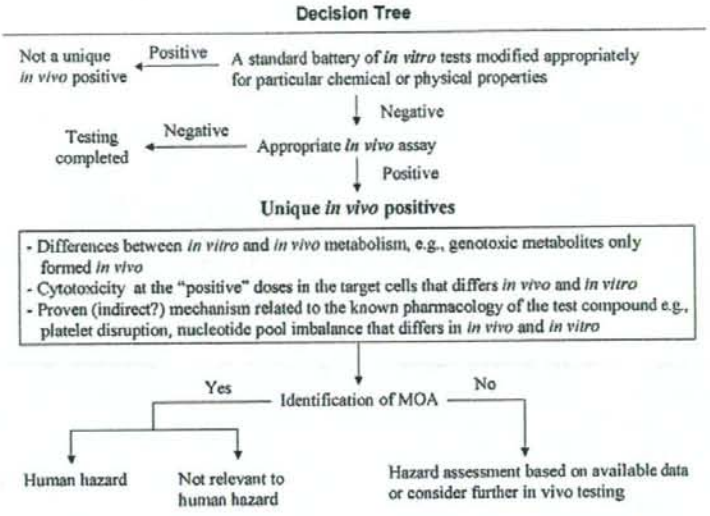

Fig. 1. Decision tree for evaluating possible 'unique' *in vivo*-positive compounds.

system can detect genotoxic activity, but for an unknown compound, the variations are not always obvious. The pharmacology of the test material may be important in limiting *in vitro* responses, e.g., depletion of nucleotide pools and/or related effects on folate metabolism (e.g., Alimta®, SASP and SP). In these cases, *in vitro* detection may be possible if chromosome damage is assessed in human peripheral lymphocytes rather than in Chinese hamster cells.

There may be differences between *in vitro* and *in vivo* metabolism, as is the case for urethane. Some compounds may be metabolized by gut flora *in vivo* (e.g., SASP) to release genotoxic metabolites. Higher exposures may be possible *in vivo* than *in vitro* (Roche example 1, Pharmacia example). Certain genotoxic receptor kinases appear to be difficult to detect as genotoxic agents *in vitro* (in established cell lines), but are clearly active in rodent bone marrow micronucleus tests (Pfizer, Merck, Pharmacia and AstraZeneca examples). From the research done at AstraZeneca with their example, it appears that such compounds may be aneugens. Further research using different primary or near-primary cells may provide a means of detecting these compounds *in vitro*. It is likely that these compounds have been selected as kinase inhibitors in *in vitro* model systems. It is feasible that the cells used may provide models where genotoxicity could be detected *in vitro*. If these compounds still prove difficult to detect *in vitro*, prudence dictates that bone marrow micronucleus tests be performed early in the toxicological programme for future drug candidates in this pharmacological class.

A decision tree is provided as a guide for the evaluation of compounds that appear to be genotoxic agents *in vivo* but not *in vitro* (Fig. 1).

5. Implications for different product classes

5.1. Pharmaceuticals

The ICH M3 guideline 'Non-clinical safety studies for the conduct of human clinical trials for pharmaceuticals' states that 'Prior to first human exposure, *in vitro* tests for the evaluation of mutations and chromosomal damage are generally needed ... The standard battery of tests for genotoxicity should be completed prior to the initiation of Phase II studies'. Based on the evidence above, the IWGT working group advises that the entire standard battery should be completed before the initiation of Phase I human studies provided that the characteristics of the compound under question imply certain mechanisms of metabolic activation, receptor interaction or pivotal cell cycle targets that were not fully taken into account under *in vitro* genotoxicity testing conditions. For pharmaceutical candidate compounds, such information is generally available from the standard metabolism and receptor panel studies normally carried out prior to initiating clinical trials.

5.2. Cosmetics

A European Union Directive prohibits the use of animal tests for the development of new cosmetics starting in 2009 (2003/15/EC). The working group feels that reliance on *in vitro*-only test batteries may run the risk of missing some important genotoxic agents. Given the nature of cosmetics, these risks may be lower than for pharmaceuticals. Cosmetics are normally much less well characterized than pharmaceuticals regarding their effects on biological systems. A conscious decision not to do *in vivo* genotoxicity tests can be made for low exposures, knowledge about closely related compounds, etc. However, for novel cosmetic chemicals the information gained from *in vivo* genotoxicity tests may be necessary for a comprehensive risk assessment. This is supported by two additional considerations: (a) such compounds are normally not tested for carcinogenicity in animals (as opposed to pharmaceuticals) and (b) novel chemicals being used in some cosmetic formulations are being designed that may have a pharmacological mechanism of action. In such cases, and when there is significant use/systemic exposure, etc. more comprehensive testing than is provided by *in vitro* genotoxicity tests may be needed.

The analysis below also suggests that in those situations where there may be such a risk, such chemicals could be avoided as development compounds for cosmetics.

5.3. Industrial chemicals

Industrial chemicals, like cosmetics are normally much less well characterized than pharmaceuticals regarding their effects on biological systems. A conscious decision not to do *in vivo* genotoxicity tests can be made on absence of or low exposure, protection measures to limit exposure, knowledge about closely related compounds, etc. Where the potential for human exposure is high, the level of concern may drive the need for *in vivo* tests despite negative *in vitro* tests, again as for cosmetics carcinogenicity tests are not normally carried out for these chemicals, so additional genotoxicity testing may be required.

5.4. Food additives

There is a potential for long-term exposure to compounds in this product class; at present *in vivo* data on key compounds is often lacking. Again, a small subset of compounds with negative *in vitro* data may be worthy of *in vivo* testing.

6. *In vitro*-only test batteries

The testing of compounds with both *in vitro* and *in vivo* assays indicates that there are small subsets of compounds where conventional *in vitro* test batteries may miss inherent genotoxicity. Therefore, there is a case for including *in vivo* tests in regulatory test guidelines to ensure that the genotoxicity of such compounds is evaluated in an adequate manner. However, it can be envisioned that most compounds from these subsets could be identified prior to *in vivo* testing. Thus, for compounds that are negative *in vitro*, but are suspected of having activity that may be more easily detected *in vivo* (i.e., where there is prior knowledge that metabolism is likely to be different *in vitro* and *in vivo*; where the compound is likely to affect nucleotide pools through folate disruption; where metabolism by gut flora is suspected; where receptor kinase inhibitors are to be tested; where cytotoxicity at the 'positive' doses in the target cells differs *in vivo* and *in vitro* such that higher exposures may be achievable *in vivo*), it is prudent to conduct *in vivo* tests. The working group points out that modified *in vitro* test batteries may be capable of identifying many of those compounds currently shown to be positive only *in vitro*, and notes that reliance solely on *in vitro* tests may be acceptable in some cases of limited exposure.

Whilst the discussion above is relevant to the sensitivity of current genotoxicity test batteries, the problem remains of the poor specificity of currently used *in vitro* test batteries for finding non-carcinogens negative [40]. At present, *in vivo* tests are often used to determine if the genotoxic potential seen *in vitro* is realised in the whole animal. All-*in vitro* test batteries would result in many compounds that in fact do not pose a genotoxic hazard in the whole animal or man being discarded or labelled as genotoxic agents. Thus, there is a major challenge to develop new *in vitro* assays with higher overall accuracy for resolving genotoxic carcinogens from non-genotoxic non-carcinogens or to modify existing assays to improve specificity.

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cology Consulting Services) and Veronique Thybaud (sanofi-aventis).

Appendix A. IWGT questionnaire: examples of 'unique' *in vivo* positives

1. Do you have data on unequivocal *in vivo* genotoxic agents, where all standard *in vitro* tests are negative? If so, please proceed to the next questions.
2. Please give details of:
   (i) Chemical structure (if not confidential);
   (ii) Relevant mode of action if known, e.g., topoisomerase inhibitor; spindle poison; alkylating agent, etc.
3. Please list *in vitro* tests used in primary screening for each chosen compound.
4. Please submit summary test data for primary *in vitro* tests, i.e., test concentrations; mean plate counts for each strain (Ames test); mean *Tk* mutant frequencies and RTG scores for each concentration and time point (mouse lymphoma assay); mean aberration counts and mitotic index scores (chromosome aberration assays) for each concentration and time point, etc.
5. Please submit summary test data for primary positive *in vivo* tests, e.g., PCE/NCE ratios and mean micronucleated PCE counts for each time point and dose (*in vivo* micronucleus test), etc.
6. If further testing was carried out to explore differences between *in vitro* and *in vivo* test results, e.g., qualitative and quantitative comparisons of metabolites *in vitro* and *in vivo*, please give summary details and outcome of test.
7. If further testing was carried out *in vitro*, e.g., variations in composition and/or nature of the metabolic activation system; tests on isolated metabolites; use of different test systems, etc., please provide details and summary data as above.

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Research Article

Mutations in the Lungs of *gpt* delta Transgenic Mice Following Inhalation of Diesel Exhaust

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Diesel exhaust (DE) is a major airborne pollutant of urban areas. It contains various polycyclic aromatic hydrocarbons (PAH) and nitrated PAHs. In this study, *gpt* delta mice were treated with inhalation of 1 or 3 mg m^{-3} DE, or a single intratracheal instillation of diesel exhaust particles (DEP) or DEP extract. In the lungs of mice treated with inhalation of 3 mg m^{-3} DE for 12 weeks, the mutant frequency (MF) was 3.2-fold higher than that of the control group (1.90×10^{-5} and 0.59×10^{-5}, respectively). An instillation of DEP and DEP extract resulted in a significant dose-dependent linear increase in MF. In mice treated with 0.5 mg DEP and 0.2 mg DEP extract, the MFs were 3.0- and 2.7-fold higher than that of the control group, respectively. The mutagenic potency (MF mg^{-1}) of DEP extract (5.6×10^{-5}) was double that of DEP (2.7×10^{-5}), suggesting that the mutagenicity of the latter is derived primarily from compounds in the extract, which itself is responsible for ca. 50% of the weight of DEP. G:C→A:T transitions were the predominant *gpt* mutation induced by all three treatments and G:C→T:A transversions were induced by DEP and DEP extract. Guanine bases centered in nucleotide sequences such as GGA, TGA, CGG, and CGT were the major mutation targets of all three treatments. Thus, our results suggest that the mutagens contained in DEP such as PAH and nitrated PAHs induce mutations and may be responsible for carcinogenesis caused by inhalation of DE. Environ. Mol. Mutagen. 48:682–693, 2007.

Key words: diesel emission; diesel exhaust particles; 6-thioguanine selection

INTRODUCTION

Diesel exhaust (DE) is generated by the combustion of light oil and is implicated as a causative agent of lung cancer and allergic respiratory disease, including bronchial asthma [Muranaka et al., 1986]. Diesel exhaust particles (DEP) contain various potent carcinogens and mutagens such as polycyclic aromatic hydrocarbons [PAHs; e.g. benzo[*a*]pyrene (B[*a*]P)] and nitrated PAHs (nitro-PAHs), e.g. 1,6-dinitropyrene (1,6-DNP) [Harris, 1983]. Although some of the compounds in DE have been identified as pulmonary carcinogens in animals [Brightwell et al., 1986], the predominant mutagens remain to be determined.

In rat and mouse lungs, exposure to DEP through inhalation or intratracheal instillation causes oxidative DNA damage [Nagashima et al., 1995; Iwai et al., 2000] and DNA adduct formation [Gallagher et al., 1994; Sato

Abbreviations: B[*a*]P, benzo[*a*]pyrene; DE, diesel exhaust; DEP, diesel exhaust particles; DNP, dinitropyrene; MF, mutant frequency; PAH, polycyclic aromatic hydrocarbons; SPM, suspended particulate matter; 6-TG, 6-thioguanine.

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et al., 2003]. Prolonged inhalation exposure results in respiratory tract tumors in rats [Mauderly et al., 1987; Nikula et al., 1995; Iwai et al., 1997; Valberg et al., 1999]. In rat lung adenomas and adenocarcinomas induced by intratracheal instillation of DEP, point mutations in Codons 12 and 13 of the K-*ras* oncogene have previously been identified [Iwai et al., 1997]. Ichinose et al. [1997] demonstrated the induction of lung tumors in ICR mice treated with 0.05 and 0.1 mg DEP once a week for 10 weeks via intratracheal instillation (30–31%). However, whether or not DE inhalation induces lung tumors in mice remains a matter of debate [Mauderly et al., 1996].

The mutagenicity of DEP extracts has been evaluated in vitro using *Salmonella typhimurium* TA98 assay without an exogenous metabolic activation system (S9 mixture), in which frameshift mutations were found to predominate [Salmeen et al., 1984; Østby et al., 1997; Rivedal et al., 2003]. Although we demonstrated the mutagenicity of DE in vivo using Big Blue® rats [Sato et al., 2000], further studies on its pulmonary effects are required for an assessment of the health risks of air pollution. The present study was undertaken to ascertain the mutant frequency (MF) and spectrum of the mutations induced by inhalation of DE or intratracheal instillation of DEP or DEP extract. To evaluate mutagenicity in vivo, we used *gpt* delta transgenic mice carrying the lambda phage EG10 as a transgene [Nohmi et al., 2000; Thybaud et al., 2003]. When rescued phages are used to infect *E. coli* expressing Cre recombinase, they are converted into plasmids harboring the chloramphenicol (Cm) resistance and guanine phosphoribosyltransferase (*gpt*) genes. *gpt* mutants are selected using plates containing Cm and 6-thioguanine (6-TG).

In this study, inhalation of 3 mg m^{-3} DE (as suspended particulate matter [SPM]) significantly increased MF in a duration-dependent manner. Instillation of DEP (0, 0.125, 0.25, and 0.5 mg) or DEP extract (0, 0.05, 0.1, and 0.2 mg) increased MF in a linear and dose-dependent manner. The mutagenic potency (MF mg^{-1}) of DEP (2.7×10^{-5}) was half of that induced by DEP extract (5.6×10^{-5}). This suggests that the mutagenicity of DEP is derived mainly from compounds in the extract, since ca. 50% of the weight of DEP is provided by the extract. These data suggest that components in the DEP extract were the primary cause of DE-induced mutagenesis in the lungs of mice.

MATERIALS AND METHODS

Treatment of Mice

gpt delta mice carry ca. 80 copies of lambda EG10 DNA on each Chromosome 17 in a C57BL/6J background [Nohmi et al., 1996]. Exposure to DE (12 hr day^{-1}, 7 day $week^{-1}$) was performed in a chamber provided by the National Institute for Environmental Studies [Takano et al., 1998]. A diagram of the chamber can be found in Sagai et al. [1993]. In brief, DE was generated by a computer-controlled light duty (3059 cc) four-cylinder diesel engine (4JG2-type, Isuzu Automobile Company, Tokyo, Japan) run at 1,500 rpm under a load of 10 torques (kg m^{-1}), using standard diesel fuel. The DE generated by this system was injected into a stainless steel dilution tunnel (300 mm diameter × 8,400 mm length), then introduced into the 2.3 m^3 chamber. The residence time of DE in the dilution tunnel was 8.48 sec, and the flow rate in the inhalation zone of the chamber was 0.81 m sec^{-1}. Mice in the control group were maintained in a chamber supplied with filtered clean air. DEP concentration in the chamber was monitored using an Anderson Air Sampler (Shibata Science Technology, Tokyo, Japan); CO concentration was monitored with a CGT-10-3-A portable gas monitor (Shimadzu, Kyoto, Japan). NO and NO_2 concentrations were measured using an NO-NO_2-NO_x analyzer model 43 (Thermo Environmental Instruments, MA). SO_2 concentration was determined by a fluorescent SO_2 analyzer model 8850 (Monitor Labs, CO). The concentrations of DEP (mg m^{-3}), CO (ppm), NO (ppm), NO_2 (ppm), and SO_2 (ppm) were as follows: in the chamber of filtered air, 0.01 ± 0.00 (mean ± SD), 0, 0, 0.15 ± 0.03, and 0.020 ± 0.002, respectively; in 1 mg m^{-3} DE, 0.97 ± 0.16, 10.1 ± 1.5, 11.8 ± 1.5, 4.45 ± 0.64, and 0.204 ± 0.032, respectively; and in 3 mg m^{-3} DE, 2.84 ± 0.47, 25.1 ± 2.0, 26.2 ± 2.4, 9.18 ± 1.83, and 0.320 ± 0.037, respectively. Over 99% (in mass) of DEP was in the 10–470 nm diameter range, and the mass peak was measured at 110 nm diameter using a scanning mobility particle size analyzer (Model 3034, TSI, Tokyo, Japan). The number of DEP particles was estimated as 1.0×10^6 cm^{-3} in 1 mg m^{-3} DE. Three to five, 7-week-old mice were exposed to 1 or 3 mg m^{-3} DE (as SPM) for 4, 12, or 24 weeks. Eleven mice (control group) were maintained in a chamber of filtered clean air. The animals were sacrificed 3 days following the last exposure and their lungs were removed, frozen in liquid nitrogen, and stored at −80°C.

DEP were collected as described previously [Sagai et al., 1993] and the DEP extract was prepared by Dr. Hayakawa [Hayakawa et al., 1997]. In brief, DEP was dispersed in benzene-ethanol (3:1, v/v) and the mixture sonicated. The precipitate was removed by filtration and the supernatant concentrated using a rotary evaporator. The dried concentrate was used as the DEP extract. DEP (0.125, 0.25, or 0.5 mg) or DEP extract (0.05, 0.1, or 0.2 mg) was suspended in 50 μL PBS at pH 7.4 (Gibco BRL, Life Technology, Grand Island, NY) containing 0.05% Tween 80 (Nacalai Tesque, Kyoto, Japan) and 1% DMSO. Each dose was administered to three mice (9-week-old) using a single intratracheal instillation. Each animal was anesthetized with 4% halothane (Hoechst Japan, Tokyo, Japan) until unresponsive to a tactile stimulus. The animal was placed on a restraining board with linen threads to hold the mouth open, and the DEP or DEP extract was instilled into the trachea via a polyethylene tube [Takano et al., 2002; Hashimoto et al., 2005]. As controls, three mice were treated with 50 μL PBS containing 0.05% Tween 80 and 1% DMSO. Mice were sacrificed 14 days after DEP or DEP extract treatment [Suzuki et al., 1999] and their lungs were removed, frozen in liquid nitrogen, and stored at −80°C.

gpt Mutation Assay

The *gpt* assay was performed as described previously [Nohmi et al., 2000]. Genomic DNA was extracted from lung tissue using the Recover-Ease DNA Isolation Kit (Stratagene, La Jolla, CA) and Lambda EG10 phages were rescued using Transpack® Packaging Extract (Stratagene). *E. coli* YG6020 was infected with the phage and spread on M9 salt plates containing Cm and 6-TG [Nohmi et al., 2000], then incubated for 72 hr at 37°C. This enabled selection of colonies harboring a plasmid carrying the gene for chloramphenicol acetyltransferase (CAT), as well as a mutated *gpt*. Isolates exhibiting the 6-TG-resistant phenotype were cultured overnight at 37°C in LB broth containing 25 μg mL^{-1} Cm, then harvested by centrifugation (7,000 rpm, 10 min), and stored at −80°C.

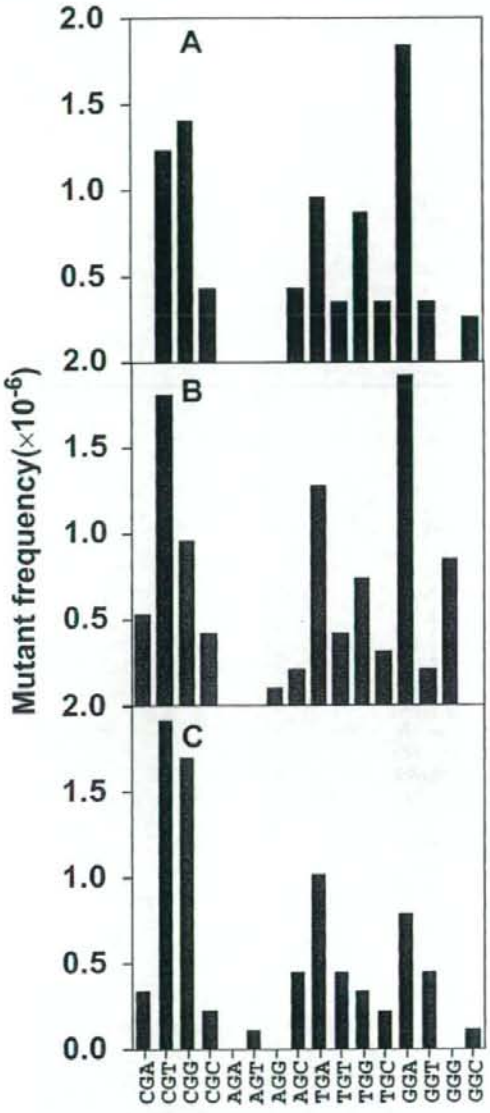

Fig. 1. CG sequence-dependence of *gpt* mutations induced by DE (A), DEP (B), and DEP extract (C).

PCR and DNA Sequencing of the 6TG-Mutants

A 739 bp DNA fragment containing *gpt* was amplified by PCR and sequenced as described previously [Nohmi et al., 2000; Hashimoto et al., 2005]. Sequencing was performed using the Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an Applied Biosystems model 3730xl DNA analyzer.

Mutant Frequency

gpt MFs were calculated by dividing the number of colonies growing on (Cm + 6-TG) agar plates by the number growing on Cm agar plates. MFs for each type of mutation in Figure 1 were calculated by dividing the total number of each type of mutant in each group by the total number of colonies growing on Cm agar plates in each group.

Statistical Analyses

All data are expressed as mean ± SD. The statistical significance of DE treatment was analyzed using the Students' t-test; that of the DEP and DEP extract treatments were analyzed using ANOVA with a post-hoc Tukey test. To evaluate the linearity of MF relative to dosage, a simple linear regression was performed. $P < 0.05$ was considered to be statistically significant. Mutational spectra were compared using the Adams–Skopek test [Adams et al., 1987; Cariello et al., 1994].

RESULTS

gpt Mutations in the Lungs of DE-Inhaled *gpt* delta Mice

To estimate the mutagenicity of DE, *gpt* delta mice were exposed to DE (1 or 3 mg m^{-3} as SPM) via inhalation and mutations in the lung were analyzed (Table I). In the lungs of control mice, the background MFs for the 4-, 12-, and 24-week treatment groups were $0.61 \pm 0.06 \times 10^{-5}$, $0.59 \pm 0.14 \times 10^{-5}$, and $0.82 \pm 0.07 \times 10^{-5}$, respectively. In the lungs of the control group, the MF at 24 weeks was a little higher than that at 4 weeks with statistical significance ($P = 0.004$). Although an age-dependent increase in spontaneous MF has been found in the liver, spleen, and adipose tissues of Big Blue® mouse [Hill et al., 2005], MutaMouse [Ono et al., 2004], and *gpt* delta mouse [Masumura et al., 2003], this is the first report of an apparent age-dependent increase in spontaneous MF in the lung. Inhalation of 3 mg m^{-3} DE for 4, 12, and 24 weeks resulted in 1.7-, 3.2-, and 2.6-fold increases in MF ($1.06 \pm 0.46 \times 10^{-5}$, $1.90 \pm 0.88 \times 10^{-5}$, and $2.11 \pm 0.08 \times 10^{-5}$, respectively) compared with the control mice during the same time period (Table I). Thus, the MF reached a plateau after 12 weeks of DE inhalation. Significant increases in MF were observed between groups treated with 3 mg m^{-3} DE via inhalation for 12 or 24 weeks, compared with the 4-week group (Table I). A 3.1-fold ($1.84 \pm 0.82 \times 10^{-5}$ vs. $0.59 \pm 0.14 \times 10^{-5}$) increase in MF was observed between mice treated for 12 weeks with 1 mg m^{-3} DE via inhalation and control mice; however, there did not appear to be a significant difference between the MF at 1 mg m^{-3} as compared to 3 mg m^{-3} DE.

Mutations Caused by Instillation of DEP and DEP Extract

Mice were treated with a single intratracheal instillation of DEP or DEP extract. Instillation of 0.125, 0.25, and 0.5 mg DEP increased the MF by 1.8-, 2.1-, and 3.0-fold ($1.16 \pm 0.01 \times 10^{-5}$, $1.40 \pm 0.05 \times 10^{-5}$, and $1.97 \pm 0.18 \times 10^{-5}$, respectively), compared with control mice ($0.66 \pm 0.08 \times 10^{-5}$) (Table II). Instillation of 0.05, 0.1, and 0.2 mg DEP extract increased the MF by 1.5-, 1.9-, and 2.7-fold ($0.97 \pm 0.10 \times 10^{-5}$, $1.28 \pm 0.11 \times 10^{-5}$, and

TABLE I. Summary of Mutant Frequency in the Lungs of *gpt* delta Mice After Inhalation of DE

DE concentration (mg m^{-3})	Exposure time (weeks)	ID of animals	Number of colonies		Mutant frequency (10^{-5})	Average mutant frequency ± SD (10^{-5})
			Mutant	Total		
Control	4	1	4	763,100	0.52	0.61 ± 0.06
		2	6	964,500	0.62	
		3	5	758,300	0.66	
		4	6	920,400	0.65	
		Total	21	3,406,300		
3	4	1	17	1,735,400	0.98	1.06 ± 0.46[a]*
		2	16	1,002,200	1.60	
		3	15	1,019,500	1.47	
		4	9	1,405,000	0.64	
		5	9	1,449,000	0.62	
		Total	66	6,611,100		
Control	12	1	3	377,600	0.79	0.59 ± 0.14
		2	2	360,500	0.55	
		3	4	852,000	0.47	
		4	2	360,000	0.56	
		Total	11	1,950,100		
1	12	1	9	309,600	2.91	1.84 ± 0.82[a]*
		2	9	448,000	2.01	
		3	9	859,200	1.05	
		4	4	289,600	1.38	
		Total	31	1,906,400		
3	12	1	15	537,600	2.79	1.90 ± 0.88[a][c]*
		2	10	438,400	2.28	
		3	8	932,000	0.86	
		4	7	654,400	1.07	
		5	12	476,800	2.52	
		Total	52	3,039,200		
Control	24	1	13	1,551,000	0.84	0.82 ± 0.07[b]*
		2	8	1,074,000	0.74	
		3	8	903,000	0.89	
		Total	29	3,528,000		
3	24	1	10	462,500	2.16	2.11 ± 0.08[a]***[c]**
		2	11	546,000	2.01	
		3	16	745,600	2.15	
		Total	37	1,754,100		

Statistical significance was determined using a Students' t-test.
[a]Significant differences between the control and DE-treated groups.
[b]Significant difference to the control group at 4 weeks.
[c]Significant differences compared with exposure to 3 mg m^{-3} DE via inhalation for 4 weeks.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

$1.78 \pm 0.19 \times 10^{-5}$, respectively), compared with control mice. The MF increased linearly for 0–0.5 mg DEP ($r^2 = 0.95$; $P < 0.001$ [Table II]) and 0–0.2 mg DEP extract ($r^2 = 0.94$; $P < 0.001$), suggesting that DEP extract as well as DEP has a potential to induce mutations in the lung.

Mutation Spectrum Induced by DE Inhalation

To determine the mutation spectrum induced by DE inhalation, we sequenced 126 and 55 *gpt* mutants from the lungs of treated and control mice, respectively. The types of mutation were analyzed and the mutations in treated and control mice are presented in Table III. In groups that inhaled DE, 55% (69/126 mutants) of the mutations were G:C → A:T transitions and 17% (21/126) were G:C → T:A transversions, whereas in control mice, 40% (22/55 mutants) of mutations were G:C → A:T transitions and 31% (17/55) were G:C → T:A transversions. In the DE-treated mice, the percentage of G:C → A:T transitions was increased and the percentage of G:C → T:A transversions was decreased by prolonged inhalation of DE for 24 weeks, whereas in the control mice the mutation types remained constant. At 24 weeks, the Adams–Skopek test showed a significant difference ($P = 0.04$) in mutation spectrum between the control and DE inhalation group; G:C → A:T transitions were elevated from 44 to 77% and G:C → T:A transversions were reduced from 28 to 8% by DE inhalation. The frequency of spontaneous

TABLE II. Summary of Mutant Frequency in the Lungs of *gpt* delta Mice Following Treatment With DEP and DEP Extract

Treatment	Amount (mg)	ID of animals	Number of colonies		Mutant frequency (10^{-5})	Average mutant frequency ± SD (10^{-5})
			Mutant	Total		
Control	0	1	7	1,016,000	0.69	0.66 ± 0.08
		2	6	836,800	0.72	
		3	3	524,200	0.57	
		Total	16	2,377,000		
DEP	0.125	1	9	780,800	1.15	1.16 ± 0.01**
		2	20	1,731,200	1.16	
		3	10	859,200	1.16	
		Total	39	3,371,200		
	0.25	1	18	1,300,800	1.38	1.40 ± 0.05***
		2	18	1,323,200	1.36	
		3	14	963,200	1.45	
		Total	50	3,587,200		
	0.5	1	10	548,800	1.82	1.97 ± 0.18***
		2	19	990,400	1.92	
		3	19	872,000	2.18	
		Total	48	2,411,200		
DEP extract	0.05	1	9	862,400	1.04	0.97 ± 0.10
		2	11	1,289,600	0.85	
		3	15	1,480,000	1.01	
		Total	35	3,632,000		
	0.1	1	12	905,600	1.33	1.28 ± 0.11**
		2	10	737,600	1.36	
		3	15	1,304,000	1.15	
		Total	37	2,947,200		
	0.2	1	11	686,400	1.60	1.78 ± 0.19***
		2	16	912,000	1.75	
		3	13	656,000	1.98	
		Total	40	2,254,400		

Statistical significance was determined using ANOVA and post hoc Tukey tests.
Significant differences between the control and DEP-treated groups are indicated (**$P < 0.01$; ***$P < 0.001$).

mutation in the lung was significantly higher at 24 weeks than at 4 weeks (Table I); however, although A:T → G:C transitions changed from 0 to 16% and 1 base deletions decreased from 29 to 8%, there was no significant difference in the mutation spectrum between the control mice at 4 and 24 weeks ($P = 0.29$, Adams–Skopek test), as demonstrated previously in several tissues of aged Big Blue® mice [Hill et al., 2005].

The spectrum of *gpt* mutations induced by DE inhalation (Table IV) indicated a prevalence of G:C → A:T transitions, with seven mutation hotspots (mutation loci identified from three or more mice at nucleotide numbers 64, 110, 115, 185, 401, 402, and 418). At nucleotide 406, both G:C → A:T transitions and G:C → T:A transversions were identified in two mice, but this nucleotide was also a mutation hotspot for G:C → T:A transversions in control mice. The predominant frameshift mutations were single-base pair deletions at G:C base pairs (9/12 = 75%).

Characteristics of the *gpt* Mutation Spectrum Induced by DEP and DEP Extract

To determine the mutation spectrum induced by DEP and DEP extract, we isolated and sequenced 127, 101, and 16 *gpt* mutants from the lungs of DEP-, DEP extract-treated, and control mice, respectively. Although G:C → A:T transitions represented the majority of the base substitutions for both DEP- and DEP extract-treated groups, G:C → T:A transversions were also common (Table V). In the mutants isolated from mice treated with DEP, 39% (50/127 mutants) of mutations were G:C → A:T transitions and 28% (35/127) were G:C → T:A transversions, whereas with DEP extract, 37% of mutations (37/101 mutants) were G:C → A:T transitions and 23% (23/101) were G:C → T:A transversions. In the instillation control mice, the majority of mutations were G:C → A:T transitions (44%, 7/16) and G:C → T:A transversions (25%, 4/16). After treatment with 0.5 mg DEP, the percentage of G:C → T:A transversions increased (from 25 to 39%), while the percentage of G:C → A:T transitions decreased (from 44 to 29%) compared to the control. It has previously been suggested that accumulation of 8-hydroxyguanine may cause an increase in G:C → T:A transversions [Arai et al., 2003]. As formation of 8-hydroxyguanine occurred in the lungs of mice after DEP treatment [Ichinose et al., 1997], the increase in the percentage of G:C → T:A transversions may be explained by DEP-catalyzed formation of 8-hydroxyguanine.

TABLE III. Classification of *gpt* Mutations Isolated From the Lungs of Control and DE-Inhalation Mice

Type of mutation in *gpt*	Control		DE		Control (weeks)						DE (weeks)					
	All		All		4		12		24		4		12		24	
	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%
Base substitution																
Transition																
G:C → A:T	22 (13)	40	69 (28)	55	9 (5)	43	2 (1)	22	11 (7)	44	31 (13)	49	18 (7)	49	20 (8)	77
(CpG site)																
A:T → G:C	4	7	3	2	0	0	0	0	4	16	2	3	1	3	0	0
Transversion																
G:C → T:A	17	31	21	17	5	24	5	56	7	28	14	22	5	14	2	8
G:C → C:G	1	2	7	6	0	0	1	11	0	0	1	2	5	14	1	4
A:T → T:A	2	4	6	5	1	5	0	0	1	4	4	6	1	3	1	4
A:T → C:G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Deletion																
−1	9	16	12	10	6	29	1	11	2	8	5	8	5	14	2	8
>2	0	0	5	4	0	0	0	0	0	0	5	8	0	0	0	0
Insertion	0	0	2	2	0	0	0	0	0	0	1	2	1	3	0	0
Other	0	0	1	1	0	0	0	0	0	0	0	0	1	3	0	0
Total	*55*	*100*	*126*	*100*	*21*	*100*	*9*	*100*	*25*	*100*	*63*	*100*	*37*	*100*	*26*	*100*

A comparison of the spectrum of mutations induced by instillation of DEP and the DEP extract (Table IV) indicates that five G:C → A:T mutation hotspots (nucleotide numbers 64, 110, 115, 401, and 418) were induced by DEP instillation. The same five G:C → A:T mutation hotspots were also induced by DE inhalation, and three mutation loci (nucleotide nos. 64, 110, and 115) were commonly induced by DEP and the DEP extract. Masumura et al. [2000] reported that *gpt* nucleotide numbers 64, 110, and 115 are mutation hotspots in nontreated mice and it is possible that components in the DE extract enhance spontaneous mutation. Therefore, mutations of nucleotide nos. 401 and 418 seem to be characteristic of DE inhalation and DEP instillation.

DISCUSSION

Following inhalation of 3 mg m^{-3} DE as SPM, the MF in the lungs of *gpt* delta transgenic mice increased with the duration of treatment, but reached a plateau by 24 weeks. Additionally, the MF of control mice was elevated at 24 weeks. The MFs in mice that inhaled DE for 4, 12, and 24 weeks were 1.7-, 3.2-, and 2.6-fold higher than the control groups, respectively (Table I). We have demonstrated that inhalation of DE induces mutations in the lungs of rats [Sato et al., 2000] and mice (this study). In the lungs of *gpt* delta mice treated with inhalation of 3 mg m^{-3} DE for 12 weeks, the MF was 3.2-fold higher than the control group (1.90×10^{-5} vs. 0.59×10^{-5}). In contrast, the MF in the lungs of Big Blue® rats treated with inhalation of 6 mg m^{-3} DE for 4 weeks was 4.8-fold higher than the control group (4.25×10^{-5} vs. 0.88 $\times 10^{-5}$) [Sato et al., 2000]. Similarly, the MF in lungs in *gpt* delta rats treated with inhalation of 3 mg m^{-3} DE for 12 weeks was 4.1-fold higher than the control group (2.70×10^{-5} vs. 0.65×10^{-5}, our unpublished results). Thus, the MF induced by inhalation of DE would appear to be lower in mice than rats. Relative to rats, the lower mutagenicity of DE in mice may be a cause of reduced tumor induction in the lungs of DE-treated mice [Mauderly et al., 1996].

We showed that MF was dependent upon the amount of DEP (from 0–0.5 mg [Table II]). The mutagenic potency (MF mg^{-1}) of DEP extract (5.6×10^{-5} mg^{-1}) was twice that of DEP (2.7×10^{-5} mg^{-1}). As DEP extract accounts for ca. 50% of the weight of DEP, this result may indicate that the mutagenicity of DEP is derived from compounds in the extract. However, unexpectedly, we found no significant difference between the MFs induced by inhalation of 1 and 3 mg m^{-3} DE for 12 weeks (Table I), whereas the DEP burden in mouse lung is known to increase linearly with respect to the period of inhalation (6–18 months) or the concentration of DE (0.35, 3.5, and 7 mg m^{-3}) [Mauderly et al., 1996]. Based on the findings of Mauderly et al. [1996], the lung burden under our exposure conditions was estimated at 0.4 and 1.3 mg DEP following 12 weeks of exposure to 1 and 3 mg m^{-3}, respectively. This estimate was obtained by correcting for the duration of exposure and DE concentration (our conditions were 12 hr day^{-1}, 7 day $week^{-1}$ vs. 7 hr day^{-1}, 5 day $week^{-1}$ for Mauderly et al. [1996]). The differences observed between the experiments may indicate that at higher DEP lung burdens, incorporation of mutagens into pulmonary tissue does not depend on the amount of DEP deposited. In fact, analysis of data

TABLE IV. DNA Sequence Analysis of *gpt* Mutations Obtained From the Lungs of DE, DEP-Treated, and Control Mice

Type of mutation	Nucleotide	Sequence change		Amino acid change	Number				
					Control (DE)	DE	Control (DEP and DEP extract)	DEP	DEP extract
Base substitution									
Transition									
G:C → A:T	3	atG → atA		Met → Ile				1	
	26	tGg → tAg		Trp → Stop		2[a]			
	27	tgG → tgA		Trp → Stop	1				
	64	Cga → Tga	CpG	Arg → Stop	6[c]	8[b]		4[b]	7[d]
	82	Caa → Taa		Gln → Stop		1			1
	87	tgG → tgA		Trp → Stop	1	1		1	1
	92	gGc → gAc		Gly → Asp		1			
	110	cGt → cAt	CpG	Arg → His	5[c]	14[d]	3[a]	16[c]	15[f]
	113	gGc → gAc		Gly → Asp		1			
	115	Ggt → Agt	CpG	Gly → Ser	1	6[d]	1	4[b]	3[b]
	116	gGt → gAt		Gly → Asp	1	1	2		2[a]
	128	gGt → gAt		Gly → Asp		1			
	145	Gaa → Aaa		Glu → Lys	1			1	1
	176	tGt → tAt		Cys → Tyr		1			
	185	aGc → aAc		Ser → Asn		3[b]			
	262	Gat → Aat		Asp → Asn	1			1	
	274	Gat → Aat		Asp → Asn	1			2[a]	1
	281	gGt → gAt		Gly → Asp		1			
	287	aCt → aTt		Thr → Ile	1				
	290	gCg → gTg	CpG	Ala → Val	1				
	346	Ccg → Tcg		Pro → Ser					1
	350	gCt → gTt		Ala → Val				1	1
	391	Caa → Taa		Gln → Stop		2[a]		1	1
	401	tGg → tAg		Trp → Stop		6[b]	1	3[b]	
	402	tgG → tgA		Trp → Stop		4[b]			
	406	Gaa → Aaa		Glu → Lys		6[a]			1
	409	Cag → Tag		Gln → Stop				1	
	416	tGg → tAg		Trp → Stop				1	
	417	tgG → tgA		Trp → Stop				7[a]	
	418	Gat → Aat		Asp → Asn	2[a]	10[c]		5[b]	2
	451	Ggt → Agt	CpG	Gly → Ser				1	
A:T → G:C	17	aTc → aCc		Ile → Thr		1			
	41	aTc → aCc		Ile → Thr			1		
	56	cTc → cCc		Leu → Pro	1	2[a]			3[a]
	146	gAa → gGa		Glu → Gly					1
	181	Tcc → Ccc		Ser → Pro					1
	269	cTg → cCg		Leu → Pro	1				1
	400	Tgg → Cgg		Trp → Arg	1				
	415	Tgg → Cgg		Trp → Arg					1
	419	gAt → gGt		Asp → Gly	1			1	
Transversion									
G:C → T:A	3	atG → atT		Met → Ile				1	
	7	Gaa → Taa	CpG	Glu → Stop	1			2	2[a]
	15	taC → taA		Tyr → Stop					1
	26	tGg → tTg		Trp → Leu				1	
	59	gCa → gAa		Ala → Glu		1			
	79	Gaa → Taa		Glu → Stop					1
	107	aGc → aTc		Ser → Ile	1				
	115	Ggt → Tgt	CpG	Gly → Cys	1				
	116	gGt → gTt		Gly → Val		1		1	
	140	gCg → gAg	CpG	Ala → Glu		1	1	1	
	143	cGt → cTt	CpG	Arg → Leu					2[a]
	145	Gaa → Taa		Glu → Stop			1	1	1
	176	tGt → tTt		Cys → Phe				2[a]	2[a]
	182	tCc → tAc		Ser → Tyr				1	
	185	aGc → aTc		Ser → Ile		1			
	189	taC → taA	CpG	Tyr → Stop	3			1	

TABLE IV. *Continued*

Type of mutation	Nucleotide	Sequence change		Amino acid change	Number				
					Control (DE)	DE	Control (DEP and DEP extract)	DEP	DEP extract
	190	Gat → Tat	CpG	Asp → Tyr				1	
	205	Cgc → Agc	CpG	Arg → Ser				1	
	208	Gag → Tag	CpG	Glu → Stop				1	
	220	Ctg → Atg		Leu → Met					1
	230	gCa → gAa		Ala → Glu				1	
	244	Gaa → Taa	CpG	Glu → Stop				1	
	262	Gat → Tat		Asp → Tyr				1	
	268	Ctg → Atg		Leu → Met				1	
	274	Gat → Tat		Asp → Tyr	1				2[a]
	287	aCt → aAt		Thr → Asn	1				1
	304	Gaa → Taa		Glu → Stop		2[a]		1	
	313	Cca → Aca		Pro → Thr				1	
	319	Gcg → Tcg		Ala → Ser		1			
	320	gCg → gAg	CpG	Ala → Glu		1	1	1	1
	346	Ccg → Acg		Pro → Thr					1
	389	cCg → cAg	CpG	Pro → Gln					1
	401	tGg → tTg		Trp → Leu	1	2[a]		1	1
	402	tgG → tgT		Trp → Cys		3[a]		1	
	406	Gaa → Taa		Glu → Stop	7[d]	3[a]		4[b]	4[c]
	409	Cag → Aag		Gln → Lys		2	1	1	
	411	caG → caT		Gln → His				1	
	412	Ccg → Acg		Pro → Thr					1
	413	cCg → cAg	CpG	Pro → Gln	1				
	418	Gat → Tat		Asp → Tyr		3[a]		6[a]	1
G:C → C:G	6	agC → agG	CpG	Ser → Arg		2		1	
	46	Gca → Cca		Ala → Pro		1			
	112	Ggc → Cgc		Gly → Arg					1
	115	Ggt → Cgt	CpG	Gly → Arg		2[a]			1
	125	cCg → cGg	CpG	Pro → Arg					1
	127	Ggt → Cgt		Gly → Arg	1			1	
	130	Gcg → Ccg		Ala → Pro				1	
	139	Gcg → Ccg		Ala → Pro		1			
	145	Gaa → Caa		Glu → Gln					1
	185	aGc → aCc		Ser → Thr					2
	190	Gat → Cat	CpG	Asp → His					1
	262	Gat → Cat		Asp → His				1	
	280	Ggt → Cgt	CpG	Gly → Arg					1
	281	gGt → gCt		Gly → Ala				1	
	290	gCg → gGg	CpG	Ala → Gly			1		
	295	Gcg → Ccg		Ala → Pro					1
	340	Gca → Cca	CpG	Ala → Pro		1			1
	402	tgG → tgC		Trp → Cys				1	
	413	cCg → cGg	CpG	Pro → Arg					1
	442	Cca → Gca		Pro → Ala			1		
	443	cCa → cGa		Pro → Arg				1	1
A:T → T:A	8	gAa → gTa		Glu → Val				1	
	10	Aaa → Taa		Lys → Stop				1	
	11	aAa → aTa		Lys → Ile				1	
	12	aaA → aaT		Lys → Asn					1
	83	cAa → cTa		Gln → Leu				1	
	134	tTa → tAa		Leu → Stop		1			
	146	gAa → gTa		Glu → Val		1			
	164	gTc → gAc		Val → Asp				1	
	187	Tac → Aac		Tyr → Asn			1		
	214	Aaa → Taa		Lys → Stop				1	
	260	aTt → aAt		Ile → Asn	1				
	311	tAt → tTt		Tyr → Phe					1
	365	gTt → gAt		Val → Asp	1				
	419	gAt → gTt		Asp → Val		4			

TABLE IV. *Continued*

Type of mutation	Nucleotide	Sequence change	Amino acid change	Number				
				Control (DE)	DE	Control (DEP and DEP extract)	DEP	DEP extract
A:T → C:G	10	Aaa → Caa	Lys → Gln					1
	17	aTc → aGc	Ile → Ser				1	
	56	cTc → cGc	Leu → Arg				4	
	106	Agc → Cgc	Ser → Arg				1	1
	188	tAc → tCc	Tyr → Ser				1	
	312	taT → taG	Tyr → Stop					1
	345	aaA → aaC	Lys → Asn					1
	419	gAt → gCt	Asp → Ala				1	
Deletion								
−1 base	8–12	gAAAAAt → gAAAAt		4[b]				1
	32	aTg → ag					1	
	34–35	gTTg → gTg			1	1		
	37	gCa → ga			2[a]			
	83–84	cAAt → cAt						1
	97	tAt → tt						1
	115–116	cGGt → cGt			1			
	137	cTg → cg			1			
	170–171	aCCg → aCg			1			
	176	tGt → tt		1				
	201–202	aCCa → aCa		1				
	237	gCg → gg					1	1
	272	gTg → gg			1			
	315–318	cAAAAg → cAAAg					1	
	321	cGc → cc			1			
	352–353	tGGt → tGt			1			
	369	aTg → ag					1	
	407–408	gAAc → gAc						1
	412–413	gCCg → gCg			1			
	416–418	tGGGa → tGGa		2[a]			1	2[a]
	426	gCg → gg			1			
	431	gTa → ga						1
	442–443	gCCa → gCa		1	1			
	444–445	cAAt → cAt					2[a]	1
	449–450	tCCg → tCg						1
	451–452	cGGt → cGt					5[d]	1
>2	97–111	tATTGCCGTAAGCCGTg → tg						1
	149–165	cTGGGTATTCGTCATGTCg → cg						1
	158–170	cGTCATGTCGATACc → cc					1	
	170–171	aCCg → ag			1			
	177–196	gTATTTCCAGCTACGATCACGa → ga			1			
	226–227	aCGc → ac			1			
	250–255	cTTCATCg → cg					1	
	252–254	tCATc → tc			1			
	262–273	tGATGACCTGGTGg → tg			1			
	292–320	gGTTGCGATTCGTGAAAT-GTATCCAAAAGCg → gg					1	
	375–377	aTGTt → at					1	
Insertion	8–12	gAAAAAt → gAAAAAAt			1			
	35	tg → tCg					1	
	83–84	cAAt → cAAAt					1	
	120–121	tGGt → tGGGt						2
	269	cTg → cTTg			1			
	301	cg → cGTGAAATg					1	
	312	tc → tGc					1	
Other	46–47	tGCa → tAGa			1			
	401–402	tGGa → tAAa						1

[a]Mutations found in 2 different mice.
[b]Mutations found in 3 different mice.
[c]Mutations found in 4 different mice.
[d]Mutations found in 5 different mice.
[e]Mutations found in 7 different mice.
[f]Mutations found in 8 different mice.

TABLE V. Classification of *gpt* Mutations From the Lungs of Control, DEP-, and DEP Extract-Treated Mice

Type of mutation in *gpt*	Control		DEP		DEP extract		DEP (mg)						DEP extract (mg)					
			All		All		0.125		0.25		0.5		0.05		0.1		0.2	
	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%
Base substitution																		
Transition																		
G:C → A:T	7 (4)	44	50 (25)	39	37 (25)	37	18 (13)	46	20 (11)	43	12 (1)	29	10 (6)	30	12 (9)	35	15 (10)	44
(CpG site)																		
A:T → G:C	1	6	1	1	7	7	0	0	1	2	0	0	3	9	2	6	2	6
Transversion																		
G:C → T:A	4	25	35	28	23	23	9	23	10	21	16	39	7	21	8	24	8	24
G:C → C:G	2	13	7	6	12	12	4	10	1	2	2	5	8	24	1	3	3	9
A:T → T:A	1	6	6	5	2	2	2	5	2	4	2	5	0	0	2	6	0	0
A:T → C:G	0	0	8	6	4	4	1	3	3	6	4	10	0	0	2	6	2	6
Deletion																		
−1	1	6	12	9	11	11	3	8	6	13	3	7	2	6	5	15	4	12
>2	0	0	4	3	2	2	1	3	3	6	0	0	1	3	1	3	0	0
Insertion	0	0	4	3	2	2	1	3	1	2	2	5	2	6	0	0	0	0
Other	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	3	0	0
Total	*16*	*100*	*127*	*100*	*101*	*100*	*39*	*100*	*47*	*100*	*41*	*100*	*33*	*100*	*34*	*100*	*34*	*100*

reported by Sun et al. [1984], Bond et al. [1986], and Yu et al. [1991] demonstrated that in the lungs the concentration of organic compounds released from DEP reaches a steady state at an early phase of exposure. Alternatively, DE-induced mutagenesis may occur via a mechanism that is easily saturated, such as DNA adduct formation, generation of reactive oxygen species, or inflammation. Further studies are required to examine whether mutagenicity is increased in DNA repair system-deficient mice by DE treatment.

The IARC classification categorizes DE as a Group 2A substance and DEP contains various potent mutagenic PAHs and nitro-PAHs such as B[*a*]P and DNPs. To assess the health risks of DE, we attempted to identify components responsible for mutagenicity in the lungs of *gpt* delta mice. As mentioned above, intratracheal instillation of DEP or DEP extract increased the MF linearly, depending on the dosage (Table II). Inhalation of DE and instillation of DEP and DEP extract induced mutations at hotspots in *gpt* (mutation loci identified from three or more mice at nucleotide numbers 64, 110, 115, 185, 401, 402, and 418 (DE inhalation); 64, 110, 115, 401, and 418 (DEP); and 64, 110, and 115 (DEP extract); Table IV). Analysis of sequences adjacent to the mutated guanine bases (Fig. 1) identified the sequences G*G*A, T*G*A, C*G*G, and C*G*T as major targets for mutation. These results indicate that DE, DEP, and DEP extract targeted the same nucleotide sequences on *gpt*, producing DNA adducts. According to the Environmental Health Criteria 171 [International Programme on Chemical Safety, 1996], the carbon black in DEP and DEP extract contributes to the carcinogenicity of DE. Our study demonstrates that DEP extract exerts potent in vivo mutagenicity in the lung and that the mutation hotspots and target sequences are similar for mice that are treated with inhalation of DE or instillation of DEP and DEP extract. These observations suggest that compounds in the DEP extract induce mutations in the lung.

We compared the mutation spectra caused by the different treatments and observed that following inhalation of DE, G:C → A:T transitions were the predominant mutation in *gpt* delta mice (Table III). This result confirms previous observations in the lungs of Big Blue® rats [Sato et al., 2000]. In the lungs of DEP and DEP extract-instilled mice, G:C → A:T transitions were also the major base substitutions but G:C → T:A transversions were induced at a higher frequency than by inhalation of DE (Table V). We expected that G:C → T:A transversions would be the predominant base substitution induced by DE, since B[*a*]P is considered to be a major mutagenic component in DE, and such transversions are a common B[*a*]P-induced mutation in the mouse lung [Hashimoto et al., 2005]. As G:C → T:A transversions were induced by DEP- and DEP extract-instillation, it is likely that B[*a*]P contributes partly to DE-induced mutagenesis. However, to understand the mechanism underlying DE-induced carcinogenesis, the mutagen responsible for G:C → A:T transitions must be identified.

A number of mutagenic compounds have been identified in DEP using a Salmonella assay [Jeffrey et al., 1990; Tokiwa et al., 1993], and Salmeen et al. [1984] suggested that mono- and dinitro-PAHs such as 1,3-, 1,6- and 1,8-DNP may account for between 30 and 40% of the mutagenic activity. We have shown that intratracheal instillation of 1,6-DNP into the lungs of *gpt* delta mice [Hashimoto et al., 2005, 2006] induced mainly G:C → A:T transitions but not G:C → T:A transversions. In addition, the mutation

hotspots induced by DE inhalation were similar to those induced by 1,6-DNP [Hashimoto et al., 2006] but not by B[*a*]P [Hashimoto et al., 2005]. These results suggest that 1,6-DNP and related compounds are likely to be among the main contributors to mutagenesis induced by DE inhalation. Further studies are required to identify all the major mutagens in DE and to understand the mechanisms of mutagenesis induced by DE inhalation.

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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

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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

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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

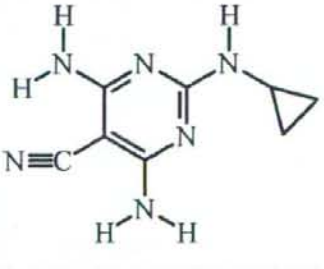

Fig. 1. Chemical structure of dicyclanil (DC).

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	$29.0 \pm 1.1^{**}$	$1.42 \pm 0.08^{*}$	$4.89 \pm 0.21^{*}$
Female	Control	5	25.0 ± 0.6	1.06 ± 0.05	4.25 ± 0.16
	Dicyclanil	5	24.0 ± 0.8	$1.27 \pm 0.07^{**}$	$5.29 \pm 0.12^{**}$

* $p < 0.05$ 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	$2.23 \pm 0.55^{*}$
		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.