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Validation study of the combined repeated-dose toxicity and genotoxicity assay using *gpt* delta rats

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Key words

Genotoxicity, *gpt* delta, mutation, reduction of animal use, repeated-dose toxicity

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Transgenic rodents carrying reporter genes to detect organ-specific *in vivo* genetic alterations are useful for risk assessment of genotoxicity that causes cancer. Thus, the Organization for Economic Co-operation and Development has established the guideline for genotoxicity tests using transgenic animals, which may be combined with repeated-dose toxicity studies. Here, we provide evidence to support equivalence of *gpt* delta and wild type (WT) rats in terms of toxicological responses to a genotoxic hepatocarcinogen, *N*-nitrosodiethylamine (DEN), and a non-genotoxic hepatocarcinogen, di(2-ethylhexyl)phthalate (DEHP). *gpt* delta rats treated with DEHP showed similar increases in liver and kidney weights, serum albumin, albumin/globulin ratios, and incidence of diffuse hepatocyte hypertrophy compared to WT F344 and Sprague–Dawley (SD) rats. DEN-treated *gpt* delta rats showed equivalent increases in the number and area of precancerous GST-P-positive foci in the liver compared to WT rats. The livers of DEN-treated *gpt* delta rats also showed increased frequencies of *gpt* and Spi$^-$ mutations; such changes were not observed in DEHP-treated *gpt* delta rats. These results indicated that *gpt* delta rats (both F344 and SD backgrounds) showed comparable DEHP-induced toxicity and DEN-induced genotoxicity to those observed in WT rats. With regard to the administration period, the general toxicity of 1.2% DEHP was evident throughout the experimental period, and the genotoxicity of 10 p.p.m. DEN could be detected after 2 weeks of administration and further increased at 4 weeks. These results suggested that combined assays using *gpt* delta rats could detect both general toxicity and genotoxicity by the canonical 4-week administration protocol. Therefore, this assay using *gpt* delta rats would be applicable for risk assessment including early detection of genotoxic carcinogens and ultimately serve to reduce cancer risks in humans from environmental chemicals.

Carcinogenicity is one of the most serious hazards of chronic exposure to chemicals. Carcinogens are classified into two major groups: genotoxic carcinogens and non-genotoxic carcinogens. As genotoxicity is not thought to have a threshold, it is important to determine the genotoxicity of chemicals for risk assessment. Therefore, a number of *in vitro* and *in vivo* genotoxicity tests have been developed. Among them, transgenic *in vivo* genotoxicity assays, which use transgenic rodents carrying reporter genes to detect genetic alterations, enable us to evaluate organ-specific *in vivo* genotoxicity. Thus, the Organisation for Economic Co-operation and Development (OECD) has established the guideline for genotoxicity testing using transgenic animals. The *gpt* delta rats and mice are considered one of the established transgenic models, which have provided sufficient data to support their use in the OECD test guideline.[1] These rodents carry the *gpt* transgene, which detects point mutations, and the *red/gam* transgenes, which detect deletion mutations.[2,3]

At present, *in vivo* genotoxicity studies are carried out independently of repeated-dose toxicity studies. Therefore, both transgenic animals (for evaluation of genotoxicity) and wild type (WT) animals (for evaluation of general toxicity) are required. Therefore, combining transgenic rodent gene mutation assays[1] with repeated-dose toxicity studies would conform to the 3Rs principle (Replacement, Refinement, and Reduction) of animal use in laboratory experiments. To accomplish this objective, data are required to ensure that transgenic gene mutation assays are efficiently sensitive in the protocol used for repeated-dose toxicity studies and to verify that the performance of the repeated-dose assay is not adversely affected by using a transgenic rodent strain rather than the parental WT strain.[1] Therefore, in this study, we compared the general toxicity of a genotoxic hepatocarcinogen, *N*-nitrosodiethylamine (DEN), and a non-genotoxic hepatocarcinogen, di(2-ethylhexyl)phthalate (DEHP), in F344/*gpt* delta and Sprague–Dawley (SD)/*gpt* delta rats with their parental WT F344 and SD rats to determine whether *gpt* delta rats were equivalent to WT rats in terms of toxicological responses. In addition to general toxicity, we also compared the carcinogenic effects of DEN between *gpt* delta and WT rats by examining the appearance of procarcinogenic GST-P-positive lesions, which are detected in the liver after genotoxic treatment.[4]

In addition to the ability of gene mutation assays in transgenic animals to detect organ-specific genotoxicity, these assays are also able to detect mutations after only a short duration of repeated exposure because clonal expansion of mutant cells is not necessary. Therefore, we also examined the appropriate administration duration for simultaneous detection of both general toxicity and genotoxicity by killing animals after 2, 4, or 8 weeks of treatment.

Materials and Methods

Experimental animals. Five-week-old male specific pathogen-free F344/WT (F344/NSlc), F344/*gpt* delta (F344/NSlc-Tg [*gpt* delta]), SD/WT (Slc:SD), and SD/*gpt* delta (Slc:SD-Tg [*gpt* delta]) rats were purchased from Japan SLC (Shizuoka, Japan) and used after a 1-week acclimatization period. The animals were housed in polycarbonate cages (five rats per cage) with soft chips for bedding. The animals were maintained in a room with a barrier system under conditions of controlled temperature (22 ± 3°C), humidity (55 ± 15%), air changes (more than 10 times/h), and lighting (12:12 h light : dark cycle) and were given free access to an MF basal diet (Oriental Yeast, Tokyo, Japan) and tap water.

Test chemicals. *N*-nitrosodiethylamine (CAS: 55-18-5, >99% pure) was purchased from Tokyo Chemical Industry (Tokyo, Japan) (Lot: PEI3F-ES). Di(2-ethylhexyl) phthalate (CAS: 117-81-7, 97% pure) was purchased from Wako Pure Chemical (Osaka, Japan) (Lot: TLM0851). For administration, DEN was dissolved in tap water before use, whereas DEHP was mixed into the powdered basal diet.

Study design. The highest doses of DEN and DEHP were determined by carcinogenic doses reported previously.[5–8] For each experiment, rats were randomly grouped into four groups per strain (n = 15 rats per group); the body weights of rats were equivalent among groups at the start of the experiment. The groups were as follows: 0 (control), 0.1, 1, and 10 p.p.m. DEN in drinking water; or 0 (control), 0.012, 0.12, and 1.2% DEHP in the diet. During the administration period, general conditions and clinical signs of the rats were monitored daily, and body weights were measured weekly. In the DEN treatment assay, the drinking water supplemented with DEN was changed more than two times per week, and water consumption was recorded. In the DEHP treatment assay, the diet supplemented with DEHP was changed two times per week, and food consumption was recorded. At 2, 4, or 8 weeks of administration, five rats per group were fasted overnight and then killed under deep anesthesia by inhalation of isoflurane. Blood samples were collected from the abdominal aorta for serum biochemistry, and the lungs, heart, thymus, liver, kidneys, spleen, adrenal glands, and testes were collected to determine changes in organ weights and abnormalities by histopathological examination. Pieces of the liver were frozen for later analysis by gene mutation assays. Because a sufficient number of F344/*gpt* delta rats could not be obtained in the DEN treatment study, only 12, 12, 12, and 11 rats were used for the 0, 0.1, 1, and 10 p.p.m. DEN groups, respectively; four animals were killed at 2 or 4 weeks, and the remaining animals were killed at 8 weeks. To compensate for the smaller number of animals in the 10 p.p.m. DEN group at 8 weeks of administration, an additional experiment was used to confirm the results as follows. Eight F344/*gpt* delta rats were randomly grouped into two groups (n = 4 rats per group) and treated with either 0 or 10 p.p.m. DEN. Only data obtained from the initial experiment are shown in figures and used for statistical analyses. Data obtained from the additional experiment were separately recorded and did not combine to the initial experiment. The study design was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences (Tokyo, Japan).

Immunohistochemical analysis for GST-P-positive liver foci. To evaluate the appearance of precarcinogenic GST-P-positive foci, formalin-fixed paraffin-embedded liver sections of DEN-treated rats were stained with anti-GST-P polyclonal antibodies (Code No. 311; Medical and Biological Laboratories, Aichi, Japan) followed by Histofine Simple Stain Rat MAX-PO (M) (Nichirei, Tokyo, Japan). GST-P expression was visualized with diaminobenzidine (Dojindo, Kumamoto, Japan). The number and area of GST-P-positive foci were analyzed with IPAP-WIN software (Sumika Technoservice, Hyogo, Japan).

***In vivo* mutation assays.** Genomic DNA was extracted from the livers of *gpt* delta rats. In the DEN treatment experiment, the DNA was purified by ethanol precipitation. In the DEHP treatment experiment, the DNA was dialyzed against TE buffer using a RecoverEase DNA Isolation Kit (Agilent Technologies, Santa Clara, CA, USA). The *gpt* and Spi$^-$ assays were carried out as described previously.[9,10] Briefly, λEG10 phage was reconstructed from genomic DNA with Transpack Packaging Extract (Agilent Technologies). For *gpt* gene mutation assays, *Escherichia coli* YG6020 were transformed with the reconstructed phage and then plated onto M9 minimum agar plates supplemented with chloramphenicol (Cm) with or without 6-thioguanine (6-TG). The transformants carrying mutant *gpt* genes, which grew on M9+Cm+6-TG plates, were subjected to colony-direct PCR with primers designed to amplify the *gpt* transgene for sequencing analysis. Frequencies of *gpt* gene mutations were calculated by the number of 6-TG^{r}Cm^r colonies harboring independent mutations divided by the number of Cm^r colonies. For Spi$^-$ mutant assays, *E. coli* XL1-Blue MRA and XL1-Blue MRA (P2) were infected with the phage and then plated onto λ-trypticase plates. The Spi$^-$ candidates, which formed plaques on the XL1-Blue MRA (P2) plates, were confirmed by respotting onto *E. coli* XL1-Blue MRA, XL1-Blue MRA (P2), and WL95 (P2) strains. Plaques that appeared on the three *E. coli* strains were counted as Spi$^-$ mutants. Spi$^-$ mutant frequencies were calculated by the number of Spi$^-$ mutants divided by the number of plaques on XL1-Blue MRA.

Statistical analysis. Significant differences in the data for body weights, organ weights, serum biochemistry, numbers and areas of GST-P-positive foci in the liver, frequencies of mutations in the *gpt* gene, and Spi$^-$ mutant frequencies in *red/gam* genes were evaluated by Tukey's test. Significant differences in the incidences of histopathological findings were evaluated by Fisher's exact probability tests.

Results

Clinical signs of toxicity prior to euthanasia. In both DEN and DEHP experiments, no clinical signs were observed throughout the experimental period. All animals survived until the scheduled necropsy. Average intakes of DEN and DEHP per body weight were considered to be dose-dependent (Tables 1,2).

As WT and *gpt* delta rats were not littermates, the F344/*gpt* rats used in DEN treatment and the SD/*gpt* delta rats used in both DEN and DEHP treatments were relatively smaller than their corresponding WT rats at the start of treatment (6 weeks old). Administration of DEN at 0.1, 1, or 10 p.p.m. in drinking water had no effect on body weight gain (Figs 1a,b,S1) in all

Table 1. Water consumption and chemical intake during the entire treatment period with *N*-nitrosodiethylamine (DEN)

Strain/genotype	DEN, p.p.m.	Water consumption, g/animal/day	Chemical intake, μg/kg BW/day
F344/WT	0	20.5	0.00
	0.1	19.9	9.00
	1	20.6	90.0
	10	20.7	906
F344/*gpt* delta	0	20.3	0.00
	0.1	19.2	9.50
	1	19.5	96.3
	10	19.1	932
	0†	18.8	0.00
	10†	18.6	923
SD/WT	0	30.6	0.00
	0.1	30.7	8.38
	1	28.1	78.8
	10	29.9	841
SD/*gpt* delta	0	30.6	0.00
	0.1	32.9	9.38
	1	29.6	85.0
	10	28.3	836

†Additional experiment. BW, body weight; SD, Sprague–Dawley.

Table 2. Food consumption and chemical intake during the entire treatment period with di(2-ethylhexyl)phthalate (DEHP)

Strain/genotype	DEHP, %	Food consumption, g/animal/day	Chemical intake, mg/kg BW/day
F344/WT	0	15.7	0.00
	0.012	16.6	8.75
	0.12	16.6	89.1
	1.2	16.8	914
F344/*gpt* delta	0	18.0	0.00
	0.012	16.9	9.05
	0.12	16.8	90.8
	1.2	16.4	920
SD/WT	0	21.0	0.00
	0.012	20.6	7.24
	0.12	21.2	73.9
	1.2	25.3	838
SD/*gpt* delta	0	19.3	0.00
	0.012	19.9	7.24
	0.12	19.5	70.6
	1.2	20.6	857

BW, body weight; SD, Sprague–Dawley.

strains. Administration of DEHP at 1.2% in food caused significant decreases (or decreasing trends) in body weight gain compared to the corresponding controls from weeks 3 to 8 (Fig. 1c,d). The magnitudes of the observed decreases were 4.7% (F344/WT), 7.6% (F344/*gpt* delta), 7.2% (SD/WT), and 6.9% (SD/*gpt* delta) of the mean control body weight at 4 weeks and 6.6% (F344/WT), 10.5% (F344/*gpt* delta), 9.5% (SD/WT), and 12.2% (SD/*gpt* delta) of the mean control body weight at 8 weeks (Fig. 1).

Relative organ weights. Administration of DEN at 0.1, 1, or 10 p.p.m. in drinking water had no significant effect on organ weights, regardless of the genotype or strain (Table S1). Administration of DEHP at 1.2% in the diet caused a significant increase in liver weight compared to the corresponding controls. The magnitudes of the increases ranged from 1.9- to 2.0-fold (F344/WT), 1.6- to 2.0-fold (F344/*gpt* delta), 1.7- to 1.9-fold (SD/WT), and 1.7- to 1.9-fold (SD/*gpt* delta) throughout the experimental period. We also observed significant increases (or increasing trends) in liver weights of rats consuming 0.12% DEHP. Administration of DEHP at 1.2% in both genotypes and strains also caused significant increases (1.1–1.3-fold) in kidney weight compared to the corresponding controls throughout the experimental period (Fig. 2, Table S2).

Serum biochemistry. Administration of DEN at 0.1, 1, or 10 p.p.m. in drinking water had no significant effect on serum biochemistry in all strains (Table S3). Regardless of the genotype and strain, administration of DEHP at 1.2% in the diet caused significant increases in serum albumin (1.1–1.3-fold) and albumin/globulin ratios (1.3–2.0-fold) compared to the corresponding controls. At 1.2%, DEHP also caused a 1.3–1.5-fold increase in serum alkaline phosphatase (ALP) at 8 weeks (Fig. 3, Table S4).

Histopathological findings. Administration of DEN at 0.1, 1, or 10 p.p.m. in drinking water had no significant effect on the incidences of lesions in the liver, spleen, kidneys, lung, heart, or stomach, as indicated by H&E staining of tissue sections (Table 3). Administration of DEHP at 1.2% in the diet caused diffuse hepatocyte hypertrophy in all rats throughout the experimental period. Significant increases in alveolar foamy cell infiltration were found in SD/WT rats treated with 1.2% DEHP at week 8. However, this may have been incidental as this lesion was also frequently found in control groups. Although significant increases in kidney weights were observed in rats treated with 1.2% DEHP, no treatment-related changes were observed under microscopic examinations (Table 4).

Immunohistochemical analysis of GST-P-positive liver foci. Due to the short duration of administration, the majority of GST-P-positive foci contained few cells, particularly at week 2. Therefore, we counted all GST-P-positive hepatocytes as GST-P-positive foci, without cell number/size thresholds. Administration of DEN at 10 p.p.m. caused a significant increase in the number of foci and a significant increase (or increasing trend) in the area of foci beginning at week 2 in all strains (Fig. 4). With regard to both the number and area of GST-P-positive foci, no significant differences were observed between *gpt* delta and WT rats for both F344 and SD strains at all doses and for all administration durations. In F344/*gpt* delta rats, although evaluation was carried out with a limited number of initial samples, statistical significance was evident. The additional F344/*gpt* delta rats treated with 0 or 10 p.p.m. DEN for 8 weeks showed comparable data for the number (2.9 or 164.4 foci/cm^2, respectively) and area (8.1×10^{-4} or 7.7×10^{-2} mm^2/cm^2, respectively) of foci (Fig. S2). It was established that GST-P-positive foci were not induced by peroxisome proliferators,[11] such as DEHP. Indeed, our previous study showed that a carcinogenic dose (12 000 p.p.m., the same as in this study) of DEHP did not induce GST-P foci in *gpt* delta rats.[9] Thus, we considered that GST-P-positive foci was not a suitable marker for comparison between *gpt* delta and WT rats on the effect of DEHP exposure. Therefore, examination of GST-P-positive foci was carried out in the DEN treatment experiment only.

***In vivo* mutation assays.** Administration of DEN induced significant increases in *gpt* mutation frequencies (Fig. 5a, Tables S5,S6) and Spi^- mutant frequencies (Fig. 5b, Tables S7,S8). In the 10 p.p.m. DEN groups, mutation frequencies of the *gpt* transgene were 1.62×10^{-5}, 4.05×10^{-5}, and 3.79×10^{-5}

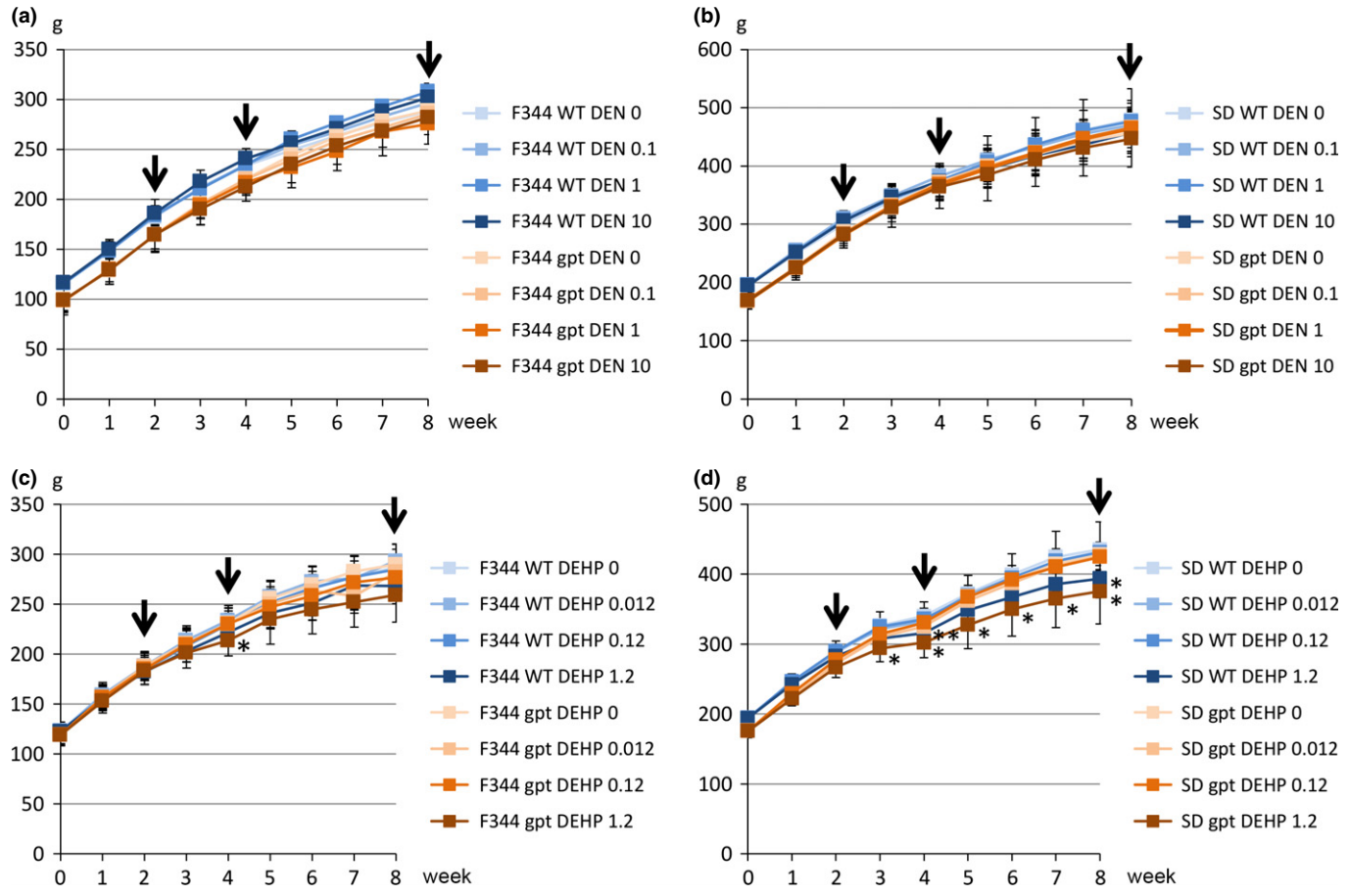

Fig. 1. Body weight gain in male rats treated with *N*-nitrosodiethylamine (DEN) or di(2-ethylhexyl)phthalate (DEHP). F344/wild type (WT) and F344/*gpt* delta rats (a) or Sprague–Dawley (SD)/WT and SD/*gpt* delta rats (b) administered 0, 0.1, 1, or 10 p.p.m. DEN. F344/WT and F344/*gpt* delta rats (c) and SD/WT and SD/*gpt* delta rats (d) administered 0, 0.012, 0.12, or 1.2% DEHP. Four or five animals from each group (except 10 ppm DEN group at week 8 in F344; n=3) were killed at the time points indicated by arrows. $*P < 0.05$, $**P < 0.01$ versus respective control group.

at 2, 4, and 8 weeks, respectively, in F344/*gpt* delta rats and 1.36×10^{-5}, 3.18×10^{-5}, and 4.06×10^{-5} at 2, 4, and 8 weeks, respectively, in SD/*gpt* delta rats. These values were 16.0- to 48.8-fold higher than those of the corresponding control groups. In the 1 p.p.m. DEN groups, although statistical significance was not achieved, the mutation frequencies were 3.9- to 8.8-fold higher than those of the corresponding control groups. Sequencing analysis of *gpt* mutants revealed that administration of DEN caused significant increases in GC-TA, AT-TA, and AT-CG transversions and GC-AT and AT-CG transitions (Fig. 6, Tables S9,S10). In addition to *gpt* mutation frequencies, Spi^- mutant frequencies, which are mainly induced by large structural mutations or frameshift mutations, were also dramatically increased by DEN administration. In the 10 p.p.m. DEN groups, Spi^- mutant frequencies were 0.92×10^{-5}, 1.59×10^{-5}, and 2.73×10^{-5} at 2, 4, and 8 weeks, respectively, in F344/*gpt* delta rats and 0.99×10^{-5}, 1.75×10^{-5}, and 2.68×10^{-5} at 2, 4, and 8 weeks, respectively, in SD/*gpt* delta rats. These values were 2.5- to 9.4-fold higher than those of control groups. Comparable results were obtained from the additional experiment. Individual data are shown in Tables S5–S10. Administration of DEHP had no effect on the *gpt* mutation frequencies, *gpt* mutation spectra, or Spi^- mutant frequencies (Fig. 5c,d, Tables S11–S16).

Additional experiment. Because a sufficient number of F344/*gpt* delta rats could not be obtained in the DEN treatment study, an additional experiment was carried out to confirm the results of 8-week treatment of the 10 p.p.m. DEN group. There were no significant differences between the initial and additional experiments, and even if data from both experiments were combined, there were no statistical changes in general toxicity or immunohistochemical analysis of GST-P-positive liver foci. The sole statistically significant change was that GC-TA transversion of the 10 p.p.m. DEN group at 8 weeks was changed from $P < 0.05$ (*) to $P < 0.01$ (**) when data from these two experiments were combined.

Discussion

In this study, we evaluated whether *in vivo* genotoxicity studies and general toxicity studies could be carried out simultaneously in *gpt* delta rats, with responses equivalent to those observed in WT rats. In the general toxicological study, there were no significant changes in DEN-treated groups with respect to body weights, relative organ weights, serum biochemistry values, and histopathological examinations. Similar results were observed in an additional set of samples collected after 8 weeks of DEN exposure. Administration of DEHP at 1.2% caused significant increases in liver weights at 2, 4, and 8 weeks. Accordingly, serum albumin levels and albumin/globulin ratios were also increased significantly. These changes were consistent with the presence of diffuse

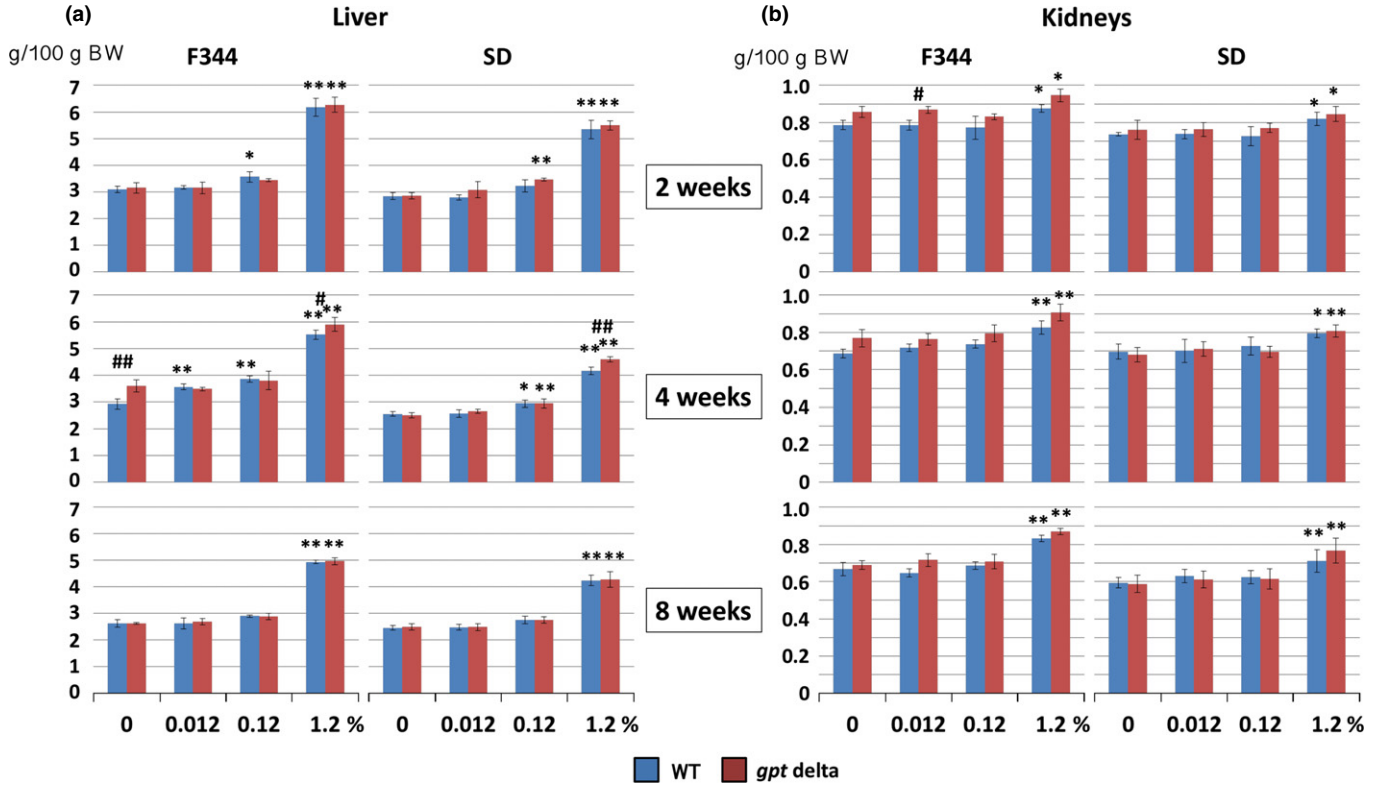

Fig. 2. Effects of di(2-ethylhexyl)phthalate (DEHP) on relative organ weights in male F344/wild type (WT), F344/*gpt* delta, Sprague–Dawley (SD)/WT, and SD/*gpt* delta rats administered 0, 0.012, 0.12, or 1.2% DEHP in the diet for 2, 4, or 8 weeks. (a) Liver; (b) kidneys. $^{*}P < 0.05$, $^{**}P < 0.01$ versus 0% DEHP group (control). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, WT versus *gpt* delta rats under the same conditions. BW, body weight.

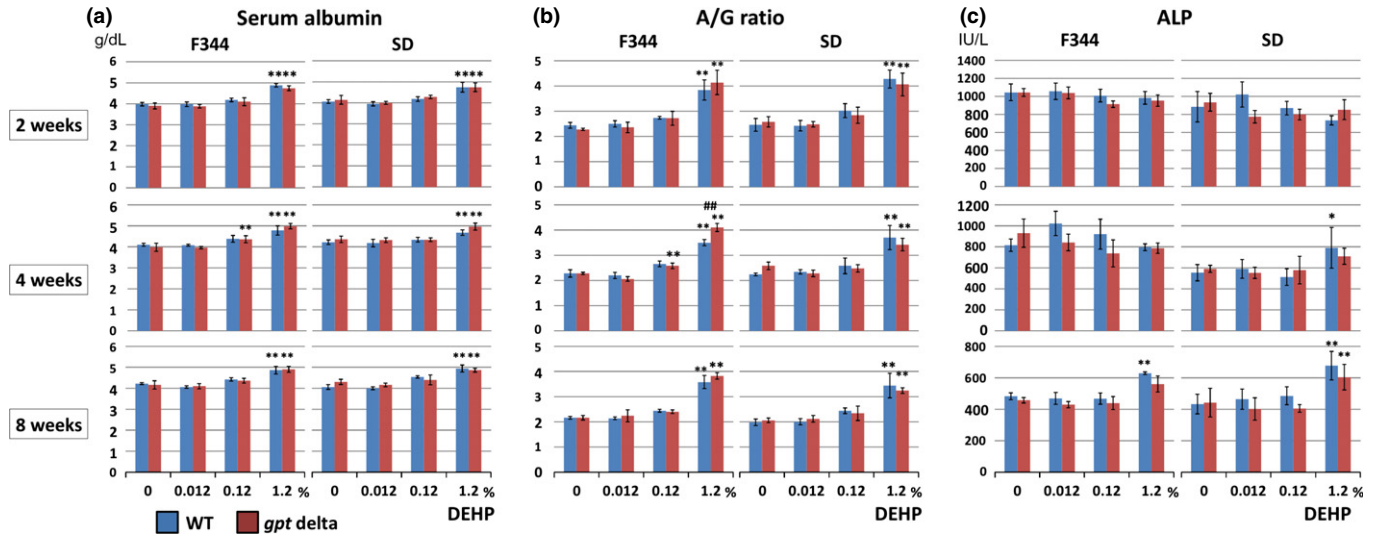

Fig. 3. Effects of di(2-ethylhexyl)phthalate (DEHP) on serum biochemistry in male F344/wild type (WT), F344/*gpt* delta, Sprague–Dawley (SD)/WT, and SD/*gpt* delta rats administered 0, 0.012, 0.12, or 1.2% DEHP in the diet for 2, 4, or 8 weeks. (a) Serum albumin, (b) albumin/globulin (A/G) ratio, and (c) alkaline phosphatase (ALP). Values are mean ± standard deviations. $^{*}P < 0.05$, $^{**}P < 0.01$ versus 0% DEHP group (control). $^{\#\#}P < 0.01$, WT versus *gpt* delta rats under the same conditions.

hepatocyte hypertrophy found in all rats treated with 1.2% DEHP. Administration of 1.2% DEHP also caused significant decreases (or decreasing trends) in body weight gain at weeks 3–8. The extents of these hepatomegaly-related changes and body weight reductions found in *gpt* delta rats were similar to those in WT rats and consistent with those reported in previous studies.[7,12] Although the testes are thought to be the most sensitive organ to DEHP,[8] for example, exposure to DEHP causes Leydig cell hyperplasia in Long–Evans rats[13] and seminiferous tubule atrophy and Sertoli cell vacuolation in SD

Table 3. Histopathological findings for F344/WT, F344/*gpt* delta, Sprague–Dawley (SD)/WT, and SD/*gpt* delta rats treated with *N*-diethylnitrosamine (DEN)

Strain/genotype	F344/WT												F344/*gpt* delta													
DEN (p.p.m.)	0			0.1			1			10			0			0.1			1			10			0†	10†
Weeks	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8	8	8
No. of animals	5	5	5	5	5	5	5	5	5	5	5	5	4	4	4	4	4	4	4	4	4	4	4	3	4	4
Organs and findings																										
Liver																										
Focus of cellular alteration	0	0	0	0	0	0	0	0	0	0	0	1 (20)	0	0	1 (25)	0	0	0	0	0	0	0	0	0	0	1 (25)
Microgranuloma	1 (20)	0	3 (60)	2 (40)	2 (40)	0	1 (20)	1 (20)	2 (40)	3 (60)	3 (60)	2 (40)	1 (25)	1 (25)	0	0	0	0	0	0	2 (50)	0	1 (25)	1 (33.3)	2 (50)	2 (50)
Lipidosis, focal	0	0	0	0	1 (20)	0	0	0	0	0	0	0	0	0	0	0	0	2 (50)	0	0	0	0	0	0	0	0
Epidermoid cyst	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pigmentation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 (33.3)	0	0
Spleen																										
Focal atrophy, acinar	0	0	0	0	0	0	0	0	0	0	0	1 (20)	0	0	0	0	0	0	0	0	0	1 (25)	0	0	0	0
Infarction	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kidneys																										
Regenerative tubules	1 (20)	1 (20)	3 (60)	0	0	0	0	1 (20)	0	0	1 (20)	2 (40)	0	1 (25)	2 (50)	0	0	0	0	0	0	1 (25)	0	0	2 (50)	1 (25)
Cell infiltration, interstitial, lymphocytic	0	0	0	0	0	0	0	0	0	0	1 (20)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lungs																										
Cell infiltration, interstitial, lymphocytic	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Heart																										
Cell infiltration, lymphocytic	0	0	1 (20)	0	0	0	0	0	0	1 (20)	0	2 (40)	0	1 (25)	1 (25)	0	0	0	0	0	0	1 (25)	1 (25)	0	1 (25)	0
Stomach																										
Cysts, glandular, glandular stomach	1 (20)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hyperplasia, squamous cell, forestomach	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 3. (continued)

Strain/genotype	SD/WT												SD/*gpt* delta											
DEN (p.p.m.)	0			0.1			1			10			0			0.1			1			10		
Weeks	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8
No. of animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Organs and findings																								
Liver																								
Focus of cellular alteration	0	0	1 (20)	0	0	0	0	0	1 (20)	0	0	1 (20)	0	0	0	0	0	0	0	0	0	0	0	0
Microgranuloma	3 (60)	3 (60)	2 (40)	3 (60)	1 (20)	2 (40)	4 (80)	3 (60)	2 (40)	3 (60)	5 (100)	4 (80)	3 (60)	2 (40)	1 (20)	1 (20)	1 (20)	0	2 (40)	4 (80)	2 (40)	1 (20)	4 (80)	2 (40)
Lipidosis, focal	0	0	0	0	0	0	0	0	0	0	0	0	2 (40)	1 (20)	0	1 (20)	0	0	0	0	0	0	0	0
Epidermoid cyst	0	0	0	0	0	0	1 (20)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pigmentation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spleen																								
Focal atrophy, acinar	0	0	0	0	0	0	0	0	0	0	0	0	0	1 (20)	0	0	0	0	0	0	0	0	0	0
Infarction	0	0	0	0	0	1 (20)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 (20)
Kidneys																								
Regenerative tubules	3 (60)	0	2 (40)	0	0	0	0	0	0	3 (60)	3 (60)	3 (60)	2 (40)	3 (60)	4 (80)	0	0	0	0	0	0	2 (40)	3 (60)	4 (80)
Cell infiltration, interstitial, lymphocytic	0	0	0	0	0	0	0	0	0	0	0	1 (20)	0	0	0	0	0	0	0	0	0	1 (20)	0	0
Lungs																								
Cell infiltration, interstitial, lymphocytic	0	0	1 (20)	0	0	0	0	0	0	0	0	0	0	0	1 (20)	0	0	0	0	0	0	0	0	1 (20)
Heart																								
Cell infiltration, lymphocytic	0	1 (20)	2 (40)	0	0	0	0	0	0	0	0	1 (20)	0	0	3 (60)	0	0	0	0	0	0	0	0	0
Stomach																								
Cysts, glandular, glandular stomach	0	1 (20)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hyperplasia, squamous cell, forestomach	0	0	0	0	0	0	0	0	0	0	0	0	0	1 (20)	0	0	0	0	0	0	0	0	0	0

†Additional experiment. Data are shown as number of cases (%).

Table 4. Histopathological findings for F344/WT, F344/*gpt* delta, Sprague–Dawley (SD)/WT, and SD/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP)

Strain/genotype	F344/WT												F344/*gpt* delta											
DEHP (%)	0			0.012			0.12			1.2			0			0.012			0.12			1.2		
Weeks	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8
No. of animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Organs and findings																								
Liver																								
Microgranuloma	1 (20)	3 (60)	0	3 (60)	1 (20)	4 (80)	2 (40)	3 (60)	2 (40)	0	0	1 (20)	2 (40)	1 (20)	1 (20)	1 (20)	1 (20)	4 (80)	1 (20)	0	1 (20)	0	0	0
Hypertrophy, diffuse	0	0	0	0	0	0	0	0	0	5 (100)**	5 (100)**	5 (100)**	0	0	0	0	0	0	0	0	0	5 (100)**	5 (100)**	5 (100)**
Single cell necrosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Focal necrosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 (20)	0	0	0	0	0	0
Bile duct proliferation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kidneys																								
Hyaline cast	1 (20)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Regenerative tubules	1 (20)	0	3 (60)	0	3 (60)	5 (100)	0	1 (20)	5 (100)	1 (20)	1 (20)	3 (60)	1 (20)	2 (40)	3 (60)	0	3 (60)	5 (100)	1 (20)	5 (100)	3 (60)	0	2 (40)	4 (80)
Mineralization, medulla	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cell infiltration, interstitial, lymphocytic	1 (20)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tubular dilatation, cortex	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tubular vacuolation, distal tubules	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyst	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lungs																								
Cell infiltration, interstitial, lymphocytic	0	0	2 (40)							1 (20)	0	1 (20)	0	0	0							0	0	0
Foamy cell infiltration, alveolar	0	0	0							0	0	0	0	0	0							0	0	0
Osseous metaplasia	0	1 (20)	0							0	0	0	0	0	0							0	0	0
Granuloma	0	0	0							0	0	0	0	0	0							0	0	0
Testes																								
Multinucleated cell	0	0	0							0	0	0	0	0	0							0	0	0
Tubular cell vacuolation	0	0	0							0	0	1 (20)	0	0	1 (20)							0	0	0
Tubular atrophy	0	0	0							0	1 (20)	1 (20)	0	0	0							0	0	0
Tubular degeneration	0	0	0							0	0	0	0	0	0							0	0	0

Table 4. (continued)

Strain/genotype	SD/WT												SD/*gpt* delta											
DEHP (%)	0			0.012			0.12			1.2			0			0.012			0.12			1.2		
Weeks	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8
No. of animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Organs and findings																								
Liver																								
Microgranuloma	2 (40)	3 (60)	2 (40)	4 (80)	3 (60)	5 (100)	4 (80)	2 (40)	2 (40)	0	1 (20)	1 (20)	3 (60)	3 (60)	4 (80)	5 (100)	4 (80)	5 (100)	4 (80)	2 (40)	3 (60)	0	0	0
Hypertrophy, diffuse	0	0	0	0	0	0	0	0	0	5 (100)**	5 (100)**	5 (100)**	0	0	0	0	0	0	0	0	0	5 (100)**	5 (100)**	5 (100)**
Single cell necrosis	0	0	0	0	0	1 (20)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Focal necrosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bile duct proliferation	0	0	0	0	0	0	0	0	0	0	1 (20)	0	0	0	0	0	0	0	0	0	0	0	0	0
Kidneys																								
Hyaline cast	0	0	0	0	0	1 (20)	0	0	1 (20)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Regenerative tubules	1 (20)	1 (20)	2 (40)	2 (40)	1 (20)	4 (80)	3 (60)	2 (40)	3 (60)	3 (60)	3 (60)	4 (80)	1 (20)	3 (60)	3 (60)	1 (20)	3 (60)	2 (40)	1 (20)	3 (60)	4 (80)	2 (40)	3 (60)	5 (100)
Mineralization, medulla	0	0	0	1 (20)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cell infiltration, interstitial, lymphocytic	0	0	0	0	0	2 (40)	2 (40)	2 (40)	1 (20)	0	0	0	1 (20)	1 (20)	0	0	1 (20)	0	1 (20)	2 (40)	0	1 (20)	0	2 (40)
Tubular dilatation, cortex	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 (20)
Tubular vacuolation, distal tubules	0	0	0	1 (20)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyst	0	0	0	1 (20)	0	0	0	1 (20)	0	0	0	0	0	0	0	0	0	0	0	0	0	1 (20)	0	0
Lungs																								
Cell infiltration, interstitial, lymphocytic	0	1 (20)	1 (20)							1 (20)	0	0	1 (20)	2 (40)	2 (40)	0						1 (20)	1 (20)	2 (40)
Foamy cell infiltration, alveolar	0	3 (60)	0							0	2 (40)	5 (100)**	0	0	0							0	2 (40)	2 (40)
Osseous metaplasia	0	0	0							0	0	0	0	0	0							0	0	0
Granuloma	0	0	0							0	0	1 (20)	0	0	1 (20)							0	0	0
Testes																								
Multinucleated cell	1 (20)	0	0							0	0	0	0	0	0							0	0	0
Tubular cell vacuolation	0	0	0							0	0	0	0	0	0							0	0	0
Tubular atrophy	0	0	1 (20)							0	1 (20)	0	0	0	0							0	0	0
Tubular degeneration	0	0	0							0	1 (20)	0	0	0	0							0	0	0

$^{**}P < 0.01$ *versus* control. Data are shown as number of cases (%).

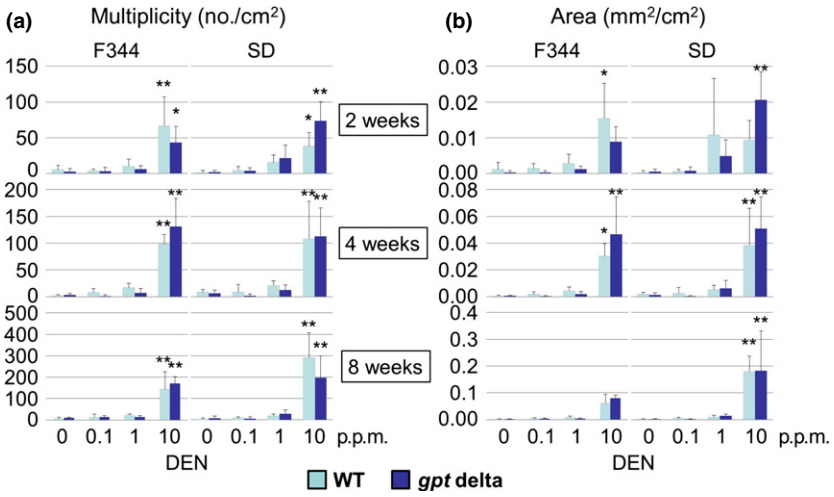

Fig. 4. Immunohistochemical analysis of GST-P-positive foci. The multiplicity (no./cm^2) (a) and area (mm^2/cm^2) (b) of GST-P-positive foci in the livers of *N*-nitrosodiethylamine (DEN)-treated rats. *$P < 0.05$, **$P < 0.01$ versus 0 p.p.m. DEN group (control). SD, Sprague–Dawley; WT, wild type.

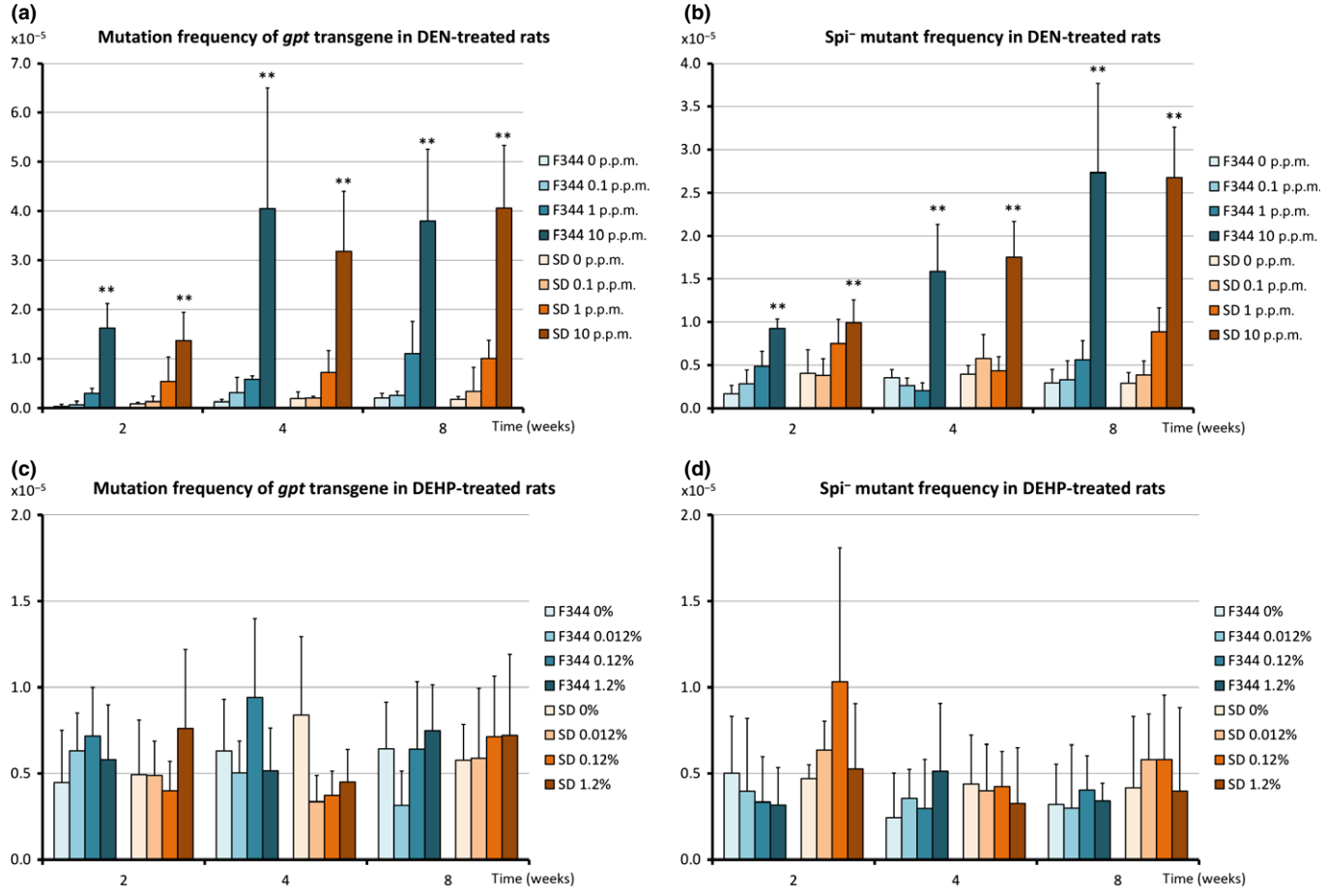

Fig. 5. (a) Frequency of mutations in the *gpt* transgene in the livers of male F344/*gpt* delta and Sprague–Dawley (SD)/*gpt* delta rats administered 0, 0.1, 1, or 10 p.p.m. *N*-nitrosodiethylamine (DEN) in drinking water.(b) Spi$^-$ mutant frequencies in the livers of male F344/*gpt* delta and SD/*gpt* delta rats administered 0, 0.1, 1, or 10 p.p.m. DEN in the drinking water. (c) Frequency of mutations in the *gpt* transgene in the livers of male F344/*gpt* delta and SD/*gpt* delta rats administered 0, 0.012, 0.12, or 1.2% di(2-ethylhexyl)phthalate (DEHP) in the diet. (d) Spi$^-$ mutant frequencies in the livers of male F344/*gpt* delta and SD/*gpt* delta rats administered 0, 0.012, 0.12, or 1.2% DEHP in the diet. **$P < 0.01$ versus 0 p.p.m. DEN group (control).

rats[14] even at lower doses than those used in this study, no significant changes were found in the present histopathological examination of both *gpt* delta and WT rats. This may have been due to the age of the rats at the beginning of administration and/or the administration durations. Although we did observe some statistically significant differences between *gpt* delta and WT rats in serum biochemistry values in the DEN exposure study and in other organ weights and

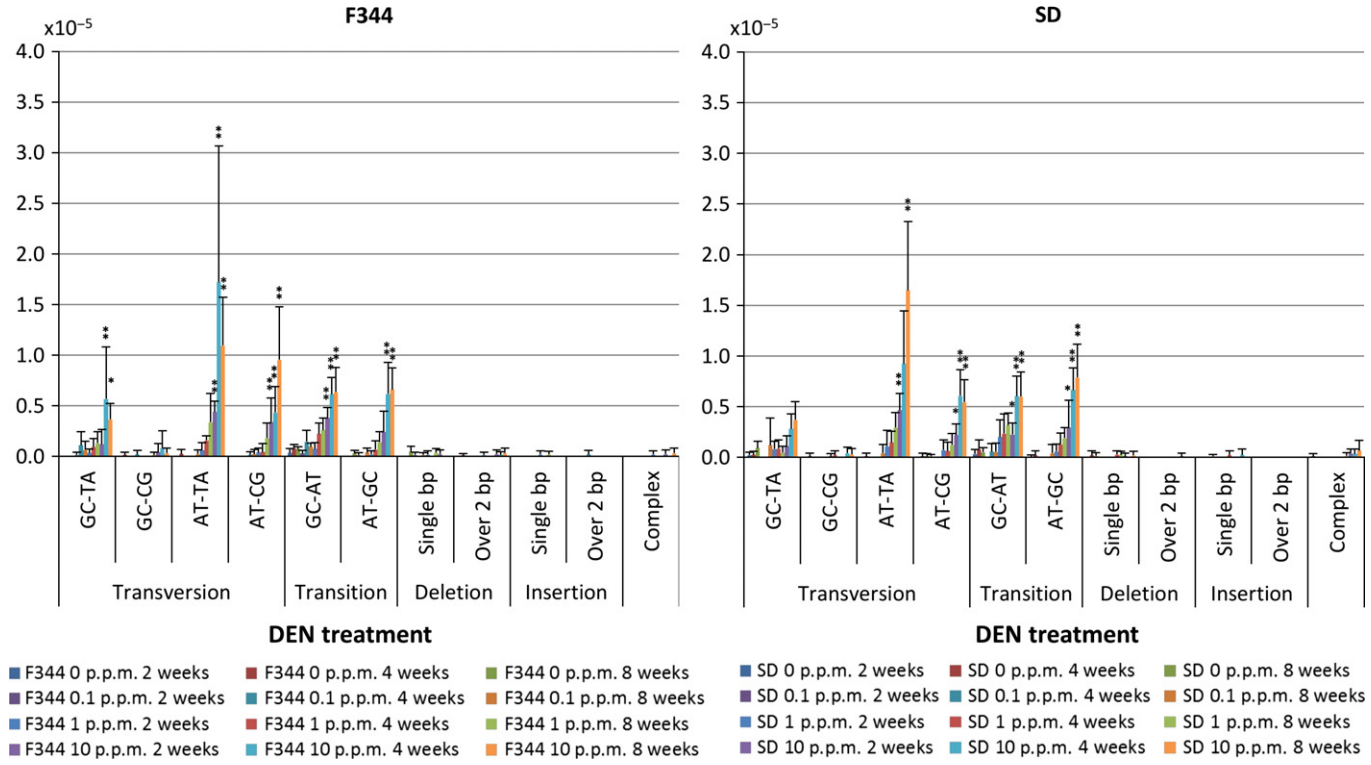

Fig. 6. Mutation spectrum of the *gpt* transgene in the livers of male F344/*gpt* delta and Sprague–Dawley (SD)/*gpt* delta rats administered 0, 0.1, 1, or 10 p.p.m. *N*-nitrosodiethylamine (DEN) in drinking water. **P* < 0.05, ***P* < 0.01 versus 0 p.p.m. DEN group (control).

serum biochemistry values in the DEHP exposure study, no dose-dependent responses were observed, and these differences were sporadic. Thus, we assumed that these differences were incidental. Taken together, our data indicated that *gpt* delta and WT rats (both F344 and SD strains) showed comparable general toxicity responses to DEN and DEHP.

With regard to the comparison of *in vivo* genotoxicity, administration of DEN induced the same level of mutations in F344/*gpt* delta and SD/*gpt* delta rats in both *gpt* and Spi$^-$ assays. The *gpt* mutation spectra were also similar for both strains. Administration of DEN is known to generate various monoalkylated lesions, that is, N^7-ethylguanine, O^4-ethylthymine, N^3-ethyladenine, and O^6-ethylguanine. N^7-ethylguanine further undergoes depurination, resulting in the formation of abasic sites.[15] These lesions cause mutations mainly as consequences of error-prone translesion synthesis. Thus, the most frequent mutations were AT-TA transversions, followed by GC-AT and AT-GC transitions and AT-CG and GC-TA transversions. However, the major mutations found in DEN-induced tumors were GC-AT and AT-GC transitions in the *Ha-ras* gene in C3H/He mice,[16] AT-GC and GC-AT transitions and GC-TA transversions in the *K-ras* gene in A/J mice,[17] and AT-GC transitions in the *H-ras* gene in B6C3F$_1$ mice.[18] The discrepancy in the mutation spectra between these studies and our results could be due to hotspots of examined genes and results of selective amplification. As endogenous *ras* genes are functionally expressed in rats, mutations responsible for clonal expansion would be selectively amplified accompanying tumorigenesis. However, the *gpt* transgene is not expressed in rats, so that it is genetically neutral, which avoids selective pressure *in vivo*.[10] Thus, *gpt* assays can detect a wide spectrum of mutations of the *gpt* transgene that result in loss of its enzymatic activity in *E. coli*. While gene mutation assays are only available in *gpt* delta rats, the equivalent formation of procarcinogenic GST-P-positive foci between *gpt* delta and WT rats implied that similar levels of genotoxicity were induced in both *gpt* delta and WT rats following administration of DEN. In the DEHP treatment group, mutation frequencies of control groups were much higher than that of DEN treatment. The reason for the difference would be that these experiments were independently carried out by different people. We think it is important that the constant protocol and criteria were kept in each experiment so that we are able to compare to mutation frequencies within respective experiments. Although DEHP is generally considered to be non-genotoxic,[12] *lac*Z plasmid-based transgenic mouse mutation assays[19] and several *in vitro* studies have suggested the genotoxic potential of DEHP.[20] Our present results clearly showed that administration of DEHP did not induce gene mutations in either *gpt* mutation frequency or Spi$^-$ mutant assays in both F344 and SD strains of *gpt* delta rats. This is consistent with our previous report.[9] Thus, we concluded that DEHP was non-genotoxic in *gpt* delta rats.

Collectively, these results suggested that *gpt* delta rats (both F344 and SD strains) showed comparable general toxicity responses, including the presence of preneoplastic liver lesions and genotoxicity, to WT rats in response to DEN and DEHP treatment. Therefore, these data supported the validity of the combined assay to detect both general toxicity and genotoxicity simultaneously.

In this study, we also examined whether these toxicities could be detected after short durations of administration. Both the general toxicity of 1.2% DEHP and the genotoxicity of 10 p.p.m. DEN could be detected after 2 weeks of administration, suggesting that the combined assay using *gpt* delta rats was capable of detecting both general toxicity and genotoxicity

after at least 2 weeks of administration. Since mutation frequencies in the *gpt* and *red/gam* transgenes at 4 weeks of administration were higher than those at 2 weeks of administration, longer administration may improve the sensitivity of the *in vivo* genotoxicity test. As 4-week repeated-dose toxicity tests have been widely used for risk assessments, as described in OECD Test Guideline 407,[21] we consider the detection capacity of the combined general toxicity and genotoxicity model at 4 weeks to be an advantage of this *gpt* delta rat model for use in risk assessment. In conclusion, we found that the combined repeated-dose toxicity and genotoxicity assay using *gpt* delta rats was applicable for simultaneous detection of genotoxicity with general toxicity, thereby serving to reduce cancer risks in humans from environmental chemicals.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Body weight gain of F344/*gpt* delta rats treated with 0 or 10 p.p.m. *N*-nitrosodiethylamine (DEN) in the additional experiment.

Fig. S2. Immunohistochemical analysis of GST-P-positive foci in the livers of F344/*gpt* delta rats treated with 0 or 10 p.p.m. of *N*-nitrosodiethylamine (DEN) for 8 weeks in the additional experiment.

Table S1. Relative organ weights of F344/wild type (WT), F344 *gpt* delta, Sprague–Dawley (SD)/WT, and SD/*gpt* delta rats treated with *N*-nitrosodiethylamine (DEN).

Table S2. Relative organ weights of F344/wild type (WT), F344 *gpt* delta, Sprague–Dawley (SD)/WT, and SD/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

Table S3. Serum biochemistry of F344/wild type (WT), F344 *gpt* delta, Sprague–Dawley (SD)/WT, and SD/*gpt* delta rats treated with *N*-nitrosodiethylamine (DEN).

Table S4. Serum biochemistry of F344/wild type (WT), F344 *gpt* delta, Sprague–Dawley (SD)/WT, and SD/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

Table S5. Frequency of mutations in the *gpt* gene in the livers of F344/*gpt* delta rats treated with *N*-nitrosodiethylamine (DEN).

Table S6. Frequency of mutations in the *gpt* gene in the livers of Sprague–Dawley (SD)/*gpt* delta rats treated with *N*-nitrosodiethylamine (DEN).

Table S7. Spi$^-$ mutant frequencies in the livers of F344/*gpt* delta rats treated with *N*-nitrosodiethylamine (DEN).

Table S8. Spi$^-$ mutant frequencies in the livers of Sprague–Dawley (SD)/*gpt* delta rats treated with *N*-nitrosodiethylamine (DEN).

Table S9. Mutation spectrum of the *gpt* gene in livers of F344/*gpt* delta rats treated with *N*-nitrosodiethylamine (DEN).

Table S10. Mutation spectrum of the *gpt* gene in livers of Sprague–Dawley (SD)/*gpt* delta rats treated with *N*-nitrosodiethylamine (DEN).

Table S11. Frequency of mutations in the *gpt* gene in livers of F344/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

Table S12. Frequency of mutations in the *gpt* gene in livers of Sprague–Dawley (SD)/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

Table S13. Spi$^-$ mutant frequencies in livers of F344/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

Table S14. Spi$^-$ mutant frequencies in livers of Sprague–Dawley (SD)/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

Table S15. Mutation spectrum of the *gpt* gene in livers of F344/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

Table S16. Mutation spectrum of the *gpt* gene in livers of Sprague–Dawley (SD)/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP).