Distinction of Mutagenic Carcinogens from a Mutagenic Noncarcinogen in the Big Blue Transgenic Mouse

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The aromatic amines 2,4-diaminotoluene (2,4-DAT) and 2,6-diaminotoluene (2,6-DAT) are structural isomers that have been extensively studied for their mutagenic and carcinogenic characteristics. Both compounds are rapidly absorbed after oral administration and are equally mutagenic in the Ames test; however 2,4-DAT is a potent hepatocarcinogen, whereas 2,6-DAT does not produce an increased incidence of tumors in rats or mice at similar doses. The Big Blue transgenic B6C3F1 mouse carries multiple copies of the lacI mutational target gene. Our studies were designed to determine whether the Big Blue system could be used to detect differences in the in vivo mutagenic activity between the carcinogen–noncarcinogen pair 2,4-DAT and 2,6-DAT and to determine whether the in vivo mutagenesis assay results correspond to the rodent carcinogen bioassay results. Male B6C3F1 transgenic mice were exposed to 2,4-DAT or 2,6-DAT at 0 or 1,000 ppm in the diet for 30 and 90 days or to dimethylnitrosamine as a positive control. Mutant frequencies were nearly identical for all three groups at 30 days, while at 90 days the mutation frequency for the hepatocarcinogen 2,4-DAT (12.1 ± 1.4 × 10−6) was significantly higher (p<0.01) as compared to both age-matched (spontaneous) controls (5.7 ± 2.9 × 10−6) and the 2,6-DAT-exposed group (5.7 ± 2.4 × 10−6). Results from this study demonstrate that the Big Blue transgenic mutation assay can distinguish differences in vivo between the mutagenic responses of hepatic carcinogens and a noncarcinogen; is sensitive to mutagens through subchronic dietary exposure; and yields a differential response depending upon the length of time mice are exposed to a mutagen. — Environ Health Perspect 104(Suppl 3):683–686 (1996)

Key words: diamino-toluene, in vivo mutation assay, dimethylnitrosamine, mutagenic noncarcinogens, Big Blue, transgenic mice, lacI

Introduction

Transgenic mouse mutagenesis assays represent a novel approach for assessing the mutagenicity of various compounds. Before the availability of these assays, the mutagenic properties of chemicals were often determined using only short term in vitro tests. The Big Blue assay represents an opportunity to examine the in vivo mutagenic properties of deleterious agents through the use of a stable genomic integration of the λ shuttle vector (λLIZ), which carries a lacI target gene and a lacZ reporter gene (1,2). After treatment of mice with the agent in question, genomic DNA is isolated from the target organ(s) and the λLIZ is recovered using in vitro phage packaging extracts. Infection of Escherichia coli SCS-8 cells followed by expression of the lacI and lacZ genes permits the detection of phage-carrying mutated lacI genes. If the normal function of the lacI repressor is disrupted, the lacZ gene product, β-galactosidase, is expressed resulting in the generation of blue plaques on plates containing the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (1). Scoring the ratio of these blue mutant plaques to colorless non-mutant plaques allows for the quantitative measure of mutant frequency (MF) in the tissue of interest.

We examined the mutagenic response of the carcinogen–noncarcinogen pair 2,4- and 2,6-diaminotoluene (DAT) (Figure 1) in the liver DNA of Big Blue mice after 30 and 90 days of exposure. Both compounds are used extensively in the synthesis of toluene dioxycanates for the production of polyurethane foams and elastomers, and the annual production of both isomers exceeds 100 million pounds (3,4). The compounds are equally mutagenic in the Ames/Salmonella assay in the presence of S9 (5), and both are readily absorbed, metabolized, and excreted (6). However, while the 2,4-DAT isomer was found to be a potent rodent hepatocarcinogen when administered at 100 and 200 ppm in the National Toxicology Program (NTP) bioassay (3), the 2,6-DAT isomer was not carcinogenic when administered at doses up to 100 ppm (4).

The Big Blue system has not been evaluated using a wide variety of exposure routes. Most Big Blue studies reported to date have used short-term, high-dose gavage or intraperitoneal exposures (ip) with potent mutagens (7,8). The Big Blue assay has been shown to be sensitive to butadiene administered by inhalation for four weeks (9), and there is one report in which the Big Blue assay was used in a subchronic

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Figure 1. Structures of 2,4-diaminotoluene and 2,6-diaminotoluene demonstrating their close structural similarity.
feeding study of the potent carcinogen, 2-acetylaminofluorene (2-AAF) at 600 ppm (10). Neither of the two DAT iso-
mers examined in our study are extremely potent in vitro mutagens; therefore, a
longer-term continuous feeding study was chosen both to increase the sensitivity of
the assay and to more closely mimic the dosing regimens used in the standard NTP
bioassay. We examined the lacI mutant frequencies induced after exposing male
B6C3F1 mice to 2,4-DAT and 2,6-DAT in feed.

Materials and Methods

Chemicals

2,4-DAT, 2,6-DAT, and dimethylnitrosa-
mine (DMN) were obtained from Aldrich
Chemical Co. (Milwaukee, WI). The
diaminotoluenes (DATs) were dissolved in
acetone before being thoroughly mixed
into ground NIH-07 feed. All chemical
and biological reagents used during DNA
isolation, packaging reactions, and plating
were supplied by Stratagene (La Jolla, CA),
with the following exceptions: NZ amine,
used in the formulation of NZY media and
agar, was supplied by Schweizerhall Inc.
(Piscataway, NJ) and agarose was purchased
from Life Technologies (Gaithersburg,
MD). All buffers, including SM buffer, TE
buffer, 10 mM MgSO4, and dounce buffer
were made using chemicals supplied by
Sigma Chemical Co. (St. Louis, MO),
except where noted.

Animals and Treatment

Age-matched male, 6-week-old B6C3F1
transgenic mice bearing multiple copies of
the lacI gene stably inserted into their
genome (Big Blue mice) were obtained from
Stratagene (Taconic Farms, Germantown,
NY). The mice were randomly housed five
per cage and were quarantined for 7 days
before treatment was initiated. All animals
were housed in rooms maintained with
12 hour on-off light cycles. For untreated
control and for DAT-exposed animals,
powdered NIH-07 feed containing either 0
ppm or the respective DAT at 1,000 ppm
was provided ad libitum for 30 or 90 days.
Mice were sacrificed at 31 or 91 days. Five
animals were assigned to each treatment
group. The positive control (DMN-treated)
animals received five daily ip injections of
6 mg/kg DMN in saline and were sacrifi-
ced 15 days following the last injection.
All animals were sacrificed by CO2 physisi-
aton and cervical dislocation. Livers were
removed, immediately frozen in liquid
nitrogen, and stored at –80°C until analysis,
as described earlier (11).

DNA Isolation

Genomic DNA was isolated as described
below using the August 1992 version of the
Stratagene Big Blue Transgenic Mouse
Mutagenesis Assay Manual (12) with minor
modifications. Briefly, small liver sections
(approximately 60–80 mg) were excised from
the frozen tissue and quickly homog-
emized on ice in 3 ml dounce buffer (1.75 g
Na2HPO4, 8.0 g NaCl, 0.2 g KCl, 0.2 g
KH2PO4, 20 ml 0.5 M EDTA [ethylene-ami-
ne tetraacetic acid] made up to 1 liter
at pH 8.0 prior to autoclaving). An equal
volume of proteinase K solution (2 mg/ml
proteinase K, 2% SDS [sodium dodecyl
sulfate], 100 mM EDTA, pH 7.5) was added
to the homogenate and the mixture
was incubated in a water bath at 50°C for
3 hr. After the incubation, an additional 3
ml of dounce buffer and 9 ml of phenol:
choloroform:isoamyl alcohol (25:24:1 v/v)
(Life Technologies) was added to extract
protein. After centrifugation at 1,000 xg
for 10 min, the lower organic phase and
protein at the interface were removed and
discarded using glass pipettes. This proce-
dure was repeated three times. After the
third centrifugation, the upper aqueous
phase containing the liver DNA was
removed using wide bore pipettes. The
DNA was then precipitated by the slow
addition of two volumes of ethanol. The
DNA was recovered by spooling onto
a glass rod and was resuspended in 200 to
500 μl TE buffer. The concentration of
DNA was measured spectrophotometri-
cally and adjusted to 0.5 mg/ml with TE
buffer (10 mM Tris–HCl and 1 M
EDTA, pH 7.5, autoclaved).

Packaging Genomic DNA

Excision and packaging of the λLIZ
vector from genomic DNA was performed
using lambda phage packaging extract
(Transpack, Stratagene) according to the
Stratagene Big Blue instruction manual
(12). Rescue efficiency was estimated by
plating serial dilutions of the packaging
reactions 1 to 2 days before plating to
quantitate mutant frequency. All diluted
bacteriophages were kept at 4°C and were
plated within 3 days of packaging.

Plating Assay

The plating procedures generally followed
the Big Blue instruction manual (12),
with the following minor modifications.
Liquid NZY media supplemented with
0.25% maltose and 12.5 mM MgSO4 was
inoculated with an aliquot of SCS-8 E. coli
bacteria (Stratagen) from an overnight
culture. After a 4-hr growth period at 37°C,
the SCS-8 cells were prepared for plating
by pelleting and resuspending in 10 mM
MgSO4 at an OD500 of 0.5. The efficiency
of each packaging reaction was determined
by plating serially diluted (1:1,000) phage
with SCS-8 E. coli in triplicate for each
template. The number of viable plaque-
forming units (pfu) was then determined by
counting overnight plating results. These
data were then used to calculate the
volume of the original packaging reaction
necessary to yield 15,000 pfu. When plating
for quantitative mutagenesis, the calcu-
lated aliquot amounting to approximately
15,000 pfu was incubated for 15 min with
2.0 ml SCS8 E. coli. The phage/bacteria
complexes were then added to 35 ml of
molten (47°C) top agar (NZY media con-
taining 0.7% agarose) containing 1.5
mg/ml X-gal (Stratagene). The top agar
was then poured onto 25 x 25 cm2 assay
trays containing 250 ml NZY bottom agar
and incubated overnight at 37°C. Serially
diluted phage were again plated in tripli-
cate on the second day to determine the
number of pfu per sample. The number of
pfu per tray was never allowed to exceed
20,000 to ensure the detection of faint blue
mutant plaques, with 15,000 plaques per
plate considered to be optimum. Mutant
frequencies were calculated based on the
extrapolation from the triplicate titer plates
generated at the time of plating for mutants
(day 2). (The number of viable phage were
not determined by counting portions of
the large plates.)

Blue plaques, indicating the mutant (lacI+)
phenotype, were scored visually
using a light box and a red-cellophane
transparency to enhance the color contrast
of the mutant plaques. All mutant plaques
were scored by two individuals. Mutant
plaques were cored into individual tubes
containing 0.5 ml SM buffer (5.8 g NaCl;
2.0 g MgSO4 [7 H2O], 50 ml 1.0 M
Tris–HCl, 5 ml 2% gelatin [w/v], made
up to 1 liter and pH 7.5 prior to autoclaving)
and stored at 4°C for future verifica-
tion. All mutant plaques were verified by
replating isolated phage on 100 mm2 plates
in the presence of X-gal.

Results

Effect of Dosing Time

Two of the five positive control animals
died during treatment, apparently due to
the toxic effects of DMN. All of the DAT-exposed mice survived the length of the study. The observed Mf in the livers of DMN-treated positive control mice was $31 \times 10^{-5}$, which is similar to published values using the same treatment regimen (8). Mutant frequencies for the untreated control mice at 10.0 and $5.65 \times 10^{-5}$ at 30 and 90 days, respectively, were similar to those previously reported (13–15). The increased mutant frequency observed in 2,4-DAT-treated animals was statistically significantly different ($p < 0.01$) from age-matched controls after feeding the chemical for 90 days, but not following 30 days of chemical exposure (Figure 2A and B). These data indicate a longer dosing regimen may be necessary to observe induced mutations in vivo following treatment with less potent carcinogens. This may be due to the cumulative effects of chemical exposure over a period of 90 days compared to 30 days, to a requirement of a longer time period necessary for the expression of the mutant phenotype in vivo, or a combination of these and other factors.

**Effects of Chemical Treatment**

The Mf ($\text{mean} \times 10^{-5} \pm \text{SD; } n = 5$) at 30 days for control, 2,4-DAT, and 2,6 DAT groups were $10.0 \pm 1.9, 9.3 \pm 1.4$, and $8.7 \pm 2.8$, respectively (Figure 2A). The Mf at 90 days were $5.7 \pm 2.9, 12.1 \pm 1.4$, and $5.6 \pm 2.4$ for control, 2,4-DAT and 2,6-DAT animals, respectively (Figure 2B). Using a one-way analysis of variance with a least significant difference test, there was a significant difference ($p \leq 0.01$) in the observed Mf at 90 days between 2,4-DAT treated animals as compared to either the control or the 2,6-DAT-treated animals. The observed increase in Mf at 90 days in the 2,4-DAT treatment group was 2.1-fold over the negative control group Mf at 90 days. As expected, the Mf calculated for the positive control DMN-treated animals was also found to be statistically significantly higher (3.12-fold over control) than all other groups at both time points.

**Discussion**

The mutagenic isomers 2,4-DAT and 2,6-DAT are of special interest because of their differing carcinogenic response in bioassays conducted by the NTP, even though they exhibit similar mutagenic potencies in Salmonella typhimurium (5,6). The 2,4-DAT isomer was shown to induce hepatocellular carcinoma in rats and mice in the 2-year bioassay, while 2,6-DAT did not give rise to any tumors in male or female rats or mice at similar doses. Thus, it was of interest to determine the in vivo mutagenic potential of these isomers in the Big Blue assay system.

In order to parallel the NTP 2-year bioassays of 2,4-DAT and 2,6-DAT, we chose to expose the animals to these chemical via the diet. Most Big Blue studies to date have used short-term exposure periods, high-potency mutagens, and parenteral dosing regimens. We reasoned that a continuous exposure study would also be more relevant for comparison to NTP bioassay results. The dose chosen (1,000 ppm) corresponds to the highest nontoxic dose used in a 90-day subchronic study (3,4). The results of the present study demonstrate that 90 days was an appropriate minimum time period for detecting mutations induced by 2,4-DAT administered by feeding. Other investigators have used the dietary route of exposure in the Big Blue model successfully but with the much more potent mutagen, 2-AAF (10). They demonstrated a 3-fold increase in Mf over background after a 28-day subchronic exposure to 600 ppm 2-AAF. The carcinogen, 2,4-DAT, at 1,000 ppm increased the observed Mf in the liver approximately 2-fold over the Mf observed in the age-matched control group after 90 days but not after 30 days. The Mf in liver of the 2,6-DAT treatment group also delivered in the diet at 1,000 ppm was not significantly different from the age-matched control group after either 30 or 90 days of chemical exposure. In this study, the positive control mutagen DMN induced a 3-fold increase in the observed Mf in the liver as compared to the Mf in the age-matched control group (Figure 2). It would appear from these data that longer exposures to some mutagens may be required to produce a detectable mutagenic response in vivo.

This observation should be considered in the design of studies assessing the in vivo mutagenic activity of chemicals of weak or unknown mutagenicity, as well as in the critical evaluation of studies that report negative results following short-term chemical treatment.

In the present study, mutation frequencies were assessed after 30- and 90-day exposures to 2,4-DAT or 2,6-DAT. We demonstrate a 2-fold increase in Mf at the 90-day time point in the 2,4-DAT-treated animals that was significant ($p < 0.01$) as compared to either the untreated controls or the 2,6-DAT-treated animals. Both these chemicals are in vivo mutagens in the Ames/Salmonella assay, although only 2,4-DAT induces unscheduled DNA synthesis (16). That 2,4-DAT but not 2,6-DAT was found to be mutagenic in vivo is interesting in light of previous studies conducted in this laboratory in which we demonstrated that 2,4-DAT but not 2,6-DAT induces hepatocellular proliferation (17). We postulate that a cytotoxic effect in target organs could produce an elevated Mf in Big Blue mice as a result of clonal expansion of chemically mutated cells. Cells carrying DNA damage from either 2,4-DAT or 2,6-DAT treatment would yield mutations if forced to replicate; however, hepatocellular proliferation is only induced by 2,4-DAT. The cytotoxicity and compensatory cell proliferation induced by 2,4-DAT treatment leads to mutation fixation during cell proliferation. Such damage effectively increases the mutation rate (mutants/cell/generation) leading to the elevated mutant frequency (mutants/viable phage) observed in 2,4-DAT-exposed mice at 90 days (Figure 2B). Such a mechanism could also be involved in the promotion and progression stages of carcinogenesis (18). After 90 days of dietary exposure to 2,4-DAT, the
Mf was significantly elevated compared to control (Figure 2B). This may reflect a sustained proliferative stimulus that may clonally expand mutant cell populations. After a 30-day exposure period, the Mf in 2,4-DAT-treated animals was not elevated, suggesting that longer exposure/period durations may be necessary to observe the in vivo mutagenic effects of chemicals, the effects of chemicals administered via dietary exposure, or both. The noncarcinogenic isomer 2,6-DAT did not increase the Mf in either the 30- or 90-day animals at a treatment dose identical to the 2,4-DAT treatment dose. Thus, 2,6-DAT may not cause an increase in DNA damage in mouse liver and may only show a mutagenic response in vitro. The proliferative effect has not been observed in 2,6-DAT-exposed animals (17). It is conceivable that DNA damage may have been induced in the liver by exposure to both 2,4-DAT and 2,6-DAT. In the absence of cellular replication in the case of 2,6-DAT-treated mice, such damage may have been repaired with a corresponding reduction of the Mf to background levels. The only known difference between these two in vitro mutagens to explain their widely different mutagenic activity in vivo lies in the ability of 2,4-DAT to induce hepatocellular proliferation. Chemically induced cell proliferation may therefore be a critical factor in fixation of mutations in vivo. Consistent with a view invoking differential DNA repair, it has been shown recently that enhanced repair of DNA adducts can prevent the carcinogenic effects of methylnitrosourea (19).

In conclusion, this study demonstrates that the Big Blue assay is able to discriminate between the in vivo mutagenic response of two compounds with differing carcinogenic properties but with similar mutagenic activity in S. typhimurium. The major difference between these two chemicals in vivo is an induced cell proliferative response following exposure to 2,4-DAT that does not occur after treatment with 2,6-DAT, suggesting an important role for chemically induced cell proliferation on the mutagenic effect of chemicals. This study also illustrates that the dietary administration of chemicals is an appropriate route of administration for in vivo mutagenesis studies, allowing for longer duration exposures. We believe that these data help to validate this transgenic mouse model as a potential indicator of carcinogenic response and suggest that the Big Blue assay is also useful for mechanistic studies of carcinogenicity.

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