In this study, the *gpt* delta rat animal model was used to determine the comprehensive carcinogenicity and *in vivo* genotoxicity of MEG at three different doses, including the reported carcinogenic dose, following medium-term exposure.

MATERIALS AND METHODS

Chemicals. MEG and methylcellulose were purchased from Wako Pure Chemical Industries (Osaka, Japan). The MEG solutions were prepared in 0.5% aqueous methylcellulose.

Animals and housing conditions. The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences (Tokyo, Japan). Five-week-old male and female F344 *gpt* delta rats carrying approximately five tandem copies of the transgene lambda EG10 per haploid genome were obtained from Japan SLC (Shizuoka, Japan). The rats were housed in polycarbonate cages (three or four rats per cage) with hardwood chips for bedding in a conventional animal facility. The animals were maintained in a controlled environment with constant temperature (23±2°C), humidity (55±5°C), air changes (12 times per hour), and lighting (12 h light/dark cycle). The animals were allowed free access to a basal diet of CRF-1 commercial pellets (Charles River Japan, Kanagawa, Japan) and tap water.

Experimental design. After a 1-week acclimatization period, the animals were divided into four groups consisting of 10 male and 10 female F344 *gpt* delta rats per group. The rats were administered an intragastric dose of 0, 10, 30, or 100 mg/kg MEG daily for 13 weeks.

The clinical signs and general appearance of the rats were observed once per day. Body weight and food consumption were measured once per week. At the end of each 13-week treatment cycle, the animals were euthanized under deep anesthesia. The left lobes of the liver were fixed in neutral-buffered formalin for histopathological and immunohistopathological examination. The remaining liver was stored at −80°C for *in vivo* mutation assays.

In vivo *mutation assays.* 6-TG and Spi⁻ (insensitive P2 interference) selection assays were performed as previously described (Nohmi *et al.*, 1996, 2000). Briefly, genomic DNA was extracted from the liver tissue, and lambda EG10 DNA (48 kb) was rescued as the lambda phage through *in vitro* packaging. For 6-TG selection, the packaged phage was incubated with *Escherichia coli* YG6020, which expresses Cre recombinase and converted to a plasmid carrying *gpt* and chloramphenicol acetyltransferase. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. To determine the total number of rescued plasmids, the infected cells were poured onto plates containing chloramphenicol without 6-TG. The plates were incubated at 37°C for selection of 6-TG-resistant colonies, and the *gpt* mutant frequency (MF) was calculated by dividing the number of *gpt* mutants after clonal correction by the number of rescued phages. To characterize the *gpt* mutations, a 739-bp DNA fragment containing the 456-bp coding region of the *gpt* gene was amplified using PCR as previously described. The PCR products were analyzed with an Applied Biosystems 3730x1 DNA Analyzer (Applied Biosystems, Japan Ltd).

For Spi⁻ selection, the packaged phage was incubated with *E. coli* XL-1 Blue MRA for survival titration and *E. coli* XL-1 Blue MRA P2 for mutant selection. The infected cells were mixed with molten lambda-trypticase agar plates. The following day, plaques (Spi⁻ candidates) were punched out with sterilized glass pipettes, and the agar plugs were suspended in SM buffer. To confirm the Spi⁻ phenotype of the candidates, the suspensions were spotted on three types of plates containing XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains and spread with soft agar. The number of mutants that exhibited clear plaques on each plate was counted as confirmed Spi⁻ mutants. The Spi⁻ MF was calculated by dividing the number of Spi⁻ mutants by the number of rescued phages. In all of the *in vivo* mutations assays, positive DNA samples were included to ensure that the assay was functioning properly.

Immunohistochemical staining. Immunohistochemical staining was performed using polyclonal antibodies against GST-P (1:1000 dilution; Medical and Biological Laboratories Co., Ltd, Nagoya, Japan), which is a marker of preneoplastic lesions in the rat liver, and monoclonal anti–mouse proliferating cell nuclear antigen (PCNA) antibodies (1:100; Dako, Glostrup, Denmark) to evaluate cell proliferation activity using the avidin–biotin peroxidase complex method. The number (No./cm²) and area (mm²/cm²) of the GST-P-positive foci (> 0.01 mm²) and the total area of each liver section were measured using an IPAP image analyzer (Sumika Technos, Osaka, Japan) (Watanabe *et al.*, 1994). The numbers of PCNA-positive cells per 600–800 intact liver cells from 10 different areas per animal were counted to determine the PCNA-positive ratio.

Statistics. The body weight measurements, food and water consumption, weight of the liver, GST-P-positive foci, PCNA-LI, *gpt* and Spi⁻ MFs, and various mutation frequencies in the spectrum analysis were expressed as the mean ± SD. Significant differences between the control and treated groups were determined using Dunnett's multiple comparison test (Dunnett, 1955) after ANOVA. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Body and Liver Weights and Histopathological Examination

The final body and liver weights are shown in Table 1. There was no suppression of body weight gain in the treated groups

TABLE 1
Final Body and Liver Weights of *gpt* Delta Rats Administered MEG for 13 Weeks

Sex	Groups	Number of animals	Final body weight (g)	Liver weight	
				Absolute (g)	Relative (g/100 g bw)
Male					
	Control	10	300.0±17.4[a]	8.09±0.49	2.45±0.07
	10 mg/kg MEG	10	325.0±10.4	8.22±0.41	2.53±0.08
	30 mg/kg MEG	10	322.4±13.7	8.54±0.41	2.65±0.08
	100 mg/kg MEG	10	310.2±35.4	9.43±0.71*	3.09±0.45*
Female					
	Control	10	189.0±5.6	4.26±0.22	2.26±0.12
	10 mg/kg MEG	9	190.3±8.3	4.34±0.22	2.28±0.14
	30 mg/kg MEG	9	187.6±5.3	4.29±0.22	2.29±0.11
	100 mg/kg MEG	9	176.5±9.7	4.38±0.29	2.48±0.08#

[a]Mean ± SD.
*Significantly different from the male control group at the levels of $p < 0.001$ (Dunnett's test).
**Significantly different from the female control group at the levels of $p < 0.001$ (Dunnett's test).

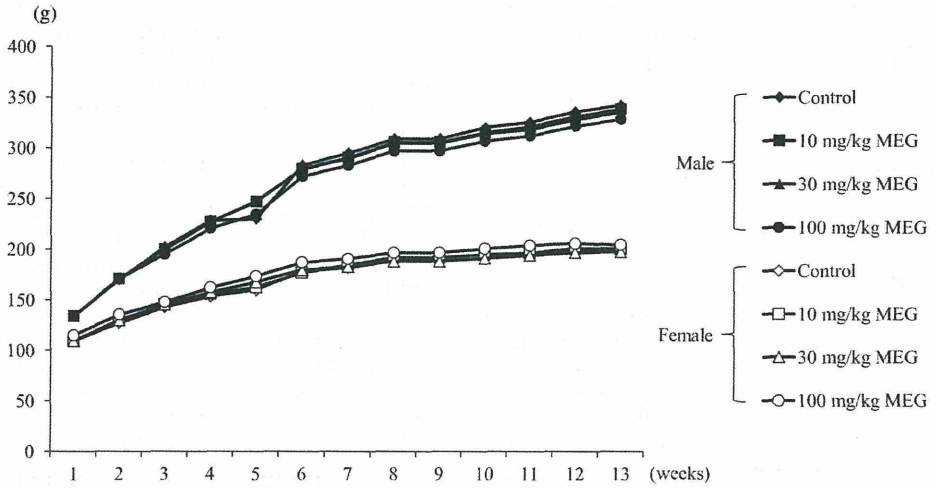

FIG. 1. Body weights of male and female *gpt* delta rats treated with MEG for 13 weeks.

TABLE 2
***gpt* MFs in the Liver of Male *gpt* Delta Rats Administered MEG for 13 Weeks**

Sex	Groups	Animal No.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	MF ($\times 10^{-5}$)	Mean ± SD
Male	Control	1	3.4	2	0.59	0.53±0.18
		2	4.5	2	0.44	
		3	4.5	3	0.67	
		4	2.9	2	0.69	
		5	7.6	2	0.27	
	10 mg/kg MEG	11	9.5	4	0.42	0.79±0.34
		12	4.8	6	1.25	
		13	3.0	2	0.66	
		14[a]	1.9	2	1.03[a]	
		15	5.3	3	0.57	
	30 mg/kg MEG	21	2.5	1	0.40	0.79±0.42
		22	5.5	8	1.45	
		23	4.9	3	0.61	
		24	7.3	7	0.96	
		25	3.6	2	0.55	
	100 mg/kg MEG	31	6.0	12	2.20	1.35±0.60*
		32	4.1	3	0.73	
		33	8.3	8	0.96	
		34	3.5	4	1.14	
		35	3.6	6	1.69	

[a]Data from animal No. 14 was excluded for the calculation of MF because of the poor packaging efficiency of the transgene.
*Significantly different from the control group at $p < 0.05$ (Dunnett's test).

during the experiment (Fig. 1). The relative weight of the liver of the male and female rats treated with 100 mg/kg and the absolute weight of the liver of the males treated with 100 mg/kg MEG were significantly increased compared with the rats in the control group. There were no histopathological changes observed in the liver of the rats treated with MEG.

In Vivo *Mutation Assays*

The *gpt* and Spi^- MFs in the liver of male and female *gpt* delta rats treated with MEG for 13 weeks are summarized in Tables 2–5. A significant increase in the *gpt* and Spi^- MFs was observed in the male and female rats treated with the carcinogenic dose of 100 mg/kg. We determined the *gpt* mutation spectra in the *gpt* mutant colonies to characterize the types of *gpt* mutations caused by exposure to MEG. A GC→CG transversion mutation in the treated male rats and an AT→TA transversion mutation in the treated female rats were observed, but the incidence rates were not statistically significant compared with the controls (Table 6).

Effects of MEG on GST-P-Positive Foci and Cell Proliferation

Under immunohistochemical examination, treatment with MEG increased the number and area of GST-P-positive foci

TABLE 3
***gpt* MFs in the Liver of Female *gpt* Delta Rats Administered MEG for 13 Weeks**

Sex	Groups	Animal No.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	MF ($\times 10^{-5}$)	Mean ± SD
Female	Control	41	10.5	1	0.10	0.21 ± 0.13
		42	14.9	2	0.14	
		43	13.1	5	0.38	
		44	13.3	4	0.30	
		45	18.0	2	0.11	
	10 mg/kg MEG	51	6.0	3	0.50	0.38 ± 0.18
		52	7.5	4	0.53	
		54	6.2	3	0.48	
		55	10.7	3	0.28	
		56	8.8	1	0.11	
	30 mg/kg MEG	61	9.5	4	0.42	0.53 ± 0.19
		62	7.8	2	0.25	
		63	10.3	7	0.68	
		64	8.8	6	0.68	
		65	4.8	3	0.62	
	100 mg/kg MEG	71	8.9	10	1.12	1.23 ± 0.59*
		72	3.1	7	2.25	
		73	5.0	4	0.81	
		74	5.6	5	0.90	
		75	5.7	6	1.05	

*Significantly different from the control group at $p < 0.05$ (Dunnett's test).

TABLE 4
Spi^- MFs in the Liver of Male *gpt* Delta Rats Administered MEG for 13 Weeks

Sex	Groups	Animal No.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within XL-1 Blue MRA (P2)	MF ($\times 10^{-5}$)	Mean ± SD
Male	Control	1	7.3	1	0.14	0.32 ± 0.27
		2	4.1	1	0.25	
		3	5.9	1	0.17	
		4	8.4	6	0.72	
		5	8.6	1	0.12	
	10 mg/kg MEG	11	9.5	4	0.42	0.31 ± 0.11
		12	7.6	2	0.27	
		13	6.9	3	0.44	
		14	8.8	2	0.23	
		15	10.4	2	0.19	
	30 mg/kg MEG	21	7.8	3	0.38	0.41 ± 0.24
		22	8.8	2	0.23	
		23	6.8	1	0.15	
		24	7.2	5	0.70	
		25	6.7	4	0.60	
	100 mg/kg MEG	31	7.0	5	0.72	0.85 ± 0.40*
		32	6.9	2	0.29	
		33	7.8	10	1.29	
		34	4.2	5	1.20	
		35	4.1	3	0.74	

*Significantly different from the control group at $p < 0.05$ (Dunnett's test).

in a dose-dependent manner compared with the control group, although the differences were not statistically significant at doses lower than 30 mg/kg (Fig. 2). The effects of MEG on cell proliferation were evaluated using immunohistochemistry for PCNA (Fig. 3). The ratio of PCNA-positive hepatocytes was significantly increased in the male and female rats treated with 100 mg/kg MEG.

DISCUSSION

MEG, a flavoring agent classified as an alkoxy-substituted allylbenzene, is present in a variety of foods, spices, teas, and essential oils. Several flavoring agents that are classified into the same chemical category, such as estragole and safrole, have been reported to be hepatocarcinogenic in rodents (Borchert

TABLE 5
Spi⁻ MFs in the Liver of Female *gpt* Delta Rats Administered MEG for 13 Weeks

Sex	Groups	Animal No.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within XL-1 Blue MRA (P2)	MF ($\times 10^{-5}$)	Mean ± SD
Female	Control	41	17.9	2	0.11	0.15±0.07
		42	24.8	2	0.08	
		43	16.5	4	0.24	
		44	20.9	4	0.19	
		45	35.1	4	0.11	
	10 mg/kg MEG	51	9.2	0	0.00	0.15±0.10
		52	18.6	4	0.22	
		54	11.3	2	0.18	
		55	18.7	2	0.11	
		56	16.1	4	0.25	
	30 mg/kg MEG	61	18.9	5	0.27	0.20±0.09
		62	13.1	4	0.31	
		63	20.1	2	0.10	
		64	14.0	2	0.14	
		65	11.2	2	0.18	
	100 mg/kg MEG	71	9.5	7	0.74	0.33±0.26*
		72	9.3	1	0.11	
		73	6.9	3	0.43	
		74	10.0	2	0.20	
		75	10.5	2	0.19	

*Significantly different from the control group at $p < 0.05$ (Dunnett's test).

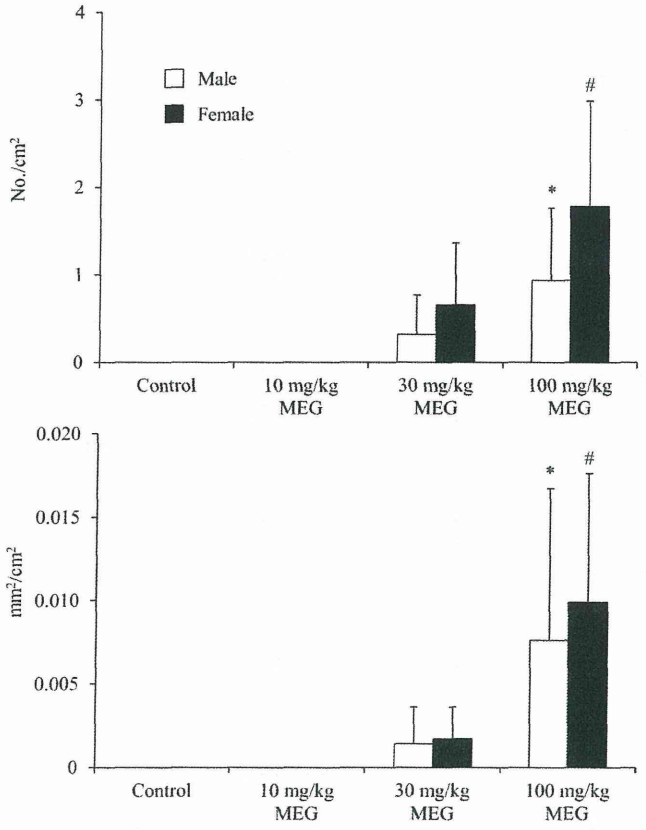

FIG. 2. Number and area of GST-P-positive foci (more than three cells) in the liver of male and female *gpt* delta rats treated with MEG for 13 weeks. Data represent the mean ± SD. * indicates a significant difference from the male controls ($p < 0.05$) using Dunnett's test. # indicates a significant difference from the female controls ($p < 0.05$) using Dunnett's test.

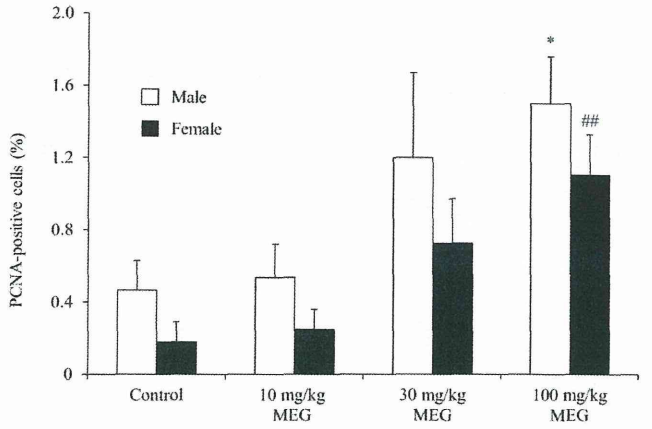

FIG. 3. Immunohistochemical staining of PCNA in the liver of male and female *gpt* delta rats treated with MEG for 13 weeks. Data represent the mean ± SD. * indicates a significant difference from the controls ($p < 0.05$) using Dunnett's test. ## indicates a significant difference from the female controls ($p < 0.01$) using Dunnett's test.

et al., 1973; Drinkwater *et al.*, 1976; IARC, 1976; Wislocki *et al.*, 1977). However, conventional mutagenicity tests have failed to demonstrate definitive results due to exposure to these chemicals (Drinkwater *et al.*, 1976; IARC, 1976; Natarajan and Darroudi, 1991; NTP, 2000; To *et al.*, 1982) even though positive results were reported using *S. typhimurium* that was modified to contain human and/or murine sulfotransferases (Herrmann *et al.*, 2012). As a result, the safety assessment of flavoring agents, including the alkoxy-substituted allylbenzenes, was pending at the 69th meeting of the JECFA. However, the Scientific Committee on Food concluded that alkenylbenzenes, such as safrole, MEG, and estragole, have genotoxic and carcinogenic effects (van den

TABLE 6
Mutation Spectra of *gpt* Mutant Colonies in the Liver of F344 *gpt* Delta Rats Administered MEG

Sex		Control		10 mg/kg MEG		30 mg/kg MEG		100 mg/kg MEG	
		Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})
Male	*Base substitution*								
	Transversions								
	GC-TA	3[a] (37.5)	0.16±0.16	2 (18.2)	0.08±0.17	3 (15.0)	0.09±0.13	4 (15.4)	0.19±0.17
	GC-CG	0	0	2 (18.2)	0.12±0.22	1 (5.0)	0.04±0.08	5 (19.2)	0.20±0.23
	AT-TA	0	0	0	0	3 (15.0)	0.10±0.16	0	0
	AT-GC	1 (12.5)	0.04±0.10	1 (9.1)	0.02±0.05	0	0	1 (3.8)	0.04±0.08
	Transitions								
	GC-TA	3 (37.5)	0.12±0.11	3 (27.3)	0.15±0.21	7 (35.0)	0.31±0.09	8 (30.8)	0.29±0.21
	AT-GC	1 (12.5)	0.04±0.10	2 (18.2)	0.06±0.09	4 (20.0)	0.16±0.10	4 (15.4)	0.15±0.17
	Deletion								
	Single base pair	0	0	1 (9.1)	0.07±0.15	2 (10.0)	0.06±0.09	1 (3.8)	0.06±0.13
	Over 2 bp	0	0	0	0	0	0	1 (3.8)	0.04±0.08
	Insertion	0	0	0	0	0	0	0	0
	Complex	0	0	0	0	0	0	2 (7.7)	0.09±0.13
	Total	8	0.53±0.18	11	0.79±0.34	20	0.79±0.42	26	1.35±0.60*
Female	*Base substitution*								
	Transversions								
	GC-TA	2 (18.2)	0.03±0.04	1 (9.1)	0.03±0.07	3 (16.7)	0.19±0.25	5 (17.9)	0.23±0.32
	GC-CG	1 (9.1)	0.01±0.03	2 (18.2)	0.06±0.08	0	0	2 (7.1)	0.13±0.29
	AT-TA	0	0	0	0	2 (11.1)	0.04±0.09	2 (7.1)	0.10±0.15
	AT-GC	0	0	0	0	1 (5.6)	0.02±0.04	0	0
	Transitions								
	GC-TA	4 (36.4)	0.06±0.07	3 (27.3)	0.08±0.08	9 (50.0)	0.23±0.18	6 (21.4)	0.21±0.17
	AT-GC	1 (9.1)	0.02±0.03	2 (18.2)	0.06±0.08	2 (11.1)	0.05±0.06	8 (28.6)	0.24±0.28
	Deletion								
	Single base pair	2 (18.2)	0.06±0.07	0	0	1 (5.6)	0.03±0.06	0	0
	Over 2 bp	0	0	2 (18.2)	0.06±0.08	0	0	2 (7.1)	0.07±0.16
	Insertion	0	0	1 (9.1)	0.02±0.04	0	0	1 (3.6)	0.04±0.09
	Complex	1 (9.1)	0.02±0.03	0	0	0	0	2 (7.1)	0.10±0.15
	Total	11	0.21±0.13	11	0.38±0.18	18	0.53±0.19	28	1.23±0.59*

[a]Number of colonies with independent mutations.
*Significantly different from the control group at $p < 0.05$.

Berg *et al.*, 2011). It has been reported that chronic oral intake of more than 37 mg/kg of MEG induced neoplasms in the liver and stomach of F344/N rats and B6C3F$_1$ mice (NTP, 2000). MEG has the potential to form a 1′-hydroxy metabolite and an epoxide on the alkenyl side chain, which are similar to the chemical changes induced by safrole. Al-Subeihi *et al.* (2012) reported that 1′-hydroxymethyleugenol glucuronide, 1′-oxomethyleugenol, and 1′-sulfooxymethyleugenol in the liver of humans and male rats were observed to be metabolites of MEG. In addition, the use of physiologically based biokinetic models to evaluate safrole, estragole, and MEG showed that the three alkenylbenzenes used similar processes to bioactivate their ultimate carcinogenic 1′-sulfoxy metabolites (Martati *et al.*, 2011). Furthermore, Chan and Caldwell (1992) reported that 1′-hydroxymethyleugenol, a metabolite of MEG, is a strong inducer of unscheduled DNA synthesis in cultured rat hepatocytes. The 1′-hydroxymetabolite and corresponding sulfate esters of allyl alkoxybenzene substances have been shown to form DNA adducts *in vivo* and *in vitro*. Herrmann *et al.* (2012) reported that hydroxylated metabolites of MEG formed the same DNA adducts as high levels of N(2)-(*trans*-methylisoeugenol-3′-yl)-2′-deoxyguanosine and modest levels of N(6)-(*trans*-methylisoeugenol-3′-yl)-2′-deoxyadenosine in *S. typhimurium* TA100-hSULT1A1 and that positive results were demonstrated in the Ames test. However, these adducts did not form in the wild-type strain, which is consistent with previous results of the micronucleus assay and several Ames tests (NTP, 2000; Sekizawa and Shibamoto, 1982). In this study, we demonstrated a significant increase in the *gpt* and Spi$^-$ MFs in male and female rats treated with 100 mg/kg MEG, which is considered a carcinogenic dose. GC→CG and AT→TA transversion mutations were observed in the treated rats, although the incidence or mutation frequencies were not significant compared with the control group. In contrast to the outcome of conventional mutagenicity tests, the positive results of the reporter gene mutation assay indicate that *in vivo* metabolism at the target site is necessary to induce MEG genotoxicity. The numbers of DNA modifications in Herrmann

et al. (2012) were not directly reflected in the mutation spectra in the *gpt* mutant colonies, which may be due to the use of different experimental systems.

In this study, the mean number and area of GST-P-positive foci were significantly increased in the liver of male and female rats treated with 100 mg/kg MEG compared with the controls. The mean number and area of GST-P-positive foci in rats in the 30 mg/kg group were slightly increased, but the results were not statistically significant. A previous study reported that the incidence of hepatocellular carcinoma was significantly increased in F344 rats and B6C3F1 mice treated with MEG at doses of 37 mg/kg and higher (NTP, 2000). The data on the quantitative analysis of GST-P-positive foci using *gpt* delta transgenic rats appear to be similar to the carcinogenicity data previously reported by NTP (2000). The quantitative analysis of PCNA-positive hepatocytes, which are an indicator of cell proliferation activity, demonstrated that there were significant increases in the ratio of PCNA-positive hepatocytes at a dose of 100 mg/kg in male and female rats, but not at doses of 30 mg/kg and lower. Similar dose-related changes were shown using the *in vivo* mutation assay. According to a previous report (Smith *et al.*, 2002), MEG-specific DNA adducts have not been detected at doses lower than 10 mg/kg. These data suggest that DNA modifications under conditions of increased cell proliferation may be required to induce gene mutations and lead to tumor formation. Although alternate modes of action have not been examined, the present data show that genotoxic mechanisms may contribute to MEG-induced hepatocarcinogenesis.

In conclusion, the MEG dose that induces preneoplastic lesions in the liver resulted in *in vivo* genotoxicity in the reporter gene mutation assay. The data presented in this study provide valuable information regarding the development of risk assessments for the flavoring agents classified as alkoxy-substituted allylbenzenes.

FUNDING

Ministry of Health, Labor and Welfare of Japan (H21-shokuhin-ippan-010).

ACKNOWLEDGMENTS

We would like to thank Ms. Ayako Kaneko and Ms. Yoshimi Komatsu for their expert technical assistance.

REFERENCES

Al-Subeihi, A. A., Spenkelink, B., Punt, A., Boersma, M. G., van Bladeren, P. J., and Rietjens, I. M. (2012). Physiologically based kinetic modeling of bioactivation and detoxification of the alkenylbenzene methyleugenol in human as compared with rat. *Toxicol. Appl. Pharmacol.* **260,** 271–284.

Borchert, P., Wislocki, P. G., Miller, J. A., and Miller, E. C. (1973). The metabolism of the naturally occurring hepatocarcinogen safrole to 1'-hydroxysafrole and the electrophilic reactivity of 1'-acetoxysafrole. *Cancer Res.* **33,** 575–589.

Cartus, A. T., Herrmann, K., Weishaupt, L. W., Merz, K. H., Engst, W., Glatt, H., and Schrenk, D. (2012). Metabolism of methyleugenol in liver microsomes and primary hepatocytes: Pattern of metabolites, cytotoxicity, and DNA-adduct formation. *Toxicol. Sci.* **129,** 21–34.

Chan, V. S., and Caldwell, J. (1992). Comparative induction of unscheduled DNA synthesis in cultured rat hepatocytes by allylbenzenes and their 1'-hydroxy metabolites. *Food Chem. Toxicol.* **30,** 831–836.

Ding, W., Levy, D. D., Bishop, M. E., Lyn-Cook Lascelles, E., Kulkarni, R., Chang, C. W., Aidoo, A., and Manjanatha, M. G. (2011). Methyleugenol genotoxicity in the Fischer 344 rat using the comet assay and pathway-focused gene expression profiling. *Toxicol. Sci.* **123,** 103–112.

Drinkwater, N. R., Miller, E. C., Miller, J. A., and Pitot, H. C. (1976). Hepatocarcinogenicity of estragole (1-allyl-4-methoxybenzene) and 1'-hydroxyestragole in the mouse and mutagenicity of 1'-acetoxyestragole in bacteria. *J. Natl. Cancer Inst.* **57,** 1323–1331.

Dunnett, C. W. (1955). A multiple comparison procedure for comparing several treatments with a control. *Amer. Statist. Assoc. J.* **50,** 1096–1121.

FAO/WHO. (2008). Summary and conclusions. *69th Meeting of the Join FAO/WHO Expert Committee on Food Additives (JECFA)*, Rome, Italy.

Hayashi, H., Kondo, H., Masumura, K., Shindo, Y., and Nohmi, T. (2003). Novel transgenic rat for in vivo genotoxicity assays using 6-thioguanine and Spi- selection. *Environ. Mol. Mutagen.* **41,** 253–259.

Herrmann, K., Engst, W., Appel, K. E., Monien, B. H., and Glatt, H. (2012). Identification of human and murine sulfotransferases able to activate hydroxylated metabolites of methyleugenol to mutagens in Salmonella typhimurium and detection of associated DNA adducts using UPLC-MS/MS methods. *Mutagenesis* **27,** 453–462.

IARC. (1976). *Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man. Safrole, isosafrole and dihydrosafrole*, Vol. **10**, pp. 231–244. International Agency for Research on Cancer, Lyon, France.

Ishii, Y., Suzuki, Y., Hibi, D., Jin, M., Fukuhara, K., Umemura, T., and Nishikawa, A. (2011). Detection and quantification of specific DNA adducts by liquid chromatography-tandem mass spectrometry in the livers of rats given estragole at the carcinogenic dose. *Chem. Res. Toxicol.* **24,** 532–541.

Jin, M., Kijima, A., Suzuki, Y., Hibi, D., Inoue, T., Ishii, Y., Nohmi, T., Nishikawa, A., Ogawa, K., and Umemura, T. (2011). Comprehensive toxicity study of safrole using a medium-term animal model with gpt delta rats. *Toxicology* **290,** 312–321.

Martati, E., Boersma, M. G., Spenkelink, A., Khadka, D. B., Punt, A., Vervoort, J., van Bladeren, P. J., and Rietjens, I. M. (2011). Physiologically based biokinetic (PBBK) model for safrole bioactivation and detoxification in rats. *Chem. Res. Toxicol.* **24,** 818–834.

Masumura, K., Horiguchi, M., Nishikawa, A., Umemura, T., Kanki, K., Kanke, Y., and Nohmi, T. (2003). Low dose genotoxicity of 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) in gpt delta transgenic mice. *Mutat. Res.* **541,** 91–102.

Miller, J. A., and Miller, E. C. (1983). The metabolic activation and nucleic acid adducts of naturally-occurring carcinogens: Recent results with ethyl carbamate and the spice flavors safrole and estragole. *Br. J. Cancer* **48,** 1–15.

Natarajan, A. T., and Darroudi, F. (1991). Use of human hepatoma cells for in vitro metabolic activation of chemical mutagens/carcinogens. *Mutagenesis* **6,** 399–403.

National Toxicology Program (NTP). (2000). Toxicology and carcinogenesis studies of Methyleugenol (CAS No. 93-15-2) in F344/N rats and B6C3F1 mice (Gavage studies). *Natl. Toxicol. Program Tech. Rep. Ser.* **491,** 1–412.

Nohmi, T., Katoh, M., Suzuki, H., Matsui, M., Yamada, M., Watanabe, M., Suzuki, M., Horiya, N., Ueda, O., Shibuya, T., *et al.* (1996). A new transgenic

mouse mutagenesis test system using Spi- and 6-thioguanine selections. *Environ. Mol. Mutagen.* **28,** 465–470.

Nohmi, T., Suzuki, T., and Masumura, K. (2000). Recent advances in the protocols of transgenic mouse mutation assays. *Mutat. Res.* **455,** 191–215.

Sekizawa, J., and Shibamoto, T. (1982). Genotoxicity of safrole-related chemicals in microbial test systems. *Mutat. Res.* **101,** 127–140.

Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., Rogers, A. E., *et al.* (2002). Safety assessment of allylalkoxybenzene derivatives used as flavouring substances - methyl eugenol and estragole. *Food Chem. Toxicol.* **40,** 851–870.

Suzuki, Y., Umemura, T., Hibi, D., Inoue, T., Jin, M., Ishii, Y., Sakai, H., Nohmi, T., Yanai, T., Nishikawa, A., *et al.* (2012). Possible involvement of genotoxic mechanisms in estragole-induced hepatocarcinogenesis in rats. *Arch. Toxicol.* **86,** 1593–1601.

To, L. P., Hunt, T. P., and Andersen, M. E. (1982). Mutagenicity of trans-anethole, estragole, eugenol, and safrole in the Ames Salmonella typhimurium assay. *Bull. Environ. Contam. Toxicol.* **28,** 647–654.

Toyoda-Hokaiwado, N., Inoue, T., Masumura, K., Hayashi, H., Kawamura, Y., Kurata, Y., Takamune, M., Yamada, M., Sanada, H., Umemura, T., *et al.* (2010). Integration of in vivo genotoxicity and short-term carcinogenicity assays using F344 gpt delta transgenic rats: In vivo mutagenicity of 2,4-diaminotoluene and 2,6-diaminotoluene structural isomers. *Toxicol. Sci.* **114,** 71–78.

Umemura, T., Tasaki, M., Kijima, A., Okamura, T., Inoue, T., Ishii, Y., Suzuki, Y., Masui, N., Nohmi, T., and Nishikawa, A. (2009). Possible participation of oxidative stress in causation of cell proliferation and in vivo mutagenicity in kidneys of gpt delta rats treated with potassium bromate. *Toxicology* **257,** 46–52.

van den Berg, S. J., Restani, P., Boersma, M. G., Delmulle, L., Rietjens, I. M. C. M. (2011). Levels of genotoxic and carcinogenic compounds in plant food supplements and associated risk assessment. *Food Nutr. Sci.* **2,** 989–1010.

Watanabe, T., Katsura, Y., Yoshitake, A., Masataki, H., and Mori, T. (1994). IPAP: Image processor for analytical pathology. *J. Toxicol. Pathol.* **7,** 353–361.

Wislocki, P. G., Miller, E. C., Miller, J. A., McCoy, E. C., and Rosenkranz, H. S. (1977). Carcinogenic and mutagenic activities of safrole, 1'-hydroxysafrole, and some known or possible metabolites. *Cancer Res.* **37,** 1883–1891.

Zhou, G. D., Moorthy, B., Bi, J., Donnelly, K. C., and Randerath, K. (2007). DNA adducts from alkoxyallylbenzene herb and spice constituents in cultured human (HepG2) cells. *Environ. Mol. Mutagen.* **48,** 715–721.

The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.37, No.4, 711-721, 2012

Original Article

In vivo genotoxicity of 1-methylnaphthalene from comprehensive toxicity studies with B6C3F1 *gpt* delta mice

Meilan Jin[1], Aki Kijima[1], Yuta Suzuki[1], Daisuke Hibi[1], Yuji Ishii[1], Takehiko Nohmi[2], Akiyoshi Nishikawa[3], Kumiko Ogawa[1] and Takashi Umemura[1]

[1]Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[2]Sciences of Genome Safety, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[3]Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

(Received February 14, 2012; Accepted April 17, 2012)

ABSTRACT — 1-Methylnaphthalene (1-MN), a constituent of the polycyclic aromatic hydrocarbons (PAHs), is a lung carcinogen in mice. However, conventional genotoxicity tests such as the Ames test and sister chromatid exchange (SCE) test have yielded equivocal results. In the present study, the *in vivo* genotoxicity of 1-methylnaphthalene (1-MN) together with its toxicological profile was investigated in a 13-week repeated dose toxicity study of 1-MN using B6C3F1 *gpt* delta mice. In the serum biochemistry, significant increases in AST and ALP were observed in males of the 0.15% 1-MN group. From histopathological examination, the incidence of single cell necrosis in the liver was significantly increased in males of the 0.15% 1-MN group; however, no changes were observed in the lungs, the target organ of 1-MN. In an *in vivo* mutation assay, no changes in mutant frequencies of *gpt* and red/gam (Spi-) in lung DNA of 1-MN treated mice were observed at 13 weeks. In addition, there were no significant differences in the proliferating cell nuclear antigen (PCNA)-positive ratios in bronchiolar epithelial cells among the groups for either sex. These results suggest that 1-MN at a carcinogenic dose not induce overt toxicity for any organs and has no *in vivo* genotoxicity in the lungs.

Key words: *gpt* delta mice, Mutagenicity, 1-methylnaphthalene

INTRODUCTION

Methylnaphthalene (MN), a constituent of the polycyclic aromatic hydrocarbons (PAHs), is widely used industrially as a solvent for pesticides and fungicides and as a mordant carrier. PAHs are formed as a result of incomplete combustion of fossil fuels (e.g. coal and oil). They are found in car and diesel exhaust, smoked or charbroiled food (Grimmer and Böhnke, 1975; Dipple, 1983; Rothman *et al.*, 1990), as well as cigarette smoke condensate and tobacco products and are suspect causative agents for human lung cancer (Hecht, 1999; Pfeifer *et al.*, 2002). While 1-MN has been found to accumulate in the body (McCain *et al.*, 1978), show toxicity, especially in the lungs (Dinsdale and Verschoyle, 1986), and among the four nucleosides, bind preferentially to adenosine in cultured cells (Harvey and Halonen, 1968), Murata *et al.* (1993) reported that no lung toxicity was observed in a 13-week repeated dose toxicity study. It has been reported that 1-MN showed lung carcinogenicity in mice fed a diet containing 0.075% or 0.15% 1-MN. Although 1-MN was positive in the sister chromatid exchange (SCE) test, it proved to be negative in the Ames test. Thus, it is unclear whether 1-MN has lung toxicity and *in vivo* genotoxicity.

In 1996, Nohmi *et al.* (1996) developed the novel transgenic *gpt* delta mouse for *in vivo* genotoxicity assays. These mice have approximately 80 copies of λ EG10 DNA at a single site in chromosome 17 of C57 BL/6J mice (Masumura *et al.*, 1999). It is well recognized that an *in vivo* mutation assay using the *gpt* delta mouse can detect not only point mutations, but also deletion mutations (Aoki *et al.*, 2007; Masumura *et al.*, 2002; Shibata *et al.*, 2009; Xu *et al.*, 2007). In addition, we confirmed that the medium-term animal model using *gpt* delta mice

Correspondence: Takashi Umemura (E-mail: umemura@nihs.go.jp)

enables 90-day repeated dose toxicity and *in vivo* mutagenicity tests to be examined in a single study (Kuroiwa *et al.*, 2007). Accordingly, the *gpt* delta mouse might also be a promising candidate for investigating the comprehensive toxicity of various agents.

In the present study, to investigate *in vivo* genotoxicity of 1-MN together with the toxicological profiles, we examined general toxicity and *in vivo* genotoxicity in a comprehensive toxicity study system using B6C3F1 *gpt* delta mice through the administration of 1-MN over 13 weeks at two doses previously determined to be carcinogenic in a 2-year carcinogenicity study.

MATERIALS AND METHODS

Chemicals

1-MN and corn oil were purchased from Wako Pure Chemical Industries (Osaka, Japan). The 1-MN was dissolved in corn oil and mixed with powdered CRF-1 diet (Charles River Japan Inc., Kanagawa, Japan) at the 0.075% and 0.15%. The final concentration of corn oil in each diet was 5%. The feed containing 1-MN was prepared every week and kept in light shielded containers at 4°C.

Animals, diet and housing conditions

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences (Tokyo, Japan). Five-week-old male and female *gpt* delta B6C3F1 mice carrying approximately 80 tandem copies of the transgene lambda EG10 per haploid genome were raised by mating C57BL/6 gpt delta and non-transgenic C3H/He mice (Japan SLC, Inc., Shizuoka, Japan). They were housed in polycarbonate cages (three or four mice per cage) with hardwood chips for bedding in a conventional animal facility. Animals were maintained under conditions of controlled temperature (23 ± 2°C), humidity (55 ± 5°C), air change (12 times per hour), and lighting (12 hr light/dark cycle). Animals were given free access to a CRF-1 basal diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water.

Experimental design

After a 1-week acclimation period, animals were divided into 3 groups consisting of 10 male and 10 female B6C3F1 *gpt* delta mice per group and were given a diet containing 0.075%, 0.15%, or 0% 1-MN for 13 weeks.

Clinical signs and general appearance were observed once a day. Body weight and food consumption were measured once a week. At the end of the study period, the animals were euthanized under deep anesthesia. Right lung lobes were fixed with neutral buffered formalin for histopathological and immunohistopathological examination. The remaining lungs were stored at -80°C for *in vivo* mutation assays. At necropsy, blood samples were collected from the abdominal aorta for hematology and serum biochemistry. Relative organ weights were calculated relative to body weight.

Hematological analysis was performed using an automated hematology analyzer, K-4500 (Sysmex Corp., Hyogo, Japan). Differential leukocyte counts and reticulocyte counts were performed with a MICROX HEG-505 (Sysmex Corp.). Parameters for serum biochemistry, shown in Table 3, were analyzed at SRL, Inc. (Tokyo, Japan) using sera frozen after centrifugation of whole blood.

At autopsy, weights of the brain, heart, lungs, liver, kidneys, spleen, thymus, adrenal glands, and testes were measured. In addition to these organs, the arteries, bone/marrow, coagulation gland, esophagus, epididymides, large intestine (cecum, colon, and rectum), lymph node, mammary glands, pancreas, peripheral nerve, prostate gland, pituitary gland, thyroid glands, salivary gland, skeletal muscle, skin, small intestine (duodenum, jejunum, and ileum), spinal cord, stomach, urinary bladder, tongue, trachea, vagina, uterus, and ovaries were fixed in 10% neutral buffered formalin. Testes were fixed in Bouin's solution overnight and then were transferred into 10% neutral buffered formalin. Tissues that needed decalcification, such as the nasal cavity, spinal cord with bones, sternum, and femur, were treated with a mixture of 10% formic acid and 10% neutral phosphate buffered formalin. These tissues were routinely embedded in paraffin, sectioned at 1 μm thick for hematoxylin and eosin staining, and examined by light microscopy. Histopathological examinations were carried out for all groups.

In vivo mutation assays

The 6-TG and Spi$^-$ (insensitive P2 interference) selection was carried out as previously described (Nohmi *et al.*, 1996, 2000). Briefly, genomic DNA was extracted from lung tissue of 4 or 5 males and females, and lambda EG10 DNA (48kb) was rescued as the lambda phage through *in vitro* packaging. For 6-TG selection, the packaged phage was incubated with E. coli YG6020, expressing Cre recombinase, and converted to a plasmid carrying *gpt* and chloramphenicol acetyltransferase. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. To determine the total number of rescued plasmids, infected cells were also poured on plates containing chloramphenicol

Table 1. Final body weight and diet consumptions and test substance intake in B6C3F1 *gpt* delta mice given diet containing 1-MN for 13 weeks

	Group	No. of animals	Final body weight (g)	Diet consumption (g/mice/day)	Daily intake of test substance (mg/kg/day)
Male	Control	10	30.3 ± 2.9[a]	5.2 ± 0.5	0.00 ± 0.00
	0.075% 1-MN	10	29.8 ± 3.1	4.9 ± 0.6	0.12 ± 0.01
	0.15% 1-MN	10	28.9 ± 2.6	4.2 ± 0.5	0.22 ± 0.03
Female	Control	10	23.6 ± 2.3	6.3 ± 1.0	0.00 ± 0.00
	0.075% 1-MN	10	23.6 ± 2.3	5.2 ± 0.5	0.17 ± 0.02
	0.15% 1-MN	10	23.2 ± 2.2	4.3 ± 0.4	0.28 ± 0.03

[a] Mean ± S.D.

without 6-TG. The plates were incubated at 37°C for the selection of 6-TG resistant colonies, and the *gpt* mutant frequency (MF) was calculated by dividing the number of *gpt* mutants after clonal correction by the number of rescued phages. To characterize *gpt* mutations, a 739 bp DNA fragment containing the 456 bp coding region of the *gpt* gene was amplified by PCR as previously described, and the PCR products were analyzed with an Applied Biosystems 3730x1 DNA Analyzer (Applied Biosystems Japan Ltd.).

For Spi$^-$ selection, the packaged phage was incubated with E. coli XL-1 Blue MRA for survival titration and E.coli XL-1 Blue MRA P2 for mutant selection. Infected cells were mixed with molten lambda-trypticase agar plates. The next day, plaques (Spi$^-$ candidates) were punched out with sterilized glass pipettes, and the agar plugs were suspended in SM buffer. To confirm the Spi$^-$ phenotype of candidates, the suspensions were spotted on three types of plates containing XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains and were spread with soft agar. The numbers of mutants that made clear plaques on each plate were counted as confirmed Spi- mutants. In all *in vivo* mutations assays, positive DNA samples were simultaneously analyzed.

Immunohistochemical staining for PCNA

Immunohistochemical staining was performed using monoclonal anti-mouse PCNA antibodies (1:100; Dako, Glostrup, Denmark) to evaluate cell proliferation using the avidin-biotin peroxidase complex (ABC) method. The numbers of PCNA-positive cells per unit area (No./cm^2) from ten different areas per animal were counted to give the PCNA-positive ratio.

Statistics

The data obtained from measurements of body weight, food and water consumption, organ weights, hematology, serum biochemistry, PCNA-positive ratio, *gpt* MFs, and Spi$^-$ MFs were expressed as the mean ± S.D. Significant differences between the control and treated groups were determined by Dunnett's multiple comparison test (Dunnett, 1955) after ANOVA. Significant differences in incidences of lesions in the histopathological examinations were evaluated using Fisher's exact probability test. P-values less than 0.05 were considered statistically significant in both analyses.

RESULTS

General condition, body weight, food consumption

Neither deaths nor remarkable changes in general appearance were observed in the treated groups during

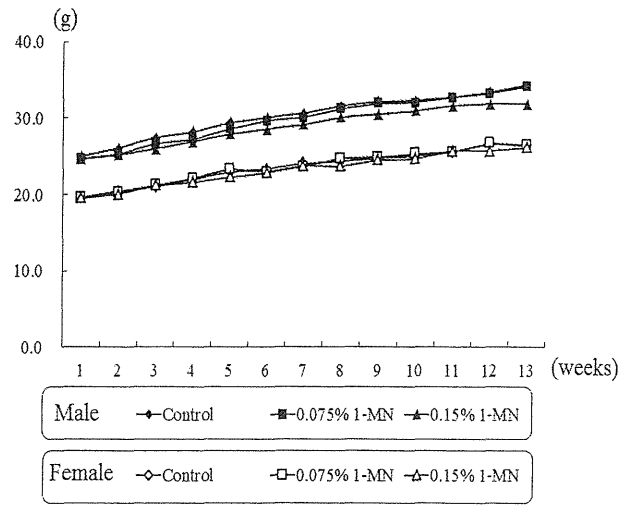

Fig. 1. Body weight curves for B6C3F1 *gpt* delta mice administered 1-MN for 13 weeks.

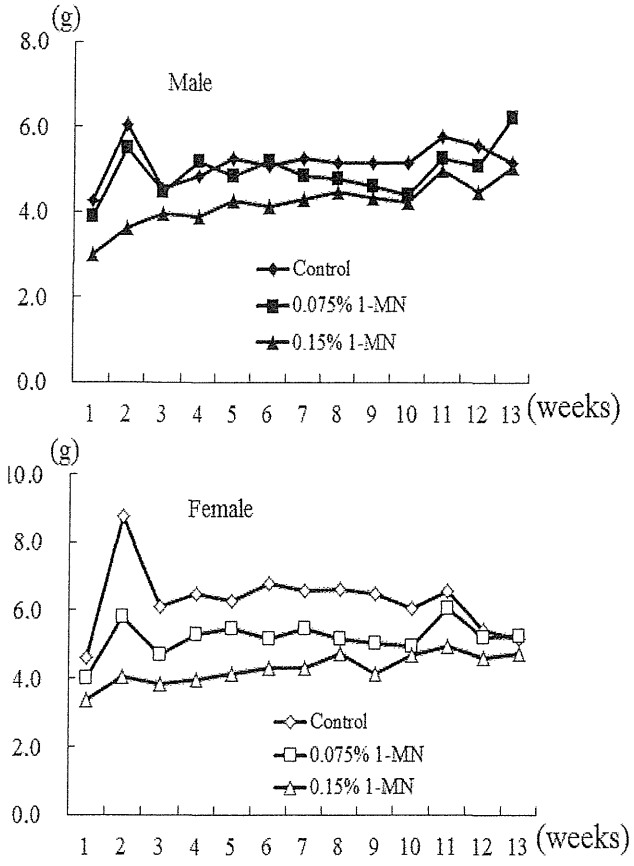

Fig. 2. Daily food intake for B6C3F1 *gpt* delta mice administered 1-MN for 13 weeks.

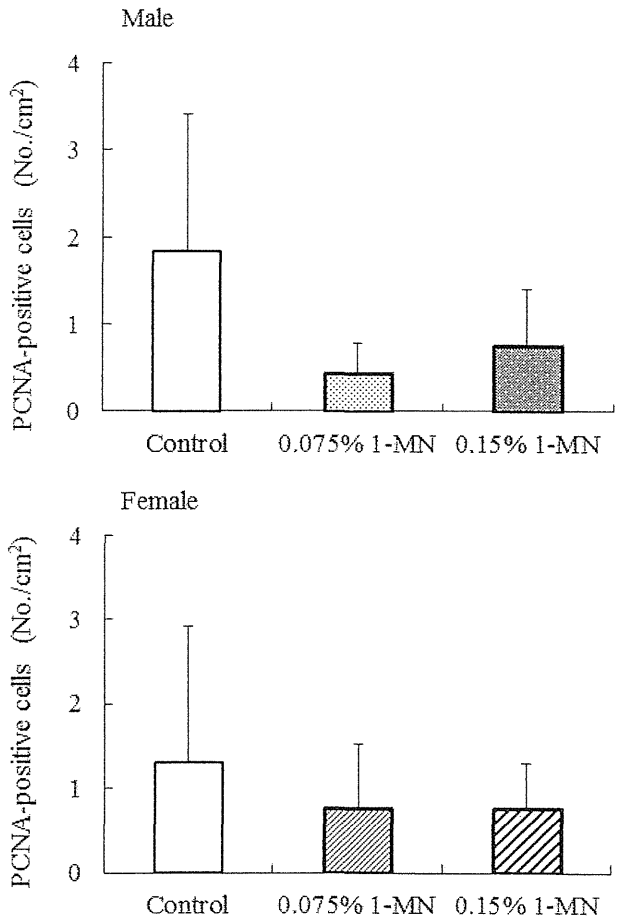

Fig. 3. Proliferating cell nuclear antigen (PCNA) positive cell ratio in the lung of male and female *gpt* delta mice administered 1-MN for 13weeks.

the experimental period. Changes in body weight during the experiment are shown in Fig. 1. There was no suppression of body weight gain in the treated groups during the experiment.

Food consumption was slightly decreased at several time points in the treatment period in females of the treated groups and males in the 0.15% 1-MN group (Fig. 2). However, as shown in Fig. 1, there were no effects on body weight gain in the treated groups during the experimental period. In addition, there were no remarkable differences in the overall average food intake between the control and treated groups for either sex (Table 1).

Hematology and serum biochemistry

The results of the hematological and serum biochemical analyses are shown in Tables 2 and 3. In hematological examinations, a significant increase in the ratio of segmented neutrophils in males of the 0.15% 1-MN group and basophils in females of all treated groups was observed. In addition, a significant decrease in the ratio of band form neutrophils was observed in males of the 0.075% 1-MN group. In serum biochemical examinations, significant decreases in phospholipid, BUN, and CRN and increases in AST and ALT were observed in males of the 0.15% 1-MN group. In addition, a significant decrease in Ca was observed in males of the treated groups. In females, a significant increase in Cl and decreases in phospholipid and TC were observed in the 0.15% 1-MN group.

Organ weights and histopathological examination

The absolute and relative organ weights are shown in Tables 4 and 5. In males of the treated groups, the absolute weights of spleen and heart were significantly decreased compared to the control group. In addition, the relative weights of these organs were also decreased in the treated

Table 2. Hematological data for male and female B6C3F1 *gpt* delta mice given diet containing 1-MN for 13 weeks

	Control	0.075% 1-MN	0.15% 1-MN
No. of animals examined	10	10	10
Male			
WBC (x10²/μl)	24.2 ± 15.0[a]	22.0 ± 9.0	15.0 ± 7.0
RBS (x10⁴/μl)	963 ± 40	959 ± 64	965 ± 63
Hb (g/dl)	13.9 ± 0.6	14.0 ± 1.0	14.1 ± 0.8
Ht (%)	50.6 ± 2.0	50.5 ± 3.2	50.5 ± 3.2
MCV (fl)	53.0 ± 0.0	52.6 ± 0.5	52.0 ± 0.0
MCH (pg)	14.4 ± 0.2	14.7 ± 0.3	14.6 ± 0.4
MCHC (g/dl)	27.5 ± 0.4	27.8 ± 0.4	27.9 ± 0.7
Plt (x10⁴/μl)	138.0 ± 8.0	134.0 ± 14.0	137.0 ± 17.0
Differential leukocyte counts (%)			
Band form neutrophils	5.3 ± 1.8	2.6 ± 0.9*	3.9 ± 2.4
Segmented neutrophils	14.8 ± 3.2	16.8 ± 3.9	27.5 ± 13.5*
Eosinophils	1.3 ± 0.9	0.6 ± 0.4	1.1 ± 0.4
Basophils	0.3 ± 0.5	0.4 ± 0.2	0.3 ± 0.3
Lymphocytes	77.0 ± 4.5	79.0 ± 3.7	66.4 ± 16.0
Monocytes	0.9 ± 0.3	0.6 ± 0.3	0.6 ± 0.5
Reticulocytes	0.7 ± 0.6	0.2 ± 0.3	0.8 ± 0.5
Female			
No. of animals examined	10	10	10
WBC (x10²/μl)	16.0 ± 8.0	17.0 ± 11.0	17.0 ± 8.0
RBS (x10⁴/μl)	998 ± 45	991 ± 67	977 ± 53
Hb (g/dl)	14.8 ± 0.6	14.7 ± 0.9	14.4 ± 0.8
Ht (%)	53.0 ± 2.4	52.9 ± 3.5	51.6 ± 2.9
MCV (fl)	53.1 ± 0.4	53.4 ± 0.5	52.9 ± 0.5
MCH (pg)	14.8 ± 0.1	14.9 ± 0.3	14.8 ± 0.4
MCHC (g/dl)	27.8 ± 0.1	27.8 ± 0.5	28.0 ± 0.5
Plt (x10⁴/μl)	115.0 ± 7.0	112.0 ± 9.0	113.0 ± 8.0
Differential leukocyte counts (%)			
Band form neutrophils	3.1 ± 1.7	2.2 ± 1.1	2.6 ± 1.5
Segmented neutrophils	10.7 ± 3.9	10.4 ± 3.2	10.9 ± 3.4
Eosinophils	1.0 ± 0.6	1.1 ± 0.7	1.1 ± 0.6
Basophils	0.1 ± 0.2	0.4 ± 0.2*	0.4 ± 0.2*
Lymphocytes	84.7 ± 5.1	85.3 ± 3.8	84.4 ± 4.2
Monocytes	0.5 ± 0.3	0.5 ± 0.3	0.4 ± 0.3
Reticulocytes	0.5 ± 0.4	0.4 ± 0.3	0.3 ± 0.3

Abbreviations: WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; Ht, hematocrit; MCV, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; Plt, platelet.

[a]Mean ± S.D. *: Significantly different from the controls at the levels of $p < 0.05$ (Dunnett's test).

Table 3. Serum biochemistry for male and female B6C3F1 *gpt* delta mice given 1-MN for 13 weeks

Group	Control	0.075% 1-MN	0.15% 1-MN
No. of animals examined	9	8	10
Males			
TP (g/dl)	5.3 ± 0.3[a]	5.3 ± 0.2	5.2 ± 0.2
Alb (g/dl)	3.1 ± 0.2	3.1 ± 0.2	3.1 ± 0.2
T-Bil (mg/dl)	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
TG (mg/dl)	99.0 ± 41.8	73.8 ± 21.6	68.1 ± 24.7
Phospholipid (mg/dl)	232.3 ± 22.8	218.9 ± 15.2	207.4 ± 5.6*
TC (mg/dl)	119.6 ± 12.5	121.3 ± 8.1	113.9 ± 5.8
BUN (mg/dl)	31.1 ± 3.8	28.6 ± 2.0	26.6 ± 3.7*
CRN (mg/dl)	0.11 ± 0.01	0.10 ± 0.01	0.09 ± 0.01**
Na (mEQ/l)	152.2 ± 1.9	151 ± 1.4	152.3 ± 1.5
Cl (mEQ/l)	115.4 ± 1.4	115.4 ± 1.3	116.9 ± 3.0
K (mEq/l)	5.3 ± 0.7	5.4 ± 1.4	5.0 ± 0.3
Ca (mg/dl)	9.2 ± 0.3	8.9 ± 0.2*	8.9 ± 0.3*
IP (mg/dl)	8.1 ± 1.0	7.5 ± 1.1	8.1 ± 0.6
AST (IU/l)	37.1 ± 2.8	37.3 ± 3.2	50.6 ± 15.6*
ALT (IU/l)	20.3 ± 2.1	20.9 ± 4.5	30.1 ± 10.4*
ALP (IU/l)	199.6 ± 19.2	209.1 ± 26.1	220.5 ± 21.0
No. of animals examined	10	10	9
Females			
TP (g/dl)	5.3 ± 0.1	5.2 ± 0.1	5.2 ± 0.1
Alb (g/dl)	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1
T-Bil (mg/dl)	0.05 ± 0.01	0.06 ± 0.02	0.07 ± 0.01
TG (mg/dl)	38.1 ± 26.6	29.9 ± 23.0	22.9 ± 17.3
Phospholipid (mg/dl)	189.2 ± 8.1	181.0 ± 7.9	172.3 ± 16.6*
TC (mg/dl)	104.6 ± 4.8	98.6 ± 7.1	97.1 ± 7.1*
BUN (mg/dl)	20.9 ± 4.1	24.4 ± 10.4	25.3 ± 5.4
CRN (mg/dl)	0.09 ± 0.01	0.11 ± 0.02	0.09 ± 0.02
Na (mEQ/l)	150.2 ± 1.2	150.9 ± 0.9	151.6 ± 2.2
Cl (mEQ/l)	115.6 ± 1.5	115.9 ± 1.4	117.6 ± 2.1*
K (mEq/l)	5.4 ± 0.4	5.4 ± 0.7	5.2 ± 0.2
Ca (mg/dl)	8.9 ± 0.2	9.0 ± 0.3	8.7 ± 0.2
IP (mg/dl)	7.5 ± 1.0	7.7 ± 1.3	7.2 ± 0.6
AST (IU/l)	39.6 ± 2.4	38.6 ± 3.4	40.3 ± 4.1
ALT (IU/l)	18 ± 2.1	16.7 ± 1.2	18.4 ± 2.5
ALP (IU/l)	344.9 ± 48.1	361.3 ± 54.7	343.6 ± 29.6

Abbreviations: TP, total protein; Alb, albumin; T-Bil, total bilirubin; TG, triglyceride; TC, Total cholesterol; BUN, blood urea nitrogen; CRN, creatinine; Na, sodium; Cl, chloride; K, potassum; Ca, calcium; IP, inorganic phosphate; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase.

*,**: Significantly different from the controls at the levels of $p < 0.05$ and $p < 0.01$, respectively (Dunnett's test) [a]Mean ± S.D.

Table 4. Organ weights in male B6C3F1 *gpt* delta mice given diet containing 1-MN for 13 weeks

Group	Control	0.075% 1-MN	0.15% 1-MN
No. of animals examined	10	10	10
Body weight	33.1 ± 1.8[a]	33.1 ± 3.7	30.7 ± 2.0
Absolute (g)			
Liver	1.35 ± 0.10	1.32 ± 0.18	1.21 ± 0.11
Lungs	0.18 ± 0.03	0.17 ± 0.02	0.17 ± 0.02
Kidneys	0.46 ± 0.08	0.45 ± 0.03	0.45 ± 0.04
Brain	0.49 ± 0.01	0.48 ± 0.01	0.48 ± 0.01
Spleen	0.09 ± 0.01	0.07 ± 0.02*	0.06 ± 0.01**
Thymus	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Heart	0.97 ± 0.06	0.81 ± 0.24*	0.72 ± 0.03**
Adrenals	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Gonads	0.21 ± 0.03	0.22 ± 0.03	0.21 ± 0.03
Relative (g/100g B.W.)			
Liver	4.09 ± 0.27	3.99 ± 0.19	3.93 ± 0.23
Lungs	0.55 ± 0.07	0.52 ± 0.07	0.51 ± 0.17
Kidneys	1.38 ± 0.24	1.38 ± 0.11	1.47 ± 0.12
Brain	1.47 ± 0.10	1.47 ± 0.15	1.57 ± 0.12
Spleen	0.27 ± 0.04	0.21 ± 0.04*	0.21 ± 0.05*
Thymus	0.09 ± 0.02	0.08 ± 0.04	0.08 ± 0.01
Heart	2.94 ± 0.21	2.48 ± 0.77	2.35 ± 0.19**
Adrenals	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.01
Gonads	0.63 ± 0.08	0.67 ± 0.08	0.67 ± 0.07

*,**: Significantly different from the controls at the levels of $p < 0.05$ and $p < 0.01$, respectively (Dunnett's test) [a] Mean ± S.D.

Table 5. Organ weights in female B6C3F1 *gpt* delta mice given diet containing 1-MN for 13 weeks

Group	Control	0.075% 1-MN	0.15% 1-MN
No. of animals examined	10	10	10
Body	25.6 ± 1.4[a]	25.5 ± 2.6	24.8 ± 1.3
Absolute (g)			
Liver	1.08 ± 0.06	1.04 ± 0.06	1.00 ± 0.07*
Lungs	0.17 ± 0.02	0.17 ± 0.02	0.17 ± 0.02
Kidneys	0.34 ± 0.02	0.33 ± 0.02	0.33 ± 0.02
Brain	0.52 ± 0.01	0.5 ± 0.02	0.51 ± 0.01
Spleen	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01
Thymus	0.04 ± 0.01	0.04 ± 0.01	0.08 ± 0.10** [b]
Heart	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
Adrenals	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Relative (g/100g B.W.)			
Liver	4.28 ± 0.43	4.12 ± 0.29	4.05 ± 0.27
Lungs	0.66 ± 0.08	0.67 ± 0.12	0.68 ± 0.09
Kidneys	1.33 ± 0.13	1.29 ± 0.11	1.32 ± 0.10
Brain	2.03 ± 0.11	1.99 ± 0.16	2.04 ± 0.10
Spleen	0.32 ± 0.03	0.30 ± 0.04	0.3 ± 0.04
Thymus	0.14 ± 0.02	0.15 ± 0.02	0.35 ± 0.44
Heart	0.51 ± 0.02	0.49 ± 0.04	0.47 ± 0.04
Adrenals	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01

*,**: Significantly different from the controls at the levels of $p < 0.05$ and $p < 0.01$, respectively (Dunnett's test).
[a] Mean ± S.D. [b] lymphoma was observed in one mouse.

Table 6. Histopathological findings observed in male and female B6C3F1 *gpt* delta mice given 1-MN for 13 weeks

			Male			Female		
		1-MN	Control	0.075%	0.15%	Control	0.075%	0.15%
	Organs and findings	No.	10	10	10	10	10	10
Surivival rate			100%	100%	100%	100%	100%	100%
Liver								
	Vacuolization		0[a] (0)[b]	0 (0)	0 (0)	0 (0)	1 (10)	3 (30)
	Focal necrosis		0 (0)	0 (0)	0 (0)	5 (50)	5 (50)	7 (70)
	Single cell necrosis		0 (0)	3 (30)	5 (50)**	7 (70)	5 (50)	5 (50)
Kidney								
	Inflammatory cell infiltration		1 (10)	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)
	Vacuolization		5 (50)	8 (80)	9 (90)	0 (0)	0 (0)	0 (0)
Pancreas								
	Inflammatory cell infiltration		0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)
Stomach								
	Squamous metaplasia		1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Aderenal gland								
	Subcapsular cell hyperplasia		1 (10)	1 (10)	3 (30)	7 (70)	8 (80)	7 (70)
Ureter								
	Papillary hyperplasia		0 (0)	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)
spinal cord cervicol								
	Cyst formation		0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)
Tongue								
	Ulcer		0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)
Lymph								
	Lymphoma		0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)

[a]: The number of animals with histopathological lesions.
[b]: The incidence(%) of histopathological lesions.
**: Significantly different from the controls at the leverls of $p < 0.05$ and $p < 0.01$, respectively (Fisher's t-test).

groups, although there was no remarkable difference in the relative heart weights of the 0.075% 1-MN group. In females of the 0.15% 1-MN group, absolute liver weights were significantly decreased. On the other hand, a significant increase of absolute thymus weights was observed, but this difference was due to one lymphoma mouse.

From the histopathological examination, the incidence of single cell necrosis of the liver was significantly increased in males of the 0.15% 1-MN group (Table 6). However, the incidences of other changes in the other organs were not significantly different between the treatment and control groups (Table 6).

In vivo mutation assays of the lungs

Data for *gpt* and Spi$^-$ MFs in the lungs of male and female *gpt* delta rats treated with safrole for 13 weeks are summarized in Tables 7 and 8, respectively. There were no significant differences in *gpt* and Spi$^-$ MF among the groups for either sex.

Effects of 1-MN treatment on cell proliferation

The effect of 1-MN on cell proliferation was evaluated by immunohistochemistry for PCNA (Fig. 3). No significant difference for the PCNA-positive ratio in lungs among the groups for either sex was observed.

DISCUSSION

1-MN is a constituent of the polyaromatic hydrocarbons that typically form by incomplete combustion

Table 7. *gpt* MFs in lungs of B6C3F1 *gpt* delta mice given 1-MN for 13 weeks

	Groups	Animal No.	Cm^R colonies($x10^5$)	6-TG^R and Cm^R colonies	Mutant Frequency ($x10^{-5}$)	Mean ± S.D.
Male	Control	1	9.8	3	0.31	
		2	12.3	5	0.41	
		3	15.2	9	0.59	
		4	15.1	2	0.13	
		5	4.0	1	0.25	0.34 ± 0.17
	0.075% 1-MN	11	11.4	5	0.26	
		12	2.1	1	0.47	
		13	5.3	1	0.19	
		14	23.5	5	0.30	
		15	18.2	6	0.33	0.33 ± 0.13
	0.15% 1-MN	21	18.0	9	0.61	
		23	6.1	4	0.49	
		24	24.5	17	0.57	
		25	22.4	11	0.49	0.54 ± 0.06
Female	Control	31	7.1	1	0.14	
		32	50.5	3	0.06	
		33	43.4	10	0.23	
		34	36.0	4	0.11	
		35	18.3	6	0.33	0.17 ± 0.10
	0.075% 1-MN	41	41.1	7	0.17	
		42	36.4	3	0.08	
		43	40.6	6	0.15	
		44	38.0	12	0.32	
		45	49.2	6	0.12	0.17 ± 0.09
	0.15% 1-MN	51	25.8	6	0.23	
		52	29.3	11	0.38	
		53	33.9	7	0.21	
		54	30.2	5	0.17	
		55	55.8	0	0.00	0.20 ± 0.14

of organic materials and are ubiquitous in the environment, in diesel exhaust, air, and aquatic creatures. Methylnaphthalenes are among the most toxic of the components in the water-soluble fraction of crude and fuel oils (Anderson *et al.*, 1974; Boylan and Tripp, 1971; Lee *et al.*, 1974; Winters *et al.*, 1976). However, significant toxicity of 1-MN was not observed in the 13-week toxicity study by Murata *et al.* (1993) in spite of the occurrence of growth retardation in both sexes due to refusal of food intake. In addition, the genotoxicity of 1-MN in conventional mutagenicity tests such as the sister chromatid exchange (SCE) test and Ames test (Florin *et al.*, 1980) has remained inconclusive. Thus, an investigation of the *in vivo* genotoxicity of 1-MN together with toxic effects induced by exposure to 1-MN was carried out through comprehensive toxicity studies performed with B6C3F1 *gpt* delta mice.

During the experimental period, a tendency to decreased food consumption at several time points during the experimental period was observed in males of the 0.15% 1-MN group and females of the treated groups compared with the corresponding control groups. However, there were no effects on body weight gain in the treated groups during the experimental period. Therefore, these fluctuations were considered to be of no toxicological significance. In the serum biochemical examination, significant increases of AST and ALT were observed in males of the 0.15% 1-MN group along with the observation of single hepatocyte necrosis histopathologically. However, these changes had not a dose-dependent manner, being considered to be of no toxicological significance. Likewise, no other changes with toxicological significance were observed in the other organs. No other changes of toxicological significance were observed, which is in

Table 8. Spi⁻ MFs in lungs of B6C3F1 *gpt* delta mice given 1-MN for 13 weeks

	Groups	Animal No.	Plaues within XL-1 Blue MRA (x10^5)	Plaque within WL95 (P2)	Mutant Frequency (x10-5)	Mean ± S.D.
Male	Control	1	15.8	1	0.06	
		2	14.0	2	0.14	
		3	19.5	4	0.21	
		4	10.9	2	0.18	0.15 ± 0.06
	0.075% 1-MN	11	14.1	6	0.43	
		12	3.4	1	0.29	
		13	15.0	10	0.67	
		14	18.4	10	0.55	
		15	15.3	5	0.33	0.45 ± 0.16
	0.15% 1-MN	21	16.5	3	0.18	
		22	22.8	7	0.31	
		23	4.9	4	0.82	
		24	18.0	4	0.22	
		25	6.3	3	0.48	0.40 ± 0.26
Female	Control	31	14.9	7	0.47	
		32	12.3	3	0.24	
		33	10.4	1	0.09	
		34	10.4	6	0.58	0.35 ± 0.22
	0.075% 1-MN	41	13.6	1	0.07	
		42	20.1	9	0.45	
		43	11.8	4	0.34	
		44	17.6	9	0.51	
		45	10.9	5	0.46	0.37 ± 0.18
	0.15% 1-MN	51	8.7	0	0.00	
		52	12.4	5	0.40	
		53	14.6	3	0.21	
		54	15.5	8	0.52	0.28 ± 0.23

agreement with Murata *et al.*'s report that no lung toxicity was observed in a 13-week repeated dose toxicity test (1993). These results show that the toxicological profiles of 1-MN in the *gpt* delta mice were in concord with those in the non-transgenic wild mice, which implies that the *gpt* delta mouse model can be used to determine the actual *in vivo* genotoxicity of 1-MN.

Murata *et al.* (1993) reported that the incidence of bronchiolar/alveolar adenoma in the lungs of male mice administered 0.075% or 0.15% 1-MN was 26.0% and 24.0%, respectively. These results were statistically significant as compared with the 4.1% incidence observed in control males. The sister chromatid exchange frequency in human lymphocyte culture was increased by 1-MN in the presence of S9 mixture (Kulka *et al.*, 1988). However, in the present study, there were no significant differences in the *gpt* and Spi⁻ MFs among the groups for either sex, which indicates that 1-MN has no *in vivo* genotoxic hazard and suggests that the involvement of genotoxic mechanisms in 1-MN lung carcinogenesis is unlikely. In addition to no toxicological effect on the lungs, the PCNA-positive ratio in lungs was not significantly different among the groups for either sex. The overall data strongly suggest the necessity for re-examination of 1-MN carcinogenicity testing.

In conclusion, the comprehensive toxicity study using B6C3F1 *gpt* delta mice demonstrated that 1-MN at a carcinogenic dose did not induce *in vivo* genotoxicity, cell proliferation at the target site, or overt toxicity for any organs. It is unlikely that 1-MN lung carcinogenesis includes genotoxic mechanisms.

ACKNOWLEDGMENTS

We thank Ms. Ayako Kaneko and Ms. Yoshimi Komatsu for their expert technical assistance. This work was supported by a Grant-in-Aid for Research on Food Sanitation from the Ministry of Health, Labor and Welfare of Japan

(H21-shokuhin-ippan-010).

REFERENCES

Anderson, J.W., Neff, J.M., Cox, B.A., Tatem, H.E. and Hightower, G.M. (1974): The effects of oil estuarine animals; toxicity, uptake and depuration, respiration, In F.J. Vernberg and W. Vernberg (ed.), Pollution and physiology of marine organisms. Academic Press, Inc., New York, pp.285-310.

Aoki, Y., Hashimoto, A.H., Amanuma, K., Matsumoto, M., Hyoshi, K., Takano, H., Masumura, K., Itoh, K., Nohmi, T. and Yamamoto, M. (2007): Enhanced spontaneous and benzo(a) pyrene-induced mutations in the lung of Nrf2-deficient *gpt* delta mice. Cancer. Res., **67**, 5643-5648.

Boylan, D.B. and Tripp, B.W. (1971) Determination of hydrocarbons in seawater extracts of crude oil and crude oil fractions. Nature (London), **230**, 44-47.

Dinsdale, D. and Verschoyle, R.D. (1986) Pulmonary toxicity of naphthalene derivatives in the rat. Arch. Toxicol. Suppl., **11**, 288-291.

Dipple, A. (1983) Formation, metabolism, and mechanism of action of polycyclic aromatic hydrocarbons. Cancer Res., **43**, 2422s-2425s.

Dunnett, C.W. (1955) A multiple comparison procedure for comparing several treatments with a control. J. Am. Stat. Assoc., **50**, 1096-1121.

Florin, I., Rutberg, L., Curvall, M. and Enzell, C.R. (1980) Screening of tobacco smoke constituents for mutagenicity using the Ames' test. Toxicology, **15**, 219-232.

Grimmer, G. and Böhnke, H. (1975) Polycyclic aromatic hydrocarbon profile analysis of high-protein foods, oils, and fats by gas chromatography. J. Assoc. Off. Anal. Chem., **58**, 725-733.

Harvey, R.C. and Halonen, M. (1968) Interaction between carcinogenic hydrocarbons and nucleosides. Cancer Res., **28**, 2183-2186.

Hecht, S.S. (1999) Tobacco smoke carcinogens and lung cancer. J. Natl. Cancer Inst., **91**, 1194-1210.

Kulka, U., Schmid, E., Huber, R. and Bauchinger, M. (1988) Analysis of the cytogenetic effect in human lymphocytes induced by metabolically activated 1- and 2-methylnaphthalene. Mutat. Res., **208**, 155-158.

Kuroiwa, Y., Umemura, T., Nishikawa, A., Kanki, K., Ishii, Y., Kodama, Y., Masumura, K., Nohmi, T. and Hirose, M. (2007) Lack of *in vivo* mutagenicity and oxidative DNA damage by flumequine in the livers of gpt delta mice. Arch. Toxicol., **81**, 63-69.

Lee, C.C., Craig, W.K. and Smith, P.J. (1974) Water-soluble hydrocarbons from crude oil. Bul. Environ. Contam. Toxicol., **12**, 212-216.

Masumura, K., Kuniya, K., Kurobe, T., Fukuoka, M., Yatagai, F. and Nohmi, T. (2002) Heavy-ion-induced mutations in the *gpt* delta transgenic mouse: comparison of mutation spectra induced by heavy-ion, X-ray, and gamma-ray radiation. Envirn. Mol. Mutagen., **40**, 207-215.

Masumura, K., Matsui, M., Katoh, M., Horiya, N., Ueda, O., Tanabe, H., Ymada, M., Suzuki, H., Sofuni, T. and Nohmi, T. (1999) Spectra of *gpt* mutations in ethylnitrosourea-treated and untreated transgenic mice. Environ. Mol. Mutagen., **34**, 1-8.

McCain, B.B., Hodgins, H.O., Gronlund, W.D., Hawkes, J.W., Brown, D.W., Myers, M.S. and Vandermeulen, J.H. (1978) Bioavailability of crude oil from experimentally oiled sediments to English sole (*Parophrys vetulus*), and pathological consequences. J. Fish. Res. Board. Can., **35**, 657-664.

Murata, Y., Denda, A., Maruyama, H., Konishi, Y. (1993) Chronic toxicity and carcinogenicity studies of 1-methylnaphthalene in B6C3F1 mice. Fundam. Appl. Toxicol., **21**, 44-51.

Nohmi, T., Katoh, M., Suzuki, H., Matsui, M., Yamada, M., Watanabe, M., Suzuki, M., Horiya, N., Ueda, O., Shibuya, T., Ikeda, H. and Sofuni, T. (1996) A new transgenic mouse mutagenesis test system using Spi- and 6-thioguanine selections. Environ. Mol. Mutagen., **28**, 465-470.

Nohmi, T., Suzuki, K. and Masumura, K. (2000) Recent advances in the protocols of transgenic mouse mutation assays. Mutat. Res., **455**, 191-215.

Pfeifer, G.P., Denissenko, M.F., Olivier, M., Tretyakova, N., Hecht, S.S. and Hainaut, P. (2002) Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. Oncogene, **21**, 7435-7451.

Rothman, N., Poirier, M.C., Baser, M.E., Hansen, J.A., Gentile, C., Bowman, E.D. and Strickland, P.T. (1990). Formation of polycyclic aromatic hydrocarbon-DNA adducts in peripheral white blood cells during consumption of charcoal-broiled beef. Carcinogenesis, **11**, 1241-1243.

Shibata, A., Maeda, D., Ogino, H., Tsutsumi, M., Nohmi, T., Nakagama, H., Sugimura, T., Teraoka, H. and Masutani, M. (2009) Role of Parp-1 in suppressing spontaneous deletion mutation in the liver and brain of mice at adolescence and advanced age. Mutat. Res., **664**, 20-27.

Winters, K., O'Donnell, R., Batterton, J.C. and Baalen, C. (1976) Water soluble components of four fuel oils: chemical characterization and effects on growth of microalgae. Mar. Biol. (N.Y.) **36**, 269-276.

Xu, A., Smilenov, L.B., He, P., Masumura, K., Nohmi, T., Yu, Z. and Hei, T.K. (2007) New insight into intrachromosomal deletions induced by chrysotile in the *gpt* delta transgenic mutation assay. Environ. Health. Perspect., **115**, 87-92.