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Lack of a Dose-response Relationship for Carcinogenicity in the Rat Liver with Low Doses of 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline or *N*-Nitrosodiethylamine

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For a long period, it has been generally considered that carcinogens, particularly genotoxic ones, have no threshold in exerting their potential for cancer induction. However, the non-threshold theory can be challenged with regard to assessment of cancer risk to humans. Here we show that a food-derived, genotoxic hepatocarcinogen, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, forms DNA adducts at low doses, but does not induce glutathione *S*-transferase placental form (GST-P)-positive foci (considered to be preneoplastic lesions) or 8-hydroxy-2′-deoxyguanosine in rat liver. Moreover a *N*-nitroso compound, *N*-nitrosodiethylamine, at low doses was also found not to induce GST-P-positive foci in rat liver. These results imply that there is a no-observed effect level for hepatocarcinogenesis by these genotoxic carcinogens.

Key words: MeIQx — NDEA — Risk assessment — Carcinogenicity threshold — Low dose carcinogenicity

For a long period, it has been generally considered that genotoxic carcinogens have no threshold in exerting carcinogenic potential.[1,2] This is because genetoxic carcinogens are mutagenic, and seem to act through interaction with DNA to produce irreversible genetic changes in target organ cells. This theory is based on acceptance of a linear relationship which approaches zero at low doses for risk assessment of exposure to man with chemicals found to be carcinogenic in animal studies. However, there are limited data available for estimation of cancer risk assessment in man exposed to genotoxic carcinogens,[3–5] and the validity of the no-threshold theory can be challenged in this case. This is because life forms may possess biological responses which decrease genotoxic activity. It is very important to resolve this point from the viewpoint of cancer risk control and management.

There are many genotoxic carcinogens occurring naturally in our environment, including the large group of heterocyclic amine mutagens.[6,7] The human daily intake of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), one of these food-derived agents, is estimated to be 0.2–2.6 μg/person.[8] MeIQx can be detected in the urine of healthy volunteers after eating cooked meat[9–11] and MeIQx-DNA adducts have been found in kidney and colon tissues in man.[12] In rats, MeIQx induces DNA adduct formation in the liver[13] and hepatocellular carcinomas develop with treatment at high doses.[14]

Recently *in vivo* medium-term bioassays for carcinogens have been accepted as possible alternatives to long-term carcinogenicity tests. In particular, a liver medium-term bioassay which is a very useful tool for detection of hepatocarcinogenicity of chemicals has been developed[15] and is appropriate for assessment of low-dose effects because of its high sensitivity. Glutathione *S*-transferase placental form (GST-P)-positive foci are established preneoplastic markers in the livers of rats[15,16] and their ready detection by immunohistochemistry underlies their acceptance as end-point lesions to assess the carcinogenic response in an established liver medium-term bioassay. In the present study, for clarification of human risk assessment of genotoxic carcinogens, we examined the low-dose

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carcinogenicity of MeIQx in detail using this bioassay with the primary aim of determining whether the response curve is indeed linear near zero.

Other relatively common carcinogens in our environment are *N*-nitroso compounds, such as *N*-nitrosodiethylamine (NDEA). While Peto *et al.*[5] reported observation of a linear relationship at low doses with this hepatocarcinogen in rats, the actual doses applied were still relatively high compared to human exposure levels. Therefore, we also examined low-dose carcinogenicity of NDEA in the rat liver using GST-P-positive foci as end-point lesions.

DNA adduct formation is considered to be an important factor in carcinogenesis with heterocyclic amines and MeIQx-DNA adducts are dose-dependently formed in rat liver.[13] 8-Hydroxy-2′-deoxyguanosine (8-OHdG) is the most abundant species of adduct associated with oxidative stress, producing DNA damage which can result in specific types of mutation.[17] 8-OHdG formation is induced in target organ DNA by genotoxic or non-genotoxic carcinogens[17, 18] and MeIQx administration to rats increases the levels in the liver in a dose-dependent manner.[19] This adduct is also thought to be involved in the initiation of rat liver carcinogenesis by low doses of NDEA.[18] Therefore, levels of MeIQx-DNA adducts and 8-OHdG were also examined in the present study to cast further light on mechanistic aspects of MeIQx carcinogenicity at low doses.

MATERIALS AND METHODS

Animals and chemicals A total of 3102 male 20-day-old F344 rats were obtained from Charles River Japan, Inc. (Atsugi). MeIQx (purity, 99.9%) was purchased from the Nard Institute, Nishinomiya, NDEA (purity, 99.5%) from Sakai Research Laboratory (Fukui), and phenobarbital sodium salt (PB, purity, 98%) from Wako Pure Chemical Industries (Osaka). The animals were housed in rooms maintained on a 12 h light/dark cycle, at constant temperature and humidity, and observed daily.

Experiment 1 A total of 885 rats were employed in the experiment, which was started when they were aged 21 days. They received MeIQx at doses of 0 (group 1, a control), 0.001 (group 2), 0.01 (group 3), 0.1 (group 4), 1 (group 5), 10 (group 6), and 100 ppm (group 7) in powdered basal diet (Oriental MF, Oriental Yeast Co., Tokyo) for 4 or 16 weeks, continuously. The lowest level, 0.001 ppm of MeIQx was established as equivalent to the daily intake of this carcinogen in humans.[8] The MeIQx diets were made by Oriental Yeast Co. and the concentration in each diet was confirmed by HPLC. Numbers of rats in groups 1 to 5 were 155 each and those in groups 6 and 7 were 55 each. The rats (150 in groups 1 to 5 and 50 in groups 6 and 7) were killed at the end of week 16 under ether anesthesia for examination of immunohistochemically demonstrable GST-P expression (50 or 150 samples), and formation of MeIQx-DNA adducts (3 samples in each group) and 8-OHdG (5 samples in each group) in the liver. Five additional rats in each of groups 1 to 7 were killed at week 4 for examination of MeIQx-DNA adducts (3 samples each) and 8-OHdG (5 samples each).

Experiment 2 A total of 260 (21-day-old at the commencement) rats received MeIQx at the same doses (no group for MeIQx at 0.001 ppm) as in experiment 1 for 32 weeks (50 rats each with 0 to 1 ppm MeIQx and 30 rats each with 10 and 100 ppm). Animals were killed at the end of week 32 under ether anesthesia for examination of GST-P-positive foci in the liver.

Experiment 3 A total of 1957 (21-day-old at the commencement) rats received NDEA at doses of 0 (controls, 325 rats), 0.0001 (326 rats), 0.001(322 rats), 0.01 (326 rats), 0.1 (251 rats), 1 (256 rats) or 10 ppm (151 rats) in drinking water for 16 weeks, continuously. The lowest level, 0.0001 ppm of NDEA was established with reference to the human daily exposure to NDEA.[20, 21] All rats were killed at the end of the experiment under ether anesthesia for examination of GST-P-positive foci in the liver.

Assessments of GST-P-positive foci, MeIQx-DNA adducts and 8-OHdG Formalin-fixed liver tissue (a total of 9 sections, 3 sections each from the left lateral lobe, medial lobe, and right lateral lobe) was embedded in paraffin wax for immunohistochemical examination of GST-P in the liver, as described previously.[5] Those hepatocellular foci comprising 2 or more positive cells were counted under a light microscope. Total areas of livers were measured using a color image processor (IPAP, Sumica Technos, Osaka) and the numbers of foci per cm^2 of liver tissue were calculated. The levels of MeIQx-DNA adducts in the liver were measured by the ^{32}P-postlabeling method under modified adduct intensification conditions using frozen samples, as previously reported.[12] Under this condition, the major MeIQx-DNA adduct, dG-C8-MeIQx, can be detected as a single spot on TLC. Measurement of 8-OHdG levels in liver DNA was performed according to the method of Nakae *et al.*[18]

Statistical analysis Statistical analysis of our data was performed using the StatView-J 5.0 program (Abacus Concepts, Inc., Berkeley, CA). Differences from the control values were evaluated for significance by the Dunnet test. Values in figures are shown on a logarithmic scale.

RESULTS

Experiments 1 and 2

General findings: All the rats survived in good condition until the scheduled sacrifices. No macroscopic lesions were apparent in any organ, including the liver. No adverse effect on average body weight gain was observed

Table I. Final Average Body Weights, Average Liver Weights, and Average Total MeIQx Intakes (Experiment 1)

Groups	MeIQx doses (ppm)	No. of rats	Final body weights (g)	Liver		Total MeIQx intake (mg/rat)
				Absolute (g)	Relative (%)	
1	0	150	327±25[a)]	9.0±1.8	2.8±0.4	0
2	0.001	150	326±22	9.2±1.4	2.8±0.4	0.00164
3	0.01	150	328±21	9.2±1.4	2.8±0.3	0.01564
4	0.1	150	326±21	9.2±1.4	2.8±0.3	0.16176
5	1	150	328±21	9.4±1.6	2.9±0.4	1.65060
6	10	50	332±17	9.5±0.8	2.9±0.2	16.5711
7	100	50	322±20	10.7±1.1**	3.3±0.3*	164.523

a) Values are mean±SD.
* $P<0.05$, ** $P<0.01$ (vs. group 1).

Table II. The Occurrence of GST-P-positive Foci in the Livers of Rats Treated with MeIQx at Various Doses for 16 Weeks (Experiment 1)

Groups	MeIQx dose (ppm)	No. of rats	Size distribution of GST-P-positive foci (No./cm^2)			
			2–4 cells	5–10 cells	≥11 cells	Total
1	0	150	0.118±0.167[a)]	0.046±0.166	0.021±0.088	0.185±0.350
2	0.001	150	0.122±0.175	0.021±0.057	0.012±0.048	0.155±0.188
3	0.01	150	0.125±0.206	0.027±0.066	0.007±0.048	0.159±0.238
4	0.1	150	0.144±0.203	0.035±0.076	0.015±0.104	0.194±0.255
5	1	150	0.155±0.199	0.037±0.075	0.015±0.065	0.207±0.237
6	10	50	0.349±0.327	0.102±0.121	0.014±0.047	0.465±0.354
7	100	50	13.864±5.109*	8.854±3.239*	6.512±4.057*	29.23±10.99*

a) Values are mean±SD.
* $P<0.01$ (vs. group 1).

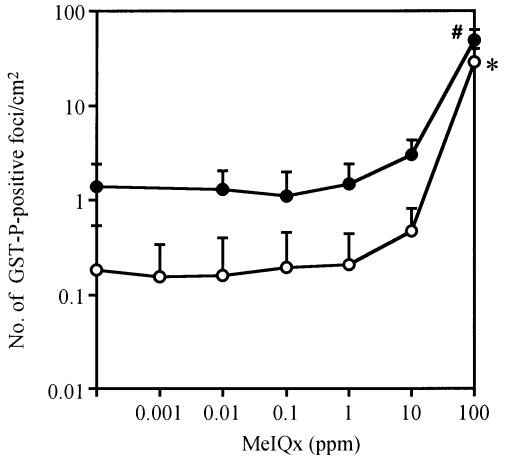

Fig. 1. Induction of GST-P-positive foci in the livers of rats treated with MeIQx at weeks 16 (experiment 1, ○) and 32 (experiment 2, ●). Significant difference from the 0 ppm group in experiment 1 at * $P<0.01$ (n=150 in 0 to 1 ppm groups, n=50 in 10 and 100 ppm groups). Significant difference from the 0 ppm group in experiment 2 at # $P<0.01$ (n=50 in 0 to 1 ppm groups, n=30 in 10 and 100 ppm groups). Bars, SD.

in rats treated with MeIQx (Table I). Average liver weights were significantly increased in groups given 100 ppm MeIQx. Average total MeIQx intake in each group was dose-dependent.

Induction of GST-P-positive foci in the liver: After 16 weeks treatment with MeIQx at various doses in the diet, numbers of GST-P-positive foci per unit area of the rat livers of groups receiving 0.001 ppm to 1 ppm of the carcinogen did not differ from the control value (non-treatment group, Table II and Fig. 1), in contrast to the increase observed with 10 ppm and the clear, significant elevation with 100 ppm MeIQx. Values in groups treated with MeIQx at doses of 0.001 and 0.01 in fact rather showed slight decrease. Numbers of GST-P-positive foci comprising 2–4 cells, 5–10 cells, and ≥11 cells in the groups given 0.001 ppm to 1 ppm MeIQx were also not different from the control values, while those with 10 ppm MeIQx, and more particularly in the group given 100 ppm MeIQx, were significantly increased.

In the livers of rats treated with MeIQx for 32 weeks, curves for numbers of GST-P-positive foci were very sim-

ilar to those observed after 16 weeks continuous treatment with the heterocyclic amine (Fig. 1).

Formations of MeIQx-DNA adducts and 8-OHdG: At both weeks 4 and 16, there were linear relationships between the various doses (0.01 ppm to 100 ppm) of MeIQx and the levels of MeIQx-DNA adducts (Fig. 2). Concerning the 8-OHdG levels in the liver DNA at week 4, no significant differences among groups receiving MeIQx from 0.001 ppm to 0.1 ppm and the control group were apparent (Fig. 3), although values were linearly elevated from 1 ppm and above, with statistical significance. On the other hand, at week 16 the level of 8-OHdG at 0.001 ppm MeIQx was not different from the control group, but the levels were linearly elevated from 0.01 ppm of MeIQx to the highest dose, and the increase was significant.

Experiment 3 No retardation was apparent in terms of average body weight gain in the NDEA treatment groups during the 16-week experimental period. All rats survived until sacrifice, as in experiment 1. Macroscopically no lesion was evident in any organ, including the liver. Average liver weights were significantly increased in the group treated with NDEA at 10 ppm (Table III).

Data for GST-P-positive foci in the liver are shown in Fig. 4. Numbers in groups receiving NDEA at 0.0001 ppm to 0.01 ppm were not different from the control value (non-treatment group). The groups given 0.1 or 1 ppm NDEA showed a significant increase of GST-P-positive foci and lesions were impossible to count at 10 ppm since they were so numerous.

DISCUSSION

To overcome the disadvantages of long-term protocols for risk assessment, medium-term bioassays have recently

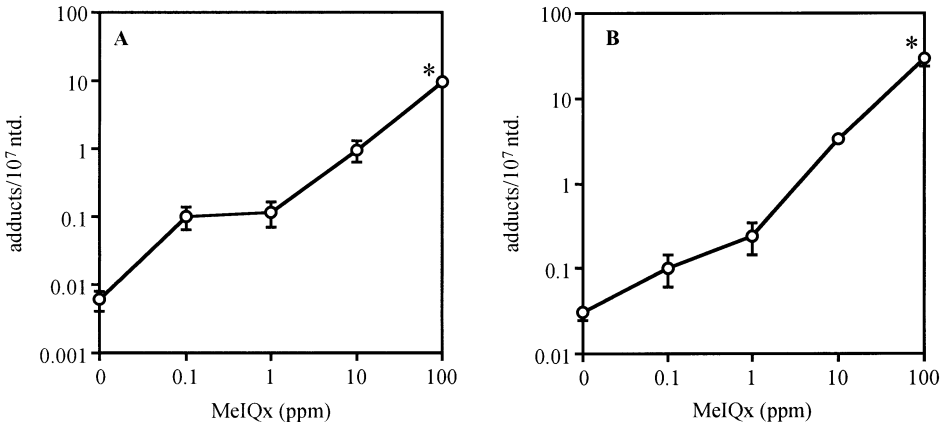

Fig. 2. MeIQx-DNA adduct formation in the livers of rats. A, 4 weeks treatment; B, 16 weeks treatment. * $P<0.01$ (vs. group 1), $n=3$ in each point; bars, SD.

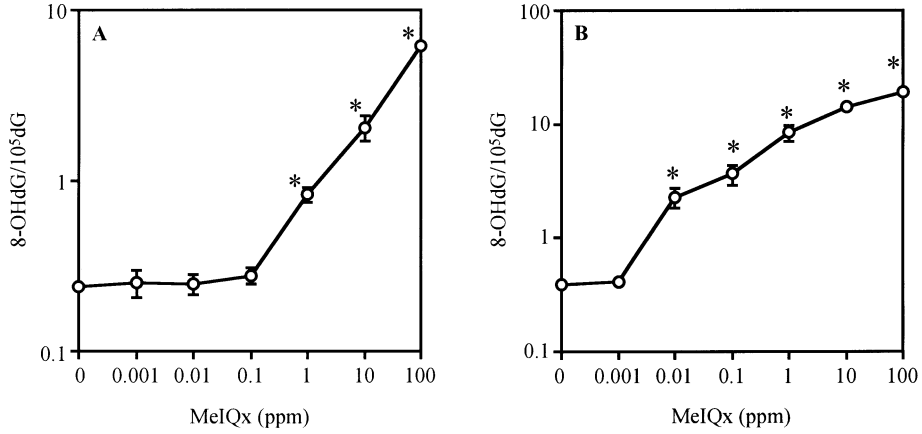

Fig. 3. 8-OHdG formation levels in the livers of rats treated with MeIQx. A, 4 weeks treatment; B, 16 weeks treatment. * $P<0.01$ (vs. group 1), $n=5$ in each point; bars, SD.

Table III. Final Average Body Weights, Average Liver Weights, and Average Total NDEA Intakes (Experiment 3)

Groups	NDEA doses (ppm)	No. of rats	Final body weights (g)	Liver		Total MeIQx intake (mg/rat)
				Absolute (g)	Relative (%)	
1	0	325	321±20[a)]	9.4±0.8	2.9±0.2	0
2	0.0001	326	324±19	9.4±0.8	2.9±0.2	0.00021
3	0.001	322	321±20	9.4±0.7	2.9±0.2	0.00208
4	0.01	326	319±21	9.2±0.8	2.8±0.1	0.02060
5	0.1	251	322±19	9.4±0.8	2.9±0.2	0.20560
6	1	256	320±21	9.5±0.7	2.9±0.2	2.07833
7	10	151	318±19	10.9±0.9*	3.4±0.1*	21.11586

a) Values are mean±SD.
* $P<0.01$ (vs. group 1).

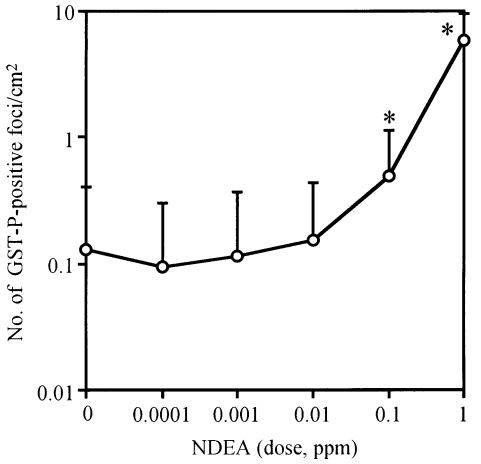

Fig. 4. Induction of GST-P-positive foci in the livers of rats treated with NDEA. * $P<0.01$ (vs. group 1). Numbers of rats are shown in Table III; bars, SD.

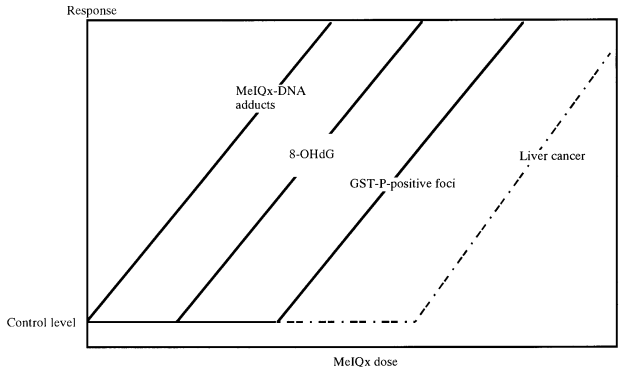

Fig. 5. Summarized relationships among various biomarkers for carcinogenesis in the livers of rats treated with MeIQx.

attracted much attention as alternatives.[15)] In the present study, the hepatocarcinogenic potential of MeIQx or NDEA was judged by counting the number of GST-P-positive foci as end-point lesions, since these foci have been shown to correlate with cancer induction.[22)] The present results clearly indicate that the plot of induction of GST-P-positive foci against dose of MeIQx or NDEA is not linear down to zero. In the liver of rats treated with MeIQx, 8-OHdG formation level is also not linear down to zero dose, whereas linear increases of MeIQx-DNA adduct levels were observed even at the low doses examined. Concerning relationships among MeIQx-DNA adducts, 8-OHdG and GST-P-positive foci in the case of MeIQx experiment, the conclusion summarized in Fig. 5 can be drawn from our findings. Levels of MeIQx-DNA adducts linearly increase from very low doses, and then, in order, curves for 8-OHdG formation and GST-P-positive foci develop from different baseline control levels. Thus, our results indicate that, in the case of exposure to genotoxic carcinogens at low doses, different no-observed effect levels may exist for different parameters relevant to carcinogenicity. In view of these findings we propose that a no-observed effect level for cancer induction due to MeIQx should also exist. Indeed, we recently found that MeIQx at doses of 0.001 and 1 ppm did not induce GST-P-positive foci or other lesions in rat liver in a 2-year carcinogenicity study (unpublished data). Therefore, it seems very likely that genotoxic carcinogens may have a no-observed effect level regarding their carcinogenic potentials.

Biological adaptive responses, resulting in physiological protection of cells against toxic agents, has recently become accepted for radiation carcinogenesis at low doses.[23)] This concept might also be useful for understanding dose effects in chemical carcinogenesis, since adaptation might be expected to occur in response to low doses of all types of DNA-damaging agents.[24, 25)] Various factors such as stimulation of the immune response, induction of detoxification and repair enzymes, and upregulation of tumor suppressor genes could result in paradoxical beneficial effects of low-dose exposure.

In both humans and rodents the importance of toxicokinetics of chemicals for exerting carcinogenicity has recently been stressed. Absorption of carcinogens into the body, distribution to target organs, metabolism to active ultimate forms which react with DNA, detoxification, and excretion, all influence DNA damage. Comparison of DNA adduct levels has demonstrated linear increase at low doses, but without a strict correlation with subsequent neoplastic development.[26, 27] In the present study, linear increases of MeIQx-DNA adduct levels in the liver were detectable even at the lowest dose examined, but this clearly was similarly not correlated with induction of preneoplastic lesions. It is noteworthy, however, that measurement of DNA adduct levels is a very good biomarker for exposure assessment, as was also indicated by Bailey *et al.*[28] in rainbow trout in tests of aflatoxin carcinogenicity.

Generation of active oxygen radicals by various carcinogens is considered to be an important factor for carcinogenesis.[29] Such radicals interact with nuclear DNA, resulting in 8-OHdG formation through oxidative processes,[30] and MeIQx is known to be associated with strong generation of hydroxy radicals.[31] In the present study, the curves for 8-OHdG levels were similar at weeks 4 and 16, with the existence of no-observed effect levels. However, the level was higher at week 4 than at week 16. This may indicate that 8-OHdG formed by MeIQx accumulates with prolonged treatment, but again the results were clearly consistent with GST-P-positive foci induction.

In conclusion, a no-observed effect level may exist for the hepatocarcinogenic potential of MeIQx and NDEA, and, by analogy, probably also for other genotoxic agents. This conclusion is very important regarding how we should view the impact of carcinogens, especially genotoxic carcinogens, in the human environment in relation to cancer risk control and management.

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