DNA Adducts and Mutagenic Specificity of the Ubiquitous Environmental Pollutant 3-Nitrobenzanthrone in Muta Mouse

Volker M. Arlt,[1]* Li Zhan,[2] Heinz H. Schmeiser,[3] Masamitsu Honma,[2] Makoto Hayashi,[2] David H. Phillips,[1] and Takayoshi Suzuki[2]

[1] Section of Molecular Carcinogenesis, Institute of Cancer Research, Surrey, United Kingdom
[2] Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo, Japan
[3] Division of Molecular Toxicology, German Cancer Research Center, Heidelberg, Germany

3-nitrobenzanthrone (3-NBA) is an extremely potent mutagen in the Salmonella reversion assay and a suspected human carcinogen identified in diesel exhaust and in ambient airborne particulate matter. To evaluate the in vivo mutagenicity of 3-NBA, we analyzed the mutant frequency (MF) in the cII gene of various organs (lung, liver, kidney, bladder, colon, spleen, and testis) in lambda/lacZ transgenic mice (Muta Mouse) after intraperitoneal treatment with 3-NBA (25 mg/kg body weight injected once a week for 4 weeks). Increases in MF were found in colon, liver, and bladder, with 7.0-, 4.8-, and 4.1-fold increases above the control value, respectively, whereas no increase in MF was found in lung, kidney, spleen, and testis. Simultaneously, induction of micronuclei in peripheral blood reticulocytes was observed. The sequence alterations in the cII gene recovered from 41 liver mutants from 3-NBA-treated mice were compared with 32 spontaneous mutants from untreated mice. Base substitution mutations predominated for both the 3-NBA-treated (80%) and the untreated (81%) groups. However, the proportion of G:C→T:A transversions in the mutants from 3-NBA-treated mice was higher (49% vs. 6%) and the proportion of G:C→A:T transitions was lower than those from untreated mice (10% vs. 66%). The increase in MF in the liver was associated with strong DNA binding by 3-NBA, whereas in lung, in which there was no increase in MF, a low level of DNA binding was observed (268.0–282.7 vs. 8.8–15.9 adducts per 10^8 nucleotides). DNA adduct patterns with multiple adduct spots, qualitatively similar to those formed in vitro after activation of 3-NBA with nitroreductases and in vivo in rats, were observed in all tissues examined. Using high-pressure liquid cochromatographic analysis, we confirmed that all major 3-NBA-DNA adducts produced in vivo in mice are derived from reductive metabolites bound to purine bases (70–80% with deoxyguanosine and 20–30% with deoxyadenosine in liver). These results suggest that G:C→T:A transversions induced by 3-NBA are caused by misreplication of adducted guanine residues through incorporation of adenine opposite the adduct (A-rule). Environ. Mol. Mutagen. 43: 186–195, 2004. © 2004 Wiley-Liss, Inc.

INTRODUCTION

Air pollution from diesel exhaust is an increasing concern as an environmental risk factor for carcinogenesis [World Health Organization, 2003]. Diesel exhaust is known to induce tumors in experimental animals and epidemiological studies have shown that occupational exposure to diesel exhaust is associated with an increased risk of lung cancer in humans [International Agenecy for Research on Cancer, 1989; Boffeta et al., 2001]. Nitropolycyclic aromatic hydrocarbons (nitro-PAHs) are widely distributed environmental pollutants found in airborne particulate matter, especially that emitted from diesel and gasoline engines [Tokiwa and Ohnishi, 1986; Yaffe et al., 2001]. Many members of this

class of compounds are potent mutagens and carcinogens and their detection in the lungs of nonsmokers with lung
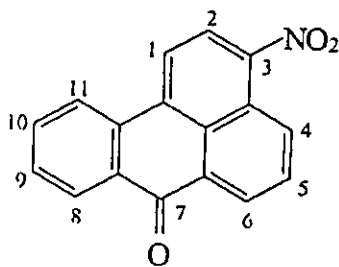
Fig. 1. Structure of 3-NBA.

cancer has led to considerable interest in assessing their potential cancer risk to humans [International Agenecy for Research on Cancer, 1989; Tokiwa et al., 1993; Purohit and Basu, 2000].

3-nitrobenzanthrone (3-NBA; 3-nitro-7H-benz[de]anthracen-7-one; Fig. 1) was recently detected in diesel exhaust and in airborne particulate matter and might originate both from incomplete combustion of fossil fuels and from reaction of the parent aromatic hydrocarbon with nitrogen oxides in the atmosphere [Enya et al., 1997; Feilberg et al., 2002; Seidel et al., 2002; Murahashi, 2003]. As a likely consequence of atmospheric washout, 3-NBA has also been detected more recently in surface soil and rainwater [Murahashi et al., 2003a, 2003b; Watanabe et al., 2003]. The uptake of 3-NBA in humans has been demonstrated by the detection of 3-aminobenzanthrone (3-ABA), a major metabolite of 3-NBA, in the urine of salt mine workers occupationally exposed to diesel emissions [Seidel et al., 2002]. 3-NBA is one of the most potent bacterial mutagens known to date, inducing 0.2 and 6.3 million revertants per nmol in *Salmonella typhimurium* TA98 and YG1024, respectively [Enya et al., 1997]. Moreover, 3-NBA induces micronuclei in mouse and in human cells and exhibits DNA strand-breaking activity in human cells [Enya et al., 1997; Phousongphouang et al., 2000; Arlt et al., 2004; Lamy et al., 2004]. Furthermore, 3-NBA is also an effective mutagen in human cells and preliminary data suggest that 3-NBA is carcinogenic in rats [Adachi et al., 2000; Phousongphouang et al., 2000].

3-NBA forms specific DNA adducts in different in vitro systems, in cells, and in vivo in rats [Bieler et al., 1999, 2003; Borlak et al., 2000; Kawanishi et al., 2000; Arlt et al., 2001, 2002, 2003a, 2003b, 2003c], and these adducts may play an important role in the initiation of mutagenesis and carcinogenesis. Although the structures of the DNA adducts remain to be characterized, the major DNA adducts formed in vitro and in rats are products derived from reductive metabolites bound to purine bases without carrying an N-acetyl group [Arlt et al., 2001, 2003a, 2003c].

Despite the strong mutagenicity of 3-NBA in bacteria, little is known about its mutagenicity in vivo. Transgenic mutation assays are a powerful tool to study chemical mutagenesis in experimental animals [Suzuki et al., 2000;

Kohara et al., 2002a, 2002b; Itoh et al., 2003]. In addition, molecular analyses of induced mutations may reveal chemical-specific mutation spectra. To evaluate the mutagenicity of 3-NBA, a transgenic mouse model, Muta Mouse, was used and mutations in the *cII* gene were assessed. In addition, DNA adduct formation was investigated using ^{32}P-postlabeling.

MATERIALS AND METHODS

Synthesis of 3-NBA

3-NBA was synthesized as described recently [Arlt et al., 2002]. The authenticity of 3-NBA was confirmed by UV, electrospray mass spectra (ES-MS), and high-field proton nuclear magnetic resonance spectroscopy.

Animal Experiments

Male Muta Mouse animals were supplied by Covance Research Products (Denver, PA) and were acclimatized for 1 week before use. 3-NBA was dissolved in olive oil (2.5 mg/ml). Five mice (7- to 8-week-old, ~ 25 g body weight) were treated with 25 mg/kg body weight once a week for 4 weeks by intraperitoneal injection (10 ml/kg). Five mice received olive oil only at the same time and in the same manner. Mice were killed 3 days after the last treatment, and lung, liver, kidney, bladder, spleen, colon, and testis tissues were collected. Tissue samples were stored at −80°C until DNA isolation. DNA was extracted by a standard phenol extraction method.

Peripheral Blood Micronucleus Assay

Forty-eight hours after the first (week 1) and second injection (week 2), peripheral blood (5 μl) was collected without anticoagulant from a tail blood vessel, placed on an acridine orange-coated glass slide, covered with a coverslip, and stained [Hayashi et al., 1990]. One thousand peripheral blood reticulocytes (RETs) per animal were analyzed by fluorescence microscopy within a few days of slide preparation, and the number of cells with micronuclei was recorded.

Lambda *cII* Mutation Analysis

The MutaPlax *cII*-Select Kit (Epicentre Technologies, Madison, WI) was used for the lambda *cII* assay. The kit contained lambda packaging extracts and cultures of *hfl⁻ Escherichia coli* G1225 for both recovered phage titer and selection of mutant phages. Lambda packaging and positive selection for *cII* mutants was performed according to the protocol of the manufacturer with minor modifications as described previously [Jakubczak et al., 1996]. Briefly, 500 μl of the packaged phage solution were incubated with 1 ml of G1225 cells (OD$_{660}$ = 1.0) at room temperature for 30 min, mixed with 14 ml Luria broth top agar, and plated on five 9 cm dishes containing 10 ml bottom agar. The plates were incubated at 25°C for 48 hr. For total virus titer, a 5 μl portion of the packaged phage was mixed with 200 μl of strain G1225 cells and 6 ml LB top agar, plated on two dishes, and incubated at 37°C for 24 hr. Wild-type phage recovered from Muta Mouse has a *cI⁻* phenotype, which permitted plaque formation on the *hfl⁻* strain at 37°C but not at 25°C. The mutant frequency (MF) was determined by dividing the number of mutants plaques by the total number of recovered phage evaluated from each animal.

Sequencing of cll Mutants

Mutations in the lambda *cll* transgene were analyzed using the DNA cycle sequencing method described previously [Suzuki et al., 2000]. The *cll* gene region (294 base pairs) was PCR-amplified directly from mutant plaques using the primer pair 5'-AAAAAGGGCATCAAATTAAACC-3' and 5'-CCGAAGTTGAGTATTTTTGCTGT-3'. A 446 base pair PCR product was purified and used for the sequencing reaction with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan). PCR amplification and DNA sequencing was performed using a Minicycler PTC-150-25 (MJ Research, Watertown, MA) and an ABI Prism 310 Genetic Analyzer (Applied Biosystems), respectively. Statistical analysis of the mutation spectra for differences between treated and control spectra was conducted using the Adams-Skopek algorithm [Cariello et al., 1994].

^{32}P-Postlabeling Analysis and High-Pressure Liquid Chromatography (HPLC) Analysis of ^{32}P-Labeled 3',5'-Deoxyribonucleoside Bisphosphate Adducts

^{32}P-postlabeling analyses using nuclease P1 digestion, butanol extraction, and autoradiography were performed as described [Arlt et al., 2002]. Chromatographic conditions for thin-layer chromatography (TLC) on polyethyleneimine-cellulose (PEI-cellulose) were D1, 1.0 M sodium phosphate, pH 6.0; D3, 4 M Li-formate, 7 M urea, pH 3.5; D4, 0.8 M LiCl, 0.5 M Tris, 8.5 M urea, pH 8.0. DNA adduct levels (relative adduct labeling; RAL) were calculated from the adduct cpm, the specific activity of [γ-^{32}P]ATP and the amount of DNA (pmol of DNA-P) used. Results were expressed as DNA adducts/10^8 nucleotides. Individual adduct spots detected by the ^{32}P-postlabeling assay, or the origins after D1 only, were excised from the TLC plates, extracted, and cochromatographed on HPLC with reference bisphosphate adducts essentially as described previously [Arlt et al., 2001].

Preparation of Reference Compounds for ^{32}P-Postlabeling

Deoxyadenosine (dA) and deoxyguanosine (dG) 3'-monophosphates or calf thymus DNA (4 μmol/ml; Sigma) were incubated with 3-NBA (0.3 mM) in a reaction containing xanthine oxidase (1 U/ml; Sigma, Gillingham, U.K.) in 50 mM potassium phosphate buffer, pH 7.0, in the presence of 1 mM hypoxanthine (Sigma) as described previously [Bieler et al., 1999; Arlt et al., 2001]. The resulting adducted deoxypurine 3'-monophosphates were used as reference compounds in ^{32}P-postlabeling experiments.

RESULTS

Micronucleus Induction in Peripheral Blood of Muta Mouse

The frequencies of micronucleus formation in peripheral blood RETs after treatment of Muta Mouse with 25 mg/kg body weight 3-NBA are shown in Table I. Significant increases in the frequency of micronucleated RETs were observed 48 hr after the first (week 1) and the second (week 2) treatment. This result confirms data on micronucleus formation observed in another mouse strain using the same amount of 3-NBA [Enya et al., 1997].

TABLE I. Micronucleus Induction in Mouse Peripheral Blood Reticulocytes of Muta Mouse Treated With 3-NBA

Treatment	Micronucleated RETs per 1,000 RETs after the first and second i.p. injection of 3-NBA (mean ± SD)[a]	
	Week 1	Week 2
Control	3.0 ± 0.8	3.0 ± 1.2
3-NBA	7.5 ± 2.8[b]	8.4 ± 3.0[b]

[a]Values represent the mean ± SD of five animals.
[b]Significantly different from untreated control animals at $P < 0.05$ (t-test).

TABLE II. Mutant Frequency in the *cII* Gene From Various Organs of Muta Mouse Treated With 3-NBA

Organ	Mean MF × 10^{-6} ± SD[a]	
	Control	3-NBA
Lung	38.1 ± 24.4	38.5 ± 14.2
Liver	30.5 ± 12.1	147.4 ± 49.4[b]
Kidney	36.2 ± 13.4	37.6 ± 13.4
Colon	36.7 ± 17.6	258.7 ± 106.4[b]
Spleen	28.8 ± 9.6	34.4 ± 11.1
Testis	15.2 ± 7.4	22.8 ± 6.3
Bladder	13.1[c]	54.4[c]

[a]Results represent the mean ± SD of five animals.
[b]Significantly different from untreated control animals at $P < 0.001$ (t-test).
[c]Results represent the mean of two animals only.

Mutagenic Specificity of 3-NBA in Muta Mouse

DNA was isolated from lung, liver, kidney, bladder, colon, spleen, and testis 3 days after the last treatment. The results of the *cII* MF analyses are shown in Table II (mouse-by-mouse data are given in the Appendix). The MF was significantly increased above spontaneous levels in colon and liver, with 7.0- and 4.8-fold increases, respectively. A 4.1-fold increase was also seen for bladder, but because only two control and two treated mice were evaluated for this tissue, this difference was not tested for significance. No increase in MF above control levels was seen in the other tissues.

Since the metabolic activation of 3-NBA due to hepatic enzymes has been intensively studied, the mutagenic specificity of 3-NBA was examined in liver tissue only. Forty-one 3-NBA-induced mutants from the liver were sequenced, together with 32 spontaneous mutants from the livers of untreated mice. The mutation spectra are summarized in Table III. Spontaneous mutations consisted mainly of base substitutions (26 of 32 mutations). Among them, G:C→A:T transitions (21 of 26 transitions) predominated and almost all of them (18 of 21 mutations) occurred at CpG sites. 3-NBA-induced mutations also consisted mainly of base substitutions (33 of 41 mutations). Comparing to the control, G:C→A:T transitions were decreased (10% vs. 66%) and G:C→T:A transversions were increased (49% vs. 6%).

TABLE III. Classification of 3-NBA-Induced and Spontaneous *cII* Mutations From Liver of Muta Mouse

Mutation type	Spontaneous		3-NBA-induced	
	Number	%	Number	%
Total	32	100	41	100
Base substitutions	26	81	33	80
Transitions	21	66	6	15
G:C→A:T (at CpG)	21 (18)	66 (56)	4 (2)	10 (5)
A:T→G:C	0	0	2	5
Transversions	5	16	27	66
A:T→T:A	2	6	4	10
A:T→C:G	0	0	1	2
G:C→T:A	2	6	20	49
G:C→C:G	1	3	2	12
Frameshifts (−1)	2	6	5	12
Frameshifts (+1)	2	6	0	0
Deletions	0	0	1	2
Insertions	0	0	0	0
Complex	2	6	2	5

The distribution of mutations in the liver is shown in Figure 2. 3-NBA-induced mutations were distributed over all the *cII* gene and no apparent hot spots were observed. Statistical analysis of the mutational spectra data demonstrated that the differences between 3-NBA-treated and control spectra were significant ($P = 0.0041$).

DNA Adduct Formation of 3-NBA in Muta Mouse

DNA adduct formation in Muta Mouse was analyzed in liver, in which MF was increased, and in lung, where no increase in MF was observed. As shown in Figure 3, 3-NBA induced essentially the same DNA adduct pattern as those observed in different in vitro activation systems, including cytosolic nitroreductases and human liver microsomes, in human cells, and in rats [Arlt et al., 2001, 2003a, 2003c]. Using butanol enrichment, the observed pattern consisted of a cluster of five adducts (spots 1–5). Analyses using nuclease P1 enrichment resulted in a cluster of four adducts (spots 1–3 and 6). No DNA adducts were observed in DNA isolated from tissues of control animals treated with vehicle (olive oil) only (data not shown). Although the structures of these adducts have yet to be elucidated, all adduct spots detected in this study on TLC plates were chromatographically indistinguishable from adduct spots found in incubations with dA (adduct 1 and 2) and dG 3'-monophosphates (adduct 3, 4, and 5) generated by 3-NBA activated by xanthine oxidase [Arlt et al., 2001]. As a second, independent chromatographic procedure, we also employed reversed-phase HPLC analysis to confirm the identities of adduct spots formed by 3-NBA (Fig. 4). The results thus obtained confirmed the findings from chromatography on TLC plates. DNA binding in lung and liver ranged from 8.8 to 15.9 and from 268.0 to 282.7 adducts per 10^8 nucleotides for total DNA adducts, respectively (Fig. 5A). Levels of individual adduct spots are given in Figure 5B. In particular, adduct spot 3 was the predominant adduct formed in both tissues.

DISCUSSION

There are increasing concerns over the carcinogenic risk of diesel exhaust. 3-NBA is a potent mutagen identified in diesel exhaust and airborne particulate matter, and preliminary data indicate that 3-NBA is carcinogenic in rodents [Enya et al., 1997; Adachi et al., 2000; Seidel et al., 2002]. 3-NBA is highly mutagenic in the *Salmonella typhimurium* strains TA98 and YG1024, producing mutagenic responses comparable to those of 1,8-dinitropyrene, one of the most mutagenic nitro-PAH reported to date [Enya et al., 1997]. In addition, previous data also indicate that 3-NBA is an efficient mutagen in human lymphoblastoid-derived MCL-5 cells at the *TK* and *HPRT* loci [Phousongphouang et al., 2000]. These observations suggest that 3-NBA may also be mutagenic in vivo. Therefore, we investigated the in vivo mutagenicity of 3-NBA after intraperitoneal treatment of transgenic Muta Mouse. The mutagenicity of 3-NBA in vivo was clearly demonstrated by this assay using the *cII* gene as target sequence. Simultaneously, the clastogenicity of 3-NBA was evaluated by the peripheral blood micronucleus assay. The assay for micronuclei showed a significant increase in the frequency of micronucleated reticulocytes, confirming previous data obtained in another mouse strain and in human cells [Enya et al., 1997; Phousongphouang et al., 2000; Arlt et al., 2004; Lamy et al., 2004].

In our study, 3-NBA increased the MF in colon, liver, and bladder, whereas no increase in MF was observed in lung, kidney, spleen, and testis. Human exposure to 3-NBA is thought to occur primarily via the respiratory tract and it is possible that the tissue-specific distributions of MF would differ after inhalation or intratracheal treatment with 3-NBA. On the other hand, different tissues will respond at different rates after exposure to a mutagen, rates of metabolism aside. Mutation fixation time may be different in different tissues, depending on cell turnover rate in a tissue, with colon being a rapid responder and lung being much slower [Heddle et al., 2003; Thybaud et al., 2003]. This could explain why 3-NBA did not induce mutations in the lung in the present study. In a recent study with rats, we found that after a single dose of 3-NBA (2 mg/kg body weight, i.p.), DNA binding by 3-NBA was higher in lung compared to liver [Arlt et al., 2003a]. Moreover, preliminary results also indicate that binding by 3-NBA in rat DNA is much higher in lung compared to liver after treatment with a single dose of 3-NBA (2 mg/kg body weight) by intratracheal instillation (data not shown). Therefore, the results of the present study may suggest that tissue-specific DNA adduct formation in mice is different to those observed in the rat model, different dosing and administration aside.
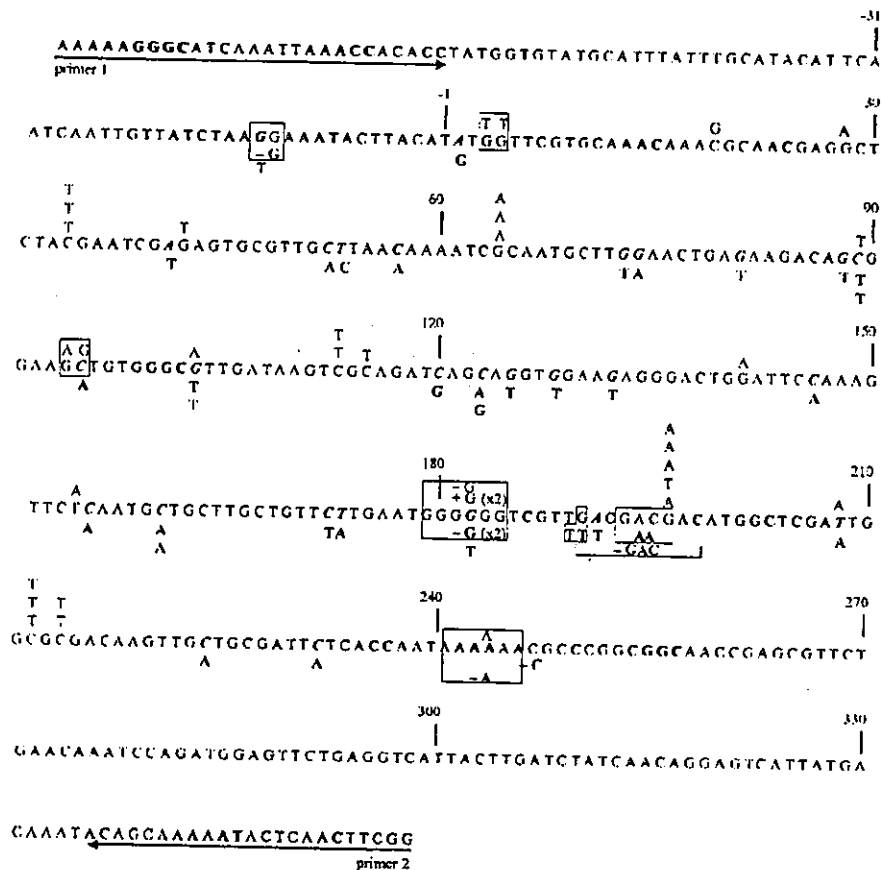
-31

AAAAAGGGCATCAAATTAAACCACACCTATGGTGTATGCATTTATTTGCATACATTCA
primer 1

-1

ATCAATTGTTATCTAAGGAAATACTTACATATGGTTCGTGCAAACAAACGCAACGAGGCT

T
T
T   T
CTACGAATCGAGAGTGCGTTGCTTAACAAAATCGCAATGCTTGGAACTGAGAAGACAGCG
T   AC   A   TA   T   TT
T

T   120   150
GAAGCTGTGGGCGTTGATAAGTCGCAGATCAGCAGGTGGAAGAGGGACTGGATTCCAAAG
A   T   G   A T   T   A
T   G

180   A
A
A
TTCTCAATGCTGCTTGCTGTTCTTGAATGGGCGGTCGTTGACGACGACATGGCTCGATTG
A   A   TA   TT T   AA   A
A   CAC

T
T T   240   270
GCGCGACAAGTTGCTGCGATTCTCACCAATAAAAACGCCCGGCGGCAACCGAGCGTTCT
A   A   A

300   330
GAACAAATCCAGATGGAGTTCTGAGGTCATTACTTGATCTATCAACAGGAGTCATTATGA

CAAATACAGCAAAAATACTCAACTTCGG
primer 2

Fig. 2. Mutations in the liver of *cII* gene obtained from control and 3-NBA-treated Muta Mouse. The sequence from top to bottom represents the amplified lambda *cII* region. Mutations shown above the strand were detected in control mice, whereas those below the strands (in bold) were detected in 3-NBA-treated mice. The boxes represent tandem (double) or complex mutations or deletions with undefined positions within the boxed region.
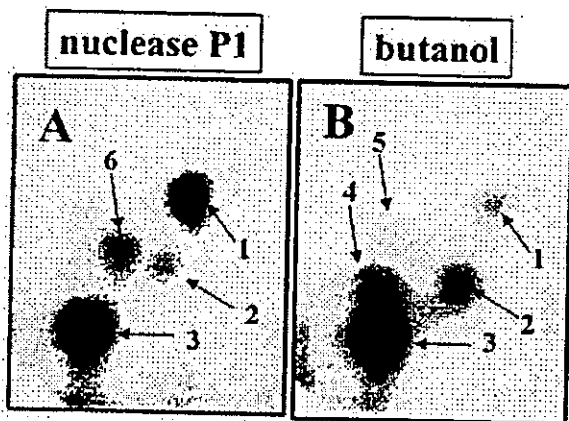
nuclease P1

A

butanol

B

Fig. 3. Autoradiographic profiles of DNA adducts obtained from digests of liver DNA of Muta Mouse treated with 3-NBA using the nuclease P1 (A) and butanol (B) enrichment versions of the ^{32}P-postlabeling assay.

The highest induced MF was found in the colon. A strong increase in MF in colon has also been observed for other mutagens, including dinitropyrenes [Kohara et al., 2002a], suggesting that the colon is a very sensitive organ for mutagenesis in the transgenic mouse mutation assay, probably because of its high proliferation rate. When the MF induced by 3-NBA in Muta Mouse is compared with those of a mixture of dinitropyrenes administered under similar conditions [Kohara et al., 2002a], it is apparent that 3-NBA has an approximately 10-fold higher potency in the main target tissue, colon. However, it is noteworthy that enteric bacteria might also play a role in the mutagenic activation of 3-NBA in colon by nitroreduction. This is in line with earlier reports in rats of strong DNA adduct formation of 3-NBA in the gastrointestinal tract, after either oral treatment or intraperitoneal injection [Arlt et al., 2001, 2003a].

Previous studies indicated that nitroreduction catalyzed by cytosolic and microsomal nitroreductases, followed by
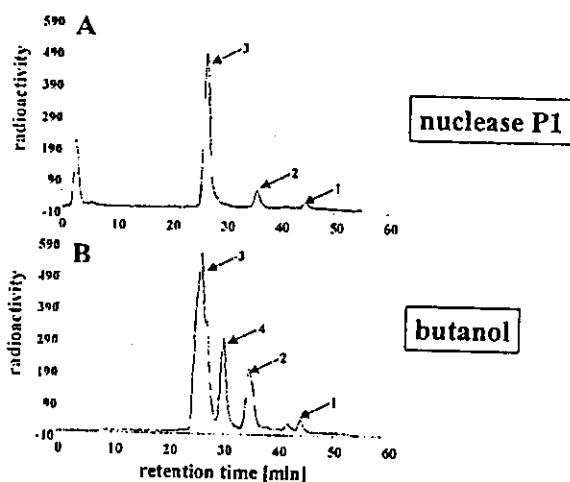
Fig. 4. Separation of ^{32}P-postlabeled 3',5'-bisphosphate adducts on a phenyl-modified reversed-phase column. HPLC autoradiograms show digests of liver DNA of Muta Mouse treated with 3-NBA using the nuclease P1 (A) and butanol (B) enrichment versions of the ^{32}P-postlabeling assay. Origins after D1 only were excised and extracted from the TLC plates, dissolved, and injected. Chromatographic conditions are described in text.

O-acetylation catalyzed by N-acetyltransferases and/or O-sulfonation catalyzed by sulfotransferases, seems to be the major pathways of bioactivation for 3-NBA leading to DNA adduct formation [Bieler et al., 1999, 2003; Borlak et al., 2000; Arlt et al., 2001, 2002, 2003a, 2003b, 2003c]. Using the ^{32}P-postlabeling assay, we observed a similar DNA adduct pattern in vivo in mice to those observed in vitro, in cell culture, and in rats [Bieler et al., 1999, 2003; Arlt et al., 2001, 2002, 2003a, 2003b, 2003c]. Comparative analyses of the major DNA adducts obtained in mice with those detected in vitro and in vivo in rats revealed that these 3-NBA-DNA adducts were chromatographically indistinguishable. Moreover, previous work has shown that the major DNA adducts are products derived from reductive metabolites bound to dA (adducts 1 and 2) or dG (adducts 3, 4, and 5) [Arlt et al., 2001, 2003c]. Further structural characterization of these 3-NBA-DNA adducts is currently being undertaken. Preliminary data indicate that 3-NBA forms the same DNA adducts in Salmonella typhimurium (data not shown). Therefore, these results support the conclusion that some or all of the major 3-NBA-DNA adducts (adducts 1–5) detected in the present study in mice represent premutagenic lesions involved in the mutagenic process, not only in Salmonella, but also in rodents.

There were variations in MFs among animals that might be derived from clonal (jackpot) mutations. The possibility of clonal mutations can be evaluated by sequencing the mutants. Sequence analysis was performed on the mutants recovered from liver, since the metabolic activation of 3-NBA due to hepatic enzymes has been intensely studied using cytosolic and microsomal enzymes from both rats and humans [Bieler et al., 1999; Arlt et al., 2002, 2003b, 2003c].

Base substitution mutations predominated for both the 3-NBA-treated and the untreated groups (about 80%). The site of mutation was distributed widely along the cII gene. The main changes in the mutation spectrum after 3-NBA treatment were a reduction in the proportion of G:C→A:T transitions (from 66% to 10%) and an increase in the proportion of G:C→T:A transversions (from 6% to 49%). Similar changes in the cII mutation spectrum were found after treatment with dinitropyrenes [Kohara et al., 2002a]. In the latter study, the authors concluded that the induction of G:C→T:A transversions correlated with the predominant formation of DNA adducts by 1,6- and 1,8-dinitropyrene at guanine residues. In contrast, aristolochic acid, another nitroaromatic compound, induces mainly A:T→T:A transversion mutations and binds preferentially to adenine residues [Kohara et al., 2002b], indicating that the mutational specificity of a compound may serve as indirect evidence for the interaction of the mutagen with specific DNA sequences. As was also observed with heterocyclic amines [Nagao et al., 1996], guanine-C8 adducts induce G:C→T:A transversion mutations probably by inserting adenine opposite to the uninformative or apurinic sites (the A-rule) [Strauss, 1991]. 3-NBA-DNA adducts at guanine residues account for around 70–80% of total DNA binding in liver DNA. Moreover, adduct 4 formed by 3-NBA (around 15% of total DNA binding at guanine residues in liver) is sensitive to digestion with nuclease P1, which is indicative of a guanine-C8 adduct [Bieler et al., 1999; Arlt et al., 2001]. Thus, the induction of G:C→T:A transversion mutations due to 3-NBA may be explained by intrinsic properties of the DNA polymerase that inserts dA opposite the lesion during DNA replication. Site-specific mutagenesis studies may provide new insights into the mutagenic activity of individual 3-NBA-DNA adducts.

It is noteworthy that the only DNA adduct derived from 3-NBA that has yet been characterized spectroscopically was synthesized by reacting the activated ester of N-acetyl-N-hydroxy-3-aminobenzanthrone (N-Ac-N-OH-ABA) with dG [Enya et al., 1998]. The adduct formed was an unusual guanine-C8 adduct, N-acetyl-3-amino-2-(2'-deoxyguanosine-8-yl)benzanthrone (dG-N-Ac-ABA). Kawanishi et al. [1998] investigated the mutagenic specificity of N-acetoxy-N-acetyl-3-aminobenzanthrone (N-Aco-N-Ac-ABA) in the supF gene in different human fibroblast cell lines. Base sequence analysis revealed that the majority of the mutations were base substitutions (around 80–90%), with G:C→T:A transversions (40–50%) being the most frequent mutation. In addition, a plasmid polymerase-stop assay in the supF gene showed that N-Aco-N-Ac-ABA preferentially bound to guanine residues [Kawanishi et al., 1998]. The results of this in vitro study have striking similarities to the results of the present in vivo study. However, it is important to point out that all 3-NBA-DNA adducts detected in the present study are dA and dG adducts that do not carry an N-acetyl group. dG-N-Ac-ABA was not detected in vivo
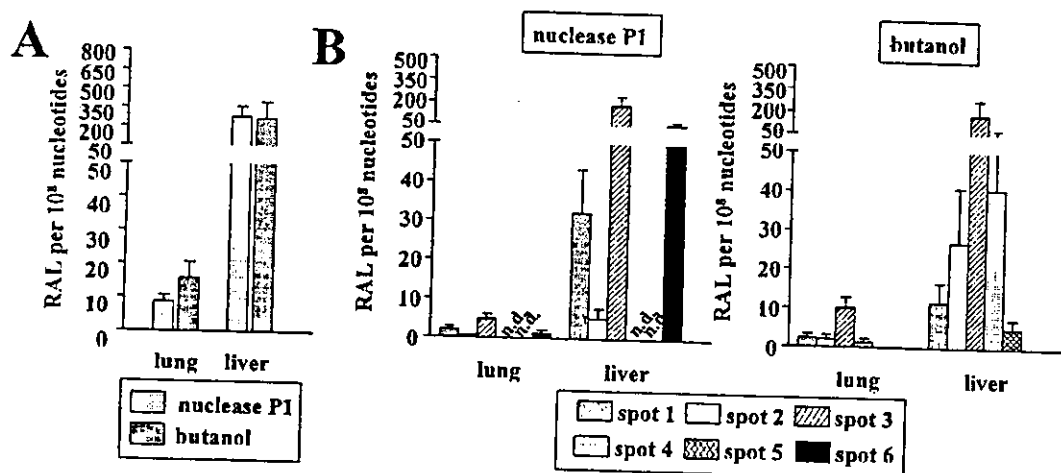
Fig. 5.   RAL of total DNA adducts in liver and lung tissue of Muta Mouse (A) treated with 3-NBA. RAL of individual DNA adducts in lung and liver tissue (B). Values represent the mean ± SD of five animals, the DNA from each organ being postlabeled twice. n.d., not detected.

either in mice (present study) or in rats or in cell culture [Arlt et al., 2001, 2002, 2003a, 2003c], suggesting that acetylated 3-NBA-DNA adducts and activation pathways reported earlier [Enya et al., 1998] based on in vitro synthesis might not entirely represent the in vivo situation.

Recently, an increased MF was reported in lung of Big Blue rats after exposure to diesel exhaust, providing important evidence for the mutagenicity of diesel exhaust in vivo [Sato et al., 2000]. A G:C→T:A transversion mutation hot spot was found at base 211 of the lacI gene. The surrounding sequence of this site (gattGgcg) is identical to the cII sequence at position 206–213, but no mutation was recovered at the corresponding guanine after treatment with 3-NBA. Only one mutation at this sequence site was observed after treatment with dinitropyrenes [Kohara et al., 2002a]. In addition, the major mutations induced by diesel exhaust in the lacI gene were A:T→G:C and G:C→A:T transitions. Therefore, as reported previously for dinitropyrenes [Kohara et al., 2002a], based on the present study there was no direct evidence for a contribution of 3-NBA to the mutagenicity of diesel exhaust. However, the different mutation spectra between diesel exhaust and 3-NBA may be attributed to different routes of exposure (inhalation vs. intraperitoneal injection), dosing, or species differences. On the other hand, transgenes may behave differently to each other. Mutation analysis in one transgene may not permit a precise extrapolation to other transgenic reporter genes [Thybaud et al., 2003]. 3-NBA induces a large variety of different DNA adducts derived both from dG and dA. To date, we can only speculate on the mutagenic specificity of 3-NBA in tumorigenesis and the mutagenic activity of individual 3-NBA-DNA adducts. However, preliminary data indicate that 3-NBA is carcinogenic in rats after intratracheal administration [Adachi et al., 2000]. Since 3-NBA has

been shown to induce specific 3-NBA-DNA adducts in various rat tissues after both oral and intraperitoneal treatment with 3-NBA [Arlt et al., 2001, 2003a], we suggest that 3-NBA-DNA adduct formation is not only critical for the mechanism of 3-NBA mutagenicity (present study) but also for its carcinogenicity.

In summary, the transgenic mouse mutation assay demonstrated that 3-NBA is mutagenic in various organs. In addition, 3-NBA induced chromosomal aberrations in blood reticulocytes. The induction of G:C→T:A transversion mutations in liver was associated with strong DNA binding by 3-NBA, predominantly at guanine residues, indicating that these mutations are probably caused by 3-NBA through an incorporation of dA opposite the adduct. This is important for the estimation of 3-NBA mutagenicity (and carcinogenicity) for humans. Our present study strongly suggests a genotoxic potential of 3-NBA for humans. To better understand the potential role of 3-NBA-DNA adducts in induction of mutations and cancer, our results require confirmation by larger animal studies that monitor the dose-dependent formation and persistence of 3-NBA-DNA adducts in susceptible target tissues.

ACKNOWLEDGMENTS

REFERENCES

Adachi S, Kawanura K, Takemoto K, Suzuki H, Hisamatsu Y. 2000. Carcinogenicity of 3-nitrobenzanthrone, a potent mutagen in diesel exhaust: preliminary results in F344 rats after intratracheal administration. In: Heinrich U, Mohr U, editors. Relationships between

respiratory disease and exposure to air pollution. Washington, DC: ILSI Press. p 315–319.

Arlt VM, Bieler CA, Mier W, Wiessler M, Schmeiser HH. 2001. DNA adduct formation by the ubiquitous environmental contaminant 3-nitrobenzanthrone in rats determined by ^{32}P-postlabeling. Int J Cancer 93:450–454.

Arlt VM, Glatt HR, Muckel E, Pabel U, Sorg BL, Schmeiser HH, Phillips DH. 2002. Metabolic activation of the environmental contaminant 3-nitrobenzanthrone by human acetyltransferases and sulfotransferase. Carcinogenesis 11:1937–1945.

Arlt VM, Sorg BL, Osborne M, Hewer A, Seidel A, Schmeiser HH, Phillips DH. 2003a. DNA adduct formation by the ubiquitous environmental pollutant 3-nitrobenzanthrone and its metabolites in rats. Biochem Biophys Res Commun 300:107–114.

Arlt VM, Glatt H, Muckel E, Pabel U, Sorg BL, Seidel A, Frank H, Schmeiser HH, Phillips DH. 2003b. Activation of 3-nitrobenzanthrone and its metabolites by human acetyltransferases, sulfotransferases and cytochrome P450 expressed in Chinese hamster V79 cells. Int J Cancer 105:583–592.

Arlt VM, Stiborova M, Hewer A, Schmeiser HH, Phillips DH. 2003c. Human enzymes involved in the metabolic activation of the environmental contaminant 3-nitrobenzanthrone: evidence for reductive activation by human NADPH:cytochrome P450 reductase. Cancer Res 63:2752–2761.

Arlt VM, Cole KJ, Phillips DH. 2004. Activation of 3-nitrobenzanthrone and its metabolites to DNA-damaging species in human B-lymphoblastoid MCL-5 cells. Mutagenesis 19:149–156.

Bieler CA, Wiessler M, Erdinger L, Suzuki H, Enya T, Schmeiser HH. 1999. DNA adduct formation from the mutagenic air pollutant 3-nitrobenzanthrone. Mutat Res 439:307–311.

Bieler CA, Arlt VM, Wiessler M, Schmeiser HH. 2003. DNA adduct formation by the environmental contaminant 3-nitrobenzanthrone in V79 cells expressing human cytochrome P450 enzymes. Cancer Lett 200:9–18.

Bofetta P, Dosemeci M, Gridley G, Bath H, Moradi T, Silverman D. 2001. Occupational exposure to diesel engine emissions and risk of cancer in Swedish men and women. Cancer Causes Control 12:365–374.

Borlak J, Hansen T, Yuan Z, Sikka HC, Kumar S, Schmidbauer S, Frank H, Jacob J, Seidel A. 2000. Metabolism and DNA-binding of 3-nitrobenzanthrone in primary rat alveolar type II cells, in human fetal bronchial, rat epithelial and mesenchymal cell lines. Polycyclic Aromat Compds 21:73–86.

Cariello NF, Piegorsch WW, Adams WT, Skopek TR. 1994. Computer program for the analysis of mutational spectra: application to p53 mutations. Carcinogenesis 15:2281–2285.

Enya T, Suzuki H, Watanabe T, Hirayama T, Hisamatsu Y. 1997. 3-nitrobenzanthrone, a powerful bacterial mutagen and suspected human carcinogen found in diesel exhausts and airborne particulates. Environ Sci Technol 31:2772–2776.

Enya T, Kawanishi M, Suzuki H, Matsui S, Hisamatsu Y. 1998. An unusual DNA adduct derived from the powerfully mutagenic environmental contaminant 3-nitrobenzanthrone. Chem Res Toxicol 11:1460–1467.

Feilberg A, Ohura T, Nielsen T, Poulsen MWB, Amagai T. 2002. Occurrence and photostability of 3-nitrobenzanthrone associated with atmospheric particles. Atmos Environ 36:3591–3600.

Hayashi M, Morita T, Kodama Y, Sofuni T, Ishidate M Jr. 1990. The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides. Mutat Res 245:245–249.

Heddle JA, Martus HJ, Douglas GR. 2003. Treatment and sampling protocols for transgenic mutation assays. Environ Mol Mutagen 41:1–6.

International Agency for Research on Cancer. 1989. Evaluation of carcinogenic risk to humans: diesel and gasoline engine exhausts and

some nitroarenes. Lyon: International Agency for Research on Cancer.

Itoh T, Kuwahara T, Suzuki T, Hayashi M, Ohnishi Y. 2003. Regional mutagenicity of heterocyclic amines in the intestine: mutation analysis of the cII gene in lambda/lacZ transgenic mice. Mutat Res 539:99–108.

Jakubczak JL, Merlino G, French JE, Muller WJ, Paul B, Adhya S, Garges S. 1996. Analysis of genetic instability during tumor progression using a novel selection-based assay for in vivo mutations in a bacteriophage λ transgene target. Proc Natl Acad Sci USA 93: 9073–9078.

Kawanishi M, Enya T, Suzuki H, Takebe H, Matsui S, Yagi T. 1998. Mutagenic specificity of a derivative of 3-nitrobenazanthrone in the supF shuttle vector plasmids. Chem Res Toxicol 11:1468–1473.

Kawanishi M, Enya, T, Suzuki H, Takebe H, Matsui S, Yagi T. 2000. Postlabelling analysis of DNA adducts formed in human hepatoma cells treated with 3-nitrobenzanthrone. Mutat Res 470:133–139.

Kohara A, Suzuki T, Honma M, Oomori T, Ohwada T, Hayashi M. 2002a. Dinitropyrenes induce gene mutations in multiple organs of the lambda/lacZ transgenic mouse (Muta Mouse). Mutat Res 515:73–83.

Kohara A, Suzuki T, Honma M, Ohwada T, Hayashi M. 2002b. Mutagenicity of aristolochic acid in the lambda/lacZ transgenic mouse (Muta Mouse). Mutat Res 515:63–72.

Lamy E, Kassie F, Gminski R, Schmeiser HH, Mersch-Sundermann V. 2004. 3-Nitrobenzanthrone (3-NBA) induced micronucleus formation and DNA damage in human hepatoma (HepG2 cells) cells. Toxicol Lett 146:103–109.

Murahashi T. 2003. Determination of mutagenic 3-nitrobenzanthrone in diesel exhaust particulate matter by three-dimensional high-performance liquid chromatography. Analyst 128:42–45.

Murahashi T, Watanabe T, Otake S, Hattori Y, Takamura T, Wakabayashi K, Hirayama T. 2003a. Determination of 3-nitrobenzanthrone in surface soil by normal-phase high-performance liquid chromatography with fluorescence detection. J Chromatogr A 992:101–107.

Murahashi T, Iwanaga E, Watanabe T, Hirayama T. 2003b. Determination of the mutagen 3-nitrobenzanthrone in rainwater collected in Kyoto, Japan. J Health Sci 49:386–390.

Nagao M, Wakabayashi K, Ushijima T, Toyota M, Totsuka Y, Sugimura T. 1996. Human exposure to carcinogenic heterocyclic amines and their mutational fingerprints in experimental animals. Environ Health Perspect 104(Suppl 3):497–501.

Phousongphouang PT, Grosovsky AJ, Eastmond DA, Covarrubias M, Arey J. 2000. The genotoxicity of 3-nitrobenzanthrone and the nitropyrene lactones in human lymphoblasts. Mutat Res 472:93–103.

Purohit V, Basu AK. 2000. Mutagenicity of nitroaromatic compounds. Chem Res Toxicol 13:673–692.

Sato H, Sone H, Sagai M, Suzuki KT, Aoki Y. 2000. Increase in mutation frequency in lung of Big Blue rat by exposure to diesel exhaust. Carcinogenesis 21:653–661.

Seidel A, Dahmann D, Krekeler H, Jacob J. 2002. Biomonitoring of polycyclic aromatic compounds in the urine of mining workers occupationally exposed to diesel exhaust. Int J Hyg Environ Health 204:333–338.

Strauss BS. 1991. The "A-rule" of mutagen specificity: a consequence of DNA polymerase bypass of non-instructional lesions? Bioessays 13:79–84.

Suzuki T, Wang X, Miyata Y, Saeki K, Kohora A, Kawazoe Y, Hayashi M, Sofuni M. 2000. Hepatocarcinogen quinoline induces G:C to C:G transversions in the cII gene in the liver of lambda/lacZ transgenic mice (Muta Mouse). Mutat Res 456:73–81.

Thybaud V, Dean S, Nohmi T, de Boer J, Douglas GR, Glickman BW,

Gorelick NJ, Heddle JA, Heflich RH, Lambert I, Martus HJ, Mirsalis JC, Suzuki T, Yajima N. 2003. In vivo transgenic mutation assays. Mutat Res 540:141–151.

Tokiwa H, Ohnishi Y. 1986. Mutagenicity and carcinogenicity of nitroarenes and their sources in the environment. CRC Crit Rev Toxicol 17:23–60.

Tokiwa H, Sera N, Horikawa K, Nakanishi Y, Shigematu N. 1993. The presence of mutagens/carcinogens in the excised lung and analysis of lung cancer induction. Carcinogenesis 14:1933–1938.

Watanabe T, Hasei T, Yoshifumi T, Otake S, Murahashi T, Takamura T, Hirayama T, Wakabayashi K. 2003. Mutagenic activity and quantification of nitroarenes in surface soil in the Kinki region of Japan. Mutat Res 538:121–131.

World Health Organization. 2003. World cancer report. Lyon: International Agenecy for Research on Cancer Press.

Yaffe D, Cohen Y, Arey J, Grosovsky AJ. 2001. Multimedia analysis of PAHs and nitro-PAH daughter products in the Los Angeles basin. Risk Anal 21:275–294.

APPENDIX. Mutant Frequencies in the *cII* Gene From Various Organs of Individual Muta Mouse Treated With 3-NBA Are as Follows.

Organ	Treatment	ID	Total plaques	Mutants	MF × 10^{-6}	Mean MF ± SD
Lung	Control	11	523500	10	19.1	
		12	332250	12	36.1	
		13	534000	9	16.9	
		14	322500	25	77.5	
		15	441000	18	40.8	38.1 ± 24.4
	3-NBA	31	475500	13	27.3	
		32	478500	28	58.5	
		33	538500	13	24.1	
		34	921000	43	46.7	
		35	307500	11	35.8	38.5 ± 14.2
Liver	Control	11	1455000	34	23.4	
		12	468000	18	38.5	
		13	576000	12	20.8	
		14	939000	45	47.9	
		15	507000	11	21.7	30.5 ± 12.1
	3-NBA	31	No packaging			
		32	111000	18	162.2	
		33	45750	4	87.4	
		34	68250	14	205.1	
		35	66750	9	134.8	147.4 ± 49.4
Kidney	Control	11	627000	25	39.9	
		12	2175000	42	19.3	
		13	2445000	62	25.4	
		14	2166000	97	44.8	
		15	2199000	113	51.4	36.2 ± 13.4
	3-NBA	31	1134000	66	58.2	
		32	1566000	41	26.2	
		33	978000	41	41.9	
		34	1368000	35	25.6	
		35	888000	32	36.0	37.6 ± 13.4
Colon	Control	11	1047000	51	48.7	
		12	2724000	60	22.0	
		13	3132000	55	17.6	
		14	672000	40	59.5	
		15	2232000	80	35.8	36.7 ± 17.6
	3-NBA	31	1119000	442	395.0	
		32	1725000	338	195.9	
		33	No packaging			
		34	2136000	333	155.9	
		35	1222500	352	287.9	258.7 ± 106.4
Spleen	Control	11	153000	51	33.3	
		12	736500	100	135.8*	
		13	1275000	26	20.4	
		14	1194000	48	40.2	
		15	708000	15	21.2	28.8 ± 9.6
	3-NBA	31	816000	30	36.8	
		32	723000	17	23.5	
		33	460500	22	47.8	
		34	2175000	49	22.5	
		35	627000	26	41.5	34.4 ± 11.1

APPENDIX. *Continued*

Organ	Treatment	ID	Total plaques	Mutants	MF × 10⁻⁶	Mean MF ± SD
Testis	Control	11	1189500	23	19.3	
		12	1119000	7	6.3	
		13	489000	4	8.2	
		14	724500	15	20.7	
		15	694500	15	21.6	15.2 ± 7.4
	3-NBA	31	633000	14	22.1	
		32	913500	28	30.7	
		33	766500	17	22.2	
		34	673500	9	13.4	
		35	664500	17	25.6	22.8 ± 6.3
Bladder	Control	12	1587000	27	17.0	
		13	873000	8	9.2	13.1
	3-NBA	32	943500	·58	61.5	
		34	1908000	90	47.2	54.4

ᵃValue was excluded for mean ± SD.