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Effects of Chronic Administration of Low Doses of 2-Amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline on Glutathione S-Transferase Placental Form-positive Foci Development in the Livers of Rats Fed a Choline-deficient Diet

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Effects of chronic administration of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) at the very low doses of 0.4 and 4 ppm, respectively 1000- and 100-fold less than the dose shown to be carcinogenic (400 ppm), on the liver of rats fed a choline-deficient (CD) diet were examined in terms of glutathione S-transferase placental form (GST-P)-positive foci. Male F344 rats were given CD diet containing 0, 0.4 or 4 ppm MeIQx for 20 or 40 weeks. As controls, rats received choline-supplemented (CS) diet in the same manner. MeIQx at 4 ppm in the CD diet significantly increased both the number and area of GST-P-positive foci, the values being 2.3- and 2.1-fold at 20 weeks and 2.0- and 3.3-fold at 40 weeks, respectively, compared with those observed for CD diet alone. MeIQx at 0.4 ppm in CD diet did not affect the development of GST-P-positive foci. No influence of the heterocyclic amine was found in the CS groups, where only very small numbers of minute lesions were observed. The level of MeIQx-DNA adducts in rats given the CD diet containing 4 ppm MeIQx was 2- to 3-fold lower than that in rats given the CS diet containing 4 ppm MeIQx at 20 and 40 weeks. This result indicates that DNA adduct formation and cell proliferation are both required for the increase of GST-P-positive foci in rats fed 4 ppm MeIQx in a CD diet. The above findings strongly suggest that MeIQx could be carcinogenic even at 4 ppm under CD conditions, where liver cell regeneration is continuously occurring.

Key words: MeIQx — Choline-deficient diet — GST-P-positive foci — Cell proliferation

Cooked protein-rich foods such as meat and fish contain heterocyclic amines which have been shown to be both mutagenic and carcinogenic.[1–5] One such compound, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) was found to induce hepatomas and lung tumors, lymphomas and leukemias in mice, and tumors in the liver, Zymbal glands, clitoral gland and skin of rats when administered at a dietary dose of 400 ppm.[6,7] However, humans are generally exposed to much lower levels of heterocyclic amines, including MeIQx, in daily life.[8,9] For instance, daily exposure levels of Japanese people to MeIQx were calculated to be 0.2–2.6 μg per person based on analysis of cooked meat and fish and also urine samples from healthy volunteers.[10] It is, therefore, important to investigate the biological action of heterocyclic amines at low doses to obtain information concerning actual risk of human cancer causation.

Increased cell division is strongly associated with a propensity for development of various kinds of human cancer.[11] In the case of liver cancer, damage induced by hepatitis virus infection or alcohol as a toxicant is indicated to be involved. Recently, several studies have been performed to elucidate biological responses to low doses of heterocyclic amines under conditions such that cycles of hepatic cell death and cell regeneration occur continuously. For instance, it was reported that a single i.p. injection of 50 or 80 mg/kg of heterocyclic amines induced enzyme-altered foci in rat liver in medium-term assays using partial hepatectomy and/or a hepatotoxin, such as carbon tetrachloride (CCl_4) or D-galactosamine.[12,13] Our previous study also demonstrated that feeding MeIQx at 40 ppm for 12 weeks greatly increased development of glutathione S-transferase placental form (GST-P)-positive foci and hyperplastic nodules in rat livers injured by CCl_4.[14] From these observations, we consider that MeIQx, even at a low dose, may contribute to neoplasia development in livers which are continuously exposed to injury.

Since the dietary dose of MeIQx (40 ppm) used in our previous study[14] was still much higher than human exposure levels to this agent in daily life, we selected much lower doses (0.4 and 4 ppm) to examine the effects of chronic administration on development of GST-P-positive foci in the livers of rats fed a choline-deficient (CD) diet for 20–40 weeks, because a CD diet induces a high level of hepatocyte proliferation in rats.[15,16] Formation of active oxygen species is considered responsible for cellular proliferation through DNA and cellular damage

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in the livers of rats fed a CD diet.[17] Alteration of methylation states of DNA has also been suggested to cause a change in differentiation of the cells under CD conditions.[16] It was reported that GST-P-positive foci consisting of more than 20 cells appeared after 8 weeks of CD dietary exposure,[18] and hepatocellular carcinomas develop in 26% of rats after 16 months on CD diet.[15]

The present paper documents a significant enhancement of GST-P-positive foci development by chronic administration of 4 ppm MeIQx to animals maintained on a CD diet.

MATERIALS AND METHODS

Chemicals MeIQx was obtained from the Nard Institute, Osaka. Vectastain-ABC kits (PK4001, ABC) for the avidin-biotin-peroxidase complex method were obtained from Vector Laboratories Inc., Burlingame, CA. Anti-GST-P rabbit antibody was kindly provided by Dr. Kimihiko Satoh of Hirosaki University. Micrococcal nuclease and spleen phosphodiesterase were both obtained from Worthington Biochemical Co., Ltd., Freehold, NJ. Nuclease P1 was from Yamasa Shoyu Co., Ltd., Choshi. [γ-^{32}P]ATP was purchased from ICN Biomedical Inc., Irvine, CA. T_4 polynucleotide kinase and potato apyrase were from Takara Shuzo Co., Ltd., Kyoto and Sigma Chemical Co., St. Louis, MO, respectively. Polyethyleneimine(PEI)-cellulose sheets were from Machery-Nagel, Düren, Germany.

Animals and treatment CD and choline-supplemented (CS) diets were from DYETS, Bethlehem, PA. CD- or CS-pellet diets containing MeIQx at the concentration of 0.4 ppm or 4 ppm were prepared by CLEA Japan, Tokyo. A total of 120 male F344 rats were purchased at 7 weeks of age from Charles River Japan Inc., Atsugi. Rats were housed 4 per wire cage and maintained in an air-conditioned animal room. After 1 week of acclimatization, they were divided into 6 groups, of 20 animals each. The experimental protocol is illustrated in Fig. 1, rats in groups 1–3 receiving CS diet and groups 4–6 being given CD diet. The CS and CD diets containing MeIQx at 0.4 ppm were given to groups 2 and 5, and those at 4 ppm to groups 3 and 6, respectively. Groups 1 and 4 served as controls not receiving MeIQx. The actual amounts of MeIQx in each diet were determined by HPLC after extraction with 50% methanol, more than 90% of the added compound being confirmed as present in all cases.

Half the animals of each group were killed at week 20 and the remaining rats at week 40. The livers were all quickly resected, and fixation and paraffinization were carried out as reported previously.[14] Five μm thick sections were cut for immunohistochemical demonstration of GST-P or hematoxylin and eosin staining. For the immunohistochemistry, deparaffinized sections were treated

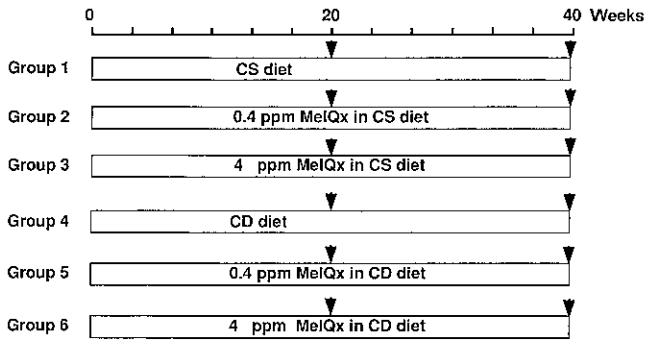

Fig. 1. Experimental protocol for examining the effects of low doses of MeIQx on the development of GST-P-positive foci in the livers of rats fed CS or CD diets. Arrows indicate killing time points.

sequentially with normal goat serum, rabbit anti-GST-P (1:1000), biotin-labeled goat anti-rabbit IgG and ABC.[19,20] As a negative control, preimmune rabbit serum was used instead of the GST-P antibody. Numbers and areas of GST-P-positive foci consisting of more than 10 cells in transection were manually measured on the entire cut surfaces of the three lobes (median right, median left and left lateral lobes) for each rat using a color video image processor (SP-500, Olympus Co., Tokyo). Both kinds of data obtained were expressed in units per cm^2 of the transection. The results were assessed by comparing the quantitative values for foci with control group data at each time point.

^{32}P-Postlabeling of MeIQx-DNA adducts DNA was isolated from the livers of the three individual rats of group 3 and group 6 by phenol extraction and digested to nucleoside 3′-monophosphates with micrococcal nuclease and spleen phosphodiesterase at 37°C for 3 h. ^{32}P-Postlabeling of MeIQx-DNA adducts was carried out using the nuclease P1 procedure with minor modifications as reported previously.[14] Briefly, digested DNA was treated with nuclease P1, then an aliquot was incubated with 0.034 nmol of [γ-^{32}P]ATP (7000 Ci/mmol) and 0.33 U/μl of T_4 polynucleotide kinase at 37°C for 1 h and subsequently with potato apyrase solution at 37°C for 45 min. Separation of MeIQx-DNA adducts on PEI-cellulose sheets, and their detection and quantitation were performed by the reported methods.[14] The levels of total adducts in each group were represented by the means ± SD of the three individual rat data.

Statistical analysis The data are all expressed as means ± SD. Student's t test was used for statistical analysis of differences in numbers and areas of GST-P-positive foci between the groups treated with CD and CS diets containing MeIQx and those given CD and CS diets alone,

respectively. The difference in MeIQx-DNA adduct levels between rats fed 4 ppm MeIQx in CD and CS diets were also statistically evaluated by using Student's *t* test.

RESULTS

Mean body and relative liver weights at weeks 20 and 40 are summarized in Table I. The body weights of rats fed the CD diet (groups 4–6) were almost the same as those fed the CS diet (groups 1–3) at both time points. Relative liver weights in groups 4–6 were significantly increased as compared to those in groups 1–3. The average MeIQx intakes of rats given 0.4 and 4 ppm MeIQx in groups fed the CS diet were 5.9 and 56 μg/day/rat, respectively. They were 6.2 and 64 μg/day/rat, respectively, for rats in the CD diet groups.

The data for GST-P-positive foci in groups 1–6 are summarized in Table II. In the livers of rats fed the CS diet (groups 1–3) at 20 and 40 weeks, numbers and areas of GST-P-positive foci were both smaller than those in rats fed CD diets. Numbers and areas of GST-P-positive foci of all groups at week 40 were larger than those of the corresponding groups at 20 weeks. The administration of MeIQx in the CS diet did not significantly alter the number or area of GST-P-positive foci. In rats fed the CD diet without MeIQx (group 4), GST-P-positive foci were clearly observed, being 2.6/cm^2 in number and 0.39 mm^2/cm^2 in area at week 20 and 6.1/cm^2 in number and 0.46 mm^2/cm^2 in area at week 40. Addition of 4 ppm MeIQx (group 6) increased 2.3-fold the number and 2.1-fold the area of GST-P-positive foci at week 20, and 2.0-fold the number and 3.3-fold the area at 40 weeks,

Table I. Body and Liver Weights of Rats Fed CS or CD Diet with or without MeIQx

Group	MeIQx (ppm)	Diet	20 weeks			40 weeks		
			No. of rats	Final body wt.[a] (g)	Liver wt.[a] (% of body wt.)	No. of rats	Final body wt.[a] (g)	Liver wt.[a] (% of body wt.)
1	0	CS	10	388±17	3.1±0.2	10	495±24	2.8±0.2
2	0.4	CS	10	396±25	3.1±0.2	10	473±34	2.9±0.2
3	4	CS	10	391±32	3.1±0.2	10	476±36	2.9±0.1
4	0	CD	10	399±24	3.5±0.3[b]	10	461±18	3.2±0.2[c]
5	0.4	CD	10	413±18	3.4±0.2[b]	10	468±39	3.3±0.3[b]
6	4	CD	10	404±26	3.7±0.3[c]	10	463±24	3.2±0.2[c]

a) Values represent mean±SD.
b), *c*) Significant difference at $P<0.01$ or $P<0.001$, respectively, from the corresponding CS group.

Table II. Numbers and Areas of GST-P-positive Foci in Livers of F344 Rats Fed CS or CD Diet with or without MeIQx

Group	MeIQx (ppm)	Diet	Occurrence of foci[a]	GST-P-positive foci[b]	
				No./cm^2	mm^2/cm^2
Week 20					
1	0	CS	2/10	0.10±0.20	0.001±0.002
2	0.4	CS	2/10	0.04±0.04	0.000±0.001
3	4	CS	1/10	0.06±0.14	0.000±0.001
4	0	CD	10/10	2.6±1.6	0.39±0.21
5	0.4	CD	10/10	3.6±2.5	0.34±0.21
6	4	CD	10/10	6.0±2.0[c]	0.80±0.50[c]
Week 40					
1	0	CS	4/10	0.26±0.25	0.005±0.0087
2	0.4	CS	6/10	0.18±0.19	0.003±0.0054
3	4	CS	6/10	0.25±0.25	0.001±0.0015
4	0	CD	10/10	6.1±3.2	0.46±0.21
5	0.4	CD	10/10	8.7±4.6	1.1±1.3
6	4	CD	10/10	12±7.1[c]	1.5±1.3[c]

a) Proportion of rats with GST-P-positive foci in liver.
b) Values represent mean±SD.
c) Significant difference at $P<0.05$ from the respective group 4 value.

as compared to group 4 values. The mean numbers of GST-P-positive foci were 6.0 and 12/cm^2 at 20 and 40 weeks and mean areas were 0.80 and 1.5 mm^2/cm^2 at 20 and 40 weeks, respectively, in group 6. However, addition of 0.4 ppm MeIQx to a CD diet (group 5) did not significantly increase the number or the area of GST-P-positive foci at 20 and 40 weeks.

With respect to histological appearance, the livers of rats fed the CS diets showed minimal focal fatty changes with ageing, but neither cellular alteration (including basophilic and eosinophilic changes) nor inflammation was observed at either 20 or 40 weeks. In the livers of rats fed the CD diet, severe fatty changes and formation of pseudo-lobuli encircled by fibrous tissue were observed with or without MeIQx administration at 20 and 40 weeks (Fig. 2A). These findings were consistent with liver cirrhosis. Some pseudo-lobuli having hyperplastic changes were stained with GST-P antibody (Fig. 2B).

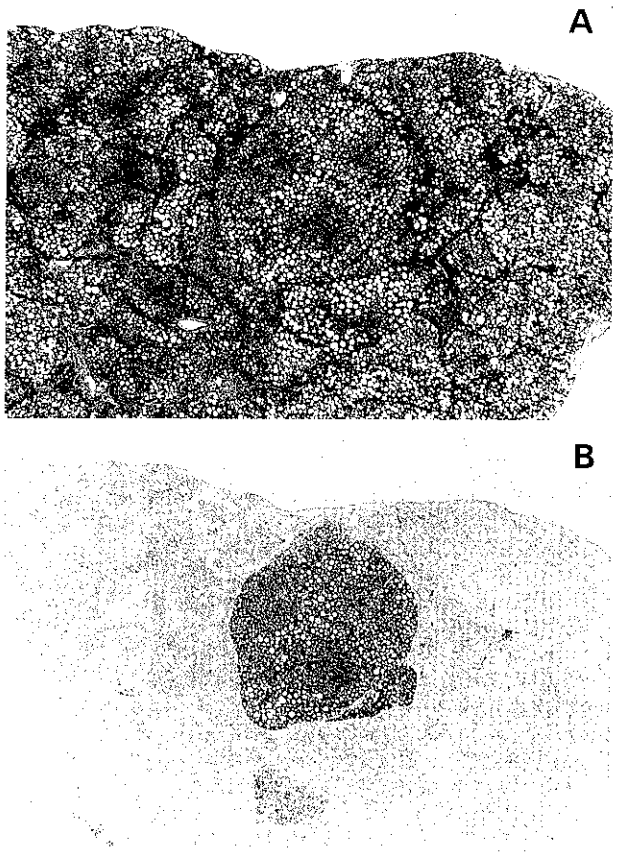

Fig. 2. Histological appearance of a typical cirrhotic liver in a rat fed CD diet containing 4 ppm MeIQx. (A) Hematoxylin and eosin staining ×40. (B) Immunohistochemical demonstration of GST-P antibody binding ×40.

MeIQx-DNA adduct levels were measured in the livers of rats in groups 3 and 6 at week 20 and week 40 using the ^{32}P-postlabeling method. Four adduct spots, the patterns of which were the same as reported previously,[14] were detected in the samples of groups 3 and 6. The levels of total adducts in group 3 and group 6, represented by means ± SD of three individual rats in each group, were $0.99 \pm 0.24/10^7$ nucleotides and $0.33 \pm 0.17/10^7$ nucleotides at week 20, and $0.75 \pm 0.15/10^7$ nucleotides and $0.33 \pm 0.19/10^7$ nucleotides at week 40, respectively. Thus, the MeIQx-DNA adduct level in rats fed CD diet containing 4 ppm MeIQx was 2 to 3 times lower ($P < 0.05$) than that in rats fed the CS diet containing 4 ppm MeIQx at both experimental time points.

DISCUSSION

The present study clearly demonstrated that chronic administration of a low dose (4 ppm) of MeIQx in a CD diet significantly increases the appearance of GST-P-positive foci in the liver of rats. In contrast, no effect in the livers of rats fed MeIQx in a CS diet was evident. The findings suggest that even 1/100th the dose of MeIQx (400 ppm) proven to be carcinogenic under normal dietary conditions could cause neoplasia under CD conditions, because a close association has been found between development of GST-P-positive foci in the liver of rats in medium-term experiments and hepatocarcinogenesis in long-term experiments.[21]

Measurement of DNA adduct levels in the livers after administration of 4 ppm MeIQx in CD and CS diets revealed values in the CD case to be 2- to 3-fold less than in the livers of rats fed the CS diet at both 20 and 40 weeks. Similarly, a previous study demonstrated that the levels of MeIQx-DNA adducts in the livers of rats treated with 40 ppm MeIQx and CCl_4 were decreased as compared with those treated with 40 ppm MeIQx alone.[14] Since the level of cytochrome P-450IA2, which is responsible for the metabolic activation of heterocyclic amines,[22] was reported to be suppressed in rat liver injured by CCl_4,[23] alteration of metabolic activation capacity in the livers of rats treated with CCl_4 may be related to the reduced levels of MeIQx-DNA adducts. This is possibly the case for rats given CD diet. From the above results, even the limited DNA adduct levels formed by 4 ppm MeIQx are probably sufficient to induce genetic alteration resulting in development of GST-P-positive foci under conditions where cell proliferation is chronically increased.

The exposure level (64 μg/day/rat) to 4 ppm MeIQx, the dose used in the present study, is still much higher than the human exposure level (0.2–2.6 μg/day/person) to MeIQx in everyday life. On the other hand, it was

earlier reported that combinations of five heterocyclic amines administered at 1/25th and 1/5th of their carcinogenic doses exert additive effects on induction of GST-P-positive foci in the livers of rats previously treated with diethylnitrosamine and subjected to partial hepatectomy.[24] Furthermore, a linear dose-response relationship was reported to exist between wide ranges of doses of MeIQx and levels of MeIQx-DNA adducts in the livers of rats and mice,[25,26] suggesting that there would be no threshold for the formation of DNA adducts by MeIQx. Thus, even at amounts as low as the human exposure level, MeIQx could produce DNA adducts in human tissues including the liver. The above observations offer some evidence that heterocyclic amines, including MeIQx, could be involved in cancer development in humans who are suffering from chronic inflammation including hepatitis.

In conclusion, estimation of the actual cancer risk from heterocyclic amines depends on their effects in combination with other factors, including proliferation. It is imperative that we clarify the nature of such additional contributory factors.

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REFERENCES

1) Sugimura, T. Carcinogenicity of mutagenic heterocyclic amines formed during the cooking process. *Mutat. Res.*, **150,** 33–41 (1985).
2) Sugimura, T. Studies on environmental chemical carcinogenesis in Japan. *Science,* **233,** 312–318 (1986).
3) Sugimura, T. Multistep carcinogenesis: a 1992 perspective. *Science,* **258,** 603–607 (1992).
4) Esumi, H., Ohgaki, H., Kohzen, E., Takayama, S. and Sugimura, T. Induction of lymphoma in CDF_1 mice by the food mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine. *Jpn. J. Cancer Res.,* **80,** 1176–1178 (1989).
5) Ito, N., Hasegawa, R., Sano, M., Tamano, S., Esumi, H., Takayama, S. and Sugimura, T. A new colon and mammary carcinogen in cooked food, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). *Carcinogenesis,* **12,** 1503–1506 (1991).
6) Ohgaki, H., Hasegawa, H., Suenaga, M., Sato, S., Takayama, S. and Sugimura, T. Carcinogenicity in mice of a mutagenic compound, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) from cooked foods. *Carcinogenesis,* **8,** 665–668 (1987).
7) Kato, T., Ohgaki, H., Hasegawa, H., Sato, S., Takayama, S. and Sugimura, T. Carcinogenicity in rats of a mutagenic compound, 2-amino-3,8-dimethylimidazo[4,5-*f*]-quinoxaline. *Carcinogenesis,* **9,** 71–73 (1988).
8) Sugimura, T., Sato, S. and Wakabayashi, K. Mutagens/carcinogens in pyrolysates of amino acids and proteins and in cooked foods: heterocyclic aromatic amines. *In* "Chemical Induction of Cancer," ed. Y.-t. Woo, D. Y. Lai, J. C. Arcos and M. F. Argus, Vol. IIIC, pp. 681–710 (1988). Academic Press, San Diego.
9) Zhang, X.-M., Wakabayashi, K., Liu, Z.-C., Sugimura, T. and Nagao, M. Mutagenic and carcinogenic heterocyclic amines in Chinese cooked foods. *Mutat. Res.*, **201,** 181–188 (1988).
10) Ushiyama, H., Wakabayashi, K., Hirose, M., Itoh, H., Sugimura, T. and Nagao, M. Presence of carcinogenic heterocyclic amines in urine of healthy volunteers eating normal diet, but not of inpatients receiving parenteral alimentation. *Carcinogenesis,* **12,** 1417–1422 (1991).
11) Preston-Martin, S., Pike, M. C., Ross, R. K., Jones, P. A. and Henderson, B. E. Increased cell division as a cause of human cancer. *Cancer Res.*, **50,** 7415–7421 (1990).
12) Tsuda, H., Takahashi, S., Yamaguchi, S., Ozaki, K. and Ito, N. Comparison of initiation potential of 2-amino-3-methylimidazo[4,5-*f*]quinoline and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline in an *in vivo* carcinogen bioassay system. *Carcinogenesis,* **11,** 549–552 (1990).
13) Kleman, M., Övervik, E., Blanck, A. and Gustafsson, J.-Å. The food-mutagens 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx) initiate enzyme-altered hepatic foci in the resistant hepatocyte model. *Carcinogenesis,* **10,** 1697–1700 (1989).
14) Sone, H., Wakabayashi, K., Kushida, H., Sugimura, T. and Nagao, M. Induction of preneoplastic lesions by a low dose of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) in the livers of rats treated with carbon tetrachloride. *Carcinogenesis,* **13,** 793–797 (1992).
15) Chandar, N. and Lombardi, B. Liver cell proliferation and incidence of hepatocellular carcinomas in rats fed consecutively a choline-devoid and a choline-supplemented diet. *Carcinogenesis,* **9,** 259–263 (1988).
16) Shinozuka, H., Katyal, S. L. and Perera, M. I. R. Choline deficiency and chemical carcinogenesis. *Adv. Exp. Med. Biol.,* **206,** 253–267 (1986).
17) Newberne, P. M. Lipotropic factors and oncogenesis. *Adv. Exp. Med. Biol.,* **206,** 223–251 (1986).
18) Yokota, K., Singh, U. and Shinozuka, H. Effects of a choline-deficient diet and a hypolipidemic agent on single glutathione *S*-transferase placental form-positive hepatocytes in rat liver. *Jpn. J. Cancer Res.,* **81,** 129–134 (1990).

19) Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I. and Sato, K. Purification, induction, and distribution of placental glutathione transferase: a new marker enzyme for preneoplastic cells in the rat chemical hepatocarcinogenesis. *Proc. Natl. Acad. Sci. USA,* **82,** 3964–3968 (1985).
20) Ito, N., Tsuda, H., Tatematsu, M., Inoue, T., Tagawa, Y., Aoki, T., Uwagawa, S., Kagawa, M., Ogiso, T., Masui, T., Imaida, K., Fukushima, S. and Asamoto, M. Enhancing effect of various hepatocarcinogens on induction of preneoplastic glutathione S-transferase placental form positive foci in rats — an approach for a new medium-term bioassay system. *Carcinogenesis,* **9,** 387–394 (1988).
21) Ogiso, T., Tatematsu, M., Tamano, S., Hasegawa, R. and Ito, N. Correlation between medium-term liver bioassay system data and results of long-term testing in rats. *Carcinogenesis,* **11,** 561–566 (1990).
22) Aoyama, T., Gonzalez, F. J. and Gelboin, H. V. Mutagen activation by cDNA-expressed $P_1$450, $P_3$450, and P450a. *Mol. Carcinog.,* **1,** 253–259 (1989).
23) Sesardic, D., Rich, K. J., Edwards, R. J., Davies, D. S. and Boobis, A. R. Selective destruction of cytochrome P-450d and associated monooxygenase activity by carbon tetrachloride in the rat. *Xenobiotica,* **19,** 795–811 (1989).
24) Ito, N., Hasegawa, R., Shirai, T., Fukushima, S., Hakoi, K., Takaba, K., Iwasaki, S., Wakabayashi, K., Nagao, M. and Sugimura, T. Enhancement of GST-P positive liver cell foci development by combined treatment of rats with five heterocyclic amines at low doses. *Carcinogenesis,* **12,** 767–772 (1991).
25) Yamashita, K., Adachi, M., Kato, S., Nakagama, H., Ochiai, M., Wakabayashi, K., Sato, S., Nagao, M. and Sugimura, T. DNA adducts formed by 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline in rat liver: dose-response on chronic administration. *Jpn. J. Cancer Res.,* **81,** 470–476 (1990).
26) Turteltaub, K. W., Felton, J. S., Gledhill, B. L., Vogel, J. S., Southon, J. R., Caffee, M. W., Finkel, R. C., Nelson, D. E., Proctor, I. D. and Davis, J. C. Accelerator mass spectrometry in biomedical dosimetry: Relationship between low-level exposure and covalent binding of heterocyclic amine carcinogens to DNA. *Proc. Natl. Acad. Sci. USA,* **87,** 5288–5292 (1990).