Mutagenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in the mammary gland of Big Blue rats on high- and low-fat diets

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Introduction

The heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is a mutagen and rodent carcinogen found in a variety of cooked meats in the human diet (1). PhIP has been under study as a possible etiological factor in certain human cancers including breast cancer (2–4). Studies in female F344 and Sprague–Dawley rats have shown that PhIP is a mammary gland carcinogen, providing further support for its possible involvement in human breast cancer (5–9).

For many years diet has been considered to modify the risk of certain human cancers, and the role of dietary fat in human cancer incidence has received considerable attention (10–12). In rodent models, high-fat diets, especially those rich in linoleic acid, have been shown to be strong promotional factors in chemically induced mammary gland cancer (13–16). Our laboratory set up a rat mammary tumor model in which multiple oral doses of PhIP are given to adolescent rats (7,8). Rats are then maintained on defined diets and tumors develop over a 25 week period. In this model, a defined high-fat diet consisting of 23.5% corn oil and fed ad libitum promotes PhIP-induced mammary gland cancer increasing the incidence of carcinoma 1.9-fold in comparison with a low-fat diet containing 5% corn oil.

Several mechanisms have been described to explain the promotional effects of high-fat diets on certain cancers (12,17–19). Dietary fat may increase the level of circulating hormones, alter cellular hormone responsiveness and modify gene expression and cell signaling pathways (17–19). In addition, lipid peroxidation induced-protein and DNA damage have also been implicated as a possible mechanism for the action of specific polyunsaturated fatty acids in carcinogenic initiation and acceleration of the carcinogenic process (12).

The objective of the current study was to use the Big Blue transgenic rat model to examine whether a high-fat diet modulates the mutagenicity of PhIP in the mammary gland. The Big Blue rat carries a lambda lacI transgene, a recoverable bacterial reporter gene that permits the detection of in vivo mutations (20). The Big Blue assay in rats and mice has been applied to studies of in vivo mutagenesis by PhIP and other heterocyclic amines in a variety of target sites including colon, prostate and mammary gland, thus supporting the utility of this assay for this family of compounds (21–24). In the current study, we test the hypothesis that the mechanism of promotion of PhIP-induced mammary gland cancer by a high-fat diet might be mediated via alteration in mammary gland mutagenesis. Several lines of evidence support this possibility. First, studies suggest that certain fatty acids may cause oxidative DNA damage (12). If a high-fat diet induces DNA damage, an increase in mutations or an alteration in mutation spectrum
would be expected. Secondly, our studies with the rat mammary model have shown that PhIP-treated rats on a high-fat diet for 6 weeks have a higher rate of proliferation in the terminal end buds of the mammary gland (25). As the rate of proliferation is regarded to be critical for mutagenesis (26), it appears plausible that a diet that increases proliferation could increase mutagenesis. In addition to studies with PhIP, we examined further whether dietary fat altered the frequency of mutants induced by 7,12-dimethylbenz[a]anthracene (DMBA), a well-established and highly potent rat mammary gland carcinogen.

Materials and methods

Chemicals

PhIP was purchased from Toronto Research Chemicals (North York, ON, Canada). DMBA was supplied by Sigma Chemical Co. (St Louis, MO). Big Blue top and bottom agar formulation (lot 088BBMA), Transpack™ packaging extract and X-gal were purchased from Stratagene (La Jolla, CA). Phenol (Gibco-BRL, Grand Island, NY) and chloroform (Mallinckrodt, Paris, KY) were obtained from the sources indicated. High- and low-fat powdered diets (23.5 and 5% corn oil, respectively) were supplied by Harlan Teklad (Madison, WI). The composition of these diets has been described previously (7).

Animals

Big Blue rats were purchased from Taconic Laboratories (Germantown, NY) at 7 weeks of age and bred to supply a sufficient number of animals. For the DMBA carcinogenicity study, 32 female F344 rats were obtained from the NIH animal supply (Animal Production Area, Frederick, MD) at 43–45 days of age. NIH guidelines for the care, housing and handling of the animals were adhered to throughout the study. Food and water were provided ad libitum throughout the study. Rats were given NIH laboratory chow prior to and during dosing with PhIP and DMBA.

Carcinogen treatment

PhIP was administered by oral gavage to 43-day-old Big Blue rats at 75 mg/kg (body wt) mixed in corn oil. Ten doses were administered as described previously for 5 consecutive days (once per day), followed by 2 days without dosing, followed by another 5 days of dosing (7,8). DMBA was given as a single gavage dose of 125 mg/kg in corn oil at 50 days of age. For each carcinogen, a control group was given the identical volume (5 ml/kg) and dosage regimen of the corn oil vehicle. Twenty-four hours after dosing with either PhIP (final dose) or DMBA, rats were placed on defined high- or low-fat diets for 6 weeks. The 6 week time point was chosen as it appeared to be sufficient for the expression of mammary gland mutations (27) and as it was the time point at which PhIP plus high-fat diet was shown to increase terminal end bud proliferation (25). At necropsy, Big Blue rats were killed by carbon dioxide inhalation and the abdominal mammary glands (numbers 4–6) carefully excised. The liver was also removed. Tissue was flash frozen and stored at ~80°C until further processing. To confirm the promotional effect of the high-fat diet on mammary tumorigenesis, a parallel animal study was carried out in 50-day-old Fischer-344 rats given 125 mg DMBA/kg, p.o. and maintained on the high- or low-fat diet for 40 weeks, or until mammary tumor size exceeded ~2 cm. Tumor multiplicity and incidence was determined at necropsy in order to include small tumors that may have evaded detection by palpation.

DNA extraction

High-molecular weight genomic DNA was isolated from 100–200 mg of the frozen mammary tissue or liver by phenol-chloroform extraction as described previously (28). The DNA concentration and purity was measured with a Beckman DU-65 spectrophotometer.

lacI mutant assay

The lambda vector was retrieved from the genomic DNA using Stratagene’s Transpack™ Packaging Extract, according to the manufacturer’s specifications. The recovered plasmid DNA was incubated with Escherichia coli SCS-8 cells in 10 mM MgSO4 for 20 min at 37°C. The samples were then combined with melted Big Blue top agar containing 1.5 mg/ml X-gal and plated onto 250 mm assay trays containing Big Blue bottom agar in accordance with the protocol in Stratagene’s Big Blue™ Transgenic Rodent Mutagenesis Assay System Instruction manual.

After overnight incubation at 37°C, the plates were scored by counting the number of mutant blue plaques. All putative mutant plaques were transferred to vials containing 0.5 ml SM buffer (0.1 M NaCl, 8 mM MgSO4, 50 mM Tris–HCl pH 7.5, 0.01% gelatin) and 50 µl chloroform using a wide-bore pipet tip. Each was diluted 1:150 in SM buffer and replated on 60 mm plates with 1.5 ml of top agar containing 1.5 mg/ml of X-gal. Plaques that remained visibly blue confirmed their mutant phenotype. The mutant frequency was then calculated as the ratio of confirmed mutant plaques to the total plaques plated. This procedure was repeated until a minimum of 100 000 plaques were scored for each mammary DNA sample.

To ensure that plating conditions were sufficiently sensitive to detect weak mutants, Stratagene’s color control mutants CM0 and CM1 were plated simultaneously with each plating. The data were not used unless the CM1 control mutants were visible. Only fully blue plaques, as determined by visual inspection, were counted as mutant plaques. The mutant plaques were further confirmed by replating and stored at ~70°C in SM buffer containing 7% DMSO.

Production of a double-stranded phagemid with lacI gene insert

In order to sequence the lacI gene harboring mutations, a double-stranded phagemid with the lacI gene mutations insert was produced in the SOLR cells. Two hundred and fifty microliters of phagemids packaged as filamentous phage particles (>1×109 plaque-forming units) were mixed with 200 µl of XL1-Blue MRF® cells (OD600 = 1.0) and 1 µl of the ExAssist helper phage (>1×106 p.f.u./µl) (Stratagene). The mixture was incubated at 37°C for 15 min followed by adding 3 ml of LB broth and continuing incubation at 37°C for 3 h with shaking. After heating at 65°C for 20 min and centrifuging, the supernatant containing the excised phagemids was collected and allowed to infect freshly growing SOLR cells (10 or 100 µl of supernatant versus 200 µl of SOLR cells) at 37°C for 15 min. The mixture was plated on LB-ampicillin agar plates and incubated overnight at 37°C.

Fig. 1. DNA adduct levels (A) and lacI mutant frequency (B) observed in mammary gland and liver of PhIP-treated rats on low- (LF) and high-fat (HF) diets. Rats were given 10 doses of PhIP (75 mg/kg, p.o.) and placed on the respective diet for 6 weeks. Values shown are means ± SEM of three animals. Bars with the same letters are not statistically different (Student’s t-test or one-way ANOVA, P > 0.05). n.d., not detected. In the intensification assay for PhIP-DNA adducts, the limit of detection for adducts 1 and 2 is 1.4 adducts/107 nucleotides and that for adduct 3 is 2.8 adducts/107 nucleotides, in both cases using a minimum of 30 c.p.m. of 32P above background as a cutoff.
PhIP, diet and mammary gland mutagenicity

Fig. 2. DNA adduct levels (A) and lacI mutant frequency (B) observed in mammary gland and liver of DMBA-treated rats on low- (LF) and high-fat (HF) diets. Rats were given single dose of 125 mg DMBA/kg, p.o., and placed on defined diet for 6 weeks. Values shown are means ± SE of three animals. Bars with the same letters are not statistically different (one-way or two-way ANOVA, P > 0.05).

Sequence analysis
A double-stranded DNA containing a lacI putative mutant was isolated from infected SOLR cells. The full length of lacI gene was sequenced with three primers: forward primer, GTATTACGCCCATGATCAG; primer #4, TGTTAAGCCCGGTTGC; and primer #6, CTGCGATGCTGGTTGCC. The mutations in the lacI gene of rat mammary gland DNA were examined by comparing the putative mutant sequence with a normal sequence provided by Stratagene.

32P-Post-labeling assay and PhIP–DNA adduct quantification
DMBA–DNA and PhIP–DNA adducts were assayed by the post-labeling method which resolves [32P]ATP labeled bisphosphonucleotide adducts as spots on autoradiograms following chromatography on polyethyleneimine-cellulose sheets. Assays for both types of adducts were run under intensification conditions, as originally described for DMBA–DNA adducts (29), and applied by us to PhIP–DNA adducts (30). Assay conditions for DMBA–DNA adducts were identical to those for PhIP–DNA adducts, except for the D1 solvent used for the initial resolution of the DMBA–DNA adducts (3.5 M lithium formate, 7.0 M urea, pH 3.5), and the D2 solvent (1.0 M magnesium chloride). DMBA–DNA adduct levels were expressed as relative adduct labeling (RAL) values. Two adduct spots were detected for DMBA (data not shown) with intensification factors 29.9 and 9.3. Intensification factors for DMBA–DNA adducts were determined as described previously (30). PhIP–DNA adduct levels were further quantified through the analysis of a PhIP–DNA adduct standard (kindly provided by Fred F.Kadlubar, NCTR, AR) containing one PhIP–DNA adduct per 10^6 nucleotides. The adduct standard was analyzed by 32P-post-labeling directly and after serial dilution with calf-thymus DNA in order to generate a standard curve relating RAL values to adduct levels per 10^6 nucleotides.

Statistical analysis
Statistical analysis was carried out using SigmaStat (Jandel Corporation) statistical software (version 2.0). Where indicated, analyses included the Student’s t-test, one-way and two-way analysis of variance (ANOVA), χ² analysis and the Z-test.

Results
PhIP–DNA adducts were detected in the mammary gland of Big Blue rats treated with PhIP and maintained on either a low- or high-fat diet (0.95 ± 0.48 and 1.38 ± 0.26 adducts/10^7 nucleotides, respectively) (Figure 1A and B). As seen in a previous study (31), only PhIP adducts to the guanine base were detected in the mammary gland, the major adduct being identified as the C8–guanine adduct. No PhIP–DNA adducts were detected in control rats (data not shown). Concomitant with the formation of PhIP–DNA adducts, the frequency of lacI mutants was 11–13-fold higher in the mammary gland of PhIP-treated rats than in the control, a difference that was statistically significant (one-way ANOVA, P < 0.05). However, diet did not significantly alter either the adduct levels or the lacI mutant frequency in the mammary gland of PhIP-treated rats. In the liver, there was a slight but statistically non-significant increase in mutant frequency following PhIP exposure (in either diet group) in comparison with control. As was seen in the mammary gland, diet did not alter the PhIP-induced mutant frequency in the liver. No PhIP–DNA adducts were detected in control rats (data not shown). Concomitantly, diet did not significantly alter either the adduct levels or the lacI mutant frequency in the mammary gland of PhIP-treated rats placed on low- or high-fat diet for 6 weeks prior to collection of tissue for DNA isolation and mutation analysis. The mutant frequency in the mammary gland of control rats (irrespective of diet) was 17.3 ± 8.9 (×10^-6), (mean (X) ± standard error of the mean (SEM)).

As described in the Materials and methods, rats were treated with PhIP or DMBA and then placed on either the low- or high-fat diet for 6 weeks prior to collection of tissue for DNA isolation and mutation analysis. The mutant frequency in the mammary gland of control rats (irrespective of diet) was 17.3 ± 8.9 (×10^-6), (mean (X) ± standard error of the mean (SEM)).

Table 1. Mutant frequency in the lacI transgene in the mammary gland of Big Blue rats exposed to PhIP and DMBA and placed on defined diet

<table>
<thead>
<tr>
<th>Treatment and diet</th>
<th>No. of plaques</th>
<th>Mutant frequency (×10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Mutant</td>
</tr>
<tr>
<td>PhIP-low fat</td>
<td>1</td>
<td>101 120</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>129 280</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>106 000</td>
</tr>
<tr>
<td>PhIP-high fat</td>
<td>1</td>
<td>106 960</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>169 240</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>110 980</td>
</tr>
<tr>
<td>DMBA-low fat</td>
<td>1</td>
<td>132 280</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>105 500</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>122 640</td>
</tr>
<tr>
<td>DMBA-high fat</td>
<td>1</td>
<td>110 600</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>141 480</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>121 800</td>
</tr>
</tbody>
</table>

As described in the Materials and methods, rats were treated with PhIP or DMBA and then placed on either the low- or high-fat diet for 6 weeks prior to collection of tissue for DNA isolation and mutation analysis. The mutant frequency in the mammary gland of control rats (irrespective of diet) was 17.3 ± 8.9 (×10^-6), (mean (X) ± standard error of the mean (SEM)).

Different superscripts indicate a statistically significant difference (two-way ANOVA, P < 0.05). DMBA induced a significantly higher mutant frequency than PhIP irrespective of diet. For each compound, there was no significant difference between diet groups.

Student’s t-test, one-way and two-way analysis of variance (ANOVA), χ² analysis and the Z-test.

Adduct levels and mutant frequency were also examined in the mammary gland and liver of DMBA-treated Big Blue rats on low- and high-fat diets for 6 weeks (Figure 2A and B). DMBA–DNA adducts were detected in both mammary gland and liver. In both tissues, however, adduct levels were not statistically different between diet groups (Student’s t-test, P > 0.05). DMBA adduct levels were ~6–7-fold higher in the
liver than in the mammary gland for both diet groups. DMBA induced a significant elevation in the lacI mutant frequency in both the mammary gland and the liver in comparison with the respective control values (one-way ANOVA, \( P < 0.05 \)). As was observed for PhIP, diet did not significantly alter mutant frequency in liver and mammary gland. In accordance with higher DMBA adduct levels in liver, mutant frequency in the lacI transgene was \( 2-3 \)-fold higher in liver than in the mammary gland of DMBA-treated rats.

Mutant frequency induced by PhIP and DMBA in the mammary gland was further compared (Table I). DMBA is a much more potent mammary gland carcinogen than PhIP (7). Although rats were exposed to a \( 6 \)-fold lower total dose of DMBA than PhIP, the mutant frequency was \( 20-30\% \) higher in the mammary gland of DMBA-treated rats than in the glands of PhIP-treated rats, irrespective of diet (two-way ANOVA, \( P < 0.05 \)).

Mutants found in the mammary gland of PhIP-treated rats were further sequenced to examine possible differences in the spectrum of mutations induced in the two diet groups (Table II). To insure that independent mutational events were analyzed (thereby avoiding inclusion of mutations that were possibly clonal expansions) only one identical mutation was counted per rat. In PhIP-treated rats, mutations were broadly distributed over the lacI gene in both the low- and high-fat diet groups. A total of 125 independent mutations were detected for both diet groups: 69 and 56 in the low- and high-fat diet groups, respectively. With both diet groups combined there were 109 sites on the lacI gene that harbored a mutation, and the majority of mutations were found in GC-rich regions. Eighty-one out of 109 sites (74\%) involved a mutation in a G:C base pair immediately adjacent to another G:C base pair. Out of 109 sites showing a mutation, 13 sites (12\%) harbored a mutation with both diet groups. The region between positions 90 and 95 (especially positions 92 and 93) was a frequent site mutation with both diet groups. The region between positions 109 sites showing a mutation, 13 sites (12\%) harbored a pair immediately adjacent to another G:C base pair. Out of 880 mutations in the lacI gene of Big Blue rats. Therefore, it appears probable that the mechanism of promotion by the high-fat diet does not involve an increase in the frequency of carcinogen-induced mutations.

The percentage of various types of mutations found in the mammary gland of PhIP-treated rats was summarized in Table III. In both diet groups, base substitution mutations at guanine were predominant, the major mutation being a G:C to T:A transversion, accounting for 35 and 43\% of mutations in the low- and high-fat diet groups, respectively. There were no statistically significant differences in the frequency of specific types of mutations between the two diet groups (\( \chi^2 \) and Z-test, \( P > 0.05 \)). G:C to C:G and G:C to A:T base substitution mutations each accounted for \( \sim 18-25\% \) of mutations, irrespective of diet. There appeared to be a higher frequency of G:C to A:T transition mutations in CpG sites in the high-fat diet group, although the difference was not statistically significant (Z-test, \( P > 0.05 \)). Specifically, 57\% (eight out of 14 G:C to A:T transition mutations) were at CpG sites in the high-fat diet group in comparison with only 29\% (five out of 17) in the low-fat diet group. Single base pair deletions at G:C base pairs constituted the next-most prevalent type of mutation after guanine base substitutions. The signature guanine deletion mutation at 5'-GGGA-3' characteristic for PhIP (21,32) was observed in both diet groups at frequencies that were not statistically significantly different from each other. Five out of 125 (4\%) total mutations (low- plus high-fat diet) harbored this specific mutation (or 28\% of all guanine deletions). Notably, 12 out of 125 (10\%) mutations involved the guanine base in the sequence 5'-CAG(Purine)-3' (Pu) at lacI positions 18, 59, 82, 296, 306, 568 and 661 (Table II).

Previous studies in our laboratory have shown that the high-fat diet containing 23.5\% corn oil promotes PhIP-induced mammary gland cancer (7,8). The promotional effect of our high-fat diet was further confirmed in studies carried out with DMBA in Fischer-344 rats, the background strain for the Big Blue rat (Table IV). In rats on high-fat diet, tumor incidence and multiplicity were, respectively, 1.9- and 3.2-fold higher than in rats on low-fat diet.

**Discussion**

Previous studies by our laboratory have shown that a high-fat diet rich in linoleic acid (specifically a 23.5\% corn oil diet) is a promotional factor for PhIP-induced rat mammary gland cancer (7,8). The current study further confirmed that such a high-fat diet is a promotional factor in DMBA-induced mammary gland carcinogenesis at the dose used with the Big Blue rats. As the mechanism of promotion by the high-fat diet is still incompletely known, the current study examined whether the high-fat diet increased the mutagenicity of these carcinogens in the mammary gland of Big Blue rats. When either PhIP or DMBA was used as the initiating agent, no significant difference was observed in the frequency of lacI mutants between the low- and high-fat diet groups. Similarly, no effect of diet was observed on the frequency of lacI mutants in liver of Big Blue rats. Therefore, it appears probable that the mechanism of promotion of chemically induced mammary gland cancer by a high-fat diet does not involve an increase in the frequency of carcinogen-induced mutations.

Analysis of the spectrum of lacI mutation found in PhIP-treated rat mammary gland further suggests that the frequency of specific mutations is not significantly altered by the high-fat diet. The principal types of mutations in the mammary gland of PhIP-treated rats were the same in the high- and low-fat diet groups (Table III), suggesting that the pattern of mutation was largely dictated by the carcinogen. The lack of increase in mutant frequency or change in mutation spectrum in animals on the high-fat diet appears to argue against the hypothesis that oxidative DNA damage is a major mechanism involved in promotion by dietary fat, at least in this animal model. Oxidative DNA damage is recognized to contribute to single base substitution mutations (33–35), a class of mutations clearly detectable by the Big Blue assay. Consistent with the conclusion that the high-fat diet did not increase mutant frequency, a previous report showed that high-fat diets (in the absence of chemical carcinogen) are not mutagenic to the colon and small intestine of Big Blue mice (36). It is noteworthy, however, that some differences in the distribution of mutations in the lacI transgene between diet groups were suggested by the sequencing data (Table II). Several sites in the lacI coding region, such as nucleotides 68–69, 82 and 234, showed more mutations in the high-fat diet group. There was a trend towards a higher percentage of G:C to A:T transition mutations at CpG sites in the high-fat diet group. The frequency of CpG site mutations out of the total G:C to A:T transition mutations was 57 and 29\% for high- and low-fat diet group, respectively, indicating nearly a 2-fold difference between the diet groups. C:G to T:A transition mutations can occur via deamination of methylcytosine (37). Speculatively, differences
<table>
<thead>
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<th>Position</th>
<th>Sequence context</th>
<th>Number of mutations</th>
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<td></td>
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</tr>
<tr>
<td>16</td>
<td>ATT C AGG</td>
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</tr>
<tr>
<td>17</td>
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<tr>
<td>18</td>
<td>TCA G GGT</td>
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<td>G → T</td>
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<tr>
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<tr>
<td>80</td>
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<td>C-del (G-del)</td>
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<tr>
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<td>G → T</td>
</tr>
<tr>
<td>221</td>
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<td>G → T</td>
</tr>
<tr>
<td>222</td>
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<td>G → T</td>
</tr>
<tr>
<td>234</td>
<td>CCT C CAG</td>
<td>C → A (G → T)</td>
</tr>
<tr>
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<tr>
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<tr>
<td>269</td>
<td>GTC G CGG</td>
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<tr>
<td>296</td>
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<tr>
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<tr>
<td>326</td>
<td>GTA G AAC</td>
<td>G → A</td>
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<tr>
<td>326</td>
<td>GTA G AAC</td>
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<tr>
<td>333</td>
<td>GAA G CGG</td>
<td>G-del</td>
</tr>
<tr>
<td>340</td>
<td>CGT C GAA</td>
<td>C → T(G → A) (CpG)</td>
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<tr>
<td>341</td>
<td>GTC G AAG</td>
<td>G → C</td>
</tr>
<tr>
<td>344</td>
<td>GAA G CCT</td>
<td>G → C</td>
</tr>
<tr>
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<td>C → T (G → A) (CpG)</td>
</tr>
<tr>
<td>375</td>
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</tr>
<tr>
<td>376</td>
<td>CGC GCAA</td>
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</tr>
<tr>
<td>379</td>
<td>GCA A CTC</td>
<td>A → T</td>
</tr>
<tr>
<td>381</td>
<td>AAC G CGT</td>
<td>G → A</td>
</tr>
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</table>
between the diet groups in the frequency of cytosine methylation or in the rates of deamination might lead to differences in the frequency of CpG site mutations. Further studies are required to confirm the difference in the frequency CpG site mutations among the diet groups and to investigate how specific diets might alter the distribution of mutations.

Our previous study showed that the rate of proliferation in the epithelium of the terminal end buds in PhIP-treated rats was highest when rats were on a high fat rather than a low-fat diet (25). In this prior study, the dosage of PhIP and the diet was identical to that used in the present study. Although a higher rate of proliferation in rats on high-fat diet would be expected to increase mutant frequency in the mammary gland, it is possible that not enough of the glandular tissue (including epithelial cells lining primary ducts, alveolar buds and lobules) was affected by the diet to cause an overall increase in mutant frequency. The effect of diet on proliferation is consistent with the hypothesis that alterations in hormone responsiveness, cell signaling and gene expression (17–19) are in a general sense the modus operandi for promotion by high-fat diet. It further appears possible that the action of the high fat diet could be restricted to specific cell types in the mammary gland.

It is recognized generally that chemical carcinogen–DNA adduct formation is necessary for carcinogen-induced mutagenesis (38). The 32P-post-labeling data herein support the conclusion that PhIP- and DMBA–DNA adducts are associated with mutagenesis in vivo in the Big Blue rat model. With both compounds, there was a correlation between adduct levels and the frequency of mutations in liver and mammary gland. The high frequency of mutations in the liver of DMBA-treated rats was associated with a high DMBA–DNA adduct level in liver in comparison with mammary gland (Figure 2). In contrast, PhIP adduct levels were higher in the mammary gland than in the liver, and accordingly, of the two tissues, the mammary gland showed the highest mutant frequency (Figure 1).

<table>
<thead>
<tr>
<th>Positiona</th>
<th>Sequence contextb</th>
<th>Mutation</th>
<th>Number of mutationsc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low fat</td>
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</tr>
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<td>418–419</td>
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<td>C-del (G-del)</td>
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</tr>
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<tr>
<td>542</td>
<td>GTG C CAG</td>
<td>G → T</td>
<td>1</td>
</tr>
<tr>
<td>566</td>
<td>CAC C AGC</td>
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<tr>
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<td>1</td>
</tr>
<tr>
<td>576</td>
<td>TCG G GCT</td>
<td>C → A (G → T)</td>
<td>1</td>
</tr>
<tr>
<td>587</td>
<td>GCG G GCC</td>
<td>G → T</td>
<td>1</td>
</tr>
<tr>
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<td>CGG G CCC</td>
<td>G → T</td>
<td>1</td>
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<tr>
<td>606</td>
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<tr>
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<td>del CTGG</td>
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<td>G → C</td>
<td>2</td>
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<tr>
<td>661</td>
<td>TCA G CCC</td>
<td>G → T</td>
<td>1</td>
</tr>
<tr>
<td>662–663</td>
<td>CAG C CGA</td>
<td>C-del (G-del)</td>
<td>1</td>
</tr>
<tr>
<td>669</td>
<td>TGT C CGG</td>
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<tr>
<td>675–677</td>
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<td>G-del</td>
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<tr>
<td>693</td>
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<td>1</td>
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<tr>
<td>714</td>
<td>AAA C CAT</td>
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<td>767</td>
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<td>794</td>
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<td>G → C</td>
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<td>795</td>
<td>GCG C CAT</td>
<td>C → A (G → T)</td>
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<tr>
<td>841</td>
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<td>G → T</td>
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<td>842</td>
<td>GTG G GAT</td>
<td>G → C</td>
<td>1</td>
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<tr>
<td>842</td>
<td>GTG G GAT</td>
<td>G → T</td>
<td>1</td>
</tr>
<tr>
<td>843</td>
<td>TGG G ATA</td>
<td>G → T</td>
<td>1</td>
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<td>877–879</td>
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<td>C-del (G-del)</td>
<td>1</td>
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<td>G-del</td>
<td>1</td>
</tr>
<tr>
<td>918</td>
<td>TGG G GCA</td>
<td>G → A</td>
<td>1</td>
</tr>
<tr>
<td>920</td>
<td>GGG C AAA</td>
<td>C-del (G-del)</td>
<td>1</td>
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<tr>
<td>986–988</td>
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<td>C-del (G-del)</td>
<td>2</td>
</tr>
<tr>
<td>993</td>
<td>TCT C ACT</td>
<td>C → A (G → C)</td>
<td>1</td>
</tr>
<tr>
<td>1038</td>
<td>CCG C CTC</td>
<td>C → G (G → C)</td>
<td>1</td>
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<td>1038</td>
<td>CCG C CTC</td>
<td>C → T (G → A)</td>
<td>1</td>
</tr>
<tr>
<td>1043</td>
<td>TCT C CCC</td>
<td>C → T (G → A)</td>
<td>1</td>
</tr>
<tr>
<td>1089</td>
<td>TTT C CCC</td>
<td>C → T (G → A)</td>
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</tr>
<tr>
<td>1089</td>
<td>TTT C CCC</td>
<td>C → G (G → C)</td>
<td>1</td>
</tr>
</tbody>
</table>

*Numbering of the lacI gene is in accordance with Stratagene.

*bThe underlined nucleotide harbors the mutation. lacI codons are not necessarily represented.

*cNumber of rats harboring an independent mutation at specific positions on the lacI gene. lacI mutants were collected from three rats on high- and low-fat diet each.
mutations indicating that 12 out of 125 (10%) mutations at the guanine base in the rats on low- and high-fat diets.

Blue rats has been reported previously (20). We also detected this mutation in a characteristic G deletion in guanines adjacent to a G:C base pair, i.e. 74% of all mutated adduct (39), the high frequency of lacI shuttle vector system (12 out of 84 mutants, 14%) the guanine position 18. Interestingly in both the DHFR gene of Chinese rats treated with PhIP is also consistent with the role of PhIP – demonstrated in a study using site-specific modifications, and in addition, 1 bp G:C deletion mutations. As followed by G:C to A:T transitions, and G:C to C:G tran-

tions in the mammary gland was characteristic for PhIP – gene in the mammary gland was characteristic for PhIP –

lacI transgene in the mammary gland of Big Blue rats has been reported previously (20).

Characteristic ‘signature’ mutation for PhIP.

Table III. Types of lacI mutations in the mammary glands of PhIP-treated rats on low- and high-fat diets

<table>
<thead>
<tr>
<th>Mutation type</th>
<th>Number of mutations (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low fat</td>
</tr>
<tr>
<td>Base substitution</td>
<td></td>
</tr>
<tr>
<td>G:C → T:A</td>
<td>24 (35%)</td>
</tr>
<tr>
<td>G:C → C:G</td>
<td>15 (22%)</td>
</tr>
<tr>
<td>G:C → A:T</td>
<td>17 (25%)</td>
</tr>
<tr>
<td>[At CpG sequences]</td>
<td>[5 (7%)]</td>
</tr>
<tr>
<td>A:T → T:A</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Single base pair deletion</td>
<td></td>
</tr>
<tr>
<td>G:C</td>
<td>12 (17%)</td>
</tr>
<tr>
<td>[At 5'–GGGA–3'–]</td>
<td>[4 (6%)]</td>
</tr>
<tr>
<td>Multiple base pair deletion</td>
<td></td>
</tr>
<tr>
<td>4 bp</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total</td>
<td>69 (100%)</td>
</tr>
</tbody>
</table>

Data were analyzed by the χ² analysis and Z-test. The spectrum of spontaneous mutations in the lacI transgene in the mammary gland of Big Blue rats has been reported previously (20).

The mutation spectrum found in the mammary gland of PhIP-treated rats is also consistent with the role of PhIP–guanine adduct formation, and specifically the C8–guanine adduct. The spectrum of mutations induced by PhIP in the lacI gene in the mammary gland was characteristic for PhIP–guanine adduct formation (39–41). These mutations are primarily base substitutions especially G:C to T:A transversions followed by G:C to A:T transitions, and G:C to C:G transversions, and in addition, 1 bp G:C deletion mutations. As demonstrated in a study using site-specifically modified oligonucleotides carrying a single deoxyguanosine (dG)–C8–PhIP adduct (39), the high frequency of lacI mutations observed at guanines adjacent to a G:C base pair, i.e. 74% of all mutated guanine nucleotides, is consistent with the formation of dG–C8–PhIP. Previous studies have also reported that PhIP induces a characteristic G deletion in 5’–GGGA–3’ sequence (21,32). We also detected this mutation in five out of 125 (4%) total mutations indicating that five of 18 (28%) G:C deletions constituted this signature mutation. In addition, we detected 12 out of 125 (10%) mutations at the guanine base in the sequence 5’–CA(T–Pu)–3’.

Four of six rats had a base substitution mutation at a 5’–CA(T–Pu)–3’ site at lacI nucleotide position 18. Interestingly in both the DHFR gene of Chinese hamster ovary cells (four out of 20 mutants) and in the supF shuttle vector system (12 out of 84 mutants, 14%) the guanine base in this same 4 bp sequence context was frequently mutated after PhIP–DNA adduct formation (40,41). These findings support the notion that 5’–CA(T–Pu)–3’ is a characteristic target site for PhIP–guanine adduct-induced base substitution mutations in vivo in the mammary gland.

The results of this study support the general notion that carcinogen-induced mutations play a role in carcinogenesis. For example, in PhIP-treated rats, the liver, a non-target organ for PhIP, showed a slight, but non-statistically significant increase in mutant frequency whereas a 12-fold increase in mutant frequency was observed in the mammary gland. However, the relationship between mutagenesis and carcinogenesis is potentially complex and other factors can obscure this association. DMBA on a dosage basis is at least a 10-fold more potent mammary gland carcinogen than PhIP (7,42). Irrespective of diet, the mutant frequency in the mammary gland for DMBA is 20–30% higher than for PhIP. Therefore, while a higher frequency of mutations is associated with a higher potency as a mammary gland carcinogen, the striking difference in carcinogenic potency is matched by only a modest difference in mutagenesis. There are several possible implications of this finding. First, it is possible that a small change in lacI mutant frequency might indeed reflect a large change in cancer incidence. Secondly, mutation spectrum, rather than mutant frequency, may be a more critical determinant of carcinogenesis. DMBA has been shown to induce a 44% incidence of adenine base substitution mutations in the mammary gland of Big Blue rats (43), a mutation type observed infrequently with PhIP. Adenine adducts appear to be more mutagenic than guanine adducts in the mammary gland (43) and speculatively, adenine mutations may be more carcinogenic than guanine mutations in this organ. Thirdly, it is also probable that additional carcinogen-specific factors play a role in susceptibility to mammary gland carcinogenesis. For example, in our hands, DMBA but not PhIP increases plasma levels of 17β-estradiol, which in turn might modulate susceptibility to mammary gland cancer (44). Finally, it is clear from our studies with diet that mutagenesis may not need to be modified significantly in order to modulate carcinogenesis. Further studies are clearly needed to better understand the relationship between carcinogen-induced mutations and cancer development in the mammary gland as well as the additional factors conferring susceptibility of the mammary gland to these mutations.

Acknowledgements

The authors thank Fred Kadlubar, NCTR, Arkansas, for providing the PhIP–DNA adduct standard, and Shada Rouhani for excellent technical assistance with the preparation of the phagmids.

References


Received November 20, 2001; revised January 23, 2002; accepted February 1, 2002.