Effects of Intermittent Exposure to Aflatoxin B₁ on DNA and RNA Adduct Formation in Rat Liver: Dose-Response and Temporal Patterns

Rene E. Sotomayor,*† Melissa Washington,* Linh Nguyen,† Rahma Nyang’anyi,† Dennis M. Hinton,* and Ming Chou‡

*Center for Food Safety and Applied Nutrition, and the †Joint Institute for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, University of Maryland, College Park, Maryland 20742; and ‡National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, Arkansas 72079

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We studied the effects of intermittent exposure to aflatoxin B₁ (AFB₁) on hepatic DNA and RNA adduct formation. Fisher-344 male rats were fed 0.01, 0.04, 0.4, or 1.6 ppm of AFB₁ intermittently for 8, 12, 16, and 20 weeks, alternating with 4 weeks of dosing and 4 weeks of rest. Other groups of rats were fed 1.6 ppm of AFB₁ continuously for 4, 8, 12, and 16 weeks. Control rats received AFB₁-free NIH-31 meal diet. AFB₁-DNA and -RNA adducts were measured by HPLC with fluorescence detection. The data are presented as total DNA or RNA adducts. The DNA and RNA adduct levels increased or decreased depending on the cycles of dosing and rest. Rats removed from treatment 1 month after 1 or 2 dosing cycles (8 and 16 weeks of intermittent exposure) showed approximately a twofold decrease in DNA adduct levels and a two- to elevenfold decrease in RNA adduct levels compared with rats euthanized immediately after the last dosing cycle (12 and 20 weeks of intermittent exposure). Our data indicate that DNA and RNA adducts increased linearly, from 0.01 ppm to 1.6 ppm of AFB₁, after 12 and 20 weeks of intermittent treatment. A linear dose response was also apparent for DNA but not for RNA adducts; liver; rats.

Key Words: intermittent exposure; aflatoxin B₁; DNA and RNA adducts; liver; rats.

Dietary exposures to carcinogens and mutagens depend on individual consumption patterns, which change with age, availability of foods, and lifestyle. Individuals are often exposed to a particular agent in an intermittent or discontinuous manner rather than in a continuous one. Despite the importance of the subject, little is known about the health risk posed by intermittent exposure to carcinogens and mutagens (Banotai et al., 1999; Kodell et al., 1987; Murdoch et al., 1992). Cancer risk estimates rely almost exclusively on the chronic, continuous-exposure rodent cancer bioassay. Cancer risk estimates for less than lifetime are generally based on the assumption that the cancer risk is proportional to the total accumulated dose or equivalently to the average dose rate (Halmes et al., 2000; Kodell et al., 1987).

Aflatoxin B₁ (AFB₁), a secondary metabolite of the fungus Aspergillus flavus, was selected as a model agent in this study to assess the effects of intermittent exposure on DNA and RNA adduct formation in rat liver. AFB₁ is the most potent carcinogen found in the food supply (Dragan and Pitot, 1994; Wogan, 1992; Wogan et al., 1974). AFB₁ is activated by CYP450 enzymes to an AFB₁-8,9-epoxide, which rapidly reacts with the N⁷ of guanine in DNA and RNA (Essigman et al., 1977; Lin et al., 1977; Swenson et al., 1977). The imidazole ring of guanine becomes susceptible to nucleophilic attack by hydroxide ions resulting in the ring-opened AFB₁-FAPyr adduct derivatives, forms 1 and 2 (Wang and Cerutti, 1980). AFB₁-FAPyr 1 has been implicated in the initiation of liver carcinoma because of its slow removal from DNA (Croy and Wogan, 1981).

Elucidating the quantitative and qualitative relationships between dose and extent of DNA adduct formation is important for cancer-risk assessment. Linear dose-response relationships for hepatic AFB₁-DNA adducts have been reported after acute exposures (see Choy 1993, for review). However, most of the evidence indicates that acute exposures to AFB₁ less readily results in liver tumor induction. Continuous dosing studies have also reported a linear dose-response relationship for hepatic AFB₁-DNA adducts in rats (Buss et al., 1990; Phillips et al., 1999). Betchel (1989) concluded that there was a linear relationship between hepatic AFB₁-DNA adducts and liver tumor induction. A linear dose response for DNA adducts may also exist for intermittent exposure to AFB₁, but the data to support this are not available.

The present study was part of a larger collaborative research project designed to study the effects of intermittent exposure to AFB₁ on genetic, immunotoxic, and metabolic endpoints in an...
hydroaflatoxin B1 (AFB1-N7-Gua) and the ring-opened imidazole derivatives, formamido-4-oxo-3,4-dihydroxypyrimid-5-yl)9-hydroxyaflatoxin B1 (AFB1-8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)9-hydroxyaflatoxin B1 (AFB1-N7-Gua). 

were fed NIH-31 meal diets premixed with AFB1 at the concentrations of 0.01, 0.04, 0.4, and 1.6 ppm of AFB1, intermittently and 1.6 ppm of AFB1 continuously. Control rats received AFB1-free diet. The markers (▼) indicate the point of sacrifice of the rats during the study.

experimental system that would mimic human exposure to mycotoxins. This paper reports results on the temporal patterns and dose-response relationships of hepatic DNA and RNA adducts in rats fed AFB1 intermittently for several weeks.

MATERIALS AND METHODS

Chemicals. AFB1 (CAS No 1162-65-8), calf-thymus DNA, RNases, and DNases were purchased from Sigma Chemical Co., St. Louis, MO. Proteinase K, Tris-saturated phenol, Tris-saturated phenol/chloroform/isoamyl alcohol, DNases were purchased from Sigma Chemical Co., St. Louis, MO. Proteinase K, Tris-saturated phenol, Tris-saturated phenol/chloroform/isoamyl alcohol, and anhydrous ethanol were purchased from Amresco, Inc., Solon, OH.

Synthesis of AFB1-guanine standards. 8,9-Dihydro-8-(7-guanyl)-9-hydroxyaflatoxin B1 (AFB1-N2-Gua) and the ring-opened imidazole derivatives, 8,9-dihydro-8-(2-amino-6-formamido-4-oxo-3,4-dihydropyrimid-5-yl)-formamido)-9-hydroxyaflatoxin B1 (AFB1-FAPyr1) and 8,9-dihydro-8-(2-amino-6-formamido-4-oxo-3,4-dihydropyrimid-5-yl)-9-hydroxyaflatoxin B1 (AFB1-FAPyr2) were synthesized as described previously (Sotomayor et al., 1999).

Animals, diets, and experimental design. Fisher-344/N male rats from the breeding colony of the National Center for Toxicological Research (NCTR, Jefferson, AR) were used in this study. The rats were weaned at 21 days of age, housed singly, acclimated to the NIH-31 meal diet for 1 week, and randomly allocated to control and treatment groups at 4 weeks of age. NIH-31 is a semi-purified, open formula, rat and mouse diet. Rats in the treatment groups were fed NIH-31 meal diets premixed with AFB1 at the concentrations of 0.01, 0.04, 0.4, and 1.6 ppm. All rats received water ad libitum. The AFB1-NH3-31 meal diets were prepared by mixing 40 kg of the NIH-31 meal with 0.4, 1.6, and 64 mg of AFB1 dissolved in 500 ml of ethanol. The concentration of AFB1, in each diet preparation was confirmed (recovery >90%) by measuring AFB1 in a 50-g diet sample according to the method of Park et al. (1990). Control rats received AFB1-free NIH-31 meal diet.

The rats were fed intermittently with AFB1 for 8, 12, 16, and 20 weeks or continuously for 4, 8, 12, and 16 weeks. The intermittent treatment consisted of 4-week dosing periods alternated with 4-week no-dosing periods. Figure 1 shows a summary of the dosing schedule. At the end of the respective treatments, rats were euthanized by CO2 asphyxiation. The livers were immediately removed, weighed, divided for different studies, quick-frozen in liquid nitrogen and stored at –80°C until analysis. Livers from three to five rats per dose group were used for the DNA and RNA adduct studies. Kidneys, testes, and spleens were also removed for different studies.

Separate groups of rats were used to estimate food consumption. Four rats per dose group were housed singly in hanging cages. Spilled food was collected and weighed. Food consumption was corrected by adding the weight of wasted food to the weight difference before and after feeding.

DNA and RNA extraction and purification. Approximately 3 g of frozen liver per rat were minced into smaller pieces on a Petri dish containing Dulbecco’s phosphate-buffered saline (PBS) and 20 mM EDTA, pH 7.8, on ice. The liver pieces were transferred to a 50-ml centrifuge tube and rinsed at least three times in PBS to remove the excess blood. The liver pieces were transferred to a fresh 50-ml centrifuge kept on ice and approximately 10 ml of cold TE buffer (50 mM Tris–HCl, 10 mM EDTA, 100 mM NaCl, pH 7.8–8.0) were added. The liver pieces were homogenized with a Polytron PT3000 homogenizer (Brinkman Instruments, Westbury, NY) at 5000 to 6000 rpm for approximately 2 min or until a homogeneous suspension was obtained. The liver suspension was then centrifuged at approximately 2000 × g for 10 min at 4°C. The supernatant was removed and kept frozen at –80°C for RNA extraction. The pellet, containing mostly nuclei, was suspended in 10 ml of cold TE buffer and recentrifuged as above. The supernatant was discarded and the nuclear pellet was re-suspended in a small volume of TE buffer by gently flicking the centrifuge tube with the fingers. Five ml of TE buffer was added and the suspension was gently mixed until no clumps were visible. RNAS A and T1 were added to a concentration of 150 µg and 1000 U per ml of suspension, respectively, vortexed, and incubated at 40°C for 1 h. Fresh proteinase K (150 µg/ml of suspension) was added and the incubation continued for 1 h. Sodium dodecyl sulfate (SDS) solution was added to final concentration of 0.5% and the nuclear suspension incubated for an additional 30 min. At the end of the incubation period, an equal volume of Tris-saturated phenol, pH 7.8, was added. The centrifuge tube was rocked gently to mix the aqueous and organic phases for 5 min. The suspension was transferred to a pre-spin Phase-Lock Gel III (PLG-III) centrifuge tube (5 Prime-3 Prime, Inc., Boulder, CO) and centrifuged at 1500 × g for 15 min at room temperature. The top aqueous phase was transferred to a fresh PLG-III tube and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and centrifuged as before. The top aqueous phase was further extracted with an equal volume of chloroform:isoamyl alcohol (24:1) and was centrifuged as before. The top phase containing the DNA was transferred to a fresh centrifuge tube and the DNA precipitated with 2.5 volumes of chilled ethanol. The DNA was spun with a sealed Pasteur pipette, rinsed three times with 70% ethanol, dried, and dissolved in distilled water.

RNA was extracted from the supernatant of the liver homogenates according to Chattopadhyay et al. (1993) with modifications. Briefly, SDS was added to the supernatant to a final concentration of 0.5%. One-tenth volume of 2 M sodium acetate, pH 4.2, was added. An equal volume of saturated phenol, pH 4.5, was added and mixed by rocking the tubes for 5 min. The suspension was centrifuged at 10,000 × g for 15 min at C. The aqueous phase was re-extracted with an equal volume of phenol:chloroform (49:1) and centrifuged as before. Approximately three volumes of chilled absolute ethanol were added to the aqueous phase containing the RNA and left overnight at –80°C. The precipitated RNA was washed three times with 80% ethanol, dried, and dissolved in distilled water.

The concentrations of DNA and RNA were measured by UV absorbance using a Beckman DU-650 spectrophotometer (Beckman Instruments, Fullerton, CA). The purity of DNA and RNA were determined by UV scanning between 200 and 300 nm and by the 260/280 nm ratio. For DNA and RNA, the ratios were 1.80 to 1.85 and 1.9 to 2.0, respectively. In addition, purity was checked by HPLC analysis of DNA and RNA nucleosides.

Hydrolysis and solid-phase extraction. DNA and RNA were acid hydrolyzed in 0.1 N HCl at 70–75°C for 30 min according to Hertzog et al. (1980). The pH was then brought to approximately 4.5 with 5 N NaOH. The DNA and RNA hydrolysates were loaded onto 3-ml C18 cartridges (Waters Corp., Milford, MA) previously conditioned with 3 ml of HPLC-grade methanol followed by 3 ml of glass-distilled water. The DNA and RNA hydrolysates were then eluted with 15 ml of 10% methanol followed by 12 ml of 85% methanol. The lipophilic AFB1-DNA or -RNA adducts were eluted during the second elution. Recovery was approximately 95%, as determined by elution of known amounts of [1H]-AFB1-N2-Gua. The 85% methanol eluate containing the adducts was evaporated using a centrifugal vacuum evaporator (Jouan, Win-
The dried adducts were either kept frozen at –20°C or were dissolved in 300 μl of 35% methanol for immediate HPLC analysis.  

**HPLC analysis of DNA and RNA adducts.** DNA and RNA adducts were measured by HPLC with fluorescence detection, using a method previously described (Sotomayor *et al.*, 1999). Briefly, a Waters HPLC system was used, consisting of a 991 model photodiode array detector, a 470 model spectrophotometer, and a 600E model system controller. A Supelcosil LC-18S column (250 × 4.6 mm, 5-μm particle size) and a Supelguard LC-18S (20 × 4.6 mm, 5-μm particle size) guard column (Supelco, Bellefonte, PA) were used. The column was eluted isocratically with 35% methanol in water at 1 ml/min for 20 min at room temperature. The spectrophotometer was set at 365 nm excitation and 428 nm emission according to Autrop *et al.* (1983) and Weaver *et al.* (1994). The gain was set at 1000 × with an attenuation of two for maximum fluorescence detection. The concentrations of the DNA and RNA adducts were calculated from standard curves using pmol concentrations of authentic AFB1-N7-Gua standards. The limit of detection was between 1 and 3 pmol for AFB1-FAPyr1 and AFB1-FAPyr2 adducts per injection. In comparison, AFB1-N7-Gua was an order of magnitude less fluorescent than the opened imidazole-ring adducts. Figure 2 shows an HPLC fluorescence profile of the standards.  

**Statistical analysis.** All the data were analyzed with the standard SAS statistical software (ver. 8.2). Tests included: means and standard errors of the mean, analysis of variance (ANOVA), and Dunnett’s multiple pairwise *t*-tests for comparisons of the dose groups to the controls (0.0 ppm). Statistical differences at a *p* value ≤ 0.05 were considered significant.  

**RESULTS**  

**Food Intake, Body Weight, and Liver Weight**  
Table 1 shows the food consumption and AFB1 intake of control and treated rats. Food consumption was measured daily throughout the 20-week study. No significant reductions in food intake were observed. Table 2 shows the body and liver weights of control and treated rats. Rats were weighed weekly throughout the duration of the study. Livers were removed immediately after euthanasia and weighed. Significantly lower body weights (*p* ≤ 0.05) compared to controls were observed after 12 and 20 weeks of intermittent exposure with 1.6 ppm of AFB1. Body weights were also significantly reduced after 16 weeks of continuous treatment with 1.6 ppm of AFB1 (Table 2). An unexpected, significant reduction in the body weights of rats fed 0.01 ppm of AFB1 intermittently for 8 weeks was also observed (Table 2). Liver weights were lower than controls after 16 weeks of continuous feeding and after 12 and 20 weeks of intermittent exposure with 1.6 ppm of AFB1. (Table 2). However, no significant differences were found when the body weight was entered as a covariable (data not shown).  

**Analysis of Hepatic DNA and RNA Adduct Formation**  
AFB1-DNA and -RNA adducts were measured by fluorescence at 365 nm excitation and 428 nm emission. In a previous study from our laboratory, a linear relationship between the concentration of AFB1-DNA adducts and fluorescence intensity was demonstrated (Sotomayor *et al.*, 1999). Under the HPLC conditions used, the AFB1-N7-guanine and AFB1-FAPyr1 adducts had similar retention times and were partially co-eluted. However, the AFB1-FAPyr2 adduct did separate completely from AFB1-N7-Gua with a different retention time. Therefore, in this paper, the terms AFB1-DNA and -RNA adducts are used to indicate total adducts, including both AFB1-N7-Gua and AFB1-FAPyr1 adducts. No peaks corresponding to AFB1-DNA or -RNA adducts were observed in livers from untreated control rats. Figure 3 shows a typical HPLC fluorescence profile of hepatic DNA and RNA adducts from rats fed intermittently for 12 weeks with 0.01 ppm of AFB1.

**DNA and RNA Adduct Formation and Removal**  
At any given dose during intermittent treatment, the levels of AFB1-DNA adducts followed a pattern characterized by in-
TABLE 1

Mean Food Consumption (g/rat/day) over AFB1 Intake (μg/rat/day), measured by weeks

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
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<th>18</th>
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</thead>
<tbody>
<tr>
<td>0.0</td>
<td>18.4±2.3</td>
<td>18.7±1.0</td>
<td>20.4±1.4</td>
<td>20.8±1.7</td>
<td>20.6±1.2</td>
<td>19.9±1.2</td>
<td>20.3±1.5</td>
<td>19.5±1.4</td>
<td>18.7±1.4</td>
<td>19.4±1.3</td>
<td>19.6±1.2</td>
<td>21.1±2.2</td>
<td>21.1±0.8</td>
<td>20.0±1.4</td>
<td>21.5±1.9</td>
<td>21.7±1.4</td>
<td>21.1±2.1</td>
<td>21.4±2.0</td>
<td>22.5±3.4</td>
<td>21.7±2.5</td>
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<td>0.01</td>
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<td>19.3±1.1</td>
<td>19.4±1.6</td>
<td>20.0±1.5</td>
<td>19.9±1.3</td>
<td>19.5±1.6</td>
<td>20.0±1.9</td>
<td>19.7±1.2</td>
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<td>20.4±1.3</td>
<td>19.6±1.2</td>
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<td>21.1±1.2</td>
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<td>20.3±1.5</td>
<td>19.0±1.4</td>
<td>19.4±1.5</td>
<td>19.4±1.1</td>
<td>19.1±0.8</td>
<td>19.7±1.1</td>
<td>19.2±1.9</td>
<td>18.8±1.2</td>
<td>19.5±1.1</td>
<td>19.7±1.3</td>
<td>20.7±1.2</td>
<td>20.4±1.3</td>
<td>20.6±1.7</td>
<td>22.1±1.8</td>
<td>22.5±2.9</td>
<td>19.9±1.7</td>
<td>19.6±0.9</td>
<td>21.2±3.8</td>
<td>20.3±3.3</td>
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<tr>
<td>0.4</td>
<td>20.2±1.9</td>
<td>20.7±2.1</td>
<td>19.6±1.3</td>
<td>20.5±0.6</td>
<td>20.5±1.1</td>
<td>20.7±1.3</td>
<td>21.3±1.6</td>
<td>19.8±1.2</td>
<td>18.6±1.5</td>
<td>19.4±1.5</td>
<td>19.9±1.3</td>
<td>20.3±1.9</td>
<td>19.5±1.4</td>
<td>20.0±1.9</td>
<td>20.7±1.4</td>
<td>20.7±1.7</td>
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<td>1.6</td>
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<td>18.8±2.1</td>
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<td>20.8±1.8</td>
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<td>16.5±1.5</td>
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<td>18.0±1.9</td>
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<td>19.6±1.8</td>
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<td>3.0</td>
<td>29.0±29.1</td>
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<tr>
<td>0.7</td>
<td>28.5±27.4</td>
<td>30.6±30.6</td>
<td>30.1±31.0</td>
<td>31.0±28.8</td>
<td>29.1±31.2</td>
<td>31.4±31.0</td>
<td>29.9±31.4</td>
<td>31.4±41.0</td>
<td>32.0±32.0</td>
<td>31.0±25.3</td>
<td>27.0±29.6</td>
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</table>
creases and decreases, depending on the cycles of dosing and rest (Fig. 4). The levels of AFB$_1$-DNA adducts decreased after 8 and 16 weeks of intermittent exposure but were significantly higher ($p < 0.05$) than those of controls. Control adduct levels are represented by zero, since no AFB$_1$-DNA or -RNA adducts were detected. Eight and 16 weeks of intermittent exposure represent one and two cycles of dosing followed by one cycle of rest before euthanasia, respectively. Each cycle lasted 4 weeks. Resumption of AFB$_1$ dosing produced significant increases of DNA adducts after 12 weeks and 20 weeks of intermittent exposure ($p < 0.05$). These treatment periods represent two dosing cycles with one intercalated no-dosing cycle and three dosing cycles with two intercalated no-dosing cycles.

TABLE 2
Mean Body (BW) and Liver (LW) Weights ± SD (g) of Rats Fed AFB$_1$ Intermittently (I) or Continuously (C) and Control Rats Fed an AFB$_1$-Free Diet

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>4 weeks BW</th>
<th>8 weeks BW</th>
<th>12 weeks BW</th>
<th>16 weeks BW</th>
<th>20 weeks BW</th>
</tr>
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<tbody>
<tr>
<td>0.0</td>
<td>201 ± 20</td>
<td>290 ± 6</td>
<td>358 ± 35</td>
<td>379 ± 26</td>
<td>400 ± 20</td>
</tr>
<tr>
<td>0.01 I</td>
<td>185 ± 16</td>
<td>80 ± 1.4</td>
<td>260 ± 18</td>
<td>339 ± 32</td>
<td>377 ± 12</td>
</tr>
<tr>
<td>0.04 I</td>
<td>181 ± 5</td>
<td>7.5 ± 0.2</td>
<td>277 ± 19</td>
<td>319 ± 17</td>
<td>371 ± 17</td>
</tr>
<tr>
<td>0.40 I</td>
<td>204 ± 28</td>
<td>9.6 ± 2.0</td>
<td>267 ± 18</td>
<td>325 ± 18</td>
<td>360 ± 21</td>
</tr>
<tr>
<td>1.60 I</td>
<td>214 ± 11</td>
<td>9.9 ± 0.3</td>
<td>278 ± 15</td>
<td>302 ± 29*</td>
<td>365 ± 24</td>
</tr>
<tr>
<td>1.60 C</td>
<td>214 ± 11</td>
<td>9.9 ± 0.3</td>
<td>277 ± 15</td>
<td>316 ± 27</td>
<td>346 ± 14*</td>
</tr>
</tbody>
</table>

Dunnett’s $t$-test $< 0.05$ compared to controls (0.0 ppm). NS, no liver samples collected for this study. The means for 1.6 I and 1.6 C are the same, since 4 weeks represent one dosing cycle.

FIG. 3. HPLC fluorescence profiles of hepatic AFB$_1$-DNA (A) and -RNA (B) adducts from rats fed intermittently for 12 weeks with 0.01 ppm of AFB$_1$. Approximately 1 mg of DNA or RNA hydrolysate was injected per analysis. Most hydrolysis products, including adenine, unmodified guanine, and partially degraded DNA and RNA eluted between 2 and 7 min. Fluorescence was measured at 365 nm excitation and 428 nm emission. Notice that the y-axis of panel B is 2.5 times larger than that of panel (A).

FIG. 4. Temporal patterns of hepatic DNA adduct formation after intermittent feeding with 0.01, 0.04, 0.4, and 1.6 ppm of AFB$_1$; three to 5 rats per group; mean ± SD.
respectively. For instance, the average adduct levels at 8 and 16 weeks of treatment with 0.4 ppm of AFB$_1$ were 3.5 and 5.9 pmol/mg DNA compared to 10.5 and 10.6 pmol/mg DNA at 12 and 20 weeks, respectively.

The pattern of RNA adduct formation was similar to that of DNA adduct formation (Fig. 5). However, the amount of AFB$_1$ adducts per mg of RNA was much larger than that of DNA. Notice that the $y$-axis of Figure 5 is approximately an order of magnitude larger than that of Figure 4. For example, after 4 weeks of treatment with 0.4 ppm of AFB$_1$, the average amounts of DNA and RNA adducts were 9.2 pmol per mg of DNA and 61 pmol per mg of RNA, respectively. These quantitative differences were observed at all doses. When the dosing was stopped after one or two cycles of intermittent treatment (8 and 16 weeks, respectively), the levels of RNA adducts declined drastically. For example, the levels of RNA adducts decreased from an average of 287 pmol per mg of RNA after 12 weeks of intermittent treatment with 1.6 ppm of AFB$_1$ to an average of 21.1 pmol per mg of RNA after 16 weeks of intermittent exposure (Fig. 5). At 8 and 16 weeks of treatment, RNA adducts also showed much more variability than DNA adducts at the same doses. Some rats showed adduct levels at the limit of resolution of the fluorescence measurements (Fig. 5, 0.4, and 1.6 ppm).

The patterns of DNA and RNA adduct formation after continuous treatment with 1.6 ppm of AFB$_1$ are shown in Figure 6. The levels of AFB$_1$-DNA adducts increased from an average of 17.4 pmol/mg DNA at 4 weeks to 73.8 pmol/mg DNA at 16 weeks. In comparison, the levels of AFB$_1$-RNA adducts increased from an average of 137.2 pmol/mg RNA at 4 weeks to 216 pmol/mg RNA at 16 weeks. The DNA and RNA adduct levels varied greatly after 8 weeks of exposure, and no significant differences were found between the adduct levels after 8 weeks of continuous treatment.

Dose-Response Relationships

The DNA and RNA adducts are shown as a function of dose and duration of treatment in Figures 7 and 8, respectively. The amount of AFB$_1$ bound to DNA appears to be a linear function of the dose after 4 weeks of continuous treatment ($r = 0.83$) and after 8, 12, 16, and 20 weeks of intermittent exposure ($r = 0.80, 0.73, 0.84$, and 0.90, respectively) (Fig. 7). Linear dose-responses were also observed for RNA adducts after 4 weeks of continuous exposure ($r = 0.91$), and after 12 and 20 weeks of intermittent exposure ($r = 0.93$ and 0.78, respectively) (Fig. 8). No dose-response relationship was observed after 8 and 16
The main objective of this study was to assess the effects of intermittent exposure to AFB\textsubscript{1} on hepatic DNA and RNA adduct formation. Intermittent exposures were chosen to generally imitate human exposure to a food-borne carcinogen. Our main interest was not to study AFB\textsubscript{1} itself but to use it as a model carcinogen whose mechanism of action is well understood. As part of this study, data on food intake (Table 1), body weights, and liver weights (Table 2) were also collected. No significant differences in food consumption were observed between control and treated rats (Table 1). Significant effects on body weights were observed mostly at 1.6 ppm of AFB\textsubscript{1}, after either intermittent or continuous treatments. Rebound effects on body weights were observed when rats were returned to an AFB\textsubscript{1}-free diet during the 8 and 16 weeks of intermittent exposure. Similar rebound effects on body weights have been reported in mice treated intermittently with 20 ppm of vomitoxin for 13 weeks (Banotai \textit{et al.}, 1999). Rats fed AFB\textsubscript{1} intermittently at doses lower than 1.6 ppm showed no major differences in body weights compared to the controls (Table 2). The only exception in our study was at 8 weeks of intermittent treatment with 0.01 ppm of AFB\textsubscript{1}. This effect was unexpected and was likely the result of normal variation in a statistically
small group of animals. Liver weights were reduced at 12 and 20 weeks of intermittent treatment and after 16 weeks of continuous exposure with 1.6 ppm of AFB1 (Table 2). However, when the data was analyzed using the body weights as a co-variable, the differences between treated and control rats were not statistically significant. Roebuck and Maxuitenko (1994) reported that, after 3 months of continuous treatment of rats with 25 µg/rat of AFB1, the liver size did not statistically differ from that of controls. Their explanation was that initially, during treatment, significant DNA synthesis and hepatocyte regeneration occurred, resulting in a normal liver size at later stages of treatment.

Our study shows that adducts remained in DNA at significant levels for at least one month after treatment (Fig. 4), thus confirming the persistence of the DNA lesion caused by AFB1 (Croy and Wogan, 1981; Kensler et al., 1986). The latter investigators reported significant levels of AFB1-N'-FAPyr1 adducts in rat liver 3 months after treatment with 0.25 mg/kg of AFB1, for 2 weeks. Although chromatographically we were not able to fully separate the FAPyr1 derivative from the AFB1-N'-Gua, it is highly likely that the FAPyr1 derivative was the most persistent adduct observed in our study. Peaks corresponding to the minor FAPyr2 adduct were not detected in the AFB1-treated DNA, despite the fact that the FAPyr2 standard was easily resolved from the AFB1-N'-Gua and the major FAPyr1 standards (Sotomayor et al., 1999). The persistence of the major FAPyr1 adduct in DNA may play an important role in the initiation of liver carcinogenesis (Croy and Wogan, 1981). However, other investigators have disputed this assertion, suggesting that initial DNA adduct levels, rather than adduct persistence, correlate with differential tumor response (Bailey, 1994).

The temporal patterns of DNA and RNA adduct formation were characterized by increases and decreases in adduct levels (Figs. 4 and 5), depending on the cycles of dosing and rest. Although no chronic or sub-chronic intermittent exposure studies have been published, other investigators have found similar effects on the levels of AFB1-DNA after a short interruption and resumption of the treatment (Kensler et al., 1986; Schrager et al., 1990). However, only minor elevations of the DNA adducts were observed after resumption of dosing. In our case, the increase in binding was substantial, especially after 0.4 and 1.6 ppm of AFB1.

The temporal pattern of AFB1-RNA adduct formation (Fig. 5) was qualitatively similar to that of DNA. However, two major differences were found. After 8 and 16 weeks of intermittent treatment, some rats showed extremely low levels of AFB1-RNA adducts (Fig. 5, 0.4 and 1.6 ppm) while others had reduced levels comparable to those of DNA. RNA degradation (Heydrick et al., 1991; Lardeux et al., 1987) and spontaneous adduct hydrolysis most likely contributed to the variation in the low adduct levels observed. The other difference is that the extent of AFB1 binding to RNA was much greater than that to DNA. Depending on the dose, binding to RNA was 3 to 9 times greater than that of DNA on a pmol/mg basis. These results indicate that AFB1-RNA binding, as a biomarker of exposure, is more sensitive than DNA binding, confirming studies by others (Appleton et al., 1982; Heinonen et al., 1996). The latter authors showed that AFB1-RNA binding was 3.5 times greater than DNA binding in rat liver slices and 7 to 28 times greater than DNA binding in human liver slices. Although little is known about the biological significance of RNA adduct formation, some early studies indicated that feeding a diet containing 1 ppm of AFB1 to Fisher rats resulted in defects during the processing of rRNA precursors (Smith et al., 1977). Alteration of the processing of rRNA precursors resulted in an increase of double-stranded RNA regions in cytoplasmic RNA (Clawson and Smuckler, 1982). The significance of these early findings is unclear and little or no further research has been published in this intriguing area.

It is well documented that glutathione S-transferase (GST) plays a major role in detoxification of AFB1 through conjugation of AFB1 active metabolites with glutathione (GSH) (Degen and Neumann, 1978, 1981; Guengerich et al., 1996, 1998). Studies from our laboratory show that the hepatic GST activity increased significantly after intermittent or continuous exposures to AFB1, depending on the dose and the length of exposure (Sahu et al., 2000). Hepatic GST activity remained high even after 4 weeks of no dosing, during which time the adducts were reduced. Unfortunately, we did not measure hepatic GSH levels simultaneously with the GST activity to determine the correlation between AFB1-GSH conjugation and DNA and RNA adduct formation. However, our results with GST are in agreement with findings by other investigators regarding the importance of GST in modulating AFB1-DNA and -RNA binding (Guengerich et al., 1996, 1998; Kensler et al., 1986; Raj et al., 1984).

Our main interest was to determine possible dose-response relationships between adduct formation and dose of AFB1, using an intermittent dosing regime. Linear dose responses for AFB1 binding to DNA and RNA have been reported after acute exposures to AFB1 using a wide dose range, including low dosages similar to those of human exposure (Appleton et al., 1982; Wild et al., 1986; see Choy, 1993, for review). Continuous, sub-chronic exposure studies have also reported linear AFB1-DNA binding curves (Buss et al., 1990; Phillips et al., 1999). Our dose-response data suggest that AFB1-DNA adducts increased linearly with the dose after intermittent exposure (Fig. 7). Although the cycles of dosing and rest influenced the linearity of the response, it is clear that the persistence of AFB1-DNA adducts caused them to accumulate during intermittent exposure. Linear dose responses were also observed for RNA adducts after 4 weeks of continuous exposure and after 12 and 20 weeks of intermittent exposure (Fig. 8). However, the dose response curves for 8 and 16 weeks were clearly nonlinear. As discussed before, in some cases, RNA degradation might have caused the loss of adducts during the no-dosing cycles and affected the dose response.


Sahu, S. C., Chou, M. W., Sotomayor, R. E., Hinton, D. M., Barton, C. N., and

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REFERENCES


Clawson, G. A., and Smuckler, E. A. (1982). Increased amounts of double-doses. Thus, our data on the dose-response relationship for which few tumors and low levels of adducts are produced at all doses. Thus, our data on the dose-response relationship for DNA adducts suggest that intermittent exposure to AFB1 may result in fewer liver tumors when compared to continuous exposure.

INTERMITTENT EXPOSURE TO AFLATOXIN B1 AND ADDUCT FORMATION

Betchel (1989) reported that hepatic cancer risk was linearly and quantitatively related with hepatic AFB1-DNA adduct formation in the rat. This relationship is based on continuous exposure to AFB1. Our data suggest a linear relationship between intermittent dosing with AFB1 and DNA adduct formation. The shallowness of the linear curves is suggestive of the D curves or low-dose-response curves coined by Poirier and Beland (1994). This type of relationship describes situations in which few tumors and low levels of adducts are produced at all doses. Thus, our data on the dose-response relationship for DNA adducts suggest that intermittent exposure to AFB1 may result in fewer liver tumors when compared to continuous exposure.


