

Original Article

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

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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 char-broiled 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

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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 ($23 \pm 2^\circ\text{C}$), humidity ($55 \pm 5^\circ\text{C}$), 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^r (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

In vivo genotoxicity of 1-methylnaphthalene in the liver**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 (g/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./cm²) from ten different areas per animal were counted to give the PCNA-positive ratio.

Statistics

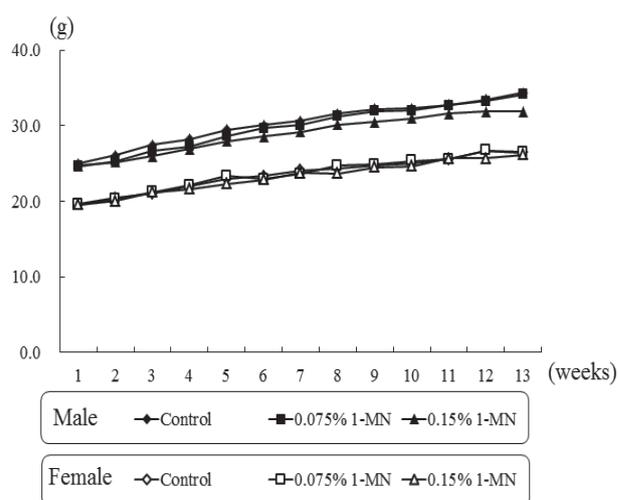
The data obtained from measurements of body weight,

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.

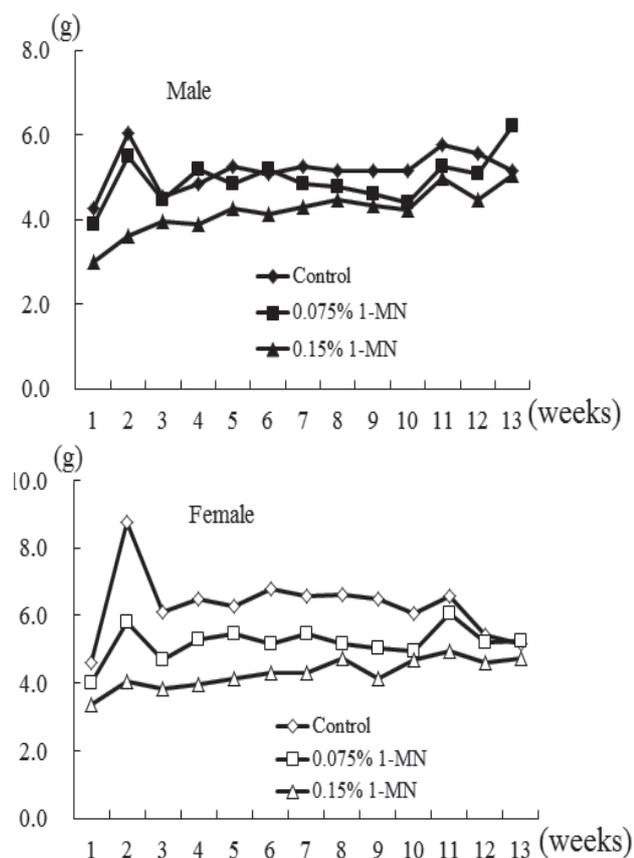


Fig. 2. Daily food intake for B6C3F1 *gpt* delta mice administered 1-MN for 13 weeks.

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

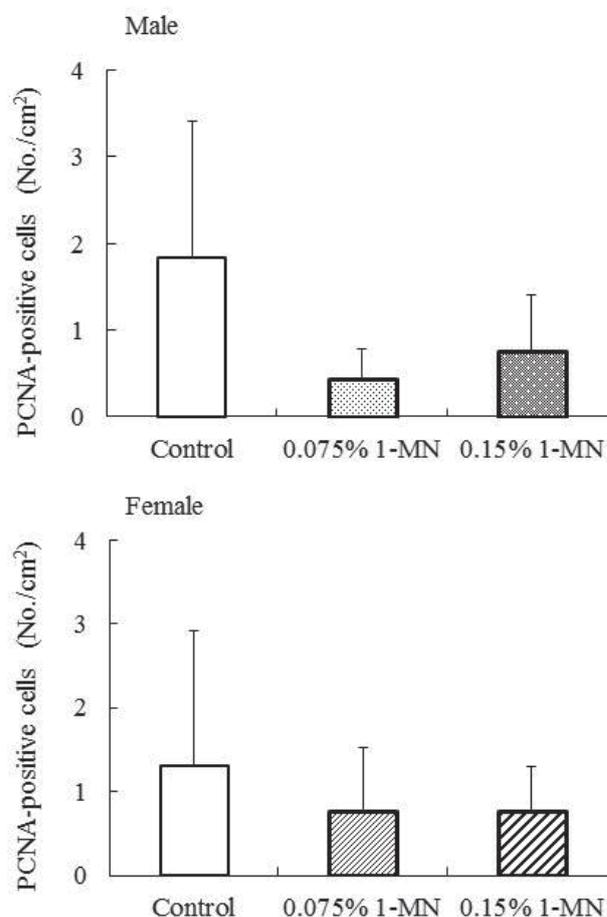


Fig. 3. Proliferating cell nuclear antigen (PCNA) positive cell ratio in the lung of male and female *gpt* delta mice administered 1-MN for 13 weeks.

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

In vivo genotoxicity of 1-methylnaphthalene in the liver**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 (x10 ³ /μl)	24.2 ± 15.0 ^a	22.0 ± 9.0	15.0 ± 7.0
RBS (x10 ⁴ /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 (x10 ⁴ /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 (x10 ³ /μl)	16.0 ± 8.0	17.0 ± 11.0	17.0 ± 8.0
RBS (x10 ⁴ /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 (x10 ⁴ /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.

^aMean ± 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, potassium; 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.

In vivo genotoxicity of 1-methylnaphthalene in the liver**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

Organs and findings	1-MN No.	Male			Female		
		Control 10	0.075% 10	0.15% 10	Control 10	0.075% 10	0.15% 10
Survival 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 levels 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

In vivo genotoxicity of 1-methylnaphthalene in the liver**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	0.34 ± 0.17
		2	12.3	5	0.41	
		3	15.2	9	0.59	
		4	15.1	2	0.13	
		5	4.0	1	0.25	
	0.075% 1-MN	11	11.4	5	0.26	0.33 ± 0.13
		12	2.1	1	0.47	
		13	5.3	1	0.19	
		14	23.5	5	0.30	
		15	18.2	6	0.33	
	0.15% 1-MN	21	18.0	9	0.61	0.54 ± 0.06
		23	6.1	4	0.49	
		24	24.5	17	0.57	
		25	22.4	11	0.49	
Female	Control	31	7.1	1	0.14	0.17 ± 0.10
		32	50.5	3	0.06	
		33	43.4	10	0.23	
		34	36.0	4	0.11	
		35	18.3	6	0.33	
	0.075% 1-MN	41	41.1	7	0.17	0.17 ± 0.09
		42	36.4	3	0.08	
		43	40.6	6	0.15	
		44	38.0	12	0.32	
		45	49.2	6	0.12	
	0.15% 1-MN	51	25.8	6	0.23	0.20 ± 0.14
		52	29.3	11	0.38	
		53	33.9	7	0.21	
		54	30.2	5	0.17	
		55	55.8	0	0.00	

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	0.15 ± 0.06
		2	14.0	2	0.14	
		3	19.5	4	0.21	
		4	10.9	2	0.18	
	0.075% 1-MN	11	14.1	6	0.43	0.45 ± 0.16
		12	3.4	1	0.29	
		13	15.0	10	0.67	
		14	18.4	10	0.55	
		15	15.3	5	0.33	
	0.15% 1-MN	21	16.5	3	0.18	0.40 ± 0.26
		22	22.8	7	0.31	
		23	4.9	4	0.82	
		24	18.0	4	0.22	
		25	6.3	3	0.48	
	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	
0.075% 1-MN		41	13.6	1	0.07	0.37 ± 0.18
		42	20.1	9	0.45	
		43	11.8	4	0.34	
		44	17.6	9	0.51	
		45	10.9	5	0.46	
0.15% 1-MN		51	8.7	0	0.00	0.28 ± 0.23
	52	12.4	5	0.40		
	53	14.6	3	0.21		
	54	15.5	8	0.52		

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 geno-

toxic 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.

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In vivo genotoxicity of 1-methylnaphthalene in the liver

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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. *Environ. 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.

