

Examination of *lacZ* Mutant Induction in the Liver and Testis of MutaTMMouse following Injection of Halogenated Aliphatic Hydrocarbons Classified as Human Carcinogens

Noriyuki HACHIYA* and Yutaka MOTOHASHI

Department of Public Health, Akita University School of Medicine, Hondo 1-chome, Akita 010-8543, Japan

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Abstract: Possible induction of *lacZ* mutation was examined in the liver and testis of MutaTMMouse following the administration of carcinogenic halogenated compounds, namely 1,2-dichloroethane (DCE), 1,2-dibromoethane (DBE), carbon tetrachloride, or 1,2-dibromo-3-chloropropane (DBCP). Slight increases were observed on the mutant frequency in the testis DNA isolated from the mice 14 days after treatment with DBCP at 40 mg/kg or with DBE at 60 mg/kg but not in the liver. Further investigation was necessary to confirm the mutation induction by these chemicals in the testis including experiments with longer sampling intervals. No increase was detected in the frequency following DCE administration of single doses of up to 150 mg/kg or of consecutive injections of up to 280 mg/kg. Marginal but biologically insignificant responses were observed in the liver from the carbon tetrachloride exposed mice. The present results suggest that these carcinogenic chemicals are less efficient for induction of gene mutation in the liver of MutaTMMouse.

Key words: Mutagenicity, *lacZ* mutation, Transgenic mice, Liver, Testis, 1,2-Dichloroethane, 1,2-Dibromoethane, Carbon tetrachloride, 1,2-Dibromo-3-chloropropane

Introduction

Induction of gene mutation and chromosome aberration by chemical carcinogens *in vivo* animal tissue is an important issue in the investigation of mechanisms in chemical carcinogenesis and for the evaluation of human risks on such chemicals. Extensive studies have been carried out on the genotoxicity of halogenated compounds that manifest carcinogenicity. Results on some carcinogenic halogenated aliphatic hydrocarbons provide little evidence for mutagenesis in laboratory rodents *in vivo* despite their carcinogenicity in the same animal species¹. 1,2-Dichloroethane (DCE, ethylene dichloride), for example, is classified in Group 2B (possible human carcinogen) by IARC² and tumorigenic to rats and mice inducing a variety

of tumors³. DCE induced chromosomal anomalies in cultured human lymphocytes but not in rodent cells *in vitro*. In studies using mice *in vivo*, no increase of micronucleated polychromatic erythrocyte frequency was observed in the bone marrow or peripheral blood after injection of DCE^{4,5}. 1,2-Dibromoethane (DBE, ethylene dibromide), a probable human carcinogen (Group 2A) of the IARC classification⁶, has been reported to induce tumors in a number of organs in rodents, including the forestomach, liver, spleen and thyroid⁷. DBE is a direct acting mutagen in bacteria and mammalian cells in culture⁸. However, no increase of chromosome aberration and micronucleus in the bone marrow^{9,10} nor of dominant lethal has been detected in rats¹¹ and mice^{12,13} after administration of DBE. Carbon tetrachloride induced liver tumors in the mouse, rat and hamster following the exposure by different routes, including inhalation and oral administration, and is classified in Group

*To whom correspondence should be addressed.

2B of human carcinogen¹⁴). Carbon tetrachloride shows little genotoxicity in bacteria, mammalian cells in culture, and rodents *in vivo*¹). On the other hand, 1,2-dibromo-3-chloropropane (DBCP) classified as IARC group 2B gives positive responses of genotoxicity not only *in vitro* but *in vivo*¹).

In recent years, transgenic animals have come to be widely used in the study of mutagenesis *in vivo*. MutaTMMouse, one of the commercially available transgenic mouse strains, is carrying *lacZ* transgenes of *E.coli* that are integrated with a vector phage λ gt10 in the genomic DNA in every cell of the mouse tissue. Because genomic DNA can be isolated from almost all the tissue of the mouse and *lacZ* mutation is detectable more easily than that of mammalian genes, the transgenic strains are very useful to investigate the induction of gene mutation in animal tissues *in vivo*. In a positive selection assay of MutaTMMouse, rare mutant phages carrying mutated *lacZ* gene are detected as plaque that can grow in a host strain *E.coli* C Δ *lac galE* on an agar plate containing phenyl β -D-galactoside (P-Gal) following *in vitro* packaging of phage λ from genomic DNA of MutaTMMouse, and total phage is scored on the plate lacking P-Gal. The present study was conducted to examine the possible induction of *lacZ* mutation in the liver and testis of MutaTMMouse following the administration of one of four carcinogenic halogenated compounds, namely DCE, DBE, carbon tetrachloride, and DBCP.

Materials and Methods

Animals and treatments

Male MutaTMMice, 7 weeks old, were purchased from Covance Research Products Inc. (Denver, PA USA) and were used at 9 weeks of age. Carbon tetrachloride, DBCP, DBE, and DCE were dissolved in olive oil as a vehicle. The animals were given a single injection, intraperitoneally (ip) or by oral gavage (po), of the following doses of chemicals: 75 or 150 mg DCE per kg, 60 mg DBE per kg, 40 mg DBCP per kg, or 700 or 1400 mg carbon tetrachloride per kg. In consecutive administration experiments, intraperitoneal injection was carried out once a day for 5 days a week. DCE was injected at 40 mg/kg/injection five times (200 mg/kg in total) or at 20 mg/kg/injection for first six injections and at 40 mg/kg/injection for the following four days (280 mg/kg in total). DBE was given at 16 mg/kg/injection for successive five days (80 mg/kg in total). The liver and testis were collected 7, 14, and 28 days after the last treatment, immediately frozen in liquid nitrogen, and stored at -80°C until used. The protocols for the animal experiments were

approved of prior to the experiments by the Animal Research Committee of Akita University School of Medicine and the protocols adhered to the "Guidelines for Animal Experimentation".

DNA isolation and analysis of lacZ mutant frequency

Preparations of crude nuclei from the liver and seminiferous tubules from the testis were described previously¹⁵. Genomic DNA was isolated by salting out-chloroform extraction¹⁶ from an aliquot of the crude nuclear preparation of the liver and from whole seminiferous tubules. *In vitro* packaging of phage λ with Transpack extract (Stratagene, La Jolla, CA USA) was performed according to the manufacture's instructions. The mutant frequency in *lacZ* locus was measured by the positive selection assay as described¹⁵).

Results

Because spontaneous frequency of the *lacZ* mutation is known to be stable in MutaMiceTM at least through 8 to 15 weeks of age, the data obtained from the tissues of mice 7 and 14 days after the injection of a vehicle were pooled and used as the baseline frequencies of *lacZ* mutation throughout the experiments. Table 1 indicates the mutant frequencies of *lacZ* in DNA isolated from the liver. The total frequency slightly increased in the liver DNA after injection of carbon tetrachloride at 700 to 1400 mg/kg. Although the difference in the total frequency was statistically significant between the pooled control and the injected groups according to Fisher's exact test, partial overlap was apparent in the range of the frequency obtained in individual mice of the control and each injected group. The frequency observed in seven out of nine mice given carbon tetrachloride was within a range between a minimum 53.0×10^{-6} and a maximum 100.4×10^{-6} of the pooled control group. Therefore, it was concluded that injection of carbon tetrachloride resulted in no biologically significant increase in the mutant frequency of the liver of MutaTMMice.

Among mice receiving DCE, the mutation frequencies in the liver of most animals were within the control level with exception to two. The frequency was extremely high and reached to 1.26% in the mouse coded 607. The same level was also observed in the frequency of the kidney and bone marrow of the same animal (data not shown). On the other hand, no increase was found if mutation in the liver was scored on *cII* locus of the vector phage λ gt10 in the same genomic DNA (data not shown). Furthermore, it was estimated that almost every cell has one *lacZ* mutation, at

Table 1. Frequencies of lacZ mutant in DNA isolated from the liver

Agent	Total dose (mg/kg)	Dosing route, schedule	Sampling time (days)	Animal code	No phage scored	No of mutant	Frequency ($\times 10^{-6}$)
O	0	p.o., single	7	601	498,000	50	100.4
				602	490,000	43	87.8
				603	160,000	14	87.5
				773	1490,000	79	53.0
				<i>Total</i>	<i>2,140,000</i>	<i>136</i>	<i>63.6</i>
DCE	75	p.o., single	14	606	230,000	11	47.8
				607	(361,000) ^{a)}	(4552)	(12610)
				621	410,000	21	51.2
				<i>Total</i>	<i>640,000</i>	<i>32</i>	<i>50.0</i>
DCE	75	p.o., single	28	622	93,000	6	64.5
				623	300,000	15	50.0
				624	443,000	24	54.2
				<i>Total</i>	<i>836,000</i>	<i>45</i>	<i>53.8</i>
DCE	150	p.o., single	7	609	93,000	7	75.3
				610	360,000	30	83.3
				<i>Total</i>	<i>453,000</i>	<i>37</i>	<i>81.7</i>
DCE	150	p.o., single	14	611	248,000	18	72.6
				612	478,000	26	54.4
				625	555,000	26	46.8
				<i>Total</i>	<i>1,281,000</i>	<i>70</i>	<i>54.6</i>
DCE	150	p.o., single	28	626	(330,000)	(150)	(454.5)
				627	534,000	45	84.3
				628	413,000	19	46.0
				<i>Total</i>	<i>947,000</i>	<i>64</i>	<i>67.6</i>
DCE	200	i.p., 40 mg/kg \times 5	14	777	655,000	40	61.1
				778	1,430,000	89	62.2
				779	3,167,000	90	28.4
				<i>Total</i>	<i>5,252,000</i>	<i>219</i>	<i>41.7</i>
DCE	200	i.p., 40 mg/kg \times 5	28	780	540,000	26	48.1
				781	1,340,000	65	48.5
				782	1,580,000	114	72.2
				<i>Total</i>	<i>3,460,000</i>	<i>205</i>	<i>59.2</i>
DCE	280	i.p., 20 mg/kg \times 6 + 40 mg/kg \times 4	14	774	1,080,000	22	20.4
				775	1,110,000	34	30.6
				776	1,150,000	59	51.3
				<i>Total</i>	<i>3,340,000</i>	<i>115</i>	<i>34.4</i>

^{a)}Data in parentheses were excluded in the calculation of total scores. See text for details. *P<0.05, **P<0.01 (Fisher's exact test).

least, in the liver, kidney, and bone marrow of the mouse, because one mutation exists in approximately every eighty *lacZ* genes, and MutaTMMouse is known to carry eighty copies of the transgene in each somatic cell. It is very likely that mouse 607 may be a germ cell mutant in which the mutation had occurred during its parental germ cell development, either in oogenesis or spermatogenesis, before fertilization. Therefore, this unusual frequency was not included in the summation of the total frequency in Table 1.

Another abnormal frequency was observed in the liver of mouse 626. The *lacZ* mutant frequency was 5 to 10 times higher in this animal than that of the other two mice in the same treatment group which show nearly the control level. No increase was detected in the frequency of *lacZ* mutant in the kidney and bone marrow, nor in the frequency of *cII* mutation in the liver of this mouse (data not shown). The source of the increased mutation was not clear in this case. It was plausible, however, that the mutation obtained in the

Table 1. Frequencies of lacZ mutant in DNA isolated from the liver (*Continued.*)

Agent	Total dose (mg/kg)	Dosing route, schedule	Sampling time (Day)	Animal code	No. phage scored	No. of mutant	Frequency ($\times 10^{-6}$)
DBE	60	i.p., single	14	767	1,410,000	86	61.0
				768	1,660,000	107	64.5
				769	1,890,000	118	62.4
				<i>Total</i>	<i>4,960,000</i>	<i>311</i>	<i>62.7</i>
DBE	80	i.p., 16 mg/kg \times 5	14	783	1,330,000	87	65.4
				784	960,000	32	33.3
				785	700,000	14	20.0
				<i>Total</i>	<i>2,990,000</i>	<i>133</i>	<i>44.5</i>
DBCP	40	i.p., single	14	770	1,410,000	62	44.0
				771	1,470,000	144	98.0
				772	2,080,000	102	49.0
				<i>Total</i>	<i>4,960,000</i>	<i>308</i>	<i>62.1</i>
CCl ₄	700	p.o., single	14	614	1,410,000	111	78.7
				615	280,000	29	103.6
				<i>Total</i>	<i>1,690,000</i>	<i>140 *</i>	<i>82.8</i>
CCl ₄	1400	p.o., single	7	617	41,000	4	97.6
				618	83,000	11	132.5
				<i>Total</i>	<i>124,000</i>	<i>15 *</i>	<i>121.0</i>
CCl ₄	1400	p.o., single	14	619	43,000	4	93.0
				620	587,000	88	149.9
				<i>Total</i>	<i>630,000</i>	<i>92 **</i>	<i>146.0</i>
CCl ₄	1400	p.o., single	28	636	340,000	33	97.1
				637	83,000	8	96.4
				638	352,000	32	90.9
				<i>Total</i>	<i>775,000</i>	<i>73 **</i>	<i>94.2</i>

liver of mouse 626 might be a consequence of proliferation of pre-existing mutation that had been generated in a somatic cell during its development after fertilization, and the frequency in the liver of this animal was also excluded in the calculation of the group total in Table 1. No increased mutation was apparent in the liver of MutaTMMice up to 28 days after a single or successive administration of DCE if the data from mice 607 and 626 are omitted. The mutation frequency did not increase in the liver of mice 14 days after injections of a single dosing of DBE or DBCP, or a successive treatment of DBE.

The *lacZ* mutant frequency was determined also in the testis DNA isolated from the same animals in which the mutation frequency was measured in the liver. Summary results are shown in Table 2. The mutant frequency slightly increased in the testis 14 days after treatment of a single dose of DBE or DBCP. The increases in total number of the mutants among scored phages were statistically significant according to Fisher's exact test. Among DBE treated mice at 60 mg/kg, however, a minimum frequency

that was obtained in the mouse coded 768 was lower than a maximum frequency of the control observed in mouse 601. A mouse coded 772 manifested a minimum frequency in the testis among DBCP injected animals, this value was nearly the same as the control mouse 601. No increase was observed in the mutation frequency after the fractionated dosing of 80 mg DBE per kg or after DCE treatments.

Discussion

The slight increases of the mutation frequency were observed in the testis of mice receiving DBCP. Although the present data is not conclusive due to the large variation of individual frequencies, it may still be possible that these results suggest mutation inductions in germ cells of MutaTMMouse by this chemical. Several reports have been published concerning in vivo mutagenesis of DBCP. In mice, DBCP induced micronuclei in the bone marrow⁵⁾ and somatic mutation detected by spot test¹⁷⁾. In germ cells of mice, DBCP did not induce dominant lethal mutation¹⁸⁾, contrary to rats in

Table 2. Frequencies of lacZ mutant in DNA isolated from the testis

Agent	Total dose (mg/kg)	Dosing route schedule	Sampling time (Day)	Animal code	No. phage scored	No. of mutant	Frequency ($\times 10^{-6}$)
O	0	p.o., single	7	601	1,180,000	16	13.6
				602	1,010,000	12	11.9
				773	844,000	2	2.4
				<i>Total</i>	<i>1,854,000</i>	<i>14</i>	<i>7.6</i>
DCE	200	i.p., 40 mg/kg \times 5	14	777	2,735,000	13	4.8
				778	2,070,000	30	14.5
				779	1,900,000	17	8.9
				<i>Total</i>	<i>6,705,000</i>	<i>60</i>	<i>8.9</i>
DCE	200	i.p., 40 mg/kg \times 5	28	780	1,280,000	25	19.5
				781	860,000	1	1.2
				782	153,000	1	6.5
				<i>Total</i>	<i>2,293,000</i>	<i>27</i>	<i>11.8</i>
DCE	280	i.p., 20 mg/kg \times 6 + 40 mg/kg \times 4	14	774	1,280,000	20	15.6
				775	2,780,000	27	9.7
				776	2,170,000	8	3.7
				<i>Total</i>	<i>6,230,000</i>	<i>55</i>	<i>8.8</i>
DBE	60	i.p., single	14	767	1,580,000	33	20.9
				768	609,000	8	13.1
				769	1,510,000	25	16.6
				<i>Total</i>	<i>3,699,000</i>	<i>66 **</i>	<i>17.8</i>
DBE	80	i.p., 16 mg/kg \times 5	14	783	1,590,000	9	5.7
				784	1,500,000	5	3.3
				785	1,460,000	4	2.7
				<i>Total</i>	<i>4,550,000</i>	<i>18</i>	<i>4.0</i>
DBCP	40	i.p., single	14	770	1,360,000	31	22.8
				771	1,080,000	44	40.7
				772	291,000	4	13.7
				<i>Total</i>	<i>2,731,000</i>	<i>79 **</i>	<i>28.9</i>

*P<0.05, **P<0.01 (Fisher's exact test).

which DBCP exposure increased dominant lethal^{11,19}). A weak response was also obtained in the testis exposed to DBE, but the observation again was insufficient to evaluate the mutation induction of DBE. The tissue was taken from the mice 14 days after the injection of DBE or DBCP in the present study. However, the sampling time of 14 days might be insufficient to achieve a full mutation fixation in the testis²⁰. It is necessary to examine the mutant frequency in the testis after 28 days or more following treatment. DBE failed to induce chromosome aberrations or micronuclei in the mouse bone marrow^{1,9,11,21}). No induction of dominant lethal mutation by this chemical was detected in mice^{12,13}) and in rats¹¹). Because, in the present study, DNA was isolated from whole testis and germ cells were not separated from supporting tissues, *lacZ* mutations scored in the testis DNA may also contain mutations in the somatic tissue that are

not inherited to next generation. However, weak mutagenicity showing up to 1.6- and 2.0 fold increases were detected on *lacI* mutant frequency in the liver of DBE treated BigBlue[®] transgenic mice and rats, respectively²²), and effects on several neurotransmitter enzymes were reported in the developing brain of the progeny of DBE exposed male rats²³). Further investigation is necessary on germ cell mutagenicity of DBE and DBCP which are known to interfere with fertility and spermatogenesis^{24,25}).

Some improvements are necessary to minimize variability of the mutation frequency data for further study. Because the methods for scoring mutant are largely dependent on growth of phage and host bacteria, it is not easy to completely eliminate the interfering factors in each experiment. There are several ways to overcome such a difficulty. For instance, increases in scored phage numbers in each animal could be

an effective way to diminish the data variability among animals, besides the addition in the number of mice used. Another approach to confirm mutation induction by the injected chemicals is the determination of the frequency of *cII* locus mutation²⁶⁾ on the same genomic DNA that is isolated from the transgenic mice as mentioned in the results. These investigations are now going on in our laboratory. Glickman and his colleagues have described the usefulness of the examination of mutation spectrum changes in a study of mutagenesis in transgenic animals on tris(2,3-dibromopropyl)phosphate which provides as much as a 50% increase in mutation frequency²⁷⁾. Another problem encountered during the study using transgenic animals was appearance of animals that seem to be outlier or jackpot having an abnormally high mutant frequency. We have experienced that the probability was not low enough^{15, 28)} to incorporate into the experiment of animals containing proliferated progeny cells of pre-existing mutation which had been generated during development before or after fertilization. The efficiency of repair on nonexpressed genes is relatively low²⁹⁾, and spontaneous mutation frequency is higher in transgenes than in endogenous locus³⁰⁾. Mouse numbered 607 was very likely to be a germ cell mutant, but sequence determination is required to confirm the clonal expansion of somatic mutation in the animal coded 626.

The marginal increase was biologically insignificant on the carbon tetrachloride exposed mice. Similarly, no increase was reported in the liver *lacZ* mutant frequency of MutaTMMice receiving carbon tetrachloride, but this chemical enhanced the mutation induction by a genotoxic hepatocarcinogen 5,9-dimethyl dibenzo[c,g]carbazole³¹⁾. It has been considered that carcinogenicity of carbon tetrachloride might be dependent on non-genotoxic mechanisms including processes such as induction of tissue damage with necrosis and of cell proliferation thereafter³²⁾. Carbon tetrachloride is also a non-mutagen to bacteria³³⁾ and to cultured mammalian cells³⁴⁾, and it did not induce micronuclei in the bone marrow cells of mice *in vivo*^{1, 21, 35)}.

It should be noted that largely negative response was observed on the induction of mutation in the liver after administration of DCE, DBE, or CCl₄. Possibility has been discussed on insufficient metabolic activation in the bone marrow might be responsible to the lack of induction of chromosome aberration in bone marrow cell by these carcinogens. The present results, however, suggest that the liver is also ineffective tissue to detect gene mutation induced by these carcinogens.

In summary, slight increases were observed on the *lacZ* mutation frequency in DNA isolated from the testis of

MutaTMMice administered DBE or DBCP. However, the present results failed to conclusively indicate gene mutation induction by these chemicals *in vivo*. The data variability observed in mutation frequency among individual animals was too large to conclude the increase in the mutation frequency in the tissues examined. Although Fisher's exact test is one of the available methods to test statistical significance of the data on very rare events such as induction of mutation or chromosome aberration, its application has limitations because data distribution is not considered in the test results. It is necessary to develop more adequate procedures for the statistical evaluation of the mutation data.

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