Liver DNA adducts in methyl-deficient rats administered a single dose of aflatoxin B1

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Using an 8 week Solt—Farber protocol with selection pressure (2-acetylaminofluorene/partial hepatectomy) applied during weeks 6 and 7, we have observed that a single oral administration of aflatoxin B1 (AFB1) to Fischer 344 rats on day 1 of the study, followed by a 3 week feeding regimen of either a methyl-deficient (CMD) or a basal (CMS) diet, results in a relative increase in hepatic preneoplastic lesions in CMD diet fed rats. It has previously been shown that a multiple dosing regimen with AFB1 started after 3 weeks of CMD diet, enhances tumor incidence. In the present study, the role of metabolic activation in the induction of preneoplastic lesions, and liver DNA adduct levels after the first dose of AFB1 in the tumorigenesis model have been investigated. AFB1—DNA adducts were determined at 2–168 h following a single non-necrogenic (100 μg/kg body wt) or necrogenic (600 μg/kg body wt) dose of AFB1 on day 1 or day 21 of a 3 week treatment with a complete basal or CMD diet. In all rats irrespective of dose, dietary treatment or time of AFB1 dosing, the patterns of adduct formation and repair did not change. In rats receiving AFB1 on day 1, total DNA adduct levels between the diet or dose groups were not significantly different, and quantitatively did not correlate with the observed increase in preneoplastic lesions, suggesting a contribution by additional factors in the initiation of these lesions. Administration of AFB1 on day 21, however, resulted in significantly reduced levels of total adducts at both dose levels in CMD diet fed rats compared to controls. Serum biochemistry data suggest that a prolonged exposure to CMD diet may cause pathological and/or biochemical alterations in hepatocytes with a resultant decrease in metabolic activation of AFB1, thus making it difficult to evaluate whether DNA damage is directly related to tumorigenesis.

Introduction

Among the many dietary components that modulate carcinogenesis (1–3), methyl group (methionine and choline) deficiency per se has recently been shown to be hepatocarcinogenic in male Fischer 344 rats (4,5). Earlier investigations have also indicated a diet, marginally methyl-deficient and high in fat, to enhance chemical carcinogenesis (6), and in particular, hepatocarcinogenesis by aflatoxin B1 (AFB1) or N-2-fluorenylacetamide (7) in rats. In addition, sequential administration of a choline-deficient diet during initiation (8) or promotion (9) increases the formation of enzyme-altered foci, and their subsequent progression to hepatocellular carcinomas (10) in rat livers initiated with carcinogens.

AFB1, a mycotoxin produced by certain strains of Aspergillus flavus, and a potent hepatotoxin and carcinogen in rats, has been implicated in the causation of human liver cancer in some tropical areas where food contamination by AFB1 exists (11). While the etiology of human liver cancer may be multifactorial (12), modification of AFB1 carcinogenesis by a methyl-deficient diet may be relevant to the human situation since protein malnutrition may coexist in populations with high incidence of liver cancer (13,14).

A number of studies have shown a multiple-dosing regimen as a requirement for tumor induction by AFB1 in rats of the Wistar, Fischer and Sprague–Dawley strains (15), though in a recent study, partial hepatectomy followed by a single dose of AFB1 induced hepatocellular carcinomas and neoplastic nodules in rats of the AS2 strain (16). Using an 8 week Solt—Farber protocol (8), we have observed that a single oral administration of AFB1 to Fischer 344 rats, followed by a 3 week feeding regimen of a methyl-deficient, amino acid-defined diet, results in an increase, compared to basal diet fed rats, in γ-glutamyl transpeptidase-positive and placent gluthatione-S-transferase-positive preneoplastic lesions when selection pressure with 2-acetylaminofluorene and partial hepatectomy are applied during weeks 6 and 7 of the study (R.Mehta et al., unpublished data). Metabolic activation of AFB1 to AFB1-8,9-oxide, which binds covalently to macromolecules, appears necessary for the potent mutagenic effects of AFB1 (17), and may represent an early and a requisite event for preneoplasia or neoplastic transformation (18). Schrager and colleagues (19), using a multiple-dose protocol, have recently shown increased levels of AFB1—DNA adducts in choline-deficient rats, suggesting that DNA damage may be related to tumor outcome. In the present study, we have investigated the role of metabolic activation in the induction of preneoplastic lesions following exposure to a methyl-deficient diet and a single dose of AFB1. In addition, using a tumor-inducing treatment schedule similar to that of Rogers et al. (7), AFB1—DNA levels after the first dose of AFB1 have been determined.

Materials and methods

Chemicals

AFB1 (from A. flavus; >98% pure; Calbiochem, San Diego, CA), [3H(HG)]-AFB1 (15–30 Ci/mmol; Moravek Biochemicals Inc., Brea, CA) and DMSO (BDH Inc., Toronto, Ontario) were purchased. The following were obtained from Sigma Chemical Co. (St Louis, MO): choline chloride, dl-methionine, folic acid, vitamin B12, dl-homocysteine, ribonuclease A (bovine pancreas, type XI-A), protease (Streptomyces griseus, type XIV), calf thymus DNA (type I), calf liver RNA (type IV), diphenylamine and orcinol. All other reagents were from Fisher Scientific Co. (Fairlawn, NJ). Authentic 8,9-dihydrodiol-8-(N4-guanyl)-9-hydroxy aflatoxin B1 (AFB1-GUA) and the two 8APY isomers were synthesized using 3-chloroperbenzoic acid (Aldrich Chemical Co. Inc., Milwaukee, WI) as a chemical catalyst, essentially as described by Garner et al. (20) and Hertzog et al., (21). The synthesized isomers were purified using chromatographic procedures described below, and identified by comparing UV spectra (22) and HPLC elution.
times with small amounts of authentic standards kindly provided by Dr R.C. Garner (University of York, UK).

**Diet**

The amino acid-defined diet used in this study has been described previously (4). The methyl-free powdered diet was prepared by Teklad Test Diets (Madison, W1; Teklad Diet no. TD 85276) and consists of a methionine-free amino acid mix, dextrose, sucrose, corn oil, mineral mix (Rogers-Harper, Teklad no. 170760), and a vitamin mix (Teklad no. TD 76283) free of choline, folic acid and vitamin B<sub>12</sub>. The nutritionally complete basal (CMS) diet was prepared in the laboratory by adding dL-methionine (5 g/kg diet), choline chloride (2 g/kg diet), folic acid (5 mg/kg diet) and vitamin B<sub>12</sub> (50 µg/kg diet) to the methyl-free diet (TD 85276). The choline/methionine-deficient (CMD) diet also mixed in the laboratory, consists of the methyl-free diet (TD 85276), dL-homocysteine (9 g/kg diet; added to the same molar concentration as methionine and choline in the complete basal diet), folic acid (5 mg/kg diet) and vitamin B<sub>12</sub> (50 µg/kg diet).

**Animals and treatments**

Male Fischer 344 rats (95–140 g; Charles River Canada Inc., Montreal) were housed individually in plastic cages under conditions meeting the requirements of the Canadian Council for Animal Care. Food and water were provided ad libitum. Rats were acclimatized upon arrival by feeding the CMS diet for 5–7 days. They were then randomly distributed to different groups and received the CMS or CMD diets for up to 3 weeks (Figure 1). On day 1 (study I) or day 21 (study II) of dietary treatment, rats received a single, oral dose of either [3H]AFB<sub>1</sub> (100–600 µg/kg body wt; 5–20 µCi/100 g body wt) in DMSO (0.1 ml/100 g body wt), or DMSO alone. Following AFB<sub>1</sub> treatment, rats in study I continued to receive their respective diets, whereas all rats in study II were returned to and maintained on the CMS diet until sacrifice. The rats were killed by ether anaesthesia, and blood was withdrawn from the inferior vena cava into serum separation tubes (Becton-Dickinson, Lincoln Park, NJ) and livers excised. Blood was allowed to clot at 4°C for a minimum of 30 min and then centrifuged at 1000 g for 10 min. Serum was collected and stored at −20°C until further analysis. Livers were stored at −70°C until further processing.

**Isolation of DNA**

Each frozen liver was thawed and homogenized in 2 vol of buffer (10 mM Tris–HCl, 0.25 M sucrose, 2 mM CaCl<sub>2</sub>, pH 7.5), and a crude nuclear pellet was prepared by the method of Hymer and Kruft (23). Liver DNA was then extracted using a modification (22) of the Marmur procedure (24). The DNA sample was then re-extracted twice with chloroform/isoamyl alcohol (24:1, v/v), and the DNA precipitated from the aqueous layer with 3 vol of cold (−4°C) absolute ethanol. The DNA was dried in vacuo and stored dry at −70°C until further analysis. This method provided purified DNA with <5% protein and <10% RNA contamination as determined by the Lowry method according to Munro and Fleck (25), and the orcinol procedure (26), respectively. The DNA purity was also assessed by the absorption ratios A<sub>260</sub>萱/A<sub>280</sub>萱 (1.65–1.85) and A<sub>260</sub>萱/A<sub>230</sub>萱 (>2.2). DNA content was determined by the diphenylamine reaction (27) and by UV absorbance at 260 nm.

**Analysis of DNA adducts**

DNA was dissolved in buffer (15 mM NaCl, 1.5 mM trisodium citrate, pH 7.0), adjusted to pH 1.0 with 0.1 N HCl and hydrolysed at 70°C for 20 min. The sample was cooled on ice, neutralized, made 5% in methanol, and loaded on a methanol-prewashed C-18 Sep-Pak cartridge (Waters/Millipore Corp., Milford, MA). The column was then washed with 5% methanol to remove unhydrolysed DNA, and eluted with 80% methanol to release the lypophilic aflatoxin adducts. After concentration, AFB<sub>1</sub> adducts were separated by HPLC on a 25 cm Partisil 5 ODS-3 column (Whatman International Ltd, Maidstone, UK), using a Perkin-Elmer Series 4 liquid chromatograph (Perkin-Elmer Corporation, Oak Brook, IL), and a 45 min, linear gradient of 15–68% methanol/water at a flow rate of 1.0 ml/min. The eluate was monitored by UV detection at 360 nm, and collected in 1 ml aliquots for quantification by scintillation counting using Atolchrome<sup>®</sup> (NEN Research Products, Du Pont Canada Inc., Mississauga, Ontario). Standards were run in the scintillant, and an LKB/Wallac Rackbeta Liquid Scintillation Counter (Wallac Oy, Turku, Finland). AFB<sub>1</sub> adducts were identified by comparison with authentic standards. Typically, the elution times were 26, 28 and 32 min for FAPY<sub>萱</sub>, FAPY<sub>萱</sub> and FAPY-GUA respectively.

**Serum analysis**

Serum glutamyl oxalo-transaminase (SGOT; EC 2.6.1.1) and serum glutamyl pyruvic transaminase (SGPT; EC 2.6.1.2) were assayed on an Astra 8 (Beckman Instruments (Canada) Inc., Mississauga, Ontario) using Beckman reagent kits.

**Statistical analysis**

The data for study I were collected from a single experiment, and were analysed using ANOVA with all main effects and interactions involving diet, dose and time. The data were first screened to determine an appropriate scale of analysis; the ANOVA residuals were tested for non-normality (28) and a test for outliers was performed (29). If the raw scale was inappropriate, the log or logit [log<sub>10</sub>(x + 1)] scale was used. SGOT, SGPT, total adducts and the amounts (pmol/mg DNA) of AFB-GUA, FAPY<sub>萱</sub> and FAPY<sub>萱</sub> were analysed on the log scale, whereas the proportions of different adducts, expressed as a percentage of total adducts, were analysed on the logit scale. Outliers were excluded from all analyses. Pairwise comparisons were carried out for significant main effects, and for simple effects when interactions were significant in the ANOVA model. The data for study II were analysed in the same manner as study I. However, the data from two separate experiments were analysed in three parts: the main effects (and all interactions) used in the ANOVA model for each part were as follows: (i) experiment 1, diet and time; (ii) experiment 2, diet, dose and time; and (iii) experiments 1 and 2, diet, experiment and time. P-values of 0.05 and less are considered significant.

**Results**

**Effect of diet on serum enzyme levels**

Stages in carcinogenesis, including induction of preneoplastic lesions (30) and tumors (31), can be influenced by compensatory cell proliferation in response to cellular damage by a chemical carcinogen. Liver toxicity following dietary and AFB<sub>1</sub> treatment has therefore been studied in order to determine (i) a dose of AFB<sub>1</sub> that causes no necrosis in hepatocytes, and (ii) whether toxicity due to CMD diet and/or AFB<sub>1</sub> affects DNA adduct formation and repair. Figure 2 illustrates the dose response of the effect of AFB<sub>1</sub> and diet on SGOT with respect to time after AFB<sub>1</sub> administration. In study I (Figure 2), significantly higher (P < 0.0001) levels of SGOT were observed in 24, 48 and 72 h after 600 µg/kg AFB<sub>1</sub> compared to the 100 µg/kg dose. Toxicity due to CMD diet (Figure 2, study I) as indicated by higher SGOT was significant (P < 0.0001) only after 168 h after AFB<sub>1</sub>. In study II (Figure 2), a significant dose–response relationship (ANOVA F-test; P < 0.0001) was observed at each time point for rats fed the CMS diet, but not those fed the CMD diet. In CMD rats, SGOT levels were higher than CMS-fed DMSO.
controls at all doses (including 0 μg/kg) of AFB₁. In CMS rats, the mean levels of SGOT for rats receiving at least 250 μg/kg AFB₁ were significantly higher (P < 0.05, t-test) than for 0 or 100 μg/kg AFB₁ dose showing that 100 μg/kg AFB₁ causes no liver necrosis over a 72 h period after administration. These data were confirmed by similar results with SGPT (data not shown), and by pathological examination of liver sections (manuscript in preparation). Therefore, in subsequent studies, 100 and 600 μg/kg AFB₁ have been compared as non-necrogenic and necrogenic dose levels respectively.

DNA adduct levels as a function of time, dose and diet

DNA adduct levels have been determined over a 2–168 h period using two doses of AFB₁ and two different experimental protocols. Study I was conducted in a single experiment. Study II consisted of three experiments: experiment 1, rats from both diet groups dosed with 100 μg/kg AFB₁ and killed at 2–72 h; experiment 2, rats from both diet groups dosed with 100 or 600 μg/kg AFB₁ and killed at 24, 48 and 72 h; and experiment 3, rats from both diet and dose groups killed at 2 and 6 h. For simplification, combined data from these experiments are presented. For statistical analysis, experiments 1 and 2, which consisted of the most data points, were first analysed separately and then combined to compare the common parts.

In study I, when [3H]AFB₁ was administered on day 1 at the start of CMS or CMD diet treatment (Figure 1), total DNA binding (Figure 3A) did not differ significantly between CMS and CMD diet groups for either 100 or 600 μg/kg AFB₁ at any time after AFB₁ administration. In study II, where [3H]AFB₁ was administered on day 21 at the end of CMS or CMD diet treatment (Figure 1), the effect of diet at 100 or 600 μg/kg AFB₁ was significant for total DNA binding, though at 600 μg/kg AFB₁, the differences between diet groups decreased over time (Figure 3B). In constrast to study I (Figure 3A) therefore, total DNA binding in study II (Figure 3B) was significantly lower in CMD rats at both AFB₁ dose levels. Significant (P < 0.001) dose group differences, however, were observed in both studies I and II, lower amounts of total binding being present at the lower dose of 100 μg/kg AFB₁ (Figure 3A and B).

In study I (data not shown) and study II (Figure 4), the formation of AFB-GUA, FAPY₆ and FAPY₉, and their repair over time followed a similar pattern in both CMS and CMD diet groups. In study I, no overall significant differences in the levels of different adducts between diet groups were observed either at 100 or 600 μg/kg AFB₁; however, as expected, significantly

![Fig. 2. Time course and dose response of the effect of diet and AFB₁ treatment on rat SGOT. Sera were obtained from rats treated according to study I and II protocols shown in Figure 1 and analysed for SGOT. Data not available for 2 and 168 h in study II. Columns, mean of data from either 3 rats (study II) or 3-6 rats (study II); bars, SD. *Significantly different (P < 0.05, t-test) from 0 or 100 μg/kg body wt AFB₁ at the corresponding time interval and within each diet group. # Significantly different (P < 0.05, t-test) from CMS diet groups at the corresponding time interval and dose group.](https://academic.oup.com/carcin/article-abstract/13/7/1241/305874)

![Fig. 3. Levels of total AFB₁-DNA adducts in livers of rats fed CMS (open symbols) and CMD (closed symbols) diets at various times after a single administration of 100 (○, ●) and 600 (▲, △) μg/kg body wt AFB₁ in (A) study I and (B) study II. Each point represents the mean ± SE of data collected from either 3 rats in a single experiment (study I), or 2–7 rats in three different experiments (study II). Asterisks indicate significant differences (t-test) between CMS and CMD groups within the same dose group and same study at each time interval; *P ≤ 0.0001, **P = 0.0002, ***P ≤ 0.05.](https://academic.oup.com/carcin/article-abstract/13/7/1241/305874)

![Fig. 4. Study II: formation and repair of AFG-GUA (○, ●), FAPY₆ (□, ■) and FAPY₉ (▲, △) in rats fed CMS (open symbols) and CMD (closed symbols) diets and administered a single dose of either (A) 100 or (B) 600 μg/kg body wt [3H]AFB₁. Each point represents the mean ± SE of data collected from 2–7 rats in three different experiments. Asterisks indicate significant differences (t-test) between CMS and CMD groups within the same dose group at each time-point; *P ≤ 0.0001, **P ≤ 0.05.](https://academic.oup.com/carcin/article-abstract/13/7/1241/305874)
higher \( P < 0.001 \) amounts of all adducts were present at the higher dose of AFB\(_1\). For example, in study I at the 24 h time interval, the average amounts of AFB-GUA adduct present at 100 \( \mu \)g/kg dose were 0.60 ± 0.09 (mean ± SD, \( n = 3 \) rats) and 1.19 ± 0.14 pmol/mg DNA in CMS and CMD rats respectively, whereas at 600 \( \mu \)g/kg AFB\(_1\) the corresponding values were 4.73 ± 2.08 and 6.98 ± 1.55 pmol/mg DNA. In contrast, the amounts of different adducts in study II were significantly lower in CMD rats compared to CMS rats at both 100 \( \mu \)g/kg (Figure 4A) and 600 \( \mu \)g/kg (Figure 4B) AFB\(_1\). Significant dose group differences \( P < 0.0001 \) were observed for all variables, the effect of dose being consistent over time in both diet groups (Figure 4A and B). The absolute amounts of AFB-GUA, FAPY\(_a\) and FAPY\(_b\) were observed to increase over 2 - 24 h and then gradually disappear over a 72 h period. Both FAPY\(_a\) and FAPY\(_b\) were repaired at a slower rate compared to AFB-GUA (Figure 4). Such similar patterns in repair rates were also observed when comparing the relative proportions of these three adducts over time in both study I (Figure 5A) and study II (Figure 5B). Thus, FAPY\(_a\) and

![Fig. 5. Effect of CMS (□) and CMD (●) diets on relative proportions of AFB-GUA, FAPY\(_a\), and FAPY\(_b\) expressed as a percentage of total adducts in hydrolysed DNA in (A) study I and (B) study II. Columns, means of data from 3 rats (study I) or 2 - 7 rats (study II); bars, SE. Within each study, columns with same letters are significantly different from each other (\( t \)-test, \( P \leq 0.05 \)). *Below the detection limit of 0.02 pmol of adduct. ND, not determined.

### Table I. Effect of diet on body weight, liver weight, DNA content and \([\text{H}]\text{AFB}_1\) activation in rat liver

| Study | AFB\(_1\) dose (\( \mu \)g/kg body wt) | Diet | Body wt at dosing*(g) | Liver wt*(g) | DNA content\( ^b \) (mg/liver) | Total AFB\(_1\)-DNA adducts\( ^b \) expressed as:
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<td></td>
<td></td>
<td></td>
<td>pmol/mg DNA</td>
</tr>
<tr>
<td>I</td>
<td>100</td>
<td>CMS</td>
<td>142.0 ± 2.6 (15)</td>
<td>6.17 ± 0.13 (15)</td>
<td>12.16 ± 1.07 (15)</td>
<td>4.65 ± 1.17 (3)</td>
</tr>
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<td></td>
<td></td>
<td>CMD</td>
<td>135.0 ± 2.2 (15)</td>
<td>5.79 ± 0.27 (15)</td>
<td>11.27 ± 0.72 (15)</td>
<td>7.07 ± 3.92 (3)</td>
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<td>600</td>
<td>CMS</td>
<td>136.0 ± 1.7 (15)</td>
<td>4.86 ± 0.20 (15)</td>
<td>17.86 ± 1.67 (15)</td>
<td>29.45 ± 11.52 (3)</td>
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<td>CMD</td>
<td>131.3 ± 2.2 (15)</td>
<td>4.60 ± 0.16 (15)</td>
<td>10.31 ± 0.72* (15)</td>
<td>27.78 ± 0.23 (3)</td>
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<tr>
<td>II</td>
<td>100</td>
<td>CMS</td>
<td>221.0 ± 2.5 (25)</td>
<td>8.08 ± 0.32 (25)</td>
<td>9.02 ± 0.61 (25)</td>
<td>15.18 ± 3.02 (3)</td>
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<td></td>
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<td>CMD</td>
<td>159.0 ± 1.6* (24)</td>
<td>10.17 ± 0.28* (24)</td>
<td>5.66 ± 0.51* (24)</td>
<td>2.13 ± 1.02* (3)</td>
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<td></td>
<td>600</td>
<td>CMS</td>
<td>212.0 ± 2.7 (15)</td>
<td>7.60 ± 0.30 (3)</td>
<td>13.71 ± 0.58 (3)</td>
<td>89.61 ± 33.86 (3)</td>
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<td></td>
<td></td>
<td>CMD</td>
<td>157.0 ± 1.9* (15)</td>
<td>11.09 ± 0.43* (3)</td>
<td>4.21 ± 0.45* (3)</td>
<td>20.40 ± 3.46* (3)</td>
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*Presented as means ± SE from data pooled from all time intervals within each diet and dose group.
\(^b\)As an example, total DNA adducts data only at the 2 h time interval is presented, means ± SE.
\(^c\)Number in brackets is the number of rats.
\(^d\)Liver weights were unavailable in all rats.
\(^e\)Significantly different from the corresponding CMS diet group \( t \)-test, \( P < 0.01 \).
FAPY₃, which are decomposition products of AFB-GUA (32), occurred in greater proportions at later times. While significant differences in relative proportions of adducts between dose and diet groups were observed in some cases (Figure 5), the pattern is inconsistent.

**Effect of diet on body weight, liver weight and liver DNA content**

In study I, body weights, liver weights and liver DNA content were similar among rats in either CMS or CMD diet groups (Table I). In study II, a longer exposure period to CMD diet resulted in significantly reduced body weights and liver DNA content, and significantly increased liver weights compared to rats fed the CMS diet (Table I). CMD rats in study II had lower levels of overall DNA adduction with AFB₁ regardless of diet-induced alteration in target organ size. This is shown by a comparison of total DNA binding expressed as either pmol/mg DNA or pmol/whole liver. As an example, total DNA adduct data only at the 2 h time interval are presented (Table I).

**Discussion**

Tumor induction by AFB₁ alone, or in combination with diet appears to require a treatment regimen of small repeated doses of AFB₁ (15). However, we have recently observed that in Fischer 344 rats, a single non-necrogenic (100 µg/kg body wt) or necrogenic (600 µg/kg body wt) dose of AFB₁, followed by a 3 week feeding regimen of CMD diet in a Solt—Farber protocol (8), results in a 97—99% increase in enzyme-altered neoplastic liver foci in the CMD diet group compared to CMS diet fed rats. (R.Mehta et al., unpublished data). The primary objective of the present study was to determine if AFB₁ interaction with hepatocellular DNA plays a role in initiation of hepatic neoplastic lesions. Study I, where AFB₁ was administered on day 1 of the dietary treatment, corresponds to the protocol used for the induction of neoplastic foci. In study I, formation and repair of AFB₁—DNA adducts was followed over a 1 week period after [³H]AFB₁ administration. The patterns of adduct formation and repair were similar to those reported previously (32,33). In general, no differences have been observed in the amount of total DNA binding, or the extent of formation and repair of the major AFB₁ adduct or its decomposition products between the CMS and CMD diet groups at both dose levels of AFB₁ studied. It would appear that metabolic activation of AFB₁ is necessary for the induction of neoplastic lesions as we have observed virtually no liver foci in CMD rats administered DMSO compared to a 99% increase in CMD rats dosed with AFB₁ (unpublished observations). However, as our data from study I indicate, any contribution by AFB₁ is not reflected by qualitative or quantitative differences in DNA adduct levels between CMS and CMD diet groups. In contrast, a positive relationship between increased AFB₁—DNA adduct levels and a higher tumor incidence has been reported when Fischer rats are given multiple doses of AFB₁ and continuously fed the CMD diet (19). It is difficult to evaluate the relationship between AFB₁ activation and tumor incidence in our single-dose protocol until it is known whether this treatment protocol can also progress into tumors. This is because while enzyme-altered hepatic lesions, which represent populations of 'initiated' cells with altered gene expression (34), have been demonstrated to be one site of origin for liver cell cancer (35,36), these lesions may not definitely predict tumor outcome since only a limited number progress into tumors (37—39).

Study II was carried out to determine the effect of CMD diet on AFB₁ activation following the first dose of AFB₁ in a multi-dose treatment protocol for tumorigenesis used by Rogers et al. (7). Following 3 weeks of feeding with CMD diet, these investigators administered 15 µg/day AFB₁ for 3—5 days a week for 7 weeks. The respective dietary treatments were continued throughout the study, which lasted for a total of 90 weeks. At the end of this study, a higher tumor incidence in CMD rats compared to CMS rats was observed. Rogers et al. (7) used Fischer rats with an initial body weight of 40—50 g. Assuming normal growth during the next 3 weeks of dietary treatment, the body weight at the time of first dose of AFB₁ would have been ~125 g, suggesting that an AFB₁ dose of 15 µg/rat/day corresponds to 120 µg/kg body wt/day, which approximates to the non-necrogenic (100 µg/kg body wt) dose of AFB₁ used in the present studies. In study II, the patterns of adduction formation and repair were unchanged between the two diet or dose groups and were similar to those observed in study I. However, we observed lower levels of total binding of AFB₁ to DNA, and lesser amounts of the different adducts in CMD diet fed rats compared to the CMS rats at both 100 and 600 µg/kg AFB₁ and for up to a 72 h period following AFB₁ administration. These data are in agreement with the reported decreased capacity of microsomal fractions from CMD rats to activate AFB₁ to mutagen(s) in vitro (40) as well as decreased cytochromes P450 and b₅, contents, reduced microsomal mixed-function oxidase activities and lower AFB₁ covalent binding to DNA and RNA in CMD rats compared to CMS rats (41—44). If metabolic activation of AFB₁ correlates positively with tumor induction, these findings from study II and other investigators are unexpected since CMD rats show an increased sensitivity to AFB₁ carcinogenesis. In a similar schedule of treatments, however, enhanced AFB₁—DNA interaction in response to CMD diet was observed only after the second and subsequent doses of AFB₁ (19). Following a single administration of AFB₁, these authors (19) reported no significant differences in total adducts between control and deficient rats. A number of experimental variables such as differences in methyl-deficient diets, strain and age of rats, and route of AFB₁ administration exist between study II and the previous study (19). Thus, Schrager et al. (19) used 40—50 g Sprague-Dawley rats dosed i.p. with AFB₁ and fed a choline/methionine-free diet according to Rogers et al. (7). Further, unlike these investigators (19), we discontinued the CMD diet treatment following AFB₁ administration in order for study II to parallel with other studies conducted in our laboratory. Therefore, the discrepancy between our DNA binding data and that of Schrager et al. (19) could be due to variations in experimental protocols, as, for example, age- and strain-related differences in phase I and phase II drug-metabolizing enzymes, and/or differences in rates of enzymatic excision or spontaneous depurination of AFB₁—DNA adducts (21,32,44—46).

Administration of a CMD diet alone to rats results in fatty infiltration of liver cells with focal necrosis over the first 4 weeks, followed by fibrosis, regeneration, cirrhosis and ultimately carcinoma when the diet is fed for 8 months or longer (47). Decreased metabolic activation of AFB₁ could be due to either changes in liver phospholipids during CMD diet induced hepatic toxicity (41) and/or selective decreases in certain isozymes of cytochrome P450 (48,49). That liver toxicity may play a role is reflected by our serum biochemistry data (Figure 2). Thus, in study I, similar levels of AFB₁—DNA binding correlate with near-control values of SGOT in CMD rats at the non-necrogenic dose of AFB₁, for up to 72 h following CMD diet and AFB₁ treatments. However, in study II, toxicity due to 3 week CMD diet treatment is apparent even in the DMSO-treated rats at the earliest (24 h) time-point analysed.

In study II, but not study I, CMD diet induced toxicity after
3 weeks was also manifested by reduced body weights, increased liver weights and a lower DNA content in CMD rats (Table I). Despite these alterations in the target organ size, CMD rats in study II show lower amounts of total AFB1-DNA adducts whether they are expressed as pmol/mg of liver DNA or as pmol/whole liver. Preliminary experiments indicated that the lower body weights in CMD rats are due to a reduction in food consumption; however, rats pair-fed with the CMS diet at the same level of food consumption as CMD rats showed a similar toxic response to AFB1 as CMS rats fed ad libitum as determined by serum biochemistry and pathological examination. Therefore, all subsequent comparisons have been carried out between CMD rats and CMS rats fed ad libitum. The increase in liver weight is probably associated with extensive triglyceride accumulation rather than increase in cell number since the initial response to CMD diet over the first 2–4 weeks appears to be liver cell death with cell proliferation occurring at a sufficient rate to compensate for cell loss (50,51). In fact increases in DNA content, which may signify changes in cellular ploidy and/or cell number, were not detectable until hepatocyte cell loss declined at around 8 weeks of CMD diet treatment (51). In study II, the lower DNA contents in CMD rats (Table I) could be attributed to continual cell death and/or reduced recoveries of liver DNA if the methods used are not adequate for extracting fragmented, small mol. wt liver DNA from fragile nuclei as would be the case in CMD rats where the dietary treatment is known to cause DNA strand breaks (52). It appears, therefore, that in study II, the lower levels of DNA adducts observed in CMD rats after the first dose of AFB1 is perhaps more indicative of diet-induced liver toxicity, making it difficult to predict the role of metabolic activation during carcinogenesis.

In summary, while metabolic activation of AFB1 seems necessary in the induction of hepatic preneoplastic lesions in CMD rats, the similar AFB1-adduct levels in CMS and CMD rats suggest a contribution by additional factors in the initiation of these lesions. Second, prolonged exposure to CMD diet, as may occur in a carcinogenesis treatment schedule, may lead to pathological and/or biochemical alterations in hepatocytes, which may then interfere with an accurate measurement of DNA adducts. The latter observation suggests a need for caution in the interpretation of biochemical data derived from studies involving the interaction of one or more factors during carcinogenesis, especially when these factors may themselves produce a toxic response.

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References


Liver DNA adducts in methyl-deficient rats


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