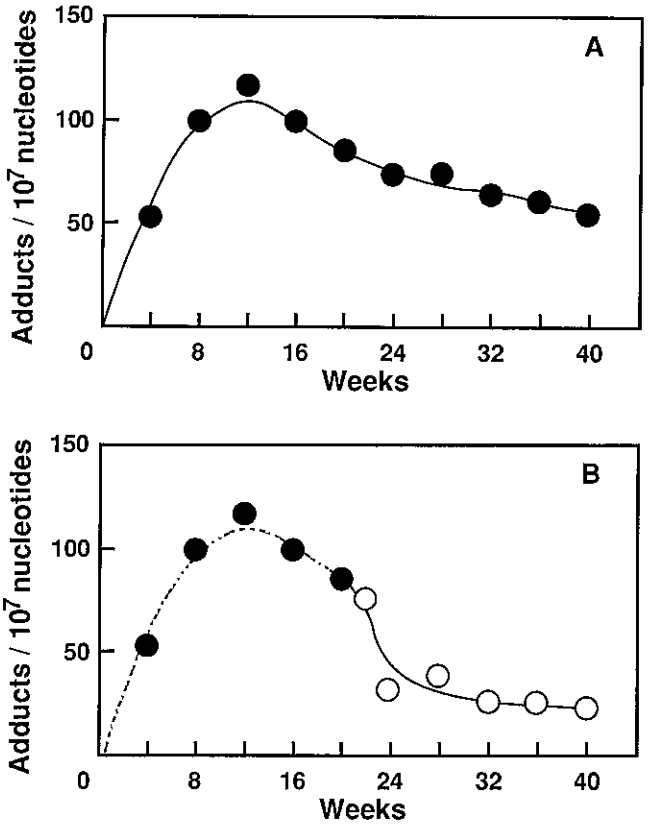

Fig. 4. Time course of change of MeIQx-DNA adduct levels in the livers of rats treated with 400 ppm MeIQx. The treatment periods with MeIQx for groups I (A) and II (B) were the same as described in Fig. 2.

values reached a maximum at week 12, and then gradually decreased with administration of a carcinogenic dose (400 ppm) of MeIQx in the diet for 40 weeks. When the 4 and 12 week levels of MeIQx-DNA adducts were plotted for the three doses (0.4, 8 and 400 ppm), a linear relationship was obtained, as previously reported.[15]

When MeIQx feeding was stopped at week 20, a biphasic decrease of MeIQx-DNA adduct levels was apparent for all three doses tested. Namely, after the cessation of MeIQx, a rapid removal phase was observed between weeks 4 and 8 for the 0.4 ppm and over the first 4 weeks for the 8 and 400 ppm MeIQx groups, followed by a slow removal phase. No change in DNA adduct patterns on TLC was evident with any dose at any time point. Thus, the biphasic kinetics suggest that there may be two DNA regions, one susceptible and the other resistant to removal of MeIQx-DNA adducts. It is well known that the liver consists of many types of cells including liver cells, Kupffer cells, bile duct epithelium and endothelium. Therefore, it is possible that there are two types of cell in the rat liver; one type which is susceptible to removal of MeIQx-DNA adducts and the other which is resistant. Biphasic removal of DNA adducts with a rapid phase for 7–14 days has also been reported in the liver of rats fed 0.02% 2-acetylaminofluorene in the diet for 28 days.[24] On the other hand, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-DNA adducts in the liver of rats have been reported to decrease to 20% over 6 days after a single p.o. dose of PhIP.[25] Furthermore, adduct levels in rat liver declined to 17% within 48 h after a single dose of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) by gavage.[26] Therefore, chronic administration of chemical carcinogens may result in prolongation of the period required for removal of DNA adducts in the liver.

It is known that the metabolic activation (phase I) capacity of carcinogens decreases and the detoxifying (phase II) capacity increases, with the development of preneoplastic and/or tumorous lesions in the liver.[16–20] Administration of 400 ppm MeIQx resulted in the appearance of GST-P-positive foci and adenomas, respectively, at weeks 12 and 28. The fact that DNA adduct levels in the liver gradually decreased after feeding of 400 ppm MeIQx for 12 weeks, therefore, suggests that alteration of the metabolism of MeIQx in the liver of rats fed 400 ppm might have been responsible for the reduction. The lack of any histological change or induction of GST-P-positive foci in the livers of rats treated with 0.4 and 8 ppm MeIQx would thus be in line with the steady-states of DNA adduct levels reached after feeding for 16 and 8 weeks, respectively. The plateaus may reflect a state of equilibrium between formation and removal of adducts.

With 400 ppm MeIQx treatment over 40 weeks (group I), the number of GST-P-positive foci reached a maximum at week 24, while the area of GST-P-positive foci continued to increase throughout the treatment period. Fusion of GST-P-positive foci might have occurred in group I. When MeIQx feeding was stopped at week 20 (group II), the areas of GST-P-positive foci gradually decreased with increase of the treatment period. Whether this may be related to the reduction of DNA adduct levels remains unclear. However, histological changes in the livers of rats fed 400 ppm for 40 weeks (group I) were almost the same as those of the 20-week administration group (group II) with adenomas and well-differentiated hepatocellular carcinomas being first found at weeks 28 and 40, respectively, in both groups. Thus, 20-week treatment is sufficient for induction of hepatocellular carcinomas with 400 ppm MeIQx. It is noteworthy that the unremoved DNA adduct levels in the liver of rats after cessation of 400 ppm MeIQx feeding (group II) were significantly higher than the levels in rats fed 0.4 and 8 ppm MeIQx for 20 and 40 weeks. Therefore,

unremoved DNA adducts may play some role in the carcinogenesis of rats fed 400 ppm MeIQx for 20 weeks (group II).

Using the same ^{32}P-postlabeling method as for the liver, DNA adduct formation and removal in the pancreas and colon were also examined in rats fed 400 ppm MeIQx for 40 (group I) and 20 (group II) weeks (data not shown). The types of DNA adducts observed in these extrahepatic tissues were identical to those in the liver, although the maximal levels reached differed among the three organs. The pattern of change of DNA adduct levels in the pancreas resembled that in the liver with 400 ppm MeIQx, while findings for accumulation and removal of DNA adducts in the colon proved similar to those for the livers of rats fed 0.4 and 8 ppm MeIQx. Thus, the enzyme(s) involved in removal of MeIQx-DNA adducts exist not only in the liver but also the extrahepatic tissues, although they have not been identified as yet.

Since humans are exposed to heterocyclic amines including MeIQx in everyday life, DNA adducts are very likely to form in human organs as a result. It is thus very important to elucidate the repair mechanisms of heterocyclic amine-DNA adducts in animals to understand human carcinogenesis by this important group of compounds.

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