Appalachian STATE UNIVERSITY BOONE, NORTH CAROLINA

Dissimilarity in aflatoxin dose-response relationships between DNA adduct formation and development of preneoplastic foci in rat liver

Authors: **Martin Root a**, Theodore Lange, T. Colin Campbell

Abstract Earlier work in this laboratory and that carried out by others demonstrated that after a single dose of aftatoxin B1 (AFB) the resulting liver AFB-DNA adduct levels were directly proportional to dose. Earlier work also showed that after ten daily doses the AFB dose response relationship with y-glutamyl transpeptidase (GGT) positive preneoplastic foci measured at 3 months was sublinear, with a threshold at a dose of about 150 μg/kg body weight/day. The objective of this study is to determine the factors influencing the shift in AFB dose-response between AFB-DNA adducts and GGT foci. Male Fisher 344 weanling rats were orally administered one or ten doses of AFB ranging from 50 to 350 μg/kg body weight/day. The animals were killed 2 or 24 h after the first AFB dose, or after the tenth AFB dose. The first and tenth doses were tritiated in these animals and 3H-AFB-guanine adducts isolated from liver DNA were measured by HPLC. Another group was killed 3 months after receiving ten doses in order to measure GGT foci development. AFB-guanine adduct levels were directly proportional to dose after the first dose, but after the tenth dose were much lower in the 200-350 μg/kg groups than after a single dose. The GGT foci response confirmed earlier work concerning a sublinear response. Among the individual animals in the 200-350 μg/kg groups there was a positive relationship, after controlling for dose, between GGT foci development and weight gained both during dosing (P = 0.018) and also to a lesser extent during the early promotional period (P = 0.066). Enzyme activity levels of GGT in liver homogenates were higher in the highest dose groups and reflected biliary proliferation rather than histological GGT stained foci. Urinary levels of AFB metabolites changed proportions in the high dosage multiply dosed animals reflecting alteration in AFB metabolism or excretion. The differences between the linear adduct and the sublinear foci dose response curves may be the result of non adduct effects of higher multiple AFB doses on foci formation including acute cytotoxicity, altered AFB metabolism and disposition, enhanced weight gains, or shortened foci latency but not through enhanced guanine adduct levels. Other studies that showed a linear relationship between AFB dose and liver tumor development used continuous feeding of maximal doses an order of magnitude less than the lowest dose in this study and thus avoided acutely toxic effects. We hypothesize that liver tumor development may mirror foci response in a IO-dose AFB regimen with doses above 100 μg/kg due to acute toxicity effects

Martin Root a, Theodore Lange, T. Colin Campbell(1997) Dissimilarity in aflatoxin dose-response relationships between DNA adduct formation and development of preneoplastic foci in rat liver. *Chemico-Biological Interactions* (vol.106 pg. 213-227)

Dissimilarity in aflatoxin dose-response relationships between DNA adduct formation and development of preneoplastic foci in rat liver

Martin Root a, Theodore Lange , T. Colin Campbell

Abstract •

Earlier work in this laboratory and that carried out by others demonstrated that after a single dose of aftatoxin B_1 (AFB) the resulting liver AFB-DNA adduct levels were directly proportional to dose. Earlier work also showed that after ten daily doses the AFB dose response relationship with y-glutamyl transpeptidase (GGT) positive preneoplastic foci measured at 3 months was sublinear, with a threshold at a dose of about 150 µg/kg body weight/day. The objective of this study is to determine the factors influencing the shift in AFB dose-response between AFB-DNA adducts and GGT foci. Male Fisher 344 weanling rats were orally administered one or ten doses of AFB ranging from 50 to 350 µg/kg body weight/day. The animals were killed 2 or 24 h after the first AFB dose, or after the tenth AFB dose. The first and tenth doses were tritiated in these animals and ^{3}H-AFB-guanine adducts isolated from liver DNA were measured by HPLC. Another group was killed 3 months after receiving ten doses in order to measure GGT foci development. AFB-guanine adduct levels were directly proportional to dose after the first dose, but after the tenth dose were much lower in the 200-350 µg/kg groups than after a single dose. The GGT foci response confirmed earlier work concerning a sublinear response. Among the individual animals in the 200-350 µg/kg groups there was a positive relationship, after controlling for dose, between GGT foci development and weight gained both during dosing ($P = 0.018$) and also to a lesser extent during the early promotional period ($P = 0.066$). Enzyme activity levels of GGT in liver homogenates were higher in the highest dose groups and reflected biliary proliferation rather than histological GGT stained foci. Urinary levels of AFB metabolites changed proportions in the high dosage multiply dosed animals reflecting alteration in AFB metabolism or excretion. The differences between the linear adduct and the sublinear foci dose response curves may be the result of non adduct effects of higher multiple AFB doses on foci formation including acute cytotoxicity, altered AFB metabolism and disposition, enhanced weight gains, or shortened foci latency but not through enhanced guanine adduct levels. Other studies that showed a linear relationship between AFB dose and liver tumor development used continuous feeding of maximal doses an order of magnitude less than the lowest dose in this study and thus avoided acutely toxic effects. We hypothesize that liver tumor development may mirror foci response in a IO-dose AFB regimen with doses above 100 µg/kg due to acute toxicity effects.

Keywords: Aflatoxin; Carcinogenesis; Cytotoxicity; Dose-response

1. Introduction

Cancer is a multistage disease. The initial insult is generally considered to be alteration of the DNA of a somatic cell. This alteration, if fixed through a cycle of cell division, may then be clonally expanded into a focus of cells of altered phenotype, then to a putatively neoplastic nodule, and finally progressing to tumor formation. Each of these stages is necessary but not sufficient for the subsequent stage.

Aflatoxin B_1 (AFB) is a potent hepatotoxin and hepatocarcinogen for certain experimental animal species [l]. AFB requires activation to the 8,9-epoxide to become carcinogenic. Other metabolic pathways yield primary and secondary metabolites of greatly reduced carcinogenic potency. The balance between activation and metabolism is critical to the process of carcinogenesis. Although AFB is highly carcinogenic, it has been shown that a single high dose produces more liver necrosis and other acutely toxic events while multiple smaller doses produce more tumor response and less liver necrosis and acute toxicity [2].

The formation of AFB-DNA adducts is linearly dose dependent over a wide range of AFB doses and in various experimental models [3-7]. In contrast to these linear dose response curves for AFB-DNA adducts, we have previously observed a sublinear dose-response curve for putatively preneoplastic y-glutamyl transpeptidase (GGT) positive foci 3 months after completion of 2 weeks of AFB dosing [8]. Foci only appear above a dose threshold of about 150 µg/kg body weight/day. Thus, even though there is a linear relationship between a single dose and adducts, there is a sublinear relationship between multiple doses and foci formation.

There are several possible reasons for this disparity. Multiple AFB doses may yield disproportionately higher adduct levels at higher dosages through induction of AFB activating pathways. Multiple higher doses may also give rise to disproportionate levels of non-adduct effects such as increased liver necrosis, lower levels of DNA repair, or other non-genotoxic effects which promote carcinogenesis. Thus, the objective of this study was to determine possible mechanisms for the change in dose response curves between adducts and foci.

2. Methods

2.1. Animals, chemicals, and diet

Male Fisher-344 rats (50-75 g), purchased from Charles River Laboratories (Burlington, MA), were individually housed in suspended stainless steel cages with wire mesh bottoms. Upon receipt they were acclimated for 8 days in a room with controlled temperature, a 12-h photoperiodic cycle, and a relative humidity between
40 and 60%. These conditions were maintained throughout the study. Animals received food and water ad libitum. AFB was purchased from Calbiochem-Behring (La Jolla, CA) and [G-^{3}H]AFB (specific activity 10-20 Ci/mmol) from Moravek Biochemicals (City of Industry, CA). Aflatoxin P_1 (AFP), and aflatoxin M_1 (AFM) were obtained from Sigma (St. Louis, MO). All other chemicals used in this study were of analytical or reagent grade. Purified AIN-76A diet in pelleted form was prepared by Dyets, Inc. (Bethlehem, PA).

2.2. Experimental protocol

The rats were randomly assigned to treatment groups after acclimation (Fig. l). The treatments were planned on the basis of (a) dose of AFB administered (50, 100, 150, 200, 250, and 350 µg/kg body weight/day); (b) time of kill following dosing (2 h, 24 h, or 12 weeks after the last of ten doses), and (c) number of doses (one or ten). Groups for adduct quantification contained five animals each. Groups for GGT foci quantification contained ten animals each. Multiple-dose groups receiving 350 µg/kg body weight/day contained 40% more animals in anticipation of acute mortality. Both radioactive and non-radioactive AFB were prepared in tricaprylin for dosing. Sufficient crystalline AFB for the entire experiment was dissolved in minimal chloroform and diluted in tricaprylin to a concentration of about 450 µg/ml. The chloroform was evaporated from the solution by stirring for 2 days in a fume hood. This stock solution was diluted with additional tricaprylin, and concentrated radioactive AFB in ethanol was added for the labeled doses, to the proper final concentrations such that the animals received tricaprylin at 1.0 ml/kg body weight. The absolute amount of radioactivity added to each labeled dosage was the same so that the specific activity was inversely proportional to the AFB concentration. Intubation commenced 8 days after receipt of the animals. Groups A and B received single doses of tritiated AFB and were sacrificed 2 and
24 h after dosing, respectively. The multiple-dose protocol groups (two adduct groups-groups C and D and one GGT foci group-group E) received ten daily doses over a 12-day period (with no dosing on the 2 middle days). The last dose for groups C and D were radiolabeled and animals were sacrificed 2 and 24 h after the last dose, respectively. Livers in all four adduct groups were immediately removed upon sacrificing and placed in ice-cold buffer (0.05 mol/l Tris-HCl pH 7.0, 0.25 mol/l sucrose, 0.025 mol/l KCl, and 0.005 mol/l $MgCl_{2)}$. Animals in the GGT foci group were sacrificed 12 weeks after the last AFB dose. Two slices of liver were removed for each animal, frozen on dry ice, and stored at – 70°. The remainder of each liver was also stored at -70°. Two animals from each dosage of groups **B** and D were kept in metabolic cages during the overnight period between dosing and sacrifice. Complete 24 h collections of urine were obtained from groups B and D and were frozen at – 70° until further analysis.

2.3. Afiatoxin-DNA adducts

The method of Croy et al. [4] was used for the isolation of liver nuclei, the isolation of nuclear DNA, the hydrolysis of DNA, and the HPLC separation of AFB-DNA adducts. A Beckman model 334 HPLC (Fullerton, CA) with gradient capability and a Perkin-Elmer model LS95 UV-visible detector (Norwalk, CT) was used for adduct separation. The eluent was monitored at 360 nm and adducts were collected in l.0-ml fractions. Scintillation cocktail (10 ml) was added to each fraction which were then counted in a Beckman LS3133T liquid scintillation

FOCI STUDY

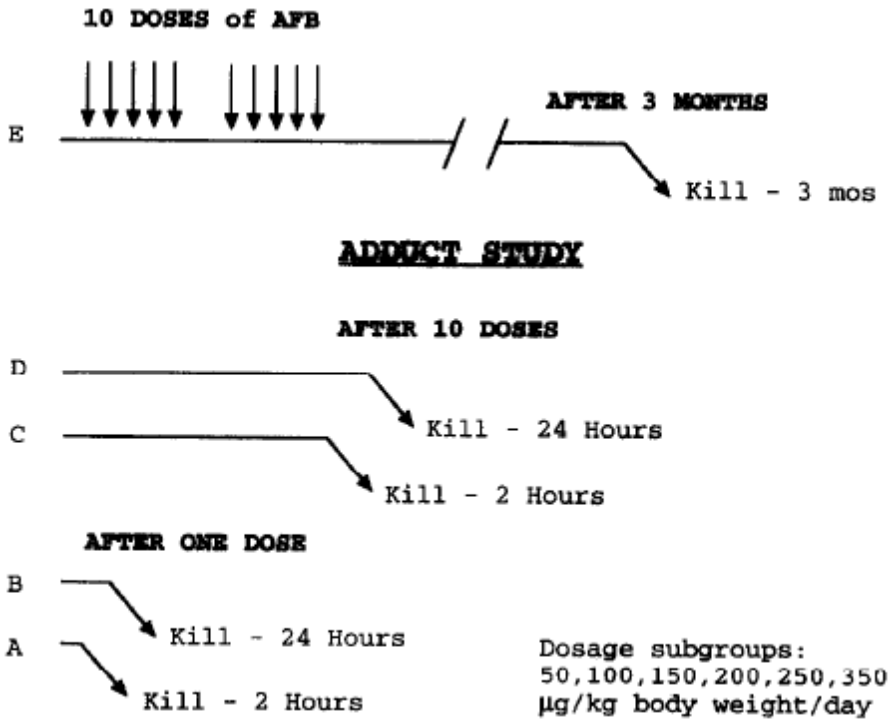

Fig. 1. Experimental protocol.

counter. The AFB-N^7-guanine standard was prepared from an AFB-modified DNA sample according to the method of Groopman et al. [9]. The ring-open AFB-DNA adduct (8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxyaflatoxin B_1) was produced by first generating AFB-DNA according to Essigmann et al. [lO] then isolated by the method of Groopman et al [9]. The levels of the two products were summed. The original intention was to measure these two separately although an uncontrolled and random conversion of N^7-guanine to the open-ring derivative during the DNA isolation and hydrolysis steps prohibited individual quantification [9]. Thus, the summation of both was used as a reliable measure of total AFB-DNA adducts.

2.4. Histochemical GGT positive liver foci

The method of Dunaif and Campbell [8] was used. Four liver sections were prepared and stained for GGT according to the method of Rutenburg et al. [ll]. Foci were counted and average diameters were measured by light microscopy. The fraction of liver occupied by foci (as expressed as parts per million of liver volume) and the number of foci per cubic centimeter were obtained by mathematical models described by Campbell et al. [12] and Nychka et al. [13].

2.5. Biochemical liver homogenate GGT

This method was a combination of the methods of Misslbeck et al. [14] and Cameron et al. [15]. A liver sample was prepared by homogenizing in a Polytron® homogenizer (Brinkman Instruments, Westbury, NY) about 0.5 g of frozen liver and 4 parts 0.1 mol/l Tris-HCI pH 8.0 (at 37°C). A 0.5-ml aliquot was placed in three 15 ml Corex® tubes in a shaking water bath at 37°C. Trichloroacetic acid (2.0 ml of an 11% solution) was added immediately to one tube. After the tubes were warmed to temperature, 1.5 ml of substrate solution was added. The substrate solution was 4 mmol/l y-glutamyl-p-nitroanilide prepared daily by dissolution at 60°C in a 50 mmol/l solution of glycylglycine and 0.1 mol/l Tris-HCI pH 8.0 (at 37°C). The reaction tubes were agitated slowly for 20 min in the water bath. The reaction was stopped with the addition of trichloroacetic acid. The tubes were centrifuged at 3500 rpm in a SB-14 rotor in a Sorvall RC-5B centrifuge (Dupont, Wilmington, DE) for 20 min. After centrifugation 2.0 ml of supernatant was transferred to a 12 x 100 mm Pyrex® tube. To each tube 1.0 ml of 0.1% $NaN0_2$ was added. After 3 min 1.0 ml of 1.0% ammonium sulfamate was added. After another 3 min, 1.0 ml of 0.052% naphthylethylenediamine was added and the tubes were shaken vigorously. The tubes were read immediately in the Beckman Instruments (Fullerton, CA) model 25 spectrophotometer at 540 nm. The activity was calculated using an molar absorptivity coefficient of 6218 for the reaction product, p-nitroanilide. If the activity was too high, a diluted sample of liver homogenate was used to repeat the assay. The protein content was determined and specific activity was calculated. Activity was expressed as μmol/min/mg protein.

2.6. Urinary acids

This method is a major modification of the method of Mattiuz et al. [16] for separating and quantifying an extensive profile of urinary acids. A urine sample was centrifuged in the Eppendorf micro-centrifuge (Brinkman Instruments, Westbury, NY) in a 1.5 ml Eppendorf capsule. An amount of urine equivalent to the urinary excretion of 2 g of body weight was diluted to 200 μl with water. Then 100 μl was injected on the HPLC. The HPLC was a Beckman model 334 with detectors and integrators in-line. The first was a Perkin-Elmer LS95 UV-Visible detector (Norwalk, CT) set at 280 nm attached to a Spectra Physics WINner® integration system (including a S/P4290 integrator) (San Jose, CA). The second was a Bioanalytical Systems (West Lafayette, IN) electrochemical detector set at +900 mV attached to a Hewlett Packard model 3396 integrator (Palo Alto, CA). The third was a Beckman filter fluorometer with fluorescamine filters attached to a Hewlett Packard model 3390A integrator. The column was a Phenomenex (Torrance, CA) Ultramex 5 μm Cl8 250 x 4.6 mm with a 2-μm Upchurch (Oak Harbor, WA) in-line prefilter. The autosampler was a Perkin-Elmer model ISSlOO with a 250-μl sample loop. The starting solvent was 0.2 mol/l sodium phosphate buffer pH 2.3 and the flow rate was 1.0 ml/min. The timed gradient program was as follows. Injections were made at time 0.

Program time (min)	Gradient of acetonitrile (% as v/v)	Duration of gradient (min)
0	0	6
6	0-6	59
65	6-11	18
83	11-13	IO
93	13-20	21
113	20-35	15
128	35-70	3
148	70-0	7
200	Ready for next injection	

Each chromatogram was 145 min long. About 165 peaks eluted.

2.7. . Urinary AFB products

When the urine samples from AFB dosed animals (Groups B and D) were subjected to the urinary acid analysis about a dozen small fluorescent peaks appeared late in the chromatogram (113-135 min). These same peaks contained radioactivity hence it was concluded that these were aflatoxin (AF)-containing peaks. Authentic AF standards were run under identical conditions. AFM and AFP

Table I
Animal growth rates during specific study periods and terminal body weightsa

AFB dose (µg/kg)	Dosing (days 1-12)	Recovery (days 13-23)	Post-recovery (days 24-99)	Terminal weight
50	4.05 ± 0.43a	3.76 ± 0.6oa	1.90 ± o.19a	326 ± 17a
JOO	3.89 ± 0.47a.b	3.74 ± 0.79"	J.98 ± 0.26a.b	327 ± 24a
150	3.59 ± 0.64b.c	3.47 ± 0.54a	1.98 ± 0.23•.b	321 ± 24"
200	3.61 ± 0.53b.c	3.62 ± 0.50"	2.02 ± 0.15"·b	330 ± 14"
250	3.34 ± 0.54c	3.80 ± 0.56"	2.05 ± O.J2•·b	332 ± 13•
350	2.94 ± 0.59d	2.88 ± 0.71b	2.10 ± 0.3Jb	317 ± 30"
Regrb	*P $;0.001*	*P =0.002*	*P =0.027*	*P =0.4*

•Growth rates (g/day ± S.D.) represent average growth rates over each study period. Significantly different values (P<0.05) within a column are denoted by different letters.
b The probability associated with a linear model of weight gain or terminal weight vs/ AFB dose.

co-eluted in one peak. Other aflatoxin-containing peaks were not identified. In order to confirm the identity of the combined AFM and AFP (AFM/P) peak, a modification of the method of Groopman et al. [17] was utilized. Samples and standards were prepared and injected into the HPLC in the same manner as for the urinary acids.

2.8. Other analytical methods

RNA was determined by the orcinol assay described by Ceriotti [18]. The method used to check the protein contamination of the DNA was that of Lowry et al. [19]. Liver homogenate protein determination for the GGT assay was with the Sigma Diagnostic Kit P5656. DNA was determined by the method of Burton [20]. All statistical procedures including ANOVA, Student's t-tests, and regression analysis, were performed with the MINITAB 6.1.1 software package (State College, PA) implemented on a personal computer.

3. Results

AFB dosing caused a linear depression in weight gain with increasing AFB dose ($R = -0.576$, $P < 0.001$) (Table 1). This dose-dependent depression continued through the 1 week recovery period following dosing ($P = 0.002$). However, from the end of the recovery period until the end of the study, the higher dose animals gained significantly more weight than their lower dose counterparts. At the end of the study, body weights were not significantly related to dose.

Fig. 2 shows the dose-response relationship of AFB-DNA adducts for the four adduct groups. A significant linear relationship existed between a single AFB dose and adducts both in animals killed 2 h post-dose (Fig. 2A) ($R = 0.89$) and in animals killed 24 h post-dose (Fig. 2B) ($R = 0.92$). The level of adducts was reduced

by about half, between 2 and 24 h, in all dosage groups. Somewhat similar results were observed for the animals administered nine doses before the radiolabeled tenth dose (groups C and D) except that adduct levels were substantially reduced at the two highest dosage levels (Fig. 2C, D) when compared with the single dose animals (groups A and B) ($P < 0.002$). Furthermore, the dose-response slope also was considerably flattened by the prior administration of nine cold AFB doses. Adduct levels were unchanged, at the two lowest dosage levels, between the first and the tenth doses ($P > 0.3$).

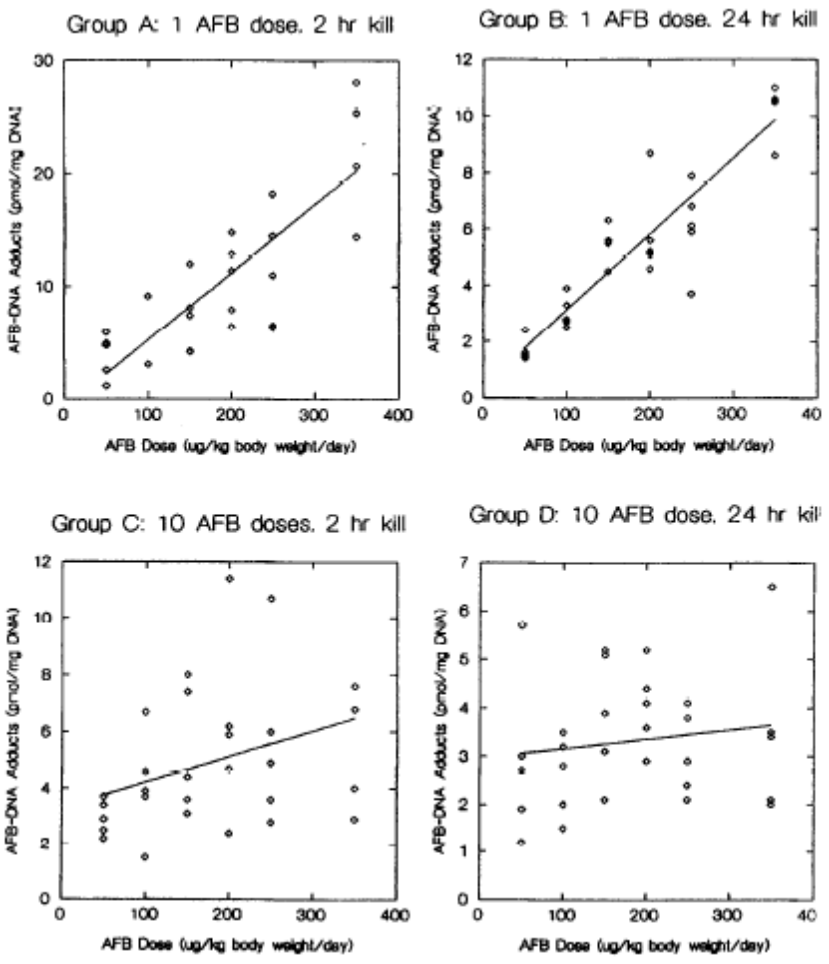

Fig. 2. AFB dose-response of AFB–DNA adducts.

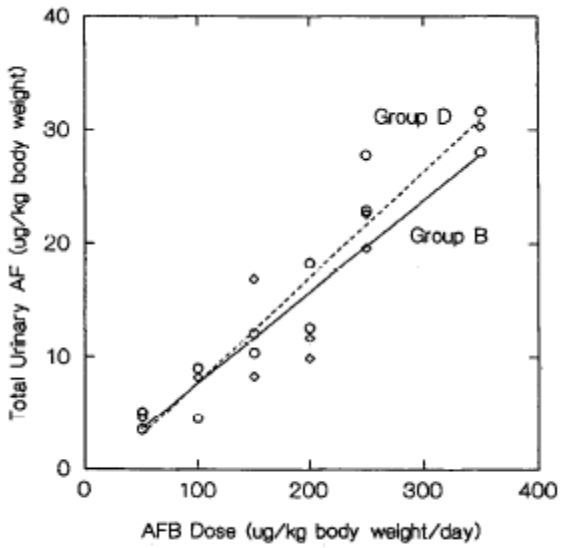

Fig. 3. AFB dose-response of total urinary radioactive AF metabolites.

A 24-h urinary excretion of total AFB products per gram of body weight (Fig. 3) produced a linear function of dose. Groups B and D were similar in their dose-response (R = 0.95 for combined groups). The percentage of total radioactivity for each HPLC AFB metabolite peak varied significantly with dose and group. For example, the percentage of total radioactivity in the AFM/P peak (Fig. 4} showed a flat dose-response relationship in group B (R = 0.13), but a positive linear

Table 2
Aflatoxin B_1 dose-response of y-glutamyl transpeptidase positive foci and y-glutamyl transpeptidase specific activity•

AFB dose (µg/kg)	Specific activity (µmol/min/mg protein)	Foci incidence	Foci number (no./cm^3)	Foci diameter *(µm)*	Foci volume (ppm)
50	10 ± 4"	5/10	10 ± 16"	108 ± 52•	15 ± 21"
100	12 ± 3•.b	4/10	10 ± 15"	99 ± 42"	16 ± 28"
150	12 ± 3a.b	7/10	24 ± *21*·	116 ± 56"	37 ± 46"
200	14 ± 2a,b	5/10	10 ± 18°	207 ± 73a.b	115 ± 180"
250	21 ± 7b	6/10	13 ± 16"	147 ± 150•,b	24 ± 36"
350	37 ± 19c	10/14	25 ± 24°	233 ± 96b	678 ± 1008b

• Values are group means ± S.D. Significantly different values within a column are denoted by different letters. For foci volume, differences were tested with fourth-root transformed values.

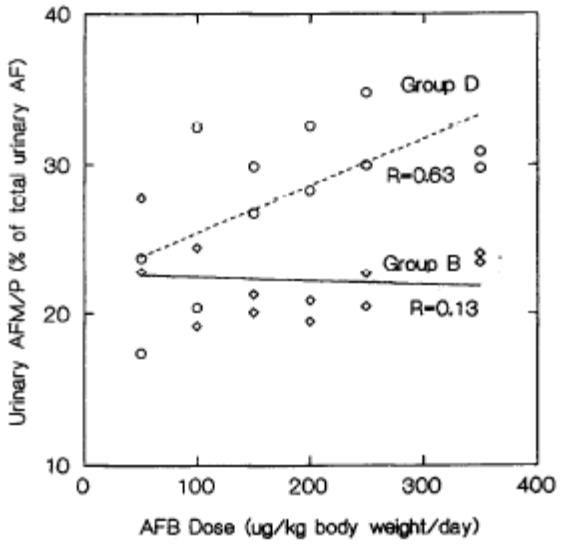

Fig. 4. AFB dose-response of percent of urinary radioactivity in AFM/P HPLC peak.

response with AFB dose in group D ($R = 0.63$). A negative linear response was seen with other radioactive peaks (data not shown) after ten doses. Thus, among the multiply dosed animals receiving higher doses, AFM/P commanded an increasing share of the urinary AFB metabolites, at the expense of the remaining AFB metabolites. The limited group D data may also suggest a curvilinear response with a maximum percentage excretion at the 250 μg dose.

At lower AFB doses GGT specific activities were about 10-12 µmol/min/mg protein, while at the two highest doses specific activities were elevated (Table 2). Histological observations were made of more extensive non-focal GGT staining, particularly biliary hyperplasia, in the higher dose groups.

The number of foci-bearing animals in each group was not significantly related to AFB dose ($P = 0.13$ with weighted logistic regression). Foci number also did not vary with dose. The mean diameter of foci among foci-bearing animals was positively and linearly related to dose although the difference between the highest and lowest group was small. Foci volume exhibited highly skewed within group distributions and showed an irregular but positive dose-response relationship (Fig. 5). Transformation of these data to the fourth root produced more normally distributed residuals and also a more linear dose-response. There appeared to be a dose threshold between about 150 and 250 µg/kg.

In the 50-150 µg/kg groups there was no correlation between body weight gain and any measure of foci response among individual rats. In the 200-350 µg/kg groups there was a positive correlation between G'GT foci volume, after adjusting

for dose, and body weight gained during the dosing period ($P = 0.018$) and also to a lesser extent with weight gained during the post-recovery period ($P = 0.066$). The relationship between weight gained during the dosing period and volume of foci was due primarily to weight gained during the last 2 days of dosing. Weight gained during dosing was also weakly correlated with number of foci ($P = 0.086$) but not with foci diameter. The relationship of foci volume with compensatory weight gain during the post recovery period was primarily due to weight gained during the third

week post-dosing. The post-dosing weight gain was also correlated with foci diameters ($P = 0.038$) but not with number of foci.

4. Discussion

Two methodological shortcomings are evident in this report: the HPLC quantitation of AFB-N^7-guanine adducts and the HPLC separation of urinary AFB metabolites. Although the high variability of the adduct data must certainly be derived, in part, from the in vitro degradation of the closed ring N^7-adduct to the open-ring form [9], we are confident in our summed data because they are in agreement with our earlier results with single dose adducts [3]. The development of the urinary acids method was incidental to this original project and the clear resolution of many AFB-containing peaks was unanticipated. Our inability to identify all of the urinary peaks or to resolve the excreted AFM and AFP does not detract from our simple assertion that these data probably reflect altered metabolism or disposition of AFB after multiple high doses of AFB.

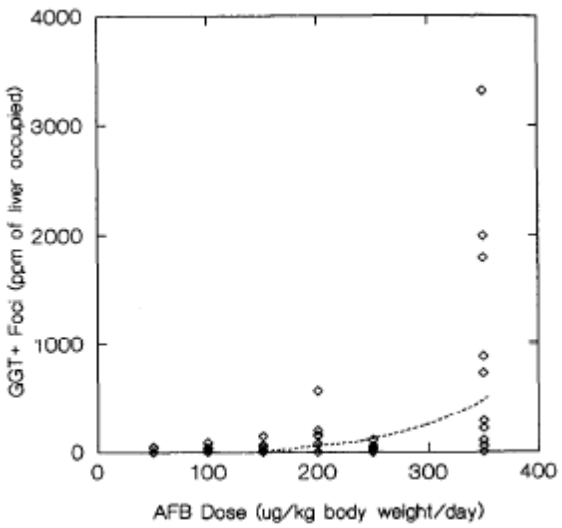

Fig. 5. AFB dose-response of GGT foci volume.

Earlier research demonstrated a linear dose-response relationship for AFB-induced rat liver DNA adducts measured 2 h after a single dose in the range from 1 ng/kg to 1 mg/kg [3,7,21,22]. We confirm these earlier findings in the range of 50-350 µg/kg. Our finding of a 50% decrease in adducts at 2 and 24 h post-dosing is less than was found by Croy et al., with 600 µg/kg administered i.p. [23]. However, at 3 h post-dosing, adduct levels from an oral dose are less than from an i.p. dose [24]. Other workers have measured the cumulative levels of DNA adducts after multiple dosing [7,21,23] and found an increase with the number of doses or the size of dose. In this study only the tenth dose was radioactive hence we measured the effect of nine previous doses on the DNA adduction of the last dose.

Prior exposure to high levels (250-350 µg/kg/day) of AFB reduced by about two-thirds adducts formed by the last dose compared to the first. Schrager et al. [25] measured AFB-N^7-guanine adducts after ten doses of about 200-250 µg/kg/day and found about a 50% decrease in accumulated adducts compared to adduct levels after a single dose.

Previous studies have shown considerable variability in AFB-induced foci response among identically treated rats [8,14,26,27]. The source of this variability is unknown but may be compensated for by a large number of animals per group. In spite of the decrease in adducts with multiple higher AFB doses, we observed a curvilinear increase in GGT foci at doses above about 150 µg/kg was supported by the results of this study in conjunction with earlier finding in this laboratory [8]. This suggests that factors other than specific AFB-DNA adduct formation enhance foci, and possibly tumor, development at higher multiple doses. Much recent research has elucidated an apparent linear relationship between AFB-DNA adducts and tumor formation. Previous work with a limited number of rats and impure AFB preparations showed an essentially linear relationship between logarithm of dose and liver tumor incidence on a probit scale [28,29]; a similar finding was reported in trout [30]. Bechtel [31] has shown that rats and trout share an identical linear dose-response relationship between adduct levels and tumors. While AFB, aftatoxicol (a metabolite of AFB), AFM, and aftatoxicol M_1 have different potencies to cause liver tumors in trout, Bailey showed that when comparing AF-DNA adducts and liver tumor response, the dose-response lines are nearly identical [32,33]) when expressed on a molecular basis.

There are two differences between these earlier studies showing linear dose response relationship and these findings showing a curvilinear response. Here, we used a 10-dose regimen and a higher dose level. The highest dose in the Wogan et al., tumor study [28], fed continuously, was about 15 µg/kg/day, using the dietary to body weight dosing conversion of Zeise et al. [34], compared to our lowest dose of 50 µg/kg body weight/day. The highest dose in the Bechtel study [31] was a continuous feeding of about 3 µg/kg body weight/day. Poirier and Beland compared nine biological models of adduct-tumor dose-responses and found only five that were linear [35]. They concluded that nonlinearity was a consequence of tissue-specific phenomena such as metabolic activation, cell proliferation, and/or cytotoxicity. These effects are accentuated at higher, more acutely toxic, doses. Lutz has suggested that, although DNA binding may be proportional to dose, cytotoxi-

city-induced cell division induced disproportionately at higher doses may enhance tumor development [36,37]. Eaton and Gallagher also suggest that tumorigenic response does not necessarily follow linearity at all doses because of the cytotoxic effects of AFB at higher doses [38].

We observed suggestive evidence of all three enhancing factors described by Poirier and Beland [35]. The changing patterns of urinary AFB metabolites reflects an increased excretion of less active metabolites (AFM or AFP) at high doses possibly through induction of altered metabolic pathways in the liver. These findings are in agreement with the less than expected levels of AFB-DNA adducts in these groups. Among multiply high dose animals there is a correlation between weight gain, a crude reflection of cellular proliferation, and foci development. The homogenate GGT activity levels were highest in animals with clearly stained biliary proliferation, a result of fibrotic regeneration from cytotoxicity.

In animal tumorigenesis models on nitrosamines, an inverse relationship between dose and time-to-response has been observed for foci [39] and tumor [40] formation. Animals administered low doses of a carcinogen take longer to develop foci than animals given higher doses. The possible carcinogenic-enhancing mechanisms at high doses observed in our study could shorten the time required for foci development, thereby enhancing the apparent threshold-dose nature of the dose-response curve.

In light of these and other findings, we suggest that AFB-induced GGT liver foci, and liver tumors, develop in a linear manner to multiple or continuous dosing at sub-toxic levels (below about 100-150 mg/kg). Above this level foci (and possibly tumors) develop in a curvilinear exponential manner at higher toxic AFB levels due to non-adduct metabolic, proliferative, and cytotoxic effects in spite of having reduced DNA adduct levels that were similar to adduct levels at much lower less acutely toxic doses.

References

[l] IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Aflatoxins, in: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Amines, and Mycotoxins. International Agency for Research on Cancer, Lyon, 1993. pp. 245-395.

[2] G.N. Wogan, P.M. Newberne, Dose-response characteristics of aflatoxin B_1 carcinogenesis in the rat, Cancer Res. 27 (1967) 2370-2376.

[3] B.S. Appleton, M.P. Goetchius, T.C. Campbell, Linear dose-response curve for the hepatic macromolecular binding of aflatoxin B_1 in rats at very low exposures, Cancer Res. 42 (1982) 3659-3662.

[4] R.G. Croy, J.M. Essigmann, V.N. Reinhold, G.N. Wogan, Identification of the principal aflatoxin B_1-DNA adduct formed in vivo in rat liver, Proc. Natl. Acad. Sci. USA 75 (1978) 1745-1749.

[5] J.D. Groopman, W.F. Busby, G.N. Wogan, Nuclear distribution of aflatoxin B_1 and its interaction with histones in rat liver in vivo, Cancer Res 40 (1980) 4343-4351.

[6] T.R. Irvin, G.N. Wogan, Quantitation of aflatoxin B_1 adduction within the ribosomal RNA gene sequences of rat liver DNA, Proc. Natl. Acad. Sci. USA 81 (1984) 664-668.

[7] P. Buss, M. Caviezel, W.K. Lutz, Linear dose-response relationship for DNA adducts in rat liver from chronic exposure to aflatoxin 8_1. Carcinogenesis 11 (1990) 2133-2135.
[8] G.E. Dunaif, T.C. Campbell, Relative contribution of dietary protein level and aflatoxin B_1 dose in generation of presumptive preneoplastic foci in rat liver, J. Natl. Cancer Inst. 78 (1987) 365-369.
[9] J.D. Groopman, R.G. Croy, G.N. Wogan, In vitro reaction of aflatoxin Bl- adducted DNA, Proc. Natl. Acad. Sci. USA 78 (1981) 5445-5449.
[10] J.M. Essigmann, R.G. Croy, A.M. Nadzan, W.F. Busby Jr, V.N. Reinhold, G. Buchi, G.N. Wogan, Structural identification of the major DNA adduct formed by aflatoxin Bl in vitro, Proc. Natl. Acad. Sci. USA 74 (1977) 1870-1874.
[l1] A.M. Rutenburg, K. Hwakyu, J.W. Fischbein, J.S. Hanker, H.L. Wasserkrug, A.M. Selegman, Histochemical and ultrastructural demonstration of y-glutamyl transpeptidase activity, J. Histochem. Cytochem. 17 (1969) 517-526.
(12] H.A. Campbell, H.C. Pitot, V.R. Potter, B.A. Laishes, Application of quantitative stereology to the evaluation of enzyme-altered foci in rat liver, Cancer Res. 42 (1982) 465-472.
(13] D. Nychka, T.D. Pugh, J.H. King, H. Koen, G. Wahba, J. Chover, S. Goldfarb, Optimal use of sampled tissue sections for estimating the number of hepatocellular foci, Cancer Res. 44 (1984) 178-183.
(14] N.G. Misslbeck, T.C. Campbell, D.A. Roe, Increase in hepatic y-glutamyl transferase (GOT) activity following chronic ethanol intake in combination with a high fat diet, Biochem. Phann. 35 (1986) 399-404.
(15] R. Cameron, J. Kellen, A. Kolin, A. Malkin, E. Farber, y-Glutamyl transferase in putative preneoplastic liver cell populations during hepatocarcinogenesis, Cancer Res. 38 (1978) 823-829.
[16] E.L. Mattiuz, J.W. Webb, S.C. Gates, High resolution separation of urinary organic acids by high performance liquid chromatography, J. Liquid Chromatogr. 5 (1982) 2343-2357.
(17] J.D. Groopman, P.R. Donahue, J. Zhu, J. Chen, G.N. Wogan, Afiatoxin metabolism in humans: detection of metabolites and nucleic acid adducts in urine by affinity chromatography, Proc. Natl. Acad. Sci. USA 82 (1985) 6492-6496.
[18] G. Ceriotti, Determination of nucleic acids in animal tissues, J. Biol. Chem. 214 (1955) 59-70.
[19] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265-275.
[20] K. Burton, Study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid, Biochem. J. 62 (1956) 315-323.
[21] C.P. Wild, R.C. Garner, R. Montesano, F. Tursi, Atlatoxin B_1 binding to plasma albumin and liver DNA upon chronic administration to rats, Carcinogenesis 7 (1986) 853-858.
(22] R.A. Bennett, J.M. Essigmann, G.N. Wogan, Excretion of an afiatoxin-guanine adduct in the urine of afiatoxin B_1-treated rats, Cancer Res. 41 (1981) 650-654.
[23] R.G. Croy, G.N. Wogan, Temporal patterns of covalent DNA adducts in rat liver after single and multiple doses of afiatoxin Bt> Cancer Res. 41 (1981) 197-203.
[24] A.D. Salbe, L.F. Bjeldanes, Effect of diet and route of administration on the DNA binding of afiatoxin B_1 in the rat, Carcinogenesis 10 (1989) 629-634.
[25] T.F. Schrager, P.M. Newberne, A.H. Pikul, J.D. Groopman, Afiatoxin-DNA adduct formation in chronically dosed rats fed a choline-deficient diet, Carcinogenesis II (1990) 177-180.
(26] D.A. Schulsinger, M.M. Root, T.C. Campbell, Effect of dietary protein quality on development of afiatoxin 8_1-induced hepatic preneoplastic lesions, J. Natl. Cancer. Inst. 81 (1989) 1241-1245.
(27] Y. He, M.M. Root, R.S. Parker, T.C. Campbell, Effects of carotenoid-rich food extracts on the development of preneoplastic lesions in rat liver and on in vivo and in vitro antioxidant status, Nutr. Cancer 27 (1997) 238-244.
[28] G.N. Wogan, S. Paglialunga, P.M. Newberne, Carcinogenic effects of low dietary levels of aflatoxin B_1 in rats, Food Cosmetic Toxicol. 12 (1974) 681-685.
(29] P.M. Newberne, Carcinogenicity of afiatoxin-contaminated peanut meal, in: G.N. Wogan (Ed.), Mycotoxins in Foodstuffs, MIT Press, Cambridge, MA, 1965, pp. 187-208.
[30] R.H. Dashwood, D.N. Arbogast, A.T. Fong, C. Pereira, J.D. Hendricks, G.S. Bailey, Quantitative inter-relationships between aflatoxin B_1 carcinogen dose, indole-3-carbinol anti-carcinogen dose, target organ DNA adduction and final tumor response, Carcinogenesis IO (1989) 175-181.

[31] D.H. Bechtel, Molecular dosimetry of hepatic aflatoxin B_1-DNA adducts: linear correlation with hepatic cancer risk, Regul. Toxicol. Pharmacol. 10 (1989) 74-81.
[32] G.S. Bailey. Role of aflatoxin-DNA adducts in the cancer process, in: D.L. Eaton, J.D. Groopman (Eds.), The Toxicology of Aflatoxins: Human Health, Veterinary and Agricultural Significance. Academic Press, New York, 1994, pp.137-148.
[33] G.S. Bailey, P.M. Loveland, C. Pereira, D. Pierce, J.D. Hendricks, J.D. Groopman, Quantitative carcinogenesis and dosimetry in rainbow trout for aflatoxin B_1 and aflatoxicol, two aflatoxins that form the same DNA adduct, Mutat. Res. 313 (1994) 25-38.
[34] L. Zeise, R. Wilson, E.A.C. Crouch, Dose-response relationships for carcinogens: a review, Environ. Health Perspect. 73 (1987) 259-308.
[35] M.C. Poirier, F.A. Beland, DNA adduct measurements and tumor incidence during chronic carcinogen exposure in rodents, Environ. Health Perspect. I02 (Suppl 6) (1994) 161-165.
[36] W.K. Lutz, Quantitative evaluation of DNA-binding data in vivo for low-dose extrapolations, Arch. Toxic. Suppl 10 (1987) 66-74.
[37] W.K. Lutz, Dose-response relationship and low dose extrapolation in chemical carcinogenesis, Carcinogenesis 11 (1990) 1243-1247.
[38] D.L. Eaton, E.P. Gallagher, Mechanisms of aflatoxin carcinogenesis, Annu. Rev. Pharmacol. Toxicol. 34 (1994) 135-172.
[39] M. Schwarz, D. Pearson, A. Buchmann, W. Kunz. The use of enzyme-altered foci for risk assessment of hepatocarcinogens, in: C.C. Travis (Ed.), Biological Based Methods for Cancer Risk Assessment. Plenum, New York, 1988, pp. 31-39.
[40] R. Peto, R. Gray, P. Brantom, P. Grasso, Effects of 4080 rats of chronic ingestion of N-nitrosodi-ethylamine or N-nitrosodimethylamine: a detailed dose-response study, Cancer Res. 51 (1991) 6415-6451.