DISCUSSION

It is well known that the expression of GGT and GST-P is used to detect hepatocellular foci and tumors in rats and mice, and histochemical and immunohistochemical methods have been developed to visualize these lesions microscopically [3, 23]). GGT has frequently been used as a marker of biochemical alteration in hepatocellular foci and tumors in rats. This membrane-bound enzyme is found at higher levels in fetal and neonatal livers than in normal adult rat livers and appears in biochemically altered hepatocellular foci soon after initiation with chemical carcinogens [9, 10, 12]. GGT catalyzes the transfer of the gammaglutamyl moiety of glutathione to an amino acid acceptor [12, 29]. It is a Phase II drug-metabolizing enzyme which may be induced by drugs, such as phenobarbital, that modify the carcinogenic process. However, it can also be influenced in normal livers by other factors such as diet, strain, age, and sex of the animal [24, 28]. Therefore, it has been suggested that GST-P may be a superior marker for biochemically altered hepatocellular foci in rats, since GST-P expression appears to be limited to hepatocellular foci and tumors in rats, with very little expression occurring in normal liver parenchyma [24, 26, 28]. On the other hand, it is generally recognized that GST-P is not immunohistochemically reactive for liver preneoplastic and neoplastic lesions of mice and there is a disadvantage that almost all the proliferative lesions are not always stained with GGT [6]. In the present study, all treated mice

had GGT-positive foci, but the number of GGT-positive foci was lower than that of hepatocellular foci observed in HE-stained sections in Experiment I. This finding means that almost all the hepatocellular foci can't be detected in GGT histochemistry. On the contrary, in Experiment II, 6 of 6 treated mice had GGT-positive foci, but the multiplicity of GGT-positive foci was two-times higher than that of hepatocellular foci observed in HE-stained sections and GGT-negative foci were also noted in 2 of 6 mice (17 %). In addition, 6 of 6 mice (100 %) had hepatocellular adenomas, while 5 of 6 treated mice (83 %) had GGT-positive adenomas. Furthermore, GGT-negative adenomas were also noted in 5 of 6 mice (83 %). These findings indicate that hepatocellular adenomas observed in HE-stained sections could not be always detected in GGT histochemistry. Regarding hepatocellular carcinomas stained with HE, 3 of 6 treated mice (50 %) had hepatocellular carcinomas, while 1 of 6 treated mice (17 %) and 2 of 6 mice (33 %) had GGT-positive carcinomas and GGT-negative carcinomas, respectively. These findings on hepatocellular carcinomas indicate that some of the carcinomas were negative for GGT. The differences in which the incidence and multiplicity of GGT-positive lesions were not the same as those observed in HE-stained sections might be attributable to the fact that GGT histochemistry can't be performed in the serial section that is continuous to the HE-stained section. However, as shown in the results of our study, we must recognize the fact that almost all the liver proliferative lesions are not always stained with GGT and some of the

carcinomas were negative for GGT.

CK8 and CK18 are known to be distributed in cytoplasmic filament networks and as bands associated with the plasma membrane [1]. A number of *in vitro* experiments and transgenic mouse model studies have shown that CK8/18 carry out essential functions in protecting hepatocytes from a variety of stresses such as griseofulvin, acetaminophen and cadmium [20, 22]. On the other hand, overexpression of CK8/18 in human hepatocellular carcinomas has been previously demonstrated by immunohistochemistry [2, 15]. In addition, CK8/18 has been reported to be a reliable marker of hepatocellular proliferative lesions during early stage of rat hepatocarcinogenesis [16]. As the possible mechanism of overexpression of CK8/18 in hepatocellular tumors in rats, it has been suggested that CK8 and CK18 complex due to CK8 phosphorylation may drive neoplastic transformation of GST-P-positive foci during rat hepatocarcinogenesis leading to the formation of hepatocellular tumors [16]. Phosphorylation of CK8 and CK18 can be increased in primary cultures of mouse hepatocytes and rat liver by some tumor promoters, such as 12-*O*-tetradecanoylphorbol-13-acetate and phenobarbital [5, 16]. Taking into account the above references, it can be suggested that CK8/18 is a useful immunohistochemical marker for hepatocellular proliferative lesions in mouse.

In our previous study, we performed a two-stage liver carcinogenesis experiment in

transgenic mice carrying human prototype of c-Ha-*ras* gene (rasH2 mice) administered fenofibrate for 8 weeks after DEN initiation and reported that the numbers of CK8/18-positive foci were significantly increased in the livers of rasH2 mice [17]. In Experiment I of the present study, hepatocellular foci observed in HE-stained sections were immunohistochemically positive for CK8/18, but foci that could not be detected in HE-stained sections were also positive for CK8/18. The multiplicity of hepatocellular foci that were observed in HE-stained sections and positive for CK8/18 was 10.17 and 18.50, respectively. These findings indicate that more hepatocellular foci could be detected in CK8/18 immunohistochemistry in addition to those observed in HE-stained sections. In Experiment II, hepatocellular foci observed in HE-stained sections were positive for CK8/18, but foci that could not be detected in HE-stained sections were also positive for CK8/18. In addition, hepatocellular foci that are negative for CK8/18 were observed in 5 of 6 mice (83 %). The total multiplicity of hepatocellular foci that were observed in HE-stained sections and positive/negative for CK8/18 was 4.47 and 23.17, respectively. These findings indicate that more hepatocellular foci could be detected in CK8/18 immunohistochemistry in addition to those observed in HE-stained sections. In Experiment II, hepatocellular adenomas observed in HE-stained sections were positive for CK8/18, while hepatocellular adenomas that are negative for CK8/18 were observed in 4 of 6 mice (67 %). The total multiplicity of

hepatocellular adenomas from the treated mice that were observed in HE-stained sections and positive/negative for CK8/18 was 11.17 and 11.17, respectively. These findings indicate that hepatocellular adenomas observed in HE-stained sections could be also detected in CK8/18 immunohistochemistry. Similarly, 3 of 6 treated mice (50 %) had hepatocellular carcinomas that were also positive for CK8/18. There were no hepatocellular carcinomas that were negative for CK8/18 in these treated mice. The total multiplicity of hepatocellular carcinomas from the treated mice that were observed in HE-stained sections and positive for CK8/18 was 1.5 and 1.5, respectively. These findings indicate that hepatocellular carcinomas observed in HE-stained sections could be also detected in CK8/18 immunohistochemistry. Based on the results of our study, it can be suggested that more hepatocellular proliferative lesions can be detected in CK8/18 immunohistochemistry in addition to those observed in HE-stained sections.

On the other hand, we observed several CK8/18-negative foci and adenomas in mice given PBO for 27 weeks after DEN initiation but not in mice subjected to 8-week tumor promoting treatment of PBO. These CK8/18-negative proliferative lesions could be also detected as hepatocellular foci or adenomas in HE-stained sections. However, it is uncertain whether these lesions are positive for GGT histochemistry, since GGT histochemistry can't be performed in the same paraffin section as HE-stained and CK8/18-immunostained sections.

Therefore, we could not clarify the biological role in these CK8/18-negative proliferative lesions, but the finding that these negative lesions can be also recognized as hepatocellular foci and tumors strongly suggests the usefulness of CK8/18 immunohistochemistry for the diagnosis of hepatocellular proliferative lesions in mice.

In conclusion, the results of our study suggest that CK8/18 immunohistochemistry can be used as a useful marker for detecting liver preneoplastic and neoplastic lesions in mice, although further studies using other hepatocarcinogens in mice are necessary to confirm the usefulness of CK8/18 immunohistochemistry for hepatocellular proliferative lesions in mice.

ACKNOWLEDEGMENTS

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LEGENDS TO FIGURES

Fig. 1. Microscopic photographs of HE-stained sections (a, d, g, j and l), CK8/18 immunohistochemistry (b, e, h, k and m) and GGT histochemistry (c, f and i) in the livers of male ICR mice given PBO for 8 or 27 weeks after DEN initiation. Hepatocellular focus in a HE-stained section obtained from a mouse given PBO for 8 weeks (a), that is positive for CK8/18 (b: serial section for Fig. 1a) and positive for GGT (c: different section from Fig. 1a). Hepatocellular adenoma in a HE-stained section obtained from a mouse given PBO for 27 weeks (d), that is positive for CK8/18 (e: serial section for Fig. 1d) and positive for GGT (f: different section from Fig. 1d). Hepatocellular carcinoma in a HE-stained section obtained from a mouse given PBO for 27 weeks (g), that is positive for CK8/18 (h: serial section for Fig. 1g) and positive for GGT (i: different section from Fig. 1g). Hepatocellular adenoma (clear cell type) in a HE-stained section obtained from a mouse given PBO for 27 weeks (j), that is negative for CK8/18 (k). Hepatocellular adenoma (basophilic cell type) in a HE-stained section obtained from a mouse given PBO for 27 weeks (l), that is negative for CK8/18 (m). Bar = 100 μm (a, b, c, g and h) Bar = 500 μm (d, e, i, j, k, l and m) Bar = 1cm (f).

Fig.1

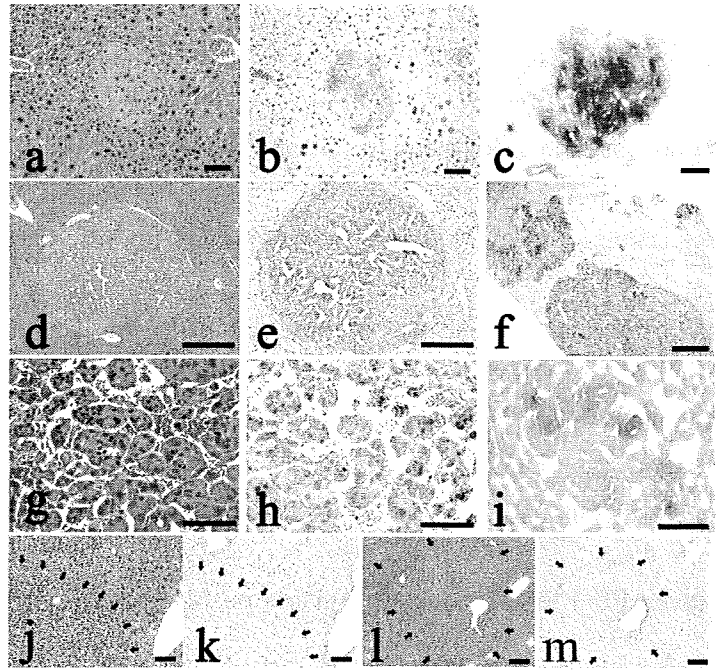

Fig. 1. Microscopic photographs of HE-stained sections (a, d, g, j and l), CK8/18 immunohistochemistry (b, e, h, k and m) and GGT histochemistry (c, f and i) in the livers of male ICR mice given PBO for 8 or 27 weeks after DEN initiation. Hepatocellular focus in a HE-stained section obtained from a mouse given PBO for 8 weeks (a), that is positive for CK8/18 (b: serial section for Fig. 1a) and positive for GGT (c: different section from Fig. 1a). Hepatocellular adenoma in a HE-stained section obtained from a mouse given PBO for 27 weeks (d), that is positive for CK8/18 (e: serial section for Fig. 1d) and positive for GGT (f: different section from Fig. 1d). Hepatocellular carcinoma in a HE-stained section obtained from a mouse given PBO for 27 weeks (g), that is positive for CK8/18 (h: serial section for Fig. 1g) and positive for GGT (i: different section from Fig. 1g). Hepatocellular adenoma (clear cell type) in a HE-stained section obtained from a mouse given PBO for 27 weeks (j), that is negative for CK8/18 (k). Hepatocellular adenoma (basophilic cell type) in a HE-stained section obtained from a mouse given PBO for 27 weeks (l), that is negative for CK8/18 (m). Bar = 100 μm (a, b, c, g and h) Bar = 500 μm (d, e, i, j, k, l and

m) Bar = 1cm (f).
210x297mm (600 x 600 DPI)

Table 1 Incidence and multiplicity of hepatocellular proliferative lesions in ICR mice given PBO for 8 weeks after DEN-initiation (Experiment I)

	HE stain	CK8/18			GGT		
		Positive	Negative	Total	Positive	Negative	Total
Incidence (%)							
Foci	6(100)	6(100)	0(0)	6(100)	6(100)	0(0)	6(100)
Adenoma	0 (0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Carcinoma	0 (0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Multiplicity (No./rat)							
Foci	10.17 ± 7.83	18.50 ± 3.45	0	18.50 ± 3.45	3.50 ± 2.43	0	3.50 ± 2.43
Adenoma	0	0	0	0	0	0	0
Carcinoma	0	0	0	0	0	0	0

DEN: N-diethylnitrosamine, PBO: piperonyl butoxide.
The data represents mean ± S.D.

Table 2 Incidence and multiplicity of hepatocellular proliferative lesions in ICR mice given PBO for 27 weeks after DEN-initiation (ExperimentII)

	HE stain	CK8/18			GGT		
		Positive	Negative	Total	Positive	Negative	Total
Incidence (%)							
Foci	6(100)	6(100)	5(83)	6(100)	6(100)	2(33)	6(100)
Adenoma	6 (100)	6(100)	4(67)	6(100)	5(83)	5(83)	6(100)
Carcinoma	3(50)	3(50)	0(0)	3(50)	1(17)	2(33)	2(33)
Multiplicity (No./rat)							
Foci	4.47 ± 2.31	19.67 ± 8.98	3.50 ± 3.45	23.17 ± 11.21	9.83 ± 5.12	0.33 ± 0.52	10.17 ± 4.96
Adenoma	11.17 ± 3.66	9.83 ± 3.06	1.33 ± 1.51	11.17 ± 3.54	2.83 ± 3.31	2.67 ± 1.50	5.50 ± 3.94
Carcinoma	1.50 ± 2.08	1.50 ± 1.97	0	1.50 ± 1.97	0.17 ± 0.41	0.29 ± 0.49	0.50 ± 0.84

DEN: N-diethylnitrosamine, PBO: piperonyl butoxide.
The data represents mean ± S.D.

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Detection of oxidative DNA damage, cell proliferation and *in vivo* mutagenicity induced by dicyclanil, a non-genotoxic carcinogen, using *gpt* delta mice

Takashi Umemura[a,*], Yuichi Kuroiwa[a], Masako Tasaki[a], Toshiya Okamura[a], Yuji Ishii[a], Yukio Kodama[b], Takehiko Nohmi[c], Kunitoshi Mitsumori[d], Akiyoshi Nishikawa[a], Masao Hirose[a]

[a] *Divisions of Pathology, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b] *Divisions of Toxicology, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[c] *Divisions of Genetics and Mutagenesis, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[d] *Laborartory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8, Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan*

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Abstract

To ascertain whether measurement of possible contributing factors to carcinogenesis concurrently with the transgenic mutation assay is useful to understand the mode of action underlying tumorigenesis of non-genotoxic carcinogens, male and female *gpt* delta mice were given dicyclanil (DC), a mouse hepatocarcinogen showing all negative results in various genotoxicity tests, at a carcinogenic dose for 13 weeks. Together with *gpt* and Spi$^-$ mutations, thiobarbituric acid-reactive substances (TBARS), 8-hydroxydeoxyguanosine (8-OHdG) and bromodeoxyuridine labeling indices (BrdU-LIs) in the livers were examined. Whereas there were no changes in TBARS levels among the groups, significant increases in 8-OHdG levels and centrilobular hepatocyte hypertrophy were observed in the treated mice of both genders. In contrast, BrdU-LIs and liver weights for the treated females, but not the males were significantly higher than those for the controls. Likewise, the *gpt* mutant frequencies (MFs) in the treated females were significantly elevated, GC:TA transversion mutations being predominant. No significant alterations were found in the *gpt* MFs of the males and the Spi$^-$ MFs of both sexes. The results for the transgenic mutation assays were consistent with DC carcinogenicity in terms of the sex specificity for females. Considering that 8-OHdG induces GC:TA transversion mutations by mispairing with A bases, it is likely that cells with high proliferation rates and a large amounts of 8-OHdG come to harbor mutations at high incidence. This is the first report demonstrating DC-induced genotoxicity, the results implying that examination of carcinogenic parameters concomitantly with reporter gene mutation assays is able to provide crucial information to comprehend the underlying mechanisms of so-called non-genotoxic carcinogenicity.

Keywords: 8-Hydroxydeoxyguanosine; Cell proliferation; *gpt* delta mice; Dicyclanil

1. Introduction

The standard battery of genotoxicity tests consisting of an *in vitro* test for gene mutations in bacteria, an *in vitro* test for chromosomal damage and/or gene

* Corresponding author. Tel.: +81 3 3700 1141; fax: +81 3 3700 1425.
E-mail address: umemura@nihs.go.jp (T. Umemura).

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mutations in mammalian cells and an *in vivo* test for chromosomal damage in rodent hematopoietic cells is usually applied in order to identify genotoxicity of environmental chemicals such as pesticides, food additives and pharmaceuticals [1]. However, the existence of discrepancies between genotoxicity and *in vivo* long-term carcinogenicity is well known [2]. There are several reasons which may explain the occurrence of false negative or positive results. For instance, although most carcinogens require biotransformation to DNA reactive species for the purpose of exerting genotoxic effects, the enzyme systems to metabolize xenobiotics in both bacteria and mammalian cells using *in vitro* assays are lacking or are expressed to only a limited extent [3]. Likewise, in *in vivo* short-term assays, it is doubtful whether target cells are exposed to test chemicals at adequate doses for a sufficient period of time, partly because of test chemical toxicity and/or a low biotransformation capacity in hematopoietic cells [3]. Thus, it is a natural consequence that alternative batteries of *in vitro* and/or *in vivo* genotoxicity tests do not fully make up the gap [4], which means we must focus our attention on the mode of action in terms of the risk assessment for environmental agents.

In this respect, reporter gene-transgenic rodents may be useful tools to predict carcinogenicity because studies can be performed with similar protocols as for the long-term bioassay [5]. Transgenic mutation assays also have the advantage of allowing a battery of other *in vivo* mutation assays such as micronucleus tests in the same animals [6]. Additionally, various proposed mechanisms underlying the actions of direct genotoxic carcinogens (e g. generation of DNA adducts) [7], indirect genotoxic carcinogens (e.g. aneugenicity or oxidative DNA damage) [8] and non-genotoxic carcinogens (e.g. methylation, mitogenicity or cytotoxicity-associated cell proliferation) [9–11] are able to be investigated concurrently with transgenic mutation assays. In fact, we have reported that simultaneous analysis of glutathione *S*-transferase placental form (GST-P) immunohistochemistry in the livers of *gpt* delta rats provided crucial information for understanding the chemical carcinogenesis of 2-amino-3-methylimidazo[4,5-*f*]quinoline, *N*-nitrosopyrrolidine and di(2-ethylhexyl)phthalate [12]. Also, finding of increases in hepatocyte proliferation together with a lack of the transgene mutations in *gpt* delta mice given flumequine, an anti-bacterial quinolone agent, helped us to define this mouse liver carcinogen as a genuine promoter [13].

Dicyclanil (4,6-diamino-2-cyclopropylaminopyrimidine-5-carbonaitrite; DC), a pyrimidine-derived insect growth regulator, has given all negative results for *in vitro* reverse mutations, gene mutations, chromosomal aberrations, unscheduled DNA synthesis, *in vivo* micronucleus formation [14] and alkaline single cell electrophoretic change [15]. However, DC has been reported to be a hepatocarcinogen in female mice [14] and recent studies revealed a possible involvement of oxidative stress [16]. In the present study, to explore the mode of action underlying DC hepatocarcinogenesis, lipid peroxidation, 8-hydroxydeoxyguanosine (8-OHdG) and hepatocyte proliferation in the livers of male and female *gpt* delta rats given DC at a carcinogenic dose were examined along with the transgenic mutation assay.

2. Materials and methods

2.1. Chemicals

Dicyclanil was kindly provided by Novartis Animal Health Co., Ltd. (Basel, Switzerland) (Fig. 1). Alkaline phosphatase and bromodeoxyuridine (BrdU) were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and nuclease P1 from Yamasa Co. (Chiba, Japan).

2.2. Animals and treatments

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Male and female B6C3F1 *gpt* delta mice carrying 80 tandem copies of the transgene lambda EG10 in a haploid genome status were raised by mating of C57BL/6 *gpt* delta and non-transgenic C3H/He mice (Japan SLC, Inc., Shizuoka, Japan). Ten male and 10 female B6C3F1 *gpt* delta mice were each randomized by weight into two groups. They were housed in a room with a barrier system, and maintained under the following constant conditions: temperature of 23 ± 2 °C, relative humidity of 55 ± 5%, ventilation frequency of 18 times/h and a 12-h light:12-h dark cycle, with free access to CRF-1 basal diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Starting at 8 weeks of age the mice were

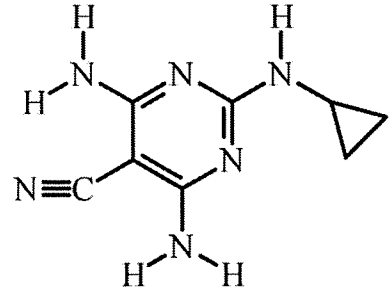

Fig. 1. Chemical structure of dicyclanil (DC).

fed diet containing 0.15% DC or maintained as non-treatment controls for 13 weeks. The dose of DC was a reported carcinogenic dose in a 18-month carcinogenicity study [14]. All mice received BrdU (100 mg/kg) by i.p. injection once a day for the final 2 days of exposure and once on the final day, 2 h before killing, as previous described [17]. All mice were killed at week 13 by exsanguination under ether anesthesia and the livers were immediately removed and weighed; slices were fixed in buffered formalin for hematoxylin and eosin (H&E) staining or BrdU immunohistochemistry. Remaining pieces of liver were frozen with liquid nitrogen and stored at −80 °C until measurement of 8-OHdG in nuclear DNA, and levels of thiobarbituric acid-reactive substances (TBARS) and performance of mutation assays.

2.3. Measurement of nuclear 8-OHdG

In order to prevent 8-OHdG formation as a byproduct during DNA isolation [18], liver DNA was extracted by a slight modification of the method of Nakae et al. [19]. Briefly, nuclear DNA was extracted with a commercially available DNA Extracter WB Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing an antioxidant NaI solution to dissolve cellular components. For further prevention of autooxidation in the cell lysis step, deferoxamine mesylate (Sigma Chemical Co.) was added to the lysis buffer [20]. DNA was digested to deoxynucleotides with nuclease P1 and alkaline phosphatase and levels of 8-OHdG (8-OHdG/10^5 deoxyguanosine) were assessed by high-performance liquid chromatography (HPLC) with an electrochemical detection system (Coulochem II, ESA, Bedford, MA, U.S.A.).

2.4. Measurement of TBARS

Malondialdehyde (MDA, nmol/g) was assessed as an index of lipid peroxidation by the method of Uchiyama and Mihara [21]. In brief, a 0.15 g portion of liver was homogenized with 1.35 mL of 1.15% KCl solution. To 0.05 mL of this homogenate, 0.2 mL 8.1% SDS and 3.0 mL 0.4% 2-thiobarbituric acid in 10% acetic acid solution (pH 3.5) were added, followed by heating in a water bath at 95 °C for 60 min. After cooling, 5.0 mL of *n*-butanol and pyridine (15:1, v/v) and 1.0 mL distilled water were added and the mixture was centrifuged at 1870 × *g* for 10 min. TBARS were measured with a Hitachi F-2500 fluorescence spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan) at 515 nm (excitation) and 553 nm (emission) in the butanol/pyridine phase.

2.5. Immunohistochemical procedures

For immunohistochemical staining of BrdU, sections were treated sequentially with normal horse serum, monoclonal mouse anti-BrdU (1:80), biotin-labeled horse anti-mouse IgG (1:400) and avidin–biotin–peroxidase complex (ABC) after denaturation of DNA with 4N HCl. The sites of peroxidase binding were demonstrated by incubation with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.). The immunostained sections were lightly counterstained with hematoxylin for microscopic examination.

2.6. Cell proliferation quantification

For each animal at least 3000 hepatocytes were counted. The labeling index (BrdU-LI) was calculated as a percentage value derived from the number of labeled cells divided by the total number of cells counted.

2.7. In vivo mutation assays

6-TG and Spi$^-$ selections were performed as previously described [5]. Briefly, genomic DNA was extracted from the livers, and lambda EG10 DNA (48 kb) was rescued as the lambda phage by *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. In order to determine the total number of rescued plasmids, 3000-fold diluted phages were used to infect YG6020, and poured on the plates containing chloramphenicol without 6-TG. The plates were then incubated at 37 °C for selection of 6-TG-resistant colonies. Positively selected colonies were counted on day 3 and collected on day 4. The mutant frequency (MF) was calculated by dividing the number of *gpt* mutants by the number of rescued phages.

For the 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. Next day, plaques (Spi$^-$ candidates) were punched out with sterilized glass pipetters and the agar plugs were suspended in SM buffer. In order to confirm the Spi$^-$ phenotype of candidates, the suspensions were spotted on three types of plates where XL-1 Blue MRA, XL-1 Blue MRA P2 or WL95 P2 strains were spread with soft agar. Real Spi$^-$ mutants, which made clear plaques on every plate, were counted.

For characterizing the mutation spectra of *gpt* mutants, a 739 bp DNA fragment containing the 456 bp coding region of the *gpt* gene was amplified by PCR as described previously [5]. DNA sequencing was performed with Big Dye™ Terminater Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems).

2.8. Statistical evaluation

For statistical analysis, the Student's *t*-test was used to compare body and liver weights, as well as quantitative data for BrdU-LIs, TBARS, 8-OHdG and MFs between groups.

3. Results

3.1. Body and liver weights

Data for final body and liver weights in male and female *gpt* delta mice given DC are shown in Table 1. Although all of the values in DC-treated male mice were significantly lower than those in the controls, liver and relative liver weights in the treated female mice were significantly increased as compared with the controls.

3.2. In vivo mutation assays

Data for *gpt* MFs analyzed by 6-TG selection are summarized in Table 2. While there were no significant differences in the MFs between the male groups, the MF in the DC-treated females was 2.23 ± 0.55, which was significantly higher than the control value (0.48 ± 0.29). To characterize *gpt* mutations due to DC exposure, they were analyzed by DNA sequencing (Table 3). In the DC-treated female mice, G:C pairs were the preferred bases for mutation, accounting for 67.3% of the mutations (70/104). In the base substitutions, the predominant type was GC:TA (34/104, 32.7%) followed by GC:AT (26/104, 25.0%) and GC:CG (10/104, 9.6%). In addition, 16.3% (17/104) of mutant colonies were identified as carrying single- or multiple deletions. As shown in Table 4, Spi^- MFs in the treated male and female mice were not significantly different from those in the relevant controls.

Table 1
Body liver and relative liver weights of *gpt* delta mice given DC

Sex	Treatment	No. of mice	BW (g)	Liver (g)	Liver/BW (%)
Male	Control	5	32.6 ± 1.6	1.66 ± 0.21	5.07 ± 0.54
	Dicyclanil	5	29.0 ± 1.1**	1.42 ± 0.08*	4.89 ± 0.21*
Female	Control	5	25.0 ± 0.6	1.06 ± 0.05	4.25 ± 0.16
	Dicyclanil	5	24.0 ± 0.8	1.27 ± 0.07**	5.29 ± 0.12**

* $p < 0.05$ vs. Control.
** $p < 0.01$ vs. Control.

Table 2
gpt MFs in the livers of *gpt* delta mice given DC

Sex	Treatment	Animal No.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies		Mutant frequency ($\times 10^{-6}$)	Mean ± S.D.
				Total	Independent		
Male	Control	1	9.0	6	5	0.56	0.42 ± 0.20
		2	10.7	7	7	0.66	
		3	6.9	4	3	0.44	
		4	9.5	4	3	0.31	
		5	12.4	2	2	0.16	
	Dicyclanil	6	11.5	6	5	0.43	0.48 ± 0.31
		7	10.7	1	1	0.09	
		8	8.9	8	8	0.90	
		9	6.1	4	4	0.66	
		10	9.7	3	3	0.31	
Female	Control	11	8.8	4	4	0.45	$0.48 + 0.29$
		12	6.5	5	2	0.31	
		13	12.6	2	2	0.16	
		14	7.6	7	7	0.93	
		15	8.7	5	5	0.57	
	Dicyclanil	16	7.0	31	19	2.72	2.23 ± 0.55*
		17	13.0	29	26	2.01	
		18	11.1	51	25	2.26	
		19	7.0	11	10	1.42	
		20	8.7	34	24	2.75	

* $p < 0.01$ vs. Control.