The Genotoxicity of Acrylamide and Glycidamide in Big Blue Rats


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Acrylamide (AA), a mutagen and rodent carcinogen, recently has been detected in fried and baked starchy foods, a finding that has prompted renewed interest in its potential for toxicity in humans. In the present study, we exposed Big Blue rats to the equivalent of ~5 and 10 mg/kg body weight/day of AA or its epoxide metabolite glycidamide (GA) via the drinking water, an AA treatment regimen comparable to those used to produce cancer in rats. After 2 months of dosing, the rats were euthanized and blood was taken for the micronucleus assay; spleens for the lymphocyte Hprt mutant assay; and liver, thyroid, bone marrow, testis (from males), and mammary gland (females) for the cII mutant assay. Neither AA nor GA increased the frequency of micronucleated reticulocytes. In contrast, both compounds produced small (approximately twofold to threefold above background) but significant increases in lymphocyte Hprt mutant frequency (MF, \( p < 0.05 \)), with the increases having dose-related linear trends (\( p < 0.05 \) to \( p < 0.001 \)). Neither compound increased the cII MF in testis, mammary gland (tumor target tissues), or liver (nontarget tissue), while both compounds induced weak positive increases in bone marrow (nontarget tissue) and thyroid (target tissue). Although the genotoxicity in tumor target tissue was weak, in combination with the responses in surrogate tissues, the results are consistent with AA being a gene mutagen in the rat via metabolism to GA.

Key Words: acrylamide; glycidamide; Big Blue rat; Hprt mutant assay; cII mutant assay.

Human exposures to acrylamide (AA) from industrial production, commercial uses, cigarette smoking, and polyacrylamide gel electrophoresis have been well documented, primarily by measurement of AA-hemoglobin adducts in blood (Bergmark, 1997). The concerns about public health risks from AA exposure have escalated recently by the finding that AA is formed in food during cooking (Rosen and Hellenas, 2002; Tareke et al., 2002). Epidemiologic studies, however, are mixed in their support for an association between the dietary intake of AA and risk of several cancers, i.e., some show no effect (Mucci et al., 2006; Pelucchi et al., 2006), while another shows increased incidences (Hogervorst et al., 2007).

AA is metabolically converted to glycidamide (GA) in both humans and experimental animals, and this conversion is an obligatory step in the formation of several AA-induced DNA adducts observed in rodent tissues (Gamboa da Costa et al., 2003; Segerback et al., 1995) and in the induction of bacterial mutagenicity (Hashimoto and Tanii, 1985), micronuclei (Paulsson et al., 2002), and dominant lethality in mouse spermatids (Ghanayem et al., 2005). Following the recognition of human exposure to AA through the diet, the dietary route of AA administration was investigated in experimental animals (Doerge et al., 2005b,c). These studies showed that AA is highly bioavailable from oral routes of administration in both rats and mice, is widely distributed to tissues, and that the dietary route, in particular, favors metabolism to GA.

AA is classified by the International Agency for Research on Cancer as a probable human carcinogen (Group 2A [International Agency for Research on Cancer, 1995]) based upon both animal cancer data and in vitro and in vivo genotoxicity studies. In two 2-year carcinogenicity studies in Fischer 344 (F344) rats (Friedman et al., 1995; Johnson et al., 1986), AA induced neoplasms at multiple organ sites, including thyroid follicular cell tumors (males and females), peritoneal mesotheliomas (males), and mammary tumors (females). In addition, tumors of the brain, oral cavity, uterus, and clitoral gland in female F344 rats were reported in the earlier study (Johnson et al., 1986). In a screening assay employing A/J mice, a strain highly susceptible to the development of lung tumors, AA administration by oral gavage or ip injection three times per week for 8 weeks resulted in an increase in lung adenomas (Bull et al., 1984a). In an initiation-promotion study, Swiss-ICR mice were administered AA by oral gavage (initiation) and then received 12-O-tetradecanoylphorbol 13 acetate (TPA) via skin application (promotion). AA with or without TPA produced significant increases in skin and lung tumors (Bull et al., 1984b).

These bioassay results, coupled with a broad body of evidence demonstrating the genotoxicity of AA (Besaratinia and Pfeifer, 2007; Dearfield et al., 1988, 1995; Rice, 2005), are
consistent with AA producing tumors through a genotoxic mode of action (MOA). Even so, questions persist regarding whether or not nongenotoxic mechanisms might be involved in the tumors induced by AA (e.g., Dourson et al., 2008; Lafferty et al., 2004). These questions have been allayed to some extent by a recent report, indicating that at least some of the alternative nongenotoxic MOAs for AA tumor induction are unlikely (Bowyer et al., 2008). However, data indicating that AA and its metabolite GA are mutagenic in animal models using treatment protocols modeled on the cancer assays would be direct evidence in support of a mutagenic MOA for AA tumorigenicity.

In a previous study, we exposed Big Blue mice to AA and GA at 1.4 and 7.0 mM, respectively, in their drinking water (equivalent to daily received doses of ~20 and 100 mg/kg body weight [bw]) for up to 28 days and observed that the high doses of both AA and GA were mutagenic in the liver cII gene (Manjanatha et al., 2006). Both compounds produced similar mutational spectra in the liver, with G:C → T:A transversion and frameshift being major mutation types. Moreover, we found that the lymphocyte Hprt mutant frequency (MF) and the frequency of micronucleated reticulocytes (MN-RETs) in peripheral blood from AA- and GA-treated mice were significantly higher (4- to 25-fold and 1.7- to 3.3-fold, respectively) than in control mice (Manjanatha et al., 2006). Although these results indicated that AA was a mutagen in mice via oral administration and was consistent with AA-producing mutations through metabolism to GA, the doses of AA used in the study were higher than those used in cancer bioassays and the target tissues for AA carcinogenesis in mice were later identified as lung, hardarian gland, and mammary gland but not liver (Beland, personal communication).

In the present study, we have continued our examination of the mutagenicity of AA in relation to its carcinogenicity by evaluating the mutagenicity of AA and GA in Big Blue rats. Our hypothesis is that AA, acting through its epoxide metabolite GA, is a genotoxic carcinogen that binds covalently to DNA and induces mutations and cell transformation, leading to cancer in multiple organs of male and female F344 rats. Thus, we have measured cII transgene mutations in cancer target tissues (thyroid, mammary gland, and testes) and non-target tissues (liver and bone marrow), Hprt mutations in spleen lymphocytes, and micronuclei in peripheral red blood cells. AA and GA were administered in drinking water at doses similar to those used in the cancer bioassays.

MATERIALS AND METHODS

Chemicals and reagents. AA (purity > 99.9%) was purchased from Sigma (St. Louis, MO), and GA (purity > 98.5%, with AA present at ~1%) was purchased from Toronto Research Chemicals (North York, Ontario). Rat MicroFlowPlus kits were purchased from Litron Laboratories (Rochester, NY). RecoverEase DNA Isolation Kits, Transpack packaging extract, and the Escherichia coli G1250 strain were obtained from Stratagene (La Jolla, CA). PCR Master Mix was purchased from Promega (Madison, WI), and CEQ Dye Terminator Cycle Sequencing kits were obtained from Beckman Coulter (Fulerton, CA).

Animals and treatments. During the course of this experiment, we followed the recommendations set forth by our Institutional Animal Care and Use Committee for the handling, maintenance, treatment, and sacrifice of the animals. Male and female Big Blue transgenic rats were obtained from Taconic Farms (Germantown, NY) as weanlings and housed two per cage. Rats were fed NIH-31IR diet (Purina Mills, Brentwood, MO), a diet selected because of its low AA content (~10 ppb; Twaddle et al., 2004). At ~50 days of age, the rats were exposed to 0, 0.7, and 1.4 mM AA or GA, dissolved in drinking water (50 or 60 mg/l and 100 or 120 mg/l, respectively), for 7 days/week and for up to 60 days. The concentrations of AA and GA in drinking water were equal to and double the maximum dose used in 2-year carcinogenesis studies of AA and GA in F344 rats (0.7 mM; Beland, personal communication). Drinking water, rather than diet, was chosen as the route of administration since GA is very unstable in diet (Doerge et al., 2005b). Chemical analysis indicated that AA and GA were stable in water for at least 1 week, and dosing solutions were prepared and changed weekly. The 60-day treatment schedule was selected in an effort to promote the accumulation of mutations in the thyroid, a tissue with a relatively low rate of cellular proliferation (Lafferty et al., 2004). Weekly, individual body weight gains and water consumption in each cage were monitored throughout the course of the experiment to estimate the amount of the test agents consumed per rat per kilogram body weight. The rats were sacrificed within 24 h of the last treatment, the peripheral blood was collected by cardiac puncture, and used for measuring MN-RETs; spleens were rapidly removed and immediately processed for the lymphocyte Hprt assay. Other tissues, including liver, testes, and thyroid, were excised, frozen quickly in liquid nitrogen, and stored at ~80°C for later assays of cII mutation.

The mammary gland was also removed from females, placed into a 50-ml centrifuge tube, and washed with ice-cold 0.9% NaCl. Approximately 5 g of mammary tissue were minced with scissors, transferred to another 50-ml centrifuge tube, and fat-free epithelial cells isolated using the method of Moon et al. (1969); the remaining mammary tissue was frozen and stored at ~80°C. The boat used for weighting the 5 g of tissue was rinsed with 20–25 ml Hank’s balanced salt solution, and this rinse was added to the tissue in the centrifuge tube. The final volume was adjusted to 45 ml: Collagenase Type 3 (Worthington Biochemical, Lakewood, NJ) was added to each sample at 35 mg/g tissue. The tissue samples were incubated at 37°C for 3 h on a rotator rack at 30%. When the digestion was complete, the samples were centrifuged at 400 × g at 14°C for 20 min. The supernatant was decanted and the inside of the tube was carefully wiped with a Kimwipe. The pellet was mixed gently into 45 ml of 0.9% NaCl for 5 min on an orbital shaker, followed by centrifugation at 400 × g at 14°C for 20 min. The NaCl wash was washed two more times, and the final cell pellet (mammary epithelial cells) was stored at ~80°C until the DNA was extracted.

Micronucleus assay. The assay was conducted by flow cytometry, following the protocol described in the instruction manual for Litron’s Rat MicroFlowPlus Kit. Briefly, 60–100 μl of the blood, collected by cardiac puncture, was mixed immediately with 350 μl of a heparin solution from the kit. A 180-μl aliquot of the heparinized blood was fixed in 2 ml of ~80°C methanol, and the fixed cells were stored at ~80°C until they were processed further. For analysis of micronuclei, the cells were washed to remove the fixative and 20-μl aliquots of each sample were added to tubes containing anti-CD71-FITC and RbNase A to stain RETs and normochromatic erythrocytes differentially. The cells then were stained with propidium iodide to label micronuclei and analyzed quantitatively using a FACSsort flow cytometer (Becton Dickinson, San Jose, CA). The stopping gate was set to analyze a total of 20,000 reticulocytes (RETs), and data on %RETs and %MN-RETs were collected. Positive and negative controls provided with the MicroFlowPlus Kit were run as controls for the flow cytometric analysis.
Lymphocyte Hprt mutant assay. Spleen lymphocytes were isolated using a Lymphocyte-R density gradient (Accurate Chemical and Scientific, Westbury, NY). The Hprt assay then was performed using a method slightly modified from the one described for mice (Dobrovolsky et al., 2005). In brief, lymphocytes isolated by gradient centrifugation were primed overnight with concanavalin A and then used to establish two sets of 96-well round-bottom microtiter plates, one containing four primed lymphocytes per well plus irradiated feeder lymphocytes in growth medium and one containing 4 × 10⁴ primed lymphocytes per well in growth medium supplemented with 2–4 μg/ml 6-thioguanine. After incubating the plates for 10–12 days, the wells were scored for colony formation using a fluorescent viability stain as described previously (Dobrovolsky et al., 2000), and the cloning efficiencies and the frequency of 6-thioguanine-resistant (6TG r) T lymphocytes were calculated using Poisson statistics.

Sequence analysis of Hprt mutants. 6TG² lymphocyte clones were expanded to ~5 × 10⁶ cells for molecular analysis of Hprt mutations, essentially as described previously (Dobrovolsky et al., 2005). Hprt sequence alterations were analyzed by reverse transcriptase-polymerase chain reaction assay of Hprt messenger RNA extracted from the expanded clones, followed by DNA sequence analysis. The PCR conditions and primers used for this analysis were described previously (Chen et al., 1999).

cII mutant assay. High–molecular weight genomic DNA was extracted from livers, testes, mammary epithelial cells, thyroid, and bone marrow using RecoverEase DNA Isolation Kits and stored at 4°C until DNA packaging was performed. The packaging of the phage, plating the packaged DNA samples, and determination of MF were carried out following the manufacturer’s instructions for the Select-cII Mutation Detection System for Big Blue Rodents (Stratagene). Briefly, the shuttle vector containing the cII target gene was rescued from total genomic DNA with phage packaging extract, and the resulting phage plated on Escherichia coli host strain G1250. To determine the total titer of packaged phages, G1250 bacteria were mixed with 1:3000 dilutions of phage, plated on TB1 plates, and incubated overnight at 37°C (nonsensitive conditions). For mutant selection, all remaining packaged phages were mixed with G1250, plated on TB1 plates, and incubated at 24°C for about 42 h (conditions for cII selection). Under selective conditions, phages with wild-type cII genes undergo lysogenization and become part of the developing bacterial lawn, whereas phages with mutated cII genes undergo lytic growth and give rise to plaques. When incubated at 37°C, phages with wild-type cII genes also undergo a lytic cycle, resulting in plaque formation. Assays were repeated until a minimum of 2 × 10⁵ plaque-forming units (pfu) from each sample was examined for mutation or the plaque DNA was exhausted. The cII MF is defined as the total number of mutant plaques (determined at 24°C) divided by the total number of plaques screened (determined at 37°C) and expressed as mutants per million pfu.

Sequence analysis of cII mutants. cII mutant plaques were isolated from the analyses conducted with thyroid DNA and replated at low density to verify the mutant phenotype. Single, well-isolated plaques were transferred from these plates to a microcentrifuge tube containing 100 μl of sterile distilled water. The tube was heated at 100°C for 5 min and centrifuged at 12,000 × g for 3 min. cII target DNA released by this procedure was amplified by PCR using primers 5′-AAAAAGGCGATCAATTAAAC-T3′ (upstream) and 5′-CCGAATGT-GAGTATTTTGGCTG-T3′ (downstream). For PCR amplification, 10 μl of the supernatant were added to 10 μl of Promega PCR Master Mix and the primers. The final concentrations of the reagents were 1X Taq polymerase reaction buffer, 0.2 μmol of each primer, 200μM of each dNTP, 1.5mM MgCl₂, and 0.25 U of Taq DNA polymerase. The PCR was performed using a PCR System 9700 (Applied Biosystems, Foster City, CA), with the following cycling parameters: 3-min denaturation at 95°C, followed by 35 cycles of 30 s at 95°C, 1 min at 60°C, and 1 min at 72°C, with a final extension of 10 min at 72°C. The PCR products were isolated using a QIAQuick PCR product purification kit (Qiagen, Chatsworth, CA). The cII mutant DNA was sequenced with a CEQ Dye Terminator Cycle Sequencing Kit and a Beckman Coulter CEQ 8000 Genetic Analysis System. The primer for cII mutation sequencing was the upstream primer used for the PCR.

Statistical analyses. Analyses were performed using SigmaStat 2.03 (SPSS, Chicago, IL). All body weight gain, daily water consumption, calculated daily dose, %MN-RET, %RET, and MF data are expressed as the mean ± SD from five to eight rats per group. After establishing the normality of the data, statistical significance was determined by one-way ANOVA followed by Dunnett’s test. Mutation spectra were compared using the computer program written by Cariello et al. (1994) for the Monte Carlo analysis of mutation spectra (Adams and Skopek, 1987).

RESULTS

Body Weight, Water Consumption, and Calculated Exposure Dose

During the 60-day treatment, the weekly individual body weight gains and water consumption were monitored for each cage and converted to per rat values. Table 1 shows the mean body weight gain (body weight after treatment minus body weight before treatment), daily water consumption, daily exposure dose, and total dose during the 60-day treatment. Both male and female rats treated with exposure concentrations of 0.7 and 1.4mM GA showed significantly less body weight gain, with the weight gains for females being less than those for males. When compared to control rats, treatment with 1.4mM GA resulted in 83 and 58% body weight gain in male and female rats, respectively. Only the female rats treated with 1.4mM AA showed significantly less body weight gain.

Daily water consumptions for male and female rats were about 40–43 and 33–36 ml, respectively. The average daily exposure doses, calculated from the weekly water consumption values and weekly body weights, indicated that the daily exposure for both doses of each agent was significantly higher (p < 0.01) for female rats than for male rats (Table 1). For example, the range for rats treated with an exposure concentration of 0.7mM AA was 3.0–5.1 mg/kg with a mean value of 3.9 mg/kg bw/day for males and 4.4–6.1 mg/kg with a mean value of 5.2 mg/kg bw/day for females. Considering the difference in the molecular weight of AA and GA, at each exposure concentration, male and female rats received similar (but not identical) daily and total doses of GA and AA.

Micronucleus Assay

With a single exception, the %RET and %MN-RET frequencies in rats treated with exposure concentrations of 0.7 and 1.4mM AA or GA for 2 months were not significantly different from the control values (Table 2). The one exception was an elevated %RET frequency in female rats treated with 1.4mM GA.

Lymphocyte Hprt Assay

Table 3 summarizes the lymphocyte Hprt MF data. Due to the length of treatment, the data were examined for outliers...
### TABLE 1
Increases in Mean Body Weight, Daily Water Consumption, and Calculated Daily Dose for Male and Female Big Blue Rats Treated with AA or GA for 2 Months$^a$

<table>
<thead>
<tr>
<th>Gender</th>
<th>Treatment</th>
<th>Exposure concentration (mM)</th>
<th>Body weight gain (g)$^b$</th>
<th>Daily water consumption (ml)</th>
<th>Daily received dose (mg/kg bw/day)</th>
<th>Total dose (mg/kg bw)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean (%) SD</td>
<td>Mean SD</td>
<td>Mean$^d$ SD</td>
<td>Mean</td>
</tr>
<tr>
<td>Male</td>
<td>Control</td>
<td>0</td>
<td>117.4 (100) 15.9</td>
<td>42.2 1.8</td>
<td>0 0 0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>0.7</td>
<td>124.3 (106) 13.3</td>
<td>42.0 2.6</td>
<td>3.9 0.7 235</td>
<td>100.7* (83) 8.0 33.9* (79) 5.5</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>0.7</td>
<td>104.6 (89) 10.0</td>
<td>40.4 2.4</td>
<td>4.6 0.6 274</td>
<td>100.7* (83) 8.0 33.9* (79) 5.5</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>1.4</td>
<td>100.7* (86) 8.0</td>
<td>42.7 2.2</td>
<td>4.6 0.6 274</td>
<td>100.7* (83) 8.0 33.9* (79) 5.5</td>
</tr>
<tr>
<td>Female</td>
<td>Control</td>
<td>0</td>
<td>42.7 (100) 8.2</td>
<td>35.7 3.9</td>
<td>0 0 0</td>
<td>42.7 (100) 8.2</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>0.7</td>
<td>42.6 (100) 4.3</td>
<td>33.7 2.0</td>
<td>5.2 0.6 309</td>
<td>42.6 (100) 4.3</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>1.4</td>
<td>30.1* (70) 6.8</td>
<td>33.2 2.8</td>
<td>10.3 1.0 616</td>
<td>33.2 2.8</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>1.4</td>
<td>33.9* (79) 5.5</td>
<td>33.6 2.2</td>
<td>5.9 0.6 356</td>
<td>33.6 2.2</td>
</tr>
</tbody>
</table>

$^a$There were eight rats in each group.  
$^b$The body weight immediately following treatment minus the body weight before treatment.  
$^c$The total dose as cumulative dose during 60-day treatment.  
$^d$There were significant differences in calculated daily dose received during the experiments between male and female groups treated with the same doses of AA or GA ($p < 0.01$).  

### TABLE 2
Percentage of %RETs and %MN-RETs in Peripheral Blood of Male and Female Rats Treated with AA or GA for 2 Months$^a$

<table>
<thead>
<tr>
<th>Exposure concentration (mM)$^b$</th>
<th>Male AA</th>
<th>Female AA</th>
<th>Male GA</th>
<th>Female GA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%RETs ± SD</td>
<td>%MN-RETs ± SD</td>
<td>%RETs ± SD</td>
<td>%MN-RETs ± SD</td>
</tr>
<tr>
<td>0</td>
<td>1.44 ± 0.33 0.06 ± 0.02</td>
<td>0.77 ± 0.26 0.03 ± 0.01</td>
<td>1.44 ± 0.33 0.06 ± 0.02</td>
<td>0.77 ± 0.26 0.03 ± 0.01</td>
</tr>
<tr>
<td>0.7</td>
<td>1.33 ± 0.22 0.05 ± 0.02</td>
<td>1.01 ± 0.19 0.04 ± 0.01</td>
<td>1.31 ± 0.21 0.05 ± 0.02</td>
<td>0.98 ± 0.15 0.04 ± 0.01</td>
</tr>
<tr>
<td>1.4</td>
<td>1.22 ± 0.22 0.05 ± 0.02</td>
<td>0.79 ± 0.17 0.04 ± 0.02</td>
<td>1.36 ± 0.17 0.05 ± 0.02</td>
<td>1.04 ± 0.15 0.05 ± 0.03</td>
</tr>
</tbody>
</table>

$^a$There were eight rats in each group.  
$^b$The actual daily doses and total doses, that were a little different based on the genders or chemicals, were calculated by the exposure concentrations and water consumptions (see Table 1).

(presumably caused by the clonal expansion of mutants) using the Grubbs-Smirnov method (Grubbs, 1969; as recommended by Thybaud et al., 2003) and values that were ≥ 3 SDs greater than the mean of the remaining values were discarded. Data from male and female rats, treated with either AA or GA, were considered separately. The MFs in the vehicle-treated rats (~4 months old at the time of assay) were similar to those seen in previous studies. For males, the control frequency of 3.7 ± 1.7 × 10^{-6} was very similar to the frequency of 2.9 ± 0.3 × 10^{-6} measured by Aidoo et al. (2003) in 5-month-old male CD rats, while the frequency of 7.5 ± 1.8 × 10^{-6} for females was similar to the frequency of 6.1 ± 0.9 × 10^{-6} detected by Manjanatha et al. (1998) in 3.5-month-old female Big Blue rats. Both AA and GA produced dose-dependent increases in lymphocyte Hprt MF (R = 0.528–0.760, p < 0.05–0.001) in both male and female rats (Table 3). The MFs in the rats treated with an exposure concentration of 1.4mM AA (male: 8.0 ± 4.3 × 10^{-6} and female: 11.6 ± 2.2 × 10^{-6}) or GA (10.1 ± 6.2 × 10^{-6} and 20.8 ± 7.7 × 10^{-6}, respectively) were significantly higher (p < 0.05 or 0.001) than those in the controls (3.7 ± 1.7 × 10^{-6} and 7.5 ± 1.8 × 10^{-6}, respectively). In addition, there was a significant difference...
of mutations from AA-treated rats was significantly different from that of control rats (p = 0.016), while the spectrum of mutations from GA-treated rats was marginally different from that of control rats (p = 0.077).

Sequence Analysis of Hprt Mutants

Hprt sequence analysis of 6TG2 clones from rats treated with exposure concentration of 1.4mM AA or GA (males and females were combined because of the relatively low number of mutations) identified 23 and 32 independent mutations, excluding the putative splicing mutations (14 and 24 mutations, respectively). Mutations that were found more than once among the mutants isolated from a single rat were assumed to be siblings resulting from the amplification of a single independent mutation. Table 4 summarizes the data for independent Hprt mutations, with the 40 independent control mutations identified in this study and 36 mutations reported previously (Aidoo et al., 2003). Note that, in this study, approximately two to four times more mutant clones were isolated in the mutant assays than the number of independent mutation. Table 4 summarizes the data for independent Hprt mutations, with the 40 independent control mutations identified in this study and 36 mutations reported previously (Aidoo et al., 2003).

Note. Significantly different from control at *p < 0.05 and **p < 0.001 (ANOVA, followed by Dunnett’s test).

The actual daily doses and total doses, that were a little different based on the genders or chemicals, were calculated by the exposure concentrations and water consumptions (see Table 1).

(p = 0.01, Bonferroni t-test) between the responses induced by the 1.4mM AA and GA treatments in females but not in males.

6.9 × 10⁻⁶, and neither AA nor GA treatment had any significant effect on liver cII MF (Fig. 1 and Supplementary tables 1 and 2). In addition, the high exposure concentration treatments with AA and GA had no significant effect on cII MFs in testes or mammary epithelial cells from male and female rats, respectively (Fig. 2 and Supplementary tables 3 and 4). The MF for mammary cells from control female rats was 23.2 ± 6.4 × 10⁻⁶, while the MF for testes from control male rats was 5.5 ± 2.1 × 10⁻⁷. Treatment with an exposure

<table>
<thead>
<tr>
<th>Exposure concentration (mM)</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>MF ± SD (×10⁻⁶)</td>
<td>n</td>
<td>MF ± SD (×10⁻⁶)</td>
<td>n</td>
</tr>
<tr>
<td>0</td>
<td>7</td>
<td>3.7 ± 1.7</td>
<td>7</td>
<td>7.5 ± 1.8</td>
</tr>
<tr>
<td>0.7</td>
<td>7</td>
<td>6.0 ± 2.0</td>
<td>7</td>
<td>9.6 ± 2.5</td>
</tr>
<tr>
<td>1.4</td>
<td>8</td>
<td>8.0 ± 4.3*</td>
<td>7</td>
<td>11.6 ± 2.2*</td>
</tr>
<tr>
<td>Trend</td>
<td>R = 0.528, p = 0.012</td>
<td>R = 0.635, p = 0.02</td>
<td>R = 0.540, p = 0.011</td>
<td>R = 0.760, p &lt; 0.001</td>
</tr>
</tbody>
</table>

Note. Significantly different from control at *p < 0.05 and **p < 0.001 (ANOVA, followed by Dunnett’s test).

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cII Mutant Assay

cII mutant assays were conducted on livers from control male rats and male rats treated with both exposure concentrations of AA and GA, while only livers from control female rats and female rats treated with the high exposure concentration of GA and AA were assayed. The MFs of the control male and female rat livers were 29.9 ± 15.9 × 10⁻⁶ and 27.7 ± 6.0 ± 3.7, respectively. Mutations that were found more than once among the mutants isolated from a single rat were assumed to be siblings resulting from the amplification of a single independent mutation. Table 4 summarizes the data for independent Hprt mutations, with the 40 independent control mutations identified in this study and 36 mutations reported previously (Aidoo et al., 2003).

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(p = 0.01, Bonferroni t-test) between the responses induced by the 1.4mM AA and GA treatments in females but not in males.

Sequence Analysis of Hprt Mutants

Hprt sequence analysis of 6TG2 clones from rats treated with exposure concentration of 1.4mM AA or GA (males and females were combined because of the relatively low number of mutations) identified 23 and 32 independent mutations, excluding the putative splicing mutations (14 and 24 mutations, respectively). Mutations that were found more than once among the mutants isolated from a single rat were assumed to be siblings resulting from the amplification of a single independent mutation. Table 4 summarizes the data for independent Hprt mutations, with the 40 independent control mutations identified in this study and 36 mutations reported previously (Aidoo et al., 2003).

Note that, in this study, approximately two to four times more mutant clones were isolated in the mutant assays than mutations identified. The number of mutations was reduced by the relatively low efficiency of expanding clones for analysis and the relatively low success in amplifying and sequencing of complementary DNA from the expanded clones. The spectrum of mutations from AA-treated rats was significantly different from that of control rats (p = 0.005) and of GA-treated rats (p = 0.016), while the spectrum of mutations from GA-treated rats was marginally different from that of control rats (p = 0.077).

<table>
<thead>
<tr>
<th>Type of mutation</th>
<th>Control/AA n (%)</th>
<th>Control/GA n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transitions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G:C → A:T</td>
<td>11 (28)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>A:T → G:C</td>
<td>3 (7)</td>
<td>8 (35)</td>
</tr>
<tr>
<td>Transversions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G:C → T:A</td>
<td>3 (7)</td>
<td>6 (26)</td>
</tr>
<tr>
<td>G:C → C:G</td>
<td>2 (5)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>A:T → T:A</td>
<td>2 (4)</td>
<td>3 (9)</td>
</tr>
<tr>
<td>Frameshifts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−1 or +2</td>
<td>1 (3)</td>
<td>2 (9)</td>
</tr>
<tr>
<td>+1 or +2</td>
<td>3 (7)</td>
<td>3 (13)</td>
</tr>
<tr>
<td>Deletions or insertions</td>
<td>4 (10)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Suspected splicing mutants</td>
<td>Not recorded</td>
<td>14 (24)</td>
</tr>
<tr>
<td>Total mutants screened</td>
<td>40</td>
<td>23</td>
</tr>
</tbody>
</table>

*Mutations detected in male and female rats for a treatment group were pooled. There were significant differences between the spectra of control and AA-treated rats (p = 0.005) and between the spectra of AA- and GA-treated rats (p = 0.016), while the spectra of control and GA-treated rats were marginally different (p = 0.077).

**Includes 4 mutations from this study plus 36 mutations previously reported by Aidoo et al. (2003).

Splicing mutants not included.
concentration of 1.4mM GA significantly increased the MF in bone marrow and the mutation frequency in thyroid from both male and female rats (Fig. 2 and Supplementary tables 5–8). While an exposure concentration of 1.4mM AA induced significantly higher mutation frequencies in female rat thyroids and MFs in male and female bone marrow, this exposure concentration of AA resulted in a higher but not significantly higher mutation frequency in male thyroid when compared to control rats (Fig. 2).

Sequence Analysis of cII Thyroid Mutants

Since AA induced thyroid tumors in both female and male rats (Friedman et al., 1995; Johnson et al., 1986), we evaluated all control and 1.4mM AA– and GA–induced thyroid cII mutants by DNA sequence analysis. Mutations that were found more than once among the mutants isolated from a single rat were assumed to be siblings resulting from the expansion of a single independent mutation. From the limited number of mutants that we collected, we identified 36 and 39 independent cII mutations from AA- and GA-treated male rats (Table 5) and 36 and 38 independent mutations for AA- and GA-treated female rats (Table 6). In addition, 25 and 34 independent mutations were identified in thyroids from control male (Table 5) and female rats (Table 6). Statistical evaluation of these spectra with the Adams and Skopek test (Adams and Skopek, 1987) indicates that the overall patterns of mutations in the AA- and GA-treated groups did not differ significantly from the controls. In addition, there were no significant differences between the spectra for AA- and GA-treated rats in either the male or the female groups.

DISCUSSION

Administration of AA in the drinking water for 2 years (up to 2 mg/kg bw/day for males and 3 mg/kg bw/day for females) is carcinogenic for Fischer 344 rats, with increases in the incidence of thyroid follicular cell tumors both in males and in females, peritesticular mesotheliomas in males, and mammary tumors in females reported in two separate studies (Friedman et al., 1995; Johnson et al., 1986). About 40% of AA is metabolized by CYP2E1 to the reactive epoxide GA in rats (60% conversion in mice) (Fennell et al., 2003), before being converted to other metabolites that are eventually excreted in the urine (e.g., GA-glutathione) or to adducts with hemoglobin and DNA (Young et al., 2007). One hypothesis is that AA produces tumors through a genotoxic mechanism, with the GA metabolite covalently binding to DNA, inducing mutations and cell transformation, which eventually leads to the tumors observed in multiple organs. This hypothesis was tested in this study by evaluating the mutagenicity of AA and GA in both surrogate and tumor target tissues of F344-Big Blue rats. The exposure was for 60 days, which was longer than the 28-day repeat dose treatment schedule recommended for transgenic in vivo mutation models (Thybaud et al., 2003). This longer treatment time was chosen to compensate for the low proliferation rate of the thyroid target tissue (Lafferty et al., 2004), with the idea of increasing the possibility that induced
mutations in thyroid and other slowly dividing tissues (e.g., liver) would accumulate to levels that could be detected.

In the present study, we exposed Big Blue rats to the equivalent of $\sim4$ to $12$ mg/kg bw/day of AA or GA via the drinking water (exposure concentrations of 0.7 and 1.4mM) (Table 1), a treatment regimen comparable to those used to produce cancer in rats ($\leq$ 5 mg/kg bw/day AA). With the same administered dose (i.e., same concentration of test agent in the drinking water), the female rats actually received about 30% more dose ($p < 0.01$) than the male rats based on the daily intake of dosed water and the weight of the animals during the experiment (Table 1), an observation similar to that made in our previous study conducted with Big Blue mice (Manjanatha et al., 2006). In addition, female rats treated with exposure concentrations of 1.4mM AA or 0.7 and 1.4mM GA had less relative body weight gain than comparably treated male rats (Table 1), suggesting that the higher effective doses resulted in greater levels of toxicity. There were no significant differences in total body weight (data not shown) or daily water consumption between the control and AA/GA-treated groups for either males or females. Previously, we observed that mice exposed to exposure concentrations of 7.0mM AA and GA consumed significantly less water when compared with those exposed to 1.4mM AA and GA (Manjanatha et al., 2006).

Among the assays conducted on surrogate tissues, neither AA nor GA produced micronuclei in peripheral blood (Table 2). There is abundant evidence that AA and GA are clastogenic in mammalian cells (Rice, 2005), and it may be that the doses of the test agents used in the present study were too low to produce a measurable positive response. Single exposure concentrations of 1.4mM AA and GA were negative for bone marrow MN induction in rats but positive in mice (Paulsson et al., 2002, 2003), indicating that rats may be relatively insensitive to MN induction by AA and GA. In addition, a recent study demonstrated that the induction of chromosome damage by AA in mouse bone marrow appears to exhibit nonlinear kinetics (Zeiger et al., 2009).

In contrast to the negative responses for MN induction, AA and GA produced dose-dependent increases in lymphocyte Hprt MF in both male and female rats ($p < 0.05$), and the MFs for the groups treated with exposure concentrations of 1.4mM of the test agents all were significantly higher than the controls (Table 3). In cII mutant assays performed on bone marrow, exposure concentrations of 1.4mM AA and GA produced relatively consistent increases in cII MF; however, the increase in AA-treated male rats was not significant (Fig. 2). In addition, Douglas reports (cited in Thybaud et al., 2003) that daily ip dosing for 7 or 28 days with 25 mg/kg AA to Muta mouse results in significant increases in bone marrow cII MF when assayed 3 days following the last dose. Although different cell types and induction mechanisms are involved in the assays, the target cells for the genotoxicity measured by the MN assay and the Hprt and cII mutation assays are all in the bone marrow (e.g., Jansen et al., 1996). These results suggest that both AA and GA are systemic genotoxins in rats under conditions similar to those of the cancer bioassays and that both compounds are more potent as gene mutagens than they are as clastogens or aneugens.

A major objective of this study was to determine cII MFs in target tissues for AA-induced tumors, thyroid, mammary gland, and testis, as well as the nontarget tissues, liver and bone marrow (bone marrow discussed above) (Figs. 1 and 2). At the exposure concentrations and treatment regimen used in this study, neither AA nor GA increased cII MF in nontarget liver or in the target mammary epithelial cells or in the testes. In the case of the testes, whole testicular tissue was assayed rather than a single cell type.
than the tunica vaginalis target, which was judged too small for analysis. In contrast to these negative responses, both AA and GA increased the mutation frequencies in the thyroid of rats (note that because all the thyroid mutants were sequenced, we have calculated mutation frequencies by correcting the MFs for potential clonal expansions). The cII mutation frequencies in the thyroid of male rats exposed to 1.4mM AA and GA were $28.2 \pm 11.7 \times 10^{-6}$ and $31.0 \pm 9.1 \times 10^{-6}$, respectively, with only the GA-induced mutation frequency being significantly greater than the control frequency of $21.3 \pm 2.5 \times 10^{-6}$ (Fig. 2A). The thyroid cII mutation frequencies in the female rats treated with an exposure concentration of 1.4mM AA or GA were $40.9 \pm 13.0 \times 10^{-6}$ and $45.4 \pm 9.7 \times 10^{-6}$, respectively, and both were significantly higher than the control frequency of $22.3 \pm 7.1 \times 10^{-6}$ (Fig. 2B). It has been reported that AA and GA treatment by single ip injection produced N7-guanine and N3-adenine GA-DNA adducts in thyroid DNA, although the adduct levels were lower than those of testes, mammary gland, leukocytes, brain, or liver (Doerge et al., 2005a). In addition, GA dosing produced much higher levels of DNA adducts than AA in all tissues examined, and there were higher levels of DNA adducts in GA-treated female rat thyroids than that in males (Doerge et al., 2005a). These later observations are consistent with the slightly higher mutation frequencies detected in thyroid DNA from GA-treated rat than in thyroid DNA from AA-treated rat and in thyroid DNA from treated female compared with treated male rats. Since carcinogenic MOA determinations are best made with data, including mutation data, from the tumor target tissue (Moore et al., 2008), these observations may be important to regulatory bodies as they assess the magnitude of human cancer risk from dietary exposure to AA in cooked foods.

Mutation spectra are often useful for confirming weak induced responses and in supporting hypotheses relating to the pathways involved in mutation induction. For instance, the cII mutation spectra in liver from AA- and GA-treated mice in our previous study (Manjanatha et al., 2006) were significantly different from the control spectrum, consistent with agent-induced increases in mutation, and the major types of mutations in the induced spectra included $G \rightarrow T$ transversion and frameshifts, mutations that could plausibly be induced by the DNA adducts produced by GA. The overall patterns of mutations from the thyroids of AA- and GA-treated rats were not significantly different from the controls (Tables 5 and 6), which could have been due to the relatively modest increase in MF in treated rats coupled with the relatively small number of mutants available for analysis. However, in both the AA- and the GA-treated female groups, the percentage of mutations with $G:C \rightarrow T:A$ transversion (19 and 29%) was higher than that in the control spectrum (9%), and in both the AA- and the GA-treated groups, the percentage of mutations with frameshifts was higher than that in the control group (22 and 23% vs. 12% for males and 25 and 24% vs. 17% for females). These observations are at least consistent with AA and GA producing cII mutation spectra in rat thyroid similar to those that they produced at higher doses in mouse liver (Manjanatha et al., 2006).

The mutation spectra analysis for the Hprt lymphocyte mutants was even less clear than that of the thyroid cII mutants because there were fewer mutations identified, which forced us to combine the data from male and female rats in an effort to increase statistical power (Table 4). This expedient differentiated the AA-induced spectrum from the control and GA-induced spectra, with relatively large differences being an increase in $G:C \rightarrow T:A$ transversion (7% for control vs. 26% for AA treated) and frameshifts (10% for controls vs. 22% for AA treated). Similar increases in $G:C \rightarrow T:A$ transversion, in particular, were not seen in the mutation spectrum from GA-treated rats. Because of the small number of Hprt mutations identified in this study, we consider any conclusions drawn from these data to be potentially misleading.

An increase in the incidence of human thyroid cancer has been detected over a recent 20-year period (Colonna et al., 2002). Thyroid tumorigenesis has two well-established MOAs, a non-genotoxic growth stimulation MOA and a mutagenic MOA. Dourson et al. (2008) suggested that these two MOAs may operate in different parts of the dose-response curve for AA-induced thyroid tumors, with a mutagenic MOA in the low end of the dose range and a growth stimulation MOA in the high end of the dose range. It has been reported that AA affects DNA integrity, as demonstrated by positive Comet assay results for DNA damage in rat thyroid cell lines (FRTL5 and PC C13) and in primary thyroid cell cultures from humans, dogs, and sheep (Chico Galdo et al., 2006). AA also produces an increase in DNA damage in rat thyroids as measured by the Comet assay (Klaunig, 2008). Subchronic AA treatments, however, increased DNA synthesis in the target tissues for tumor development (Lafferty et al., 2004). Considering the possibility of AA causing endocrine disruption, it has been reported that there were slight dose-dependent increases in plasma thyroxin and decreases in plasma thyroid-stimulating hormone after acute AA exposure in rats (Khan et al., 1999). However, treatment of rats with up to 50 mg/kg/day AA for 2 weeks produced no evidence of a systematic alteration in the hypothalamic-pituitary-thyroid axis and thyroid hormone dysregulation (Bowyer et al., 2008). Our finding that AA and GA are systemic mutagens in rats and produce cII mutations in rat thyroid, using treatment conditions similar to those used in the cancer bioassays, lends support to the hypothesis that AA operates through a genotoxic MOA to produce thyroid tumors in the rat.

Interpreting our data with regard to the testicular and mammary gland tumors produced by AA in rats is more difficult. The degree of certainty with which a negative response is demonstrated by any toxicology assay, including genetic toxicity assays, is limited by the sensitivity of the assay and its statistical power, and transgenic mutation assays are regarded as having limited sensitivity to small increases in MF due to their relatively high background MFs (e.g., Walker et al., 2009). In addition, the cII assay, as well as the Hprt
assay, only detect a subset of the possible types of gene mutations; for instance, neither detects the loss of heterozyosity or recombination events that can be detected with an autosomal reporter gene (e.g., Dobrovolsky et al., 1999). Finally, as mentioned above, it was deemed impractical to assay for mutation in the actual target cells for peritesticular mesothelioma induction, which may have compromised this measurement further. Within the limits of the assay, however, we were unable to demonstrate mutation induction in two target tissues for AA carcinogenesis in the rat, which leaves open the possibility of nongenotoxic MOAs for tumor induction in these tissues. Zeiger et al. (2009) recently demonstrated the potential problems associated with analyzing results from tests with a very weak clastogen and suggested the need of using biologically effective dose rather than administered dose as the relevant metric of exposure.

In conclusion, these results indicate that AA and GA, under exposure conditions comparable to those known to produce tumors in 2-year rat cancer bioassays, are weak systemic gene mutagens (cII rat bone marrow assay and Hprt lymphocyte assay), with female rats having marginally greater responses than male rats, probably due to higher delivered doses of AA and GA to females than males at the same exposure concentration (e.g., at an exposure concentration of 1.4mM AA or GA, the average daily doses in female rats were about 1.3-fold greater than those in male rats). Under these treatment conditions, both GA and AA also were weakly mutagenic in the thyroid cII gene, which is consistent with a genotoxic MOA for tumor induction in this tissue. This study provides additional support to a growing body of evidence for a genotoxic (mutagenic) mechanism for AA carcinogenesis in rodents.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES


