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H. 知的所有権の取得状況

なし

平成21年度 厚生労働科学研究費補助金(食品の安心・安全確保推進研究事業)
分担研究報告書

研究課題名:食品添加物等における遺伝毒性発がん物質の評価法に関する研究

分担研究課題名:国際的な「遺伝毒性物質の閾値」に関する情報収集

分担研究者:長尾 美奈子 慶応義塾大学薬学部客員教授

研究要旨

In vitro において遺伝毒性発現に閾値を示さない発がん物質の発がん性については、発がん標的臓器によって閾値の有無は異なることが示されている。一方遺伝毒性に閾値を示す物質の存在も示されていることから、遺伝毒性で閾値を示す物質は、発がん性においては標的臓器の如何を問わず閾値を示すことが想定される。すなわち遺伝毒性において閾値を示す物質を明確に把握することが重要である。 Hoffmann-La Roche から発売された Viracept (HIV のプロテアーゼ阻害剤)に、遺伝毒性発がん物質 Ethyl methanesulfonate (EMS) が混入していることが判明した。EMS は種々の *in vitro* 遺伝毒性試験で閾値の存在が示されていた。*In vitro* で遺伝毒性に閾値を示さない ethyl nitrosourea (ENU)を対照に用いて、種々の臓器における *in vivo* 遺伝毒性に閾値が存在するかを検討した。Comet assay, micronucleus test, *Lac Z* 変異で検討した結果、いずれのテスト系でも、また、テストしたいずれの臓器でも閾値の存在が示された。発がんにおいても閾値の存在が強く示唆された。EMS のマウスおよびヒトにおける体内動態に検討を加え、ヒトにおける Viracept からの EMS 実質暴露量が遺伝毒性閾値以下であること示した一連の論文を紹介する。わが国の遺伝毒性発がん物質の取り扱いに参考となる事例と考える。

キーワード:EMS, 遺伝毒性閾値、ヒト暴露、リスク評価

A. 研究目的

遺伝毒性発がん物質の遺伝毒性における閾値の評価法に関する国際的な動向を調べる目的で文献検索を行なった。本年度は、DNA に直接作用するが、DNA 修復に基づく閾値の存在が示されているアルキル化剤の医薬品への混入が欧州製薬会社製品で生じた。その混入に対するリスク評価が当該製薬会社で行われた。この評価方法は閾値のある遺伝毒性発がん物質の取り扱いに参考となる事例であるので詳細に紹介する。

２００７年３～５月に Hoffmann-La Roche により、米国、カナダ、日本以外で販売された HIV 治療薬「Viracept」に、遺

伝毒性、催奇性、発がん性のあることが知られているエチルメタンスルホン酸（EMS）が混入した。この事故はメタンスルホン酸の貯蔵タンクをエタノール洗浄した際、その除去が十分行われなかったことに起因している。この製品中の EMS 濃度は 1000 ppm に達した。HIV 患者の EMS 最大摂取量は 0.055 mg/kg day、3ヶ月間の合計摂取量は 5.2 mg/kg と算出されている。Hoffmann-La Roche により行われたこの EMS 混入のリスク評価を以下に示す。

B. 研究方法

文献検索により情報を収集した。

（倫理面への配慮）

本研究では該当するものはない。

C. 研究結果

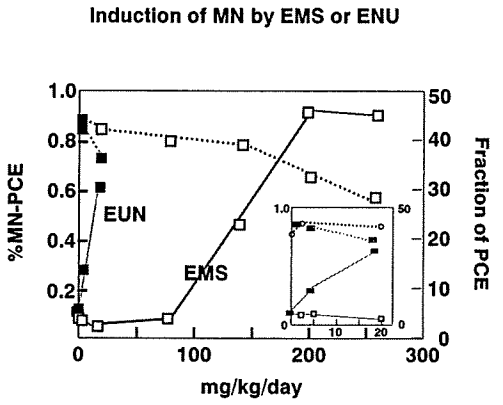
1. 遺伝毒性

a) In vitro における遺伝毒性

平成19年度の厚生労働省食品の安心・安全確保推進研究事業「食品添加物等における遺伝毒性評価のための戦略構築に関する研究」に報告したとおり、ヒトリンパ芽球細胞を用いて、EMS は小核誘発、HPRT 遺伝子変異誘発において閾値が存在することが報告されている。閾値は夫々1.35 μg/ mL および 1.25 μg/mL であった。Hoffman-La Roche では以下の in vivo 実験を行い閾値の存在を確認した。

b) In vivo における遺伝毒性

マウスに EMS を7日間投与（0, 1.25, 2.5, 5.0, 20, 80, 140, 200, 260 mg/kg/day）における骨髄小核誘発試験の結果、EMS では明らかな閾値の存在が示された。その値は 89. 8 mg/kg であった。 一方 ENU では閾値は認められなかった。図-1に結果を示す。



図—1 EMS および ENU による骨髄小核誘発

Muta™ マウスにおける LacZ 変異で閾値の有無を検討している。EMS（0, 1.56, 3.13, 6.2 5, 25, 50, 100 mg/kg day）を28日間投与し、31日目に骨髄、肝臓、消化管における突然変異率を調べた。いずれの臓器でも閾値が認められ夫々35.4, 51.3 および24.5 mg/kg day であった。一方 ENU はいずれの臓器でも閾値は認められなかった。

2. 閾値の算出

上記 EMS の種々の遺伝毒性検出系における閾値の算出には Lutz and Lutz（2009）の Hockey Stick software （www.r-project.org よりダウンロードできる）を用いて統計的に求めている。このモデルに合う用量相関は2本の直線で表される。

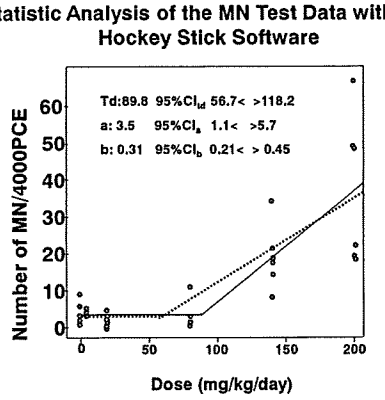
$y = a + b\,(d-td)$

y: 遺伝毒性

a: 遺伝毒性マーカーの背景レベル

b: 直線の勾配

td の用量までは y = a の勾配（b）が0の直線ではじまり、用量 td 以上では 勾配 b の直線に合う線形の得られるものである。EMS の MN テストの結果を Hockey Stick で表すと図－2のようになる。



表－1に示すのはこのモデルに従って算出した種々の遺伝毒性試験における閾値である。

表-1 EMS の種々の遺伝毒性テストにおける閾値

テスト	臓器	閾値 td (mg/kg/day)	95% CI (mg/kg/day)
MN	骨髄	89.8	56.7 - 118.2
LacZ	骨髄	35.4	21.5 - 45.7
LacZ	肝臓	51.3	25.7 - 100.0
LacZ	消化管	24.5	13.0 - 38.5

以上の結果からマウス・遺伝毒性から見た閾値は 25 mg/kg day であることがわかった。

3. リスク評価

a) 従来の方法による、用量相関が直線であると仮定しての評価

EMS には遺伝毒性、発がん性および催奇性以外の毒性に関する報告はない。

EMS の終生投与実験の報告はないので同じアルキル化剤メチルメタンスルホン酸をマウスに飲水投与したときの肺腫瘍誘発率を用いて発がん率を推定し場合 0.0031%となる。AIDS 患者では発がん感受性は約3倍であるという報告に基づく補正を行うと 0.009%になる。さらにヒトにおける AUC（下記参照）はマウスの12倍であることに基づく補正を行うと 0.11%になる。数千人の AIDS 患者が摂取したので数人が犠牲になることになる。

ラットに EMS（3-20 mM）を 0.5-3 ヶ月飲水投与したで乳がん誘発実験からの外挿では、ヒトにおけるリスクは 0.12%となる。AUC および感受性の増加の補正を行うと%の桁数で発がんを誘発したことになる。

催奇性に関するマウスのデータの外挿からは、AUC の補正なしで 0.15%の増加が推定された。BG のヒト奇形発生率は 2.68%である。AUC の補正を行うと 1.5%となり影響はかなり大きくなる。

b) 遺伝毒性における閾値存在に基づく評価

遺伝毒性物質で閾値のある EMS とない ENU のリスク評価での重要な差異は、EMS は閾値以下の低濃度に分割投与した場合には遺伝毒性は認められないが、ENU の場合は加算されることである。従ってマウスに EMS を28日間投与で得られた閾値は、より長期の投与でも変わらないことが考えられる。

EMS のリスク評価を行うにあたり先ず、暴露量で直接比較した。

1）Viracept 中の EMS は 1068ppm で、Viracept 摂取期間中のヒトの暴露量は最大で 0.055mg/kg day（体重60kg）であった。マウス in vivo における遺伝毒性閾値は 25 mg/kg/day である。これらの値から安全係数を計算すると 454 になる。

2）実験動物およびヒトにおける薬物動態を考慮した暴露評価を検討した。EMS は肝臓ミクロソームやグルタチオン転移酵素による分解は殆ど受けず、主として化学的加水分解によりエタノールとメタンスルホン酸に分解される。緩衝液、血漿、腸管液中の半減期は37℃で12時間である。経口投与した EMS は63－73%が消化管から速やかに吸収され10分後に最

高血漿濃度に達する。

^{14}C－EMS 投与実験より得られた in vivo における半減期は、マウスで 10 - 24 分、ラットおよびサル(cynomolgus)で 2.5 - 5 時間であった。そこで以下の仮定を適用した。

1. AUC は可能な限り高い値とし、排泄除去(clearance)は in vitro の緩衝液中での化学分解値を用いた。
2. マウスでは EMS の低用量では排泄除去は増加していたが、ヒトでは低用量でも増加しないとした。
3. ヒトでの吸収は速やかで100%であると仮定した。
4. EMS 体内分布の容量(distribution volume)は動物種によらず一定で0. 521 L/kg とした。

ヒトでの半減期は 10.7 h と算出された。

マウスに EMS を経口投与し得られた血中濃度－時間曲線を用いて測定したマウスにおける遺伝毒性閾値(25mg/kg)の C_{maxThd} は 315μM であった。C_{max} は排泄除去の影響は殆ど受けず、また、EMS の場合は動物種によって分布容量(distribution volume)が変わらないので、動物種が異なっても用量にのみ依存するとした。その結果、Viracept を摂取したヒトの AUC は 13μMh, C_{max} は 0.85 μM と算出された。

表-2 Viracep を摂取したヒトと閾値 EMS を投与したマウスにおける EMS の体内動態の比較

マウス		ヒト	
実測半減期	10-24 min	推定半減期	10.7 h
Thd	25mg/kg/day	Max dose	0.055 mg/kg/day
C_{maxThd}	315 μM	C_{max}	0.85 μM
AUC_{Thd}	350 μMh	AUC_{max}	13 μMh

C_{max} からの安全係数は 370 と算出された。

実質暴露量(AUC)から算出した安全係数は27であった。

D. 考 察

以上に示したように Viracept に混入していた EMS は、閾値のない LNT(Linear Non-threshold)モデルでは、マウスとヒトの代謝の違いを考慮するとかなりのリスクを伴うものであると算定される。しかし、遺伝毒性における閾値を考慮した場合は、ヒトにおける EMS の吸収、代謝をより安全側に仮定してのリスク評価で、安全であると算定された。これは事故による事例であるが、遺伝毒性発がん物質の取り扱いが現状のままで良いかを、再検討するには有用な事例であると考える。

E. 結論

化学物質の有効利用を考える上で、遺伝毒性発がん物質で閾値を有するものの情報を収集する必要がある。

F. 健康危険情報

特になし。

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なし

G. 知的所有権の取得状況

なし

別紙４

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Regular article

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

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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

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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.

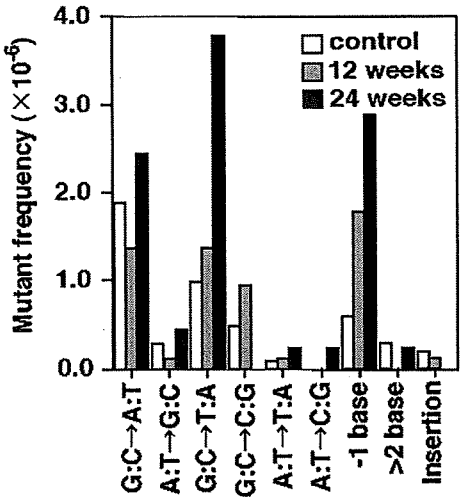

Fig. 1. Comparison of the 6-TG-resistant mutation spectra in control and DE inhaled *gpt* delta mice. The mutant frequencies of control mice and those exposed to DE for 12 weeks and 24 weeks were calculated by dividing the number of each type of *gpt* mutations in the Control all, DE 12 weeks and DE 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			
					Control		DE	
	Nucleotide number	Sequence Change			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.

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

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