Lessons learned from 40,000-animal cancer dose-response studies

George. Bailey, David Williams, Gayle Orner, Jerry Hendricks, and Cliff Pereira

Oregon State University, USA

We have conducted two 40,000-animal cancer dose-response studies, one with dibenzo(def,p)chrysene (DBC, formerly called dibenzo(a,l)pyrene DBP) and a more recent study with aflatoxin B_1 (AFB_1). These experiments used rainbow trout, an animal model well suited to ultra low-dose carcinogenesis research, to explore dose-response down to a targeted 10 excess liver tumors per 10,000 animals (ED_{001}). In study one, 42,000 trout were fed 0-225 ppm DBC for four weeks, sampled for biomarker analyses, and returned to control diet for nine months prior to gross and histologic examination. Suspect tumors were confirmed by pathology, and resulting incidences were modeled and compared to the default EPA LED_{10} linear extrapolation method. The study provided observed incidence data down to 2 above-background liver tumors per 10,000 animals at lowest dose (that is, an un-modeled ED_{0002} measurement). Among nine statistical models explored, three were determined to fit the liver data well - linear probit, quadratic logit, and Ryzin-Rai. None of these fitted models is compatible with the LED_{10} default assumption, and all fell increasingly below the default extrapolation with decreasing DBC dose. Low-dose tumor response was also not predictable from hepatic DBC-DNA adduct biomarkers, which accumulated as a power function of dose (adducts = $100*DBC^{1.31}$). Two-order extrapolations below the modeled tumor data predicted DBC doses producing one excess cancer per million individuals (ED_{10-6}) that were 500-1500-fold higher than that predicted by the five-order LED10 extrapolation. Study two was of similar design, but using AFB_1. Analysis of the results is underway, and complicated by several differences from study 1, especially presence in some quartiles and treatment groups of a fatty liver syndrome. Preliminary logistic regression analysis excluding fish with this syndrome did not support the EPA linear default assumption (i.e., logistic slope 1.0), rather indicated a sublinear dose-response with slope of 1.42 (95% CI 1.23 – 1.61), and an extrapolated ED_{10-6} that is 32-fold greater than the LED_{10} default extrapolation. Inclusion of all fish also yielded a sublinear dose-response, with slope 1.31 (95%CI 1.13 – 1.50), and an extrapolated ED_{10-6} 17-fold greater than the LED_{10} default extrapolation. Thus two genotoxins with differing biological properties yielded ultra-low dose-response curves in the same animal model that are not compatible with the linear default assumption. These results are considered specific to the animal model, carcinogen, and protocol used. They provide the first experimental estimations in any model of the degree of conservatism that may exist for the EPA default linear assumption for a genotoxic carcinogen.

Urinary Bladder Carcinogenesis by DNA Reactive and Non-Reactive Chemicals: Non-linearity's and Thresholds

Samuel M. Cohen

Department of Pathology and Microbiology, University of Nebraska Medical Center, Nebraska, USA

Cancer is due to multiple alterations of DNA occurring in a single stem cell. Chemicals can increase cancer risk by directly damaging DNA (DNA reactivity) or by increasing cell proliferation (DNA replications), increasing the number of opportunities for spontaneous DNA damage. Both types of chemicals have been shown to induce urinary bladder cancer in animal models and in humans. For DNA reactive carcinogens, such as aromatic amines like 4-aminobiphenyl (ABP), the dose response for carcinogenesis is non-linear because of the interaction of DNA reactive effects and cytotoxicity with regenerative proliferation at higher doses. The synergy between DNA reactivity and cell replication occurs commonly, such as in response to cigarette smoking. The DNA reactive effect operates through formation of DNA adducts, and the dose response can be linear or non-linear, depending on metabolic activation processes. For DNA reactive carcinogens, the distinction between threshold and level of detection in the assay system needs to be distinguished. In contrast, for non-DNA reactive carcinogens, a threshold is present. Increased cell proliferation can occur either by cytotoxicity and regeneration, by direct mitogenesis, or by decreasing cell death (e.g. inhibiting apoptosis or differentiation). In animal models, cytotoxicity can be produced by formation of urinary solids or generation of reactive metabolites which are excreted and concentrated in the urine. In humans, arsenic is an example of a bladder carcinogen which acts by formation of reactive metabolites. The threshold is dependent on the presence in the urine of a cytotoxic concentration of the metabolite(s). A defined true threshold is involved in the formation of the urinary solids, dependent on the physical-chemical property of solubility. Melamine is such an example. DNA reactive carcinogens have a non-linear dose response with respect to carcinogenicity and frequently have non-linear responses for DNA effects due to competing metabolic and repair processes, some of which are saturable. In contrast, non-DNA reactive carcinogens induce cancer as a consequence of a precursor toxic biologic effects which have thresholds.

A threshold for the murine T-cell lymphoma induction by N-ethyl-N-nitrosourea and/or radiation.

Shizuko Kakinuma, Tomoko Sawai, Yoshiko Amasaki, Mayumi Nishimura, and Yoshiya Shimada

Radiobiology for Children's Health Research Program, National Institute of Radiological Sciences, Chiba, Japan

Current strategy for estimating the risks of genotoxic substances at low-dose is a linear extrapolation from the effects observed at high doses. Since cancer is a genetic disease with stepwise accumulation of multiple mutations, it is generally considered that genotoxic carcinogens have no threshold in exerting their potential for cancer induction. However, a few recent animal studies reported the existence of a threshold for carcinogenicities. We previously observed that there existed a threshold for the N-ethyl-N-nitrosourea (ENU) and fractionated X-rays in induction of T-cell lymphoma in B6C3F1 mice, and that its magnitude was modified by their combined exposure; threshold for ENU was reduced by co-exposure with sub-carcinogenic dose of X rays.

In order to determine the contribution of mutation induction to threshold dose of ENU lymphomagenesis, we examined the mutation frequency and its spectra in the thymic cells, focusing on the point mutations, of B6C3F1 gpt-delta mice after exposure to ENU or the co-exposure with X rays. First, we found that ENU even below threshold dose for lymphomagenesis increased point mutation frequency significantly in a dose dependent manner, and that threshold dose for mutation induction was smaller than that for lymphoma development. However, mutant cells developed by ENU below threshold dose could progress into malignant lymphoma cells after co-exposure with sub-carcinogenic dose X rays, which may be ascribed to the expansion of mutant cells during the regeneration process. As a result, threshold dose of ENU lymphomagenesis decreased. In contrast, X irradiation below threshold dose for lymphomagenesis decreased the frequency of point mutations in cells of untreated cells, suggesting anti-mutagenic effect. X irradiation below threshold dose also reduced point mutation in the cells of ENU-treated mice, but that above threshold dose increased. This suggests that threshold of ENU mutagenesis could be influenced by the dose of co-exposed X rays.

In summary, threshold of ENU for lymphomagenesis is determined not by failure to induce mutation, but by the condition of mutant cells to progress into malignancy.

Exposure to ethylating agents: Where do the thresholds for mutagenic/clastogenic effects arise?

Elmar Gocke, Lutz Müller, and Thomas Singer

Preclinical Research, F. Hoffmann-La Roche Ltd, Basel, Switzerland

The presence of EMS (ethyl methanesulfonate) in tablets of a HIV medication triggered non-clinical studies into the dose response for mutation analysis after chronic dosing. Although there is a multitude of in vitro and in vivo studies on the genotoxic activity of EMS, no lifetime carcinogenicity studies, repeat dose mutation data or exposure analysis are available to serve as solid basis for risk assessment. For alkylators like EMS it is generally assumed that the dose response for mutagenicity (and by default for carcinogenicity) is linear – indicating that no 'safe' dose does exist. A recent in vitro genotoxicity study provided evidence, however, that the dose response curve for mutagenic and clastogenic activity was thresholded. We sought to verify the existence of thresholds for mutagenic and clastogenic activity in vivo. Dose levels ranging from 1.25 to 260 mg/kg/day were applied for up to 28 days. The studies were further supported by in depth metabolism and exposure analyses and a general toxicity study in rats.

Our studies provided unambiguous evidence that daily doses of up to 25 mg/kg/day did not induce any increase of mutations in the lacZ gene in the three organs tested (bone marrow, liver, GI tract, liver) or of micronuclei in bone marrow. Only at higher dose levels the genotoxic activity of EMS became apparent. Toxicokinetic assessment of the threshold doses showed AUC and Cmax values which were orders of magnitude higher than the maximal exposure of the patients and, therefore, it could be concluded that the ingestion to the genotoxic contaminant did not confer any genotoxic/carcinogenic risk to the patients.

Further, these studies showed that ethylation of cellular molecules (proteins, DNA) increased approximately linear with dose. We calculated that each liver cell experienced 380'000 ethylations per day at the threshold dose, indicating that the absence of clastogenic/mutagenic effects up to this dose must be due to error-free repair of vast numbers of DNA lesions rather than scavenging of the reactive molecules prior to reaching the DNA target.

Our investigations unambiguously demonstrated thresholded dose relations for mutagenic/clastogenic effects by EMS but gave no evidence of a threshold after exposure to the ethylating agent ENU (ethylnitroso urea), which was included into our studies for correlation purposes. As this observation is conceptually difficult to interpret it was important that subsequent studies with in depth analysis of the very low dose region revealed the likely presence of a threshold also for ENU.

These findings have important implications for the risk assessment of low dose exposures to genotoxic agents, and should impact on impending new regulation, e.g. on the limitation of PGI's (potentially genotoxic impurities) in pharmaceuticals.

Oxidative stress-induced tumorigenesis in the small intestine of *Mutyh*-deficient mice: the effect of low-level exposure to $KBrO_3$

Teruhisa Tsuzuki

Department of Medical Biophysics and Radiation Biology, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan

Oxygen radicals are produced through normal cellular metabolism, and the formation of such radicals is further enhanced by exposure to either ionizing radiation or various chemicals. The oxygen radicals attack DNA and its precursor nucleotides, and consequently induce various oxidized forms of bases in DNA within normally growing cells. Among such modified bases, 8-oxo-7, 8-dihydroguanine (8-oxoG) and 2-hydroxyadenine (2-OH-A) are highly mutagenic lesions, if not repaired. MUTYH is a DNA glycosylase that excises adenine or 2-OH-A incorporated opposite either 8-oxoG or guanine, respectively, thus considered to prevent G:C to T:A transversions in mammalian cells. The Mutyh-deficient mice showed a marked predisposition to spontaneous tumorigenesis in various tissues when examined at 18 months of age. The incidence of adenoma/carcinoma in the intestine significantly increased in Mutyh-deficient mice, as compared with wild-type mice. This high susceptibility of the intestinal tumor-development was well correlated with the condition observed in MAP (MUTYH-associated polyposis) patient. The intestinal tumor susceptibility of Mutyh-deficient mice was further enhanced by treatment with $KBrO_3$, a known oxidative renal carcinogen associated with 8-oxo-G accumulations. Oral administration of $KBrO_3$ at a dose of 0.2% in drinking water dramatically increased the formation of intestinal tumors in Mutyh-deficient mice.

Using this experimental system, we have been investigating the tumorigenic effect of $KBrO_3$. With relevance to the assessment of health risks, the exposure to lower dose in the range of 0.05 % to 0.1% of $KBrO_3$ reduced the frequency of intestinal tumor formation in Mutyh-deficient mice. These results suggest that cells are able to correctly repair oxidative DNA lesions resulting from exposures to a certain level of low doses of endogenous and exogenous chemicals with oxidizing property, and thus are less likely to be transformed to the neoplastic phenotype.

References:

Sakamoto, K., Tominaga, Y., Yamauchi, K., Nakatsu, Y., Sakumi, K., Yoshiyama, K., Egashira, A., Kura, S., Yao, T., Tsuneyoshi, M., Maki, H., Nakabeppu, Y. & Tsuzuki, T. MUTYH-null mice are susceptible to spontaneous and oxidative stress-induced intestinal tumorigenesis. Cancer Res., 67, 6599-6604 (2007).

Tsuzuki, T., Nakatsu, Y. & Nakabeppu, Y.: Significance of error-avoiding mechanisms for oxidative DNA damage in carcinogenesis. Cancer Sci., 98, 465-470 (2007).

Tsuzuki, T., Piao, J.-S., Isoda, T., Sakumi, K., Nakabeppu, Y. & Nakatsu, Y.: Oxidative stress-induced tumorigenesis in the small intestines of DNA repair-deficient mice. Health Phys., 100, 293-294 (2011).

How do thresholds for mutagenicity and clastogenicity arise for DNA damaging agents?

G.E. Johnson[1], J. Mikhail[1], K. Chapman[1], S.H. Doak[1], A. Seager[1], K. Shah[1], A.T. Thomas[1], Z. M. Zair[1], A. Scott[1], A. Doherty[1], G. J. Jenkins[1]

[1]Swansea University, Swansea College of Medicine, United Kingdom, [2]Unilever, United Kingdom; [3]Astra Zeneca, United Kingdom.

There has been a recent shift by the scientific and regulatory community, towards accepting genotoxic thresholds. Nevertheless, there are still many unanswered questions. Such as, what is the biological basis for thresholded responses to genotoxic agents? The mechanisms responsible for 'genotoxic tolerance' at low doses are wide ranging but poorly understood. However, this information is essential for hazard and risk assessment in order to fully accept the concept that genotoxic thresholds exist. For DNA reactive genotoxins, non-linear dose responses can arise from many different biological mechanisms. These include lack of bioavailability and nuclear exclusion, detoxification/activation, DNA repair and other homeostatic defence enzymes. Our recent work has been to investigate the roles of DNA repair in genotoxic thresholds for alkylating agents and pro-oxidant chemicals. Specific DNA repair enzymes have been shown to be up-regulated by low dose alkylating agents, and knocking down specific DNA repair enzymes in vitro alters the shape of the dose response e.g. to EMS. Conversely, for pro-oxidants, we have recently shown that antioxidant defences and specifically the presence of Glutathione, are perhaps more important in genotoxic tolerance at low doses of pro-oxidants. Other mechanisms that impact on the dose response are linked to secondary effects such as dose fractionation and metabolic activity.

Health risk assessment of air pollutants: Air pollutant genotoxicity and its enhancement on suppression of phase II drug-metabolizing enzymes

Yasunobu Aoki

National Institute for Environmental Studies, Japan

In health risk assessment, carcinogenic chemicals are generally categorized according to whether or not they are genotoxic. For example, when the risk assessment value of a carcinogenic hazardous air pollutant is calculated, the air pollutant is generally assigned to one of the following three categories: Category-1, in which the carcinogenicity of the chemical involves genotoxicity; Category-2, in which the involvement of genotoxicity in the carcinogenicity of the chemical is uncertain; and Category-3, in which the carcinogenic chemical is not genotoxic. In Category-1, the genotoxic chemical is judged to be carcinogenic without a threshold, and the risk assessment value is determined from the unit risk. On the other hand, if the carcinogenicity has a threshold, as in Category-3, the assessment value is determined from No-Observed Adverse Effect Level (NOAEL). Weak genotoxic chemicals may have a practical threshold and be categorized in Category-2.

Genotoxic potency is a factor in judging whether there is a threshold in the carcinogenicity; it is determined by not only the reactivity of the chemical to DNA but also the capability of the system that protects the body against chemical toxicity. This protective system is governed by processes such as phase I and II drug-metabolism, excretion, and DNA repair. We asked how the genotoxicity of an air pollutant is affected when part of this protective system is suppressed. Our research focused on phase II drug-metabolizing enzymes, whose constitutive and inducible gene expression is regulated by the essential transcription factor Nrf2. We hypothesized that, in Nrf2-knockout (KO) mice, if the levels of phase II drug-metabolizing enzymes and antioxidant proteins were suppressed, genotoxicity of air pollutants would be enhanced.

We examined the genotoxic potency of air pollutants under Nrf2-deficient conditions by using diesel exhaust (DE) and benzo[a]pyrene (BaP) as model pollutants. After exposing mice to DE for 4 weeks, the levels of bulky-DNA adduct and 8-OHdG in the lungs of Nrf2-KO mice were higher than those in the lungs of Nrf2-bearing control mice. Intratracheal administration of BaP elevated the in vivo mutation frequency (MF) in the lungs of both Nrf2-KO and Nrf2-bearing control mice, but the increase in MF induced by BaP was enhanced in Nrf2-KO mice. These results indicate that the level of phase II-drug metabolizing enzymes is a determinant of the genotoxic potency of air pollutants such as DE and BaP. A possible application of genotoxic potency (in vivo mutagenicity) data for predicting the carcinogenicity of chemicals will be discussed.

Toxicity testing strategy based on the concept of the threshold of toxicological concern (TTC)

Akihiko Hirose

Division of Risk Assessment, National Institute of Health Sciences, Japan

The Threshold of Toxicological Concern (TTC) is a principle that refers to the establishment of a generic human exposure threshold value for groups of chemicals below which there would be no appreciable risk to human health. The concept proposes that such values can be identified for a group of chemicals, including those of unknown toxicity when considering their chemical structures. As for the risk assessment of chemicals used for plastics products of Food Containers, Packaging and Apparatus, most of toxicological profiles of those chemicals have not been evaluated yet. Although, ideally, toxicity testing should be conducted based on the migration levels derived from plastics, it is not realistic to assess fully the potential risks of a large amount of chemicals. Some regulatory authorities (i.e. FDA, EFSA) had developed the comprehensive safety assessment guideline of food-contact materials prior to the application, using tiered toxicity testing strategy based on the migration levels. In Japan, although there is no official comprehensive guidance, several industry associations have introduced independently the self-regulated guidelines like that of FDA or EFSA. The frame works of all guidelines are similar, and necessary set of toxicity tests is required stepwisely depending on a few thresholds of the migration levels. The lowest threshold of 0.5 ppb (1.5 μg/person) was developed by only FDA in 1995. This lowest threshold had been derived from the carcinogenic potency database, and greatly discussed so far at that time. However, scientific bases of higher thresholds (i.e. 50 ppb or 1 ppm) in these guidelines are unclear. For example, only genotoxicity tests are required for the case of for the migration level below 50 ppb for safety evaluation. In order to assure the safety of exposure levels corresponding to below 50 ppb, the comparison of the threshold for all non-genotoxic endpoints with this exposure level should be discussed. Meanwhile, the concept of TTC has been examined and expanded for more general toxicity endpoints (Kroes et al. 2000, 2004) than carcinogenic endpoint. The concept is considered to be helpful for establishment of the threshold corresponding to the specific toxicity risk. Therefore, we examined the possibility for the replacement of these higher thresholds in these guidelines to the exposure threshold derived from the concept of TTC. These works support to establish the scientifically more transparent schema of toxicity testing for the risk assessment for plastics products of Food Containers, Packaging and Apparatus.

第二回 遺伝毒性発がん物質の閾値に関する国際シンポジウム

International Symposium on Genotoxic and Carcinogenic Thresholds

平成２３年度厚生労働科学研究費補助金 食品の安全確保推進研究事業

助成：財団法人 飯島記念食品科学振興財団

世話人：国立医薬品食品衛生研究所　変異遺伝部

部長　能美　健彦

日本バイオアッセイ研究センター

所長　福島　昭治

事務局：〒158-8501 東京都世田谷区上用賀 1-18-1
国立医薬品食品衛生研究所　変異遺伝部
TEL: 03-3700-9872 / FAX: 03-3700-2348
E-mail: threshold2011@nihs.go.jp

Genes and Environment, Vol. 31, No. 1 pp. 1–8 (2009)

Regular article

In Vivo Mutagenesis Caused by Diesel Exhaust in the Testis of *gpt* delta Transgenic Mice

Akiko H. Hashimoto[1], Kimiko Amanuma[1], Kenichi Masumura[2], Takehiko Nohmi[2] and Yasunobu Aoki[1,3]

[1]Research Center for Environmental Risk, National Institute for Environmental Studies, Ibaraki, Japan
[2]Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo, Japan

(Received June 30, 2008; Revised August 11, 2008; Accepted August 20, 2008)

Diesel exhaust (DE) is a major airborne pollutant in urban areas. In this study, we estimated the systemic effect of diesel exhaust inhalation by investigating mutations in extraplumonary organs such as the testis and liver. *gpt* delta Transgenic mice carrying the guanine phosphoribosyltransferase (*gpt*) transgene for the detection of mutations in genomic DNA were exposed to inhalation of 3 mg m^{-3} diesel exhaust (as suspended particulate matter) for 12 or 24 weeks. Compared to the control mice, DE resulted in a 2.0-fold increase in mutant frequency in the testis of mice that were exposed to DE for 24 weeks (inhaled group, 1.17×10^{-5}; control group, 0.57×10^{-5}), but not in the testis of mice exposed for 12 weeks (0.61×10^{-5}). The mutant frequency in the lungs was 2.6-fold higher in mice exposed to DE for 24 weeks than the control group, but it was not elevated in the liver (0.67×10^{-5}). In the testis, the major mutations on the *gpt* gene were G:C→T:A transversions, 1 base deletions and G:C→A:T transitions, while the major mutation in the lung was G:C→A:T transitions. The mutations on nucleotide nos. 402, 406, 409 and 416–418 in the *gpt* gene in testis seemed to be characteristic of DE inhalation in the testis. Our results suggest that inhalation of diesel exhaust is genotoxic to the testis as well as respiratory organs.

Key words: diesel exhaust emission, testis, *gpt* delta transgenic mouse, 6-thioguanine selection

Introduction

Diesel exhaust (DE) emission is a major source of air pollutant in urban areas, and has been implicated in causing allergic respiratory disease and lung cancer (1,2). Diesel exhaust particles (DEP) have been known to contain potent carcinogens and mutagens, such as polyaromatic hydrocarbons (PAH; e.g., benzo[*a*]pyrene (B[*a*]P)) and nitrated PAH (e.g., 1,6-dinitropyrene (1,6-DNP)), of which mutagenicity has been evaluated *in vitro* using a *Salmonella typhimurium* TA98 assay (3,4). Exposure to DEP through inhalation or intratracheal instillation have been shown to cause oxidative DNA damage (5,6) and DNA adduct formation (7,8) in rat and mouse lungs, and long periods of inhalation of DE resulted in respiratory tract tumors in rats (9–12). These observations suggest that mutagens in DE induce mutations in the lung, a primary target organ of inhalation, and are responsible for inducing lung cancer. Furthermore, we have previously demonstrated that typical mutagens such as B[*a*]P (13) and 1,6-DNP (14), as well as inhalation of DE (15,16), caused mutations in the lungs using transgenic rodents for analyzing *in vivo* mutagenesis (Big Blue® rat and *gpt* delta mouse). Metabolites of PAH contained in suspended particulate matter in ambient air have been detected in human urine (17), suggesting that mutagenic PAH in DE are absorbed in the lungs and transported to extrapulmonary organs, such as the testis and liver, where they could exert possible genotoxicity. Watanabe *et al.* showed that the number of daily sperm and Sertoli cells in fetuses and male rats was decreased by DE exposure (18,19). However, the mutagenic effect of DE on the extrapulmonary organs has remained unclear.

We intended to evaluate the *in vivo* mutagenicity of DE in testis and liver to obtain fundamental data for assessing the health risks of air pollution. In order to evaluate *in vivo* mutagenicity, we used the *gpt* delta transgenic mice carrying the lambda phage EG10 as a transgene for detecting mutations on genomic DNA (20,21). When the rescued phage is infected into *E. coli* expressing Cre recombinase, the phage DNA is converted into plasmids harboring the chloramphenicol (Cm)-resistance gene and guanine phosphoribosyltransferase (*gpt*) gene. The *gpt* mutants can be positively detected as colonies arising on plates containing Cm and 6-thioguanine (6-TG). Our study revealed an elevated mutant frequency and alterations in the mutation spectrum in the testis of DE-inhaled *gpt* delta transgenic mice in which

[3]Correspondence to: Yasunobu Aoki, Research Center for Environmental Risk, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan. Tel: +81-29-850-2390, Fax: +81-29-850-2588, E-mail: ybaoki@nies.go.jp

© The Japanese Environmental Mutagen Society

the mutant frequency in the lung has already been reported to increase (16).

In this study, we show that inhalation of 3 mg m^{-3} DE (as suspended particulate matter (SPM)) for 24 weeks resulted in a 2.0-fold increase in mutant frequency in the testis of *gpt* delta mice compared to the controls, but the inhalation for 12 weeks did not elevate the mutant frequency in the testis. The mutant frequency in the liver was not increased by inhalation of DE under conditions where the mutant frequency in the testis and lungs were significantly increased. The predominant mutation spectrum in the testis in response to DE inhalation included G:C→T:A transversions, 1-base deletions and G:C→A:T transitions, while the major mutations in the lungs were G:C→A:T transitions (16). These data suggest that DE inhalation exerts genotoxicity on testis systemically.

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 (22). Exposure to DE (12 h d^{-1}, 7 d $week^{-1}$) was performed in chambers equipped by the National Institute for Environmental Studies (16,23) under the same conditions as those in our previous report on *in vivo* mutations in the lung (16). Three to five 7-week-old mice were exposed to 3 mg m^{-3} DE (as SPM) for 12 or 24 weeks. Seven mice were maintained in filtered clean air (control group). The animals were sacrificed 3 days after the last exposure and their testis and liver were removed, frozen in liquid nitrogen and stored at −80°C for this study.

***Gpt* mutation assay:** The *gpt* assay was performed as described previously (20). Genomic DNA was extracted from the testis and liver tissue using the RecoverEase DNA Isolation Kit (Stratagene Co., La Jolla, CA) and Lambda EG10 phages were rescued using the Transpack® Packaging Extract (Stratagene). *E. coli* YG6020 was infected with the phage, spread on M9 salt plates containing Cm and 6-TG (19), and then incubated for 72 h at 37°C for selection of the colonies harboring a plasmid carrying the chloramphenicol acetyltransferase (CAT) gene and a mutated *gpt* gene. Isolates from the 6-TG-resistant phenotype were cultured in LB broth containing 25 $\mu g/mL^{-1}$ Cm at 37°C overnight, harvested by centrifugation (7,000 rpm, 10 min) and stored at −80°C.

PCR and DNA sequencing of the 6-TG-resistant mutants: A 739 bp DNA fragment containing the *gpt* gene was amplified by PCR and sequenced as described previously (13,20). Sequencing was performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an Applied Biosystems model 3730xl DNA analyzer.

Statistical analysis: All of the data are expressed as the mean ± SD. The statistical significance of the DE treatment was analyzed using the Student's *t*-test. $p<0.05$ was considered to be statistically significant. Mutational spectra were compared using the Adams-Skopek test (24,25).

Results

***Gpt* mutations in the testis, lung and liver of DE inhaled *gpt* delta mice:** In order to estimate the mutagenicity of DE, *gpt* delta mice inhaled DE (3 mg m^{-3} as SPM) for 12 or 24 weeks and mutations in the testis and liver were analyzed (Table 1). While the mutant frequencies in the testis of the control mice for 12 and 24 weeks inhalation were $0.57 \pm 0.04 \times 10^{-5}$ and $0.58 \pm 0.07 \times 10^{-5}$, respectively, inhalation of DE for 12 and 24 weeks resulted in 1.1 and 2.0-fold increases in mutant frequency ($0.61 \pm 0.08 \times 10^{-5}$ and $1.17 \pm 0.45 \times 10^{-5}$, respectively) compared with the controls (Table 1). Significant increases in the mutant frequency in the testis were observed in the group that inhaled DE for 24 weeks compared with the control group and the group that inhaled DE for 12 weeks. Our previous report demonstrated that inhalation of 3 mg m^{-3} DE for 24 weeks resulted in a 2.6-fold increase in the mutant frequency in the lung (Table 1) (16); however, the mutant frequency in the liver ($0.67 \pm 0.23 \times 10^{-5}$) was not elevated even after inhalation for 24 weeks compared with the control ($0.56 \pm 0.14 \times 10^{-5}$).

Alterations in the mutation spectrum in testis are induced by DE inhalation: In order to determine the mutation spectrum induced by DE inhalation, 170 6-TG-resistant mutants in a total were sequenced. As shown in Table 2, mutations of the *gpt* gene were detected in 149 mutants obtained from the testis of DE-inhaled and control mice (Table 1). The mutation type analysis indicated that the percentages of G:C→T:A transversions and 1-base deletions were increased in DE-inhaled mice (DE all) comparing to control mice (Control all). To characterize DE-induced mutagenesis precisely, the frequency of each mutation was calculated from data in Table 2 (Fig. 1). In the groups that inhaled DE for 24 weeks, the mutant frequency of G:C→T:A transversions, 1-base deletions and G:C→A:T transitions was 3.8×10^{-6}, 2.9×10^{-6} and 2.4×10^{-6}, whereas that of the control mice was, 1.0×10^{-6}, 0.6×10^{-6} and 1.9×10^{-6}, respectively. DE inhalation for 24 weeks caused a significant difference in the types of mutation in the control and DE inhalation groups ($p=0.04$, Adams-Skopek test).

The spectrum of *gpt* mutations in the testis that were induced by DE inhalation for 12 weeks and 24 weeks (Table 3) indicated a prevalence of G:C→T:A transversions with three mutation sites (nucleotide nos. 402, 406 and 409) being identified as hotspots in three or more mice, as well as G:C→A:T transition hotspots on

Table 1. Summary of mutant frequencies in the testis, lung and liver of *gpt* delta mice after inhalation of DE

Organ	DE concentration (mg m^{-3})	Exposure time (weeks)	ID of animals	Number of colonies		Mutant frequency ($\times 10^{-5}$)	Average mutant frequency ± SD ($\times 10^{-5}$)
				Mutant	Total		
Testis	0	12	1	7	1,265,600	0.55	
			2	9	1,428,800	0.63	
			3	10	1,792,000	0.56	
			4	10	1,820,800	0.55	
			Total	36	6,307,200		0.57 ± 0.04
	3	12	1	12	1,996,800	0.60	
			2	12	1,984,800	0.61	
			3	14	1,881,600	0.74	
			4	14	2,318,400	0.60	
			5	7	1,374,400	0.51	
			Total	59	9,556,000		0.61 ± 0.08
	0	24	1	9	1,676,800	0.54	
			2	5	756,800	0.66	
			3	7	1,291,200	0.54	
			Total	21	3,724,800		0.58 ± 0.07
	3	24	1	9	1,409,600	0.64	
			2	29	1,910,400	1.52	
			3	16	1,176,000	1.36	
			Total	54	4,496,000		1.17 ± 0.45*
Lung†	0	24	1	13	1,551,000	0.84	
			2	8	1,074,000	0.74	
			3	8	903,000	0.89	
			Total	29	3,528,000		0.82 ± 0.07
	3	24	1	10	462,500	2.16	
			2	11	546,000	2.01	
			3	16	745,600	2.15	
			Total	37	1,754,100		2.11 ± 0.08**
Liver	0	24	1	4	952,000	0.42	
			2	8	1,148,800	0.70	
			3	4	724,800	0.55	
			Total	16	2,825,600		0.56 ± 0.14
	3	24	1	2	275,200	0.73	
			2	2	483,200	0.41	
			3	8	937,600	0.85	
			Total	12	1,696,000		0.67 ± 0.23

Significant differences were detected between the control and DE-treated group (*: $p<0.05$, **: $p<0.001$).
†: data from our previous study (16).

another three sites (nucleotide nos. 64, 110 and 115). The predominant frameshift mutations induced by DE were single-base pair deletions in run sequences (22/29 = 76%); in this case the hotspot was located at nucleotide nos. 416–418. Therefore, the mutations on nucleotide nos. 402, 406, 409 and 416–418 seem to be characteristic of DE inhalation in testis, but were not hotspots in the lungs of DE-inhaled mice, while nucleotide no. 402 was a hotspot of G:C→A:T transitions in the lung.

Discussion

In this study we demonstrate that, as a result of inhalation of 3 mg m^{-3} DE, the mutant frequency in the testis of *gpt* delta mice increased with the duration of treatment (Table 1), but the mutant frequency in the liver was not elevated, indicating that DE inhalation exerts genotoxicity systemically on testis as well as on respiratory organs. This article is the first report on an increase in the mutant frequency in testis in response to DE inhalation (Table 1). Indeed, DE inhalation has also been

Table 2. Classification of *gpt* mutations from the testis of control and DE-inhaled mice

Type of mutation in the *gpt* gene	Control (weeks)			DE (weeks)		
	12	24	all	12	24	all
		%			%	
Base substitution						
Transition						
G:C→A:T	42	31	39	24	24	24
A:T→G:C	6	6	6	2	4	3
Transversion						
G:C→T:A	12	38	20	24	37	30
G:C→C:G	12	6	10	17	0	9
A:T→T:A	3	0	2	2	2	2
A:T→C:G	0	0	0	0	2	1
Deletion						
−1	12	13	12	30	28	29
>2	6	6	6	0	2	1
Insertion	6	0	4	2	0	1
Other	0	0	0	0	0	0
Total	100	100	100	100	100	100
Total number of mutants*	33	16	49	54	46	100

*: 149 of 170 6-TG-resitant mutants have mutation in the *gpt* gene.

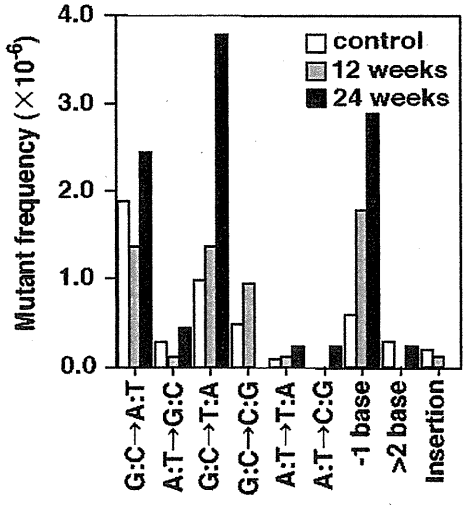

Fig. 1. Comparison of the 6-TG-resistant mutation spectra in control and DE inhaled *gpt* delta mice. The mutant frequencies of control mice and those exposed to DE for 12 weeks and 24 weeks were calculated by dividing the number of each type of *gpt* mutations in the Control all, DE 12 weeks and DE 24 weeks, respectively, by the corresponding total number of colonies (shown in Table 1).

shown to cause a decrease in the number of daily sperm and Sertoli cells in fetuses and male rats (18,19). Daily sperm production in the testis decreased dose-dependently in response to DE exposure for 6 months; a 53% reduction in sperm production was observed in rats exposed to DE (26) at the same concentration (3 mg m^{-3}) used in this study. These observations indicate that DE inhalation induces an increase in mutant frequency in the testis under the same conditions in which the reproduction of sperm was suppressed.

A significant increase in mutant frequency was observed in the testis after DE inhalation for 24 weeks but not for 12 weeks, while the mutant frequency in the lungs was elevated after inhalation for 24 weeks as well as 12 weeks (16). Delayed mutagenesis in germ cells has been observed in *lacZ* transgenic mice after 35 days treatment with ethyl nitrosourea (ENU) (27). Mutagens contained in DE were absorbed in the lung, systemically transported to the testis and possibly caused DNA adduct formation in spermatogonial stem cells and spermatogonia. These DNA adducts may be fixed as delayed mutations in germ cells through errors in DNA replication in continuous cell division during germ-cell development from spermatogenic cells to sperm. On the other hand, DNA adducts may be formed in the liver, but might not be fixed as mutations because of the low rate of cell division and/or high degree of DNA repair. However, Masumura *et al.* (28) showed that a heterocyclic amine, PhIP, was metabolically activated and induced point mutations in the liver but not the testis of *gpt* delta mice, suggesting that any factors governing the distribution and metabolism of mutagens in the body may determine the tissue specificity of mutagenesis.

The predominant mutation spectrum in the testis in response to DE included G:C→T:A transversions, 1-base deletions and G:C→A:T transitions (Table 2 and Fig. 1) as well as mutation hotspots on nucleotide nos. 402, 406 and 409, nos. 416–418 and nos. 64, 110 and 115, respectively (Table 3), while mutations in the lung were predominantly only G:C→A:T transitions (16). Mutation hotspots on nucleotide no. 406 and nos. 416–418 were identified in the testis of mice that inhaled DE for 12 weeks, in which the mutant frequency did not significantly increase, suggesting that DE acts as mutagenic agent even after inhalation for 12 weeks. G:C→T:A transversions have been known to be induced in *gpt* delta mice by B[*a*]P treatment (13) and 8-hydroxy-deoxyguanine (8-OHdG) generated by reactive oxygen species (ROS) (29). We have shown that the G:C→T:A transversion was a predominant mutation in Nrf2 deficient mice (30), in which the levels of the phase II detoxification enzymes and ROS-scavenging enzymes were suppressed (31,32) and DNA adduct formation was accelerated in the lung (33). These observations suggest some contribution of ROS to inducing mutation hotspots of G:C→T:A transversions (nucleotide nos. 402, 406 and 409) in the testis of mice subjected to DE inhalation. Nucleotide nos. 64, 110 and 115 were mutation hotspots of G:C→A:T transitions in the testis of DE-inhaled mice as well as in 1,6-dinitropyrene (DNP)-instilled lungs of *gpt* delta mice (14), and were also mutation hotspots in non-treated mice (34). The compo-

Table 3. DNA sequence analysis of *gpt* mutations obtained from the testis of DE-treated and control mice

Type of mutation	Mutation			Amino acid change	Number			
	Nucleotide number	Sequence Change			Control		DE	
					12 weeks	24 weeks	12 weeks	24 weeks
Base substitution								
Transition								
G:C→A:T	3	atG → atA		Met → Ile	1			
	64	Cga → Tga	CpG	Arg → Stop	1		4†	
	82	Caa → Taa		Gln → Stop	1			
	86	tGg → tAg		Trp → Stop	1			
	107	aGc → aAc		Ser → Asn		1		
	110	cGt → cAt	CpG	Arg → His	2*		2	2*
	113	gGc → gAc		Gly → Asp		1	1	
	115	Ggt → Agt	CpG	Gly → Ser	1		3*	3*
	116	gGt → gAt		Gly → Asp	2*	1	1	1
	145	Gaa → Aaa		Glu → Lys			1	
	176	tGt → tAt		Cys → Tyr	1			
	202	Cag → Tag		Gln → Stop				1
	401	tGg → tAg		Trp → Stop	1			
	402	tgG → tgA		Trp → Stop			1	1
	418	Gat → Aat		Asp → Asn	3†	1		3*
	451	Ggt → Agt	CpG	Gly → Ser		1		
A:T→G:C	56	cTc → cCc		Leu → Pro	1			1
	410	cAg → cGg		Gln → Arg				1
	415	Tgg → Cgg		Trp → Arg	1			
	419	gAt → gGt		Asp → Gly		1	1	
Transversion								
G:C→T:A	7	Gaa → Taa	CpG	Glu → Stop				1
	59	gCa → gAa		Ala → Glu				1
	110	cGt → cTt	CpG	Arg → Leu				1
	127	Ggt → Tgt		Gly → Cys				1
	140	gCg → gAg	CpG	Ala → Glu		2	1	1
	145	Gaa → Taa		Glu → Stop				1
	189	taC → taA	CpG	Tyr → Stop		1	1	
	208	Gag → Tag	CpG	Glu → Stop				1
	287	aCt → aAt		Thr → Asn			1	
	304	Gaa → Taa		Glu → Stop	1		1	1
	401	tGg → tTg		Trp → Leu	1			1
	402	tgG → tgT		Trp → Cys	1		2*	1
	406	Gaa → Taa		Glu → Stop			4†	4*
	409	Cag → Aag		Gln → Lys			1	2*
	413	cCg → cAg	CpG	Pro → Gln	1	2*		1
	418	Gat → Tat		Asp → Tyr		1	2*	
G:C→C:G	3	atG → atC		Met → Ile			1	
	6	agC → agG	CpG	Ser → Arg	1			
	109	Cgt → Ggt	CpG	Arg → Gly			1	
	143	cGt → cCt	CpG	Arg → Pro	1			
	145	Gaa → Caa		Glu → Gln			1	
	262	Gat → Cat		Asp → His	1			
	289	Gcg → Ccg		Ala → Pro			1	
	340	Gca → Cca	CpG	Ala → Pro			2*	
	401	tGg → tCg		Trp → Ser			1	
	402	tgG → tgC		Trp → Cys		1		
	413	cCg → cGg	CpG	Pro → Arg			2*	
	418	Gat → Cat		Asp → His	1			
A:T→T:A	35	tTg → tAg		Leu → Stop				1
	146	gAa → gTa		Glu → Val			1	
	179	aTt → aAt		Ile → Asn	1			
A:T→C:G	106	Agc → Cgc		Ser → Arg				1

Table 3. cont.

Type of mutation	Nucleotide	Sequence Change			Number			
					Control		DE	
					12 weeks	24 weeks	12 weeks	24 weeks
Deletion	8–12	gAAAAAt	→	gAAAAt	1		2	1
−1 base	126–128	cGGGt	→	cGGt			1	
	133–134	gTTa	→	gTa			1	
	155–156	aTTc	→	aTc	1			
	179–181	aTTTc	→	aTTc				2
	230	gCa	→	ga				1
	237	gCg	→	gg	1			1
	244	cGa	→	ca			1	
	249	gCt	→	gt	1			
	277	tAc	→	tc			1	
	387–389	tCCCg	→	tCCg			1	
	416–418	tGGGa	→	tGGa		1	7†	5†
	420	aTa	→	aa				1
	426	gCg	→	gg			1	
	431	gTa	→	ga		1		
	442–443	gCCa	→	gCa				1
	451–452	cGGt	→	cGt				1
	454	tCg	→	tg			1	
>2	26–34	tGGGACATGTTg	→	tg		1		
	170–171	aCCg	→	ag	1			
	238–249	cGATGGCGAAGGCt	→	ct	1			1
Insertion	75	ct	→	cAt	1			
	107	ag	→	aTg	1			
	214–216	taaag	→	tAaaag			1	
Total					33	16	54	46

* and †: Mutations found in 2 and 3 different mice, respectively.

nents in DE, such as 1,6-DNP and related compounds, may also contribute to enhance spontaneous mutations *via* the generation of ROS in the lung and also in the testis in response to DE inhalation.

Potent mutagens such as B[*a*]P and 1,6-DNP in DE are suspected to cause tumors in the lung, but their effect on the germline remains to be investigated. Previously, B[*a*]P was shown to induce a dominant-lethal mutation in the germ cells of male mice (35). We show that inhalation of DE, a major air pollutant in urban air, induces mutations in the testis, suggesting that mutagenic PAH and other mutagenic compounds in DE cause germline mutations. Previously, a germline mutation has been reported to occur in herring gulls living in an urban area (36). Recently, heritable DNA mutations in micro-satellite DNA were identified in mice that inhaled polluted ambient air in an industrial area (37,38); exposure to polluted ambient air for 10 weeks, followed by 6 weeks in the laboratory, was required for a significant increase in the sperm mutant frequency in these mice (38). This observation (38) corresponds to the delayed induction of point mutations in the testis in our study. Mutagenic compounds in ambient air may contribute to the induction of germline mutations. However, further studies are required to confirm that DE and other air pollutants cause mutations in germline cells, which are good markers for assessing the health risk of air pollution.

Acknowledgements: We thank Drs. Hiroaki Shiraishi and Michi Matsumoto (National Institute for Environmental Studies) for their support and advice. This research was supported in part by a Grant-in-Aid from the Japan Society for the Promotion of Sciences (#14207100).

References

1 International Programme on Chemical Safety. Diesel fuel and exhaust emission. Environmental Health Criteria. Vol 171, Geniva: World Health Organization; 1996.
2 United States Environmental Protection Agency. Diesel engine exhaust. Integrated Risk Information System. (http://www.epa.gov/ncea/iris/subst/0642.htm). 2008.
3 Østby L, Engen S, Melbye A, Eide I. Mutagenicity testing of organic extracts of diesel exhaust particles after fractionation and recombination. Arch Toxicol. 1997; 71: 314–9.
4 Rivedal E, Myhre O, Sanner T, Eide I. Supplemental role

of the Ames mutation assay and gap junction intercellular communication in studies of possible carcinogenic compounds from diesel exhaust particles. Arch Toxicol. 2003; 77: 533–42.

5 Nagashima M, Kasai H, Yokota J, Nagamachi Y, Ichinose T, Sagai M. Formation of an oxidative DNA damage, 8-hydroxydeoxyguanosine, in mouse lung DNA after intratracheal instillation of diesel exhaust particles and effects of high dietary fat and beta-carotene on this process. Carcinogenesis. 1995; 16: 1441–5.

6 Iwai K, Adachi S, Takahashi M, Möller L, Udagawa T, Mizuno S, Sugawara I. Early oxidative DNA damages and late development of lung cancer in diesel exhaust-exposed rats. Environ Res. 2000; 84: 255–64.

7 Gallagher J, Heinrich U, George M, Hendee L, Phillips DH, Lewtas J. Formation of DNA adducts in rat lung following chronic inhalation of diesel emissions, carbon black and titanium dioxide particles. Carcinogenesis. 1994; 15: 1291–9.

8 Sato H, Suzuki KT, Sone H, Yamano Y, Kagawa J, Aoki Y. DNA-adduct formation in lungs, nasal mucosa, and livers of rats exposed to urban roadside air in Kawasaki City, Japan. Environ Res. 2003; 93: 36–44.

9 Mauderly JL, Jones RK, Griffith WC, Henderson RF, McClellan RO. Diesel exhaust is a pulmonary carcinogen in rats exposed chronically by inhalation. Fundam Appl Toxicol. 1987; 9: 208–21.

10 Nikula KJ, Snipes MB, Barr EB, Griffith WC, Henderson RF, Mauderly JL. Comparative pulmonary toxicities and carcinogenicities of chronically inhaled diesel exhaust and carbon black in F344 rats. Fundam Appl Toxicol. 1995; 25: 80–94.

11 Iwai K, Higuchi K, Udagawa T, Ohtomo K, Kawabata Y. Lung tumor induced by long-term inhalation or intratracheal instillation of diesel exhaust particles. Exp Toxicol Pathol. 1997; 49: 393–401.

12 Valberg PA, Crouch EA. Meta-analysis of rat lung tumors from lifetime inhalation of diesel exhaust. Environ Health Perspect. 1999; 107: 693–9.

13 Hashimoto AH, Amanuma K, Hiyoshi K, Takano H, Masumura K, Nohmi T, Aoki Y. *In vivo* mutagenesis induced by benzo[*a*]pyrene instilled into the lung of *gpt* delta transgenic mice. Environ Mol Mutagen. 2005; 45: 365–73.

14 Hashimoto AH, Amanuma K, Hiyoshi K, Takano H, Masumura K, Nohmi T, Aoki Y. *In vivo* mutagenesis in the lungs of *gpt* delta transgenic mice treated intratracheally with 1,6-dinitropyrene. Environ Mol Mutagen. 2006; 47: 277–83.

15 Sato H, Sone H, Sagai M, Suzuki KT, Aoki Y. Increase in mutation frequency in lung of Big Blue rat by exposure to diesel exhaust. Carcinogenesis. 2000; 21: 653–61.

16 Hashimoto AH, Amanuma K, Hiyoshi K, Sugawara Y, Goto S, Yanagisawa R, Takano H, Masumura K, Nohmi T, Aoki Y. Mutations in the lungs of *gpt* delta transgenic mice following inhalation of diesel exhaust. Environ Mol Mutagen. 2007; 48: 682–93.

17 Sørensen M, Autrup H, Hertel O, Wallin H, Knudsen LE, Loft S. Personal exposure to PM2.5 and biomarker of DNA damage. Cancer Epidemiol Biomarkers Prev. 2003; 12: 191–6.

18 Watanabe N, Oonuki Y. Inhalation of diesel engine exhaust affects spermatogenesis in growing male rats. Environ Health Perspect. 1999; 107: 539–44.

19 Watanabe N. Decreased number of sperms and Sertoli cells in mature rats exposed to diesel exhaust as fetuses. Toxicol Lett. 2005; 155: 51–8.

20 Nohmi T, Suzuki T, Masumura K. Recent advances in the protocols of transgenic mouse mutation assays. Mutat Res. 2000; 455: 191–215.

21 Thybaud V, Dean S, Nohmi T, de Boer J, Douglas GR, Glickman BW, Gorelick NJ, Heddle JA, Heflich RH, Lambert I, Martus H, Mirsalis JC, Suzuki T, Yajima N. *In vivo* transgenic mutation assays. Mutat Res. 2003; 540: 141–51.

22 Nohmi T, Katoh M, Suzuki H, Matsui M, Yamada M, Watanabe M, Suzuki M, Horiya N, Ueda O, Shibuya T, Ikeda H, Sofuni T. A new transgenic mouse mutagenesis test system using Spi- and 6-thioguanine selections. Environ Mol Mutagen. 1996; 28: 465–70.

23 Takano H, Ichinose T, Miyabara Y, Shibuya T, Lim H, Yoshikawa T, Sagai M. Inhalation of diesel exhaust enhances allergen-related eosinophil recruitment and airway hyperresponsiveness in mice. Toxicol Appl Pharmacol. 1998; 150: 328–37.

24 Adams WT, Skopek TR. Statistical test for the comparison of samples from mutational spectra. J Mol Biol. 1987; 194: 391–6.

25 Cariello NF, Piegorsch WW, Adams WT, Skopek TR. Computer program for the analysis of mutational spectra: application to *p53* mutations. Carcinogenesis. 1994; 15: 2281–5.

26 Yoshida S, Sagai M, Oshio S, Umeda T, Ihara T, Sugamata M, Sugawara I, Takeda K. Exposure to diesel exhaust affects the male reproductive system of mice. Int J Androl. 1999; 22: 307–15.

27 Douglas GR, Jiao J, Gingerich JD, Gossen JA, Soper LM. Temporal and molecular characteristics of mutations induced by ethylnitrosourea in germ cells isolated from seminiferous tubules and in spermatozoa of *lacZ* transgenic mice. Proc Natl Acad Sci USA. 1995; 92: 7485–9.

28 Masumura K, Matsui K, Yamada M, Horiguchi M, Ishida K, Watanabe M, Ueda O, Suzuki H, Kanke Y, Tindall KR, Wakabayashi K, Sofuni T, Nohmi T. Mutagenecity of 2-amino-1-methyl-6-phenylomidazo[4,5-b]pyridine (PhIP) in the new *gpt* delta transgenic mouse. Cancer Lett. 1999; 143: 241–4.

29 Arai T, Kelly VP, Komoro K, Minowa O, Noda T, Nishimura, S. Cell proliferation in liver of *mmh/ogg1*-deficient mice enhances mutation frequency because of the presence of 8-hydroxyguanine in DNA. Cancer Res. 2003; 63: 4287–92.

30 Aoki Y, Hashimoto AH, Amanuma K, Matsumoto M, Hiyoshi K, Takano H, Masumura K, Itoh K, Nohmi T, Yamamoto M. Enhanced spontaneous and benzo[*a*]pyrene-induced mutations in the lung of Nrf2-deficient *gpt* delta mice. Cancer Res. 2007; 67: 5643–8.

31 Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun. 1997; 236: 313–22.

32 Motohashi H, Yamamoto M. Nrf2-Keap1 defines a physiologically important stress response mechanism. Trends Mol Med. 2004; 10: 549–57.

33 Aoki Y, Sato H, Nishimura N. Takahashi S, Itoh K, Yamamoto M. Accelerated DNA adduct formation in the lung of the Nrf2 knockout mouse exposed to diesel exhaust. Toxicol Appl Pharmacol. 2001; 173: 154–60.

34 Masumura K, Matsui K, Yamada M, Horiguchi M, Ishida K, Watanabe M, Wakabayashi K, Nohmi T. Characterization of mutations induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in the colon of *gpt* delta transgenic mouse: novel G:C deletions beside runs of identical bases. Carcinogenesis. 2000; 21: 2049–56.

35 Generoso WM, Cain KT, Hellwig CS, Cacheiro NLA. Lack of association between induction of dominant-lethal mutations and induction of heritable translocations with benzo[*a*]pyrene in postmeiotic germ cells of male mice. Mutat Res. 1981; 94: 155–63.

36 Yauk CL, Fox GA, McCarry BE, Quinn JS. Induced minisatellite germline mutations in herring gulls *(Larus argentatus)* living near steel mills. Mutat Res. 2000; 452: 211–8.

37 Somers CM, Yauk CL, White PA, Parfett CL, Quinn JS. Air pollution induces heritable DNA mutations. Proc Natl Acad Sci USA. 2002; 99: 15904–7.

38 Yauk C, Polyzos A, Rowan-Carroll A, Somers CM, Godschalk RW, Van Schooten FJ, Berndt ML, Pogribny IP, Koturbash I, Williams A, Douglas GR, Kovachuk O. Germ-line mutations, DNA damage, and global hypermethylation in mice exposed to particulate air pollution in urban/industrial location. Proc Natl Acad Sci USA. 2008; 105: 605–10.

In: DNA Adducts: Formation, Detection and Mutagenesis ISBN: 978-1-60741-433-9
Editors: E. Alvarez, R. Cunha, pp. 143-153 © 2010 Nova Science Publishers, Inc.

Chapter VII

Potency of Air Pollutants at DNA Adduct Formation and Assessment by *In Vivo* Mutagenesis

Yasunobu Aoki, Akiko H. Hashimoto, Hiromi Sato and Michi Matsumoto
National Institute for Environmental Studies, Research Center for Environmental Risk
16-2 Onogawa, Tsukuba, Ibaraki 305-8506 Japan.

Abstract

Various mutagenic polycyclic aromatic hydrocarbons (PAHs) including nitro-PAHs, associated with suspended particulate matter (SPM), are possibly deposited in the lungs and other organs of residents in urban areas. To assess the mutagenicity of air pollution and its carcinogenic risk in the lung and other organs, it is necessary to assess the genotoxicity *in vivo* of the various PAHs and nitro-PAHs in urban air. In this chapter, we review the genotoxicity, such as DNA adduct formation, of air pollutants and its assessment by *in vivo* mutagenesis.

Abbreviations

PAHs	polycyclic aromatic hydrocarbons
BaP	benzo[a]pyrene
SPM	suspended particulate matter
$PM_{2.5}$ and PM_{10}	SPM < 2.5 and 10 μm diameter, respectively
DNP	dinitropyrene
3NBA	3-nitrobenzanthrone
DEP	diesel exhaust particle
BPDE	benzo[a]pyrene diol-epoxide
RAL	relative adduct level

WBCs	white blood cells
DE	diesel engine exhaust
MF	mutant frequency

1. Introduction: Mutagenic PAHs in Urban Air

PAHs and nitro-PAHs are generated by the combustion of fossil fuels and are found in diesel and boiler exhaust. Many PAHs are recognized as mutagens and carcinogens, and are associated with SPM in ambient air. SPM < 2.5 μm in diameter ($PM_{2.5}$) tends to be easily trapped in pulmonary bronchia, where it possibly induces mutations that lead to tumor formation. Benzo[a]pyrene (BaP) is a typical environmental pollutant contained in SPM. Its mutagenic potency has been well examined by means of *in vitro* assay systems, such as the Ames test (*Salmonella* revertant test). BaP was evaluated as Class 2A (probably carcinogenic to humans) in IARC classification [1], and has been recently upgraded to Class 1 (carcinogenic to humans) [2]. BaP is used as a marker for carcinogenic risk of PAHs in ambient air, and unit risk of PAHs expressed as BaP for carcinogenesis is estimated to be 9×10^{-2} per μg/m^3 inhaled air (based on human studies) by the WHO Regional Office for Europe [3]. Unit risk of BaP in drinking water is also estimated to be 2.1×10^{-4} per μg/L drinking water (based on animal experiments) by the United States Environmental Protection Agency [4]. Thus, a concentration of BaP of 0.1 ng/m^3 in air and 50 ng/L in drinking water provides a cancer risk of 10^{-5} (1 in 100,000). If the daily intake of air and drinking water is set at 15 m^3 and 2 L, respectively, according to the standard default values for adults, the daily intake of BaP for a cancer risk level of 10^{-5} is estimated to be 1.5 ng for inhaled air and 100 ng for drinking water. These estimations suggest that exposure via air is a more sensitive pathway for the induction of cancer by BaP than exposure via drinking water. The target value for BaP in ambient air is 1 ng/m^3 in the EU [5], and the air quality standard for BaP as an annual average is 0.25 ng/m^3 in the UK; this value is derived from the lowest exposure level for the occurrence of lung cancer by occupational inhalation [6].

BaP has been detected in ambient air in association with SPM in many countries, especially in urban and industrial areas, and the concentrations of individual PAHs in the air range from <0.1 to 100 ng/m^3 [7]. In Japan, a report by the Ministry of the Environment documents the air concentrations of BaP at monitoring stations (366 points in 2007) in urban areas as part of a systematic nationwide monitoring managed by the Ministry and local government. The average concentration of BaP was 0.78 ng/m^3 (range, 0.05–8.1 ng/m^3) in 1998, but gradually decreased to 0.26 ng/m^3 (0.00038–1.8 ng/m^3) in 2007 [8].

In addition to BaP, various mutagenic and carcinogenic PAHs and nitro-PAHs are also released into the environment, especially in urban areas, as combustion products associated with diesel exhaust particles (DEP) and SPM. Several PAHs—including BaP, benzo[b]fluoranthene, benzo[j]fluoranthene, benzo[k]fluoranthene, chrysene, dibenzo[a,h] anthracene, and indeno[1,2,3,-cd]pyrene—were identified as carcinogens in intralung application experiments in rats [6]. For example, dibenzo[a,h]anthracene was shown to be 2.54 times more carcinogenic than BaP [6]. Among the nitro-PAHs, the dinitropyrene (DNP) isomers have been well tested for mutagenicity and carcinogenicity. For example, 1,3-, 1,6-,

and 1,8-DNP show higher mutagenic activity than BaP as determined by the Ames test [9], and intratracheal administration of 1,6-DNP induced lung tumors in hamsters [10] and rats [11]. The mutagenic activity of BaP requires cytochrome P450-mediated metabolic activation, whereas DNPs are mutagenic without metabolic activation [9], thus suggesting that BaP and DNPs have different modes of mutagenicity. Investigations of the *in vivo* mutagenicity of BaP and 1,6-DNP by intratracheal administration in transgenic *gpt* delta mice (a transgenic mouse model for detecting mutations; see section 3) show that the *in vivo* mutagenicity of 1,6-DNP [12] was about 20 times higher than that of BaP [13]. The concentration of 1,6-DNP in DEP is estimated to be about one-tenth that of BaP [14], indicating that the mutagenic potency of 1,6-DNP in DEP (*in vivo* mutagenicity × concentration) may be comparable to that of BaP. Apart from the DNP isomers, several nitro-PAH compounds, e.g., nitro-benzanthrone and nitro-benzopyrene, are mutagenic and carcinogenic. The intratracheal administration of 3-nitrobenzanthrone (3NBA) produces DNA adducts [15] and induces tumors in rat lungs [16]. 3,6-Dinitrobenzo[e]pyrene, which was originally detected in soil in Japan, shows potent mutagenic activity *in vitro* that is comparable to those estimated for 1,6- and 1,8-DNP [17, 18].

2. DNA Adduct Formation in the Lung of Experimental Animals by *In Situ* Exposure to Urban Air

PAHs and nitro-PAHs incorporated into lung, liver, and other target organs become genotoxic when converted to reactive electrophilic metabolites, known as reactive intermediates, by cytochrome P450 (CYP)-mediated monooxygenation. BaP is oxidized to BaP diol-epoxide (BPDE) by inducible CYP1A1 and CYP1A2 [19]. These reactive intermediates covalently bind genomic DNA, mainly on guanine, resulting in the formation of DNA adducts [20]. DNA repair systems can remove adducts, but adducts that are not removed can induce mismatching of DNA base pairs during DNA replication and thus cause gene mutations [21]. BaP, which forms BPDE-DNA adducts with guanine, induces G:C to T:A transversions *in vivo* [13, 22]. Mutations induced in cell growth or cell cycle genes can transform a normal somatic cell into a cancer cell, thus initiating tumor formation.

DNA adduct formation induced by environmental pollutants is a key event in mutagenesis and carcinogenesis. A simple test of the genotoxicity of air pollutants is to assess the formation of DNA adducts in the lungs of experimental animals exposed on site to polluted air ('*in situ* exposure'). In one such study, from 1995 to 1996, Wistar rats were maintained in a small-animal housing facility beside a main highway intersection in the Tokyo metropolitan area, and were exposed to the ambient air for up to 60 weeks; control rats were exposed to HEPA/charcoal-filtered ambient air [23]. The average monthly concentration of SPM < 2 μm was 51.8 μg/m^3 (range, 29.1–78.8 μg/m^3), whereas that for SPM < 11 μm was 76.5 μg/m^3 (50.4–108.3 μg/m^3). This experiment was originally designed for evaluating the effects of urban air on the respiratory system. After exposure to the ambient air, DNA adducts were analyzed by the ^{32}P-postlabeling assay, which involves nuclease P1 treatment and the separation of ^{32}P-postlabeled nucleotides by thin-layer