

# Reaction of Fumonisin with Glucose Prevents Promotion of Hepatocarcinogenesis in Female F344/N Rats while Maintaining Normal Hepatic Sphinganine/Sphingosine Ratios

Hongjun Liu,<sup>†</sup> Yun Lu,<sup>†</sup> Joseph S. Haynes,<sup>‡</sup> Joan E. Cunnick,<sup>§</sup> Patricia Murphy,<sup>†</sup> and Suzanne Hendrich<sup>\*,†</sup>

Department of Food Science and Human Nutrition, Department of Veterinary Pathology, and Department of Microbiology, Iowa State University, Ames, Iowa 50011

The reaction of the primary amine of fumonisin B<sub>1</sub> (FB<sub>1</sub>) with glucose was hypothesized to detoxify this mycotoxin. Eighty 10-day-old female F344/N rats were injected intraperitoneally with diethylnitrosamine (DEN; 15 mg/kg of body weight). At 4 weeks of age, the weaned rats were randomly assigned to one of four treatment groups with 20 rats each. At 9 weeks of age, four rats from each treatment group were killed. At 12 weeks, another five rats from each group were killed. At 20 weeks of age, the remaining rats were killed. In comparison with the rats fed basal diet or FB<sub>1</sub>-glucose (containing 25 ppm of FB<sub>1</sub>), rats fed 8 ppm (residual amount of free FB<sub>1</sub> in the FB<sub>1</sub>-glucose mixture) or 25 ppm of FB<sub>1</sub> had greater alanine aminotransferase activity at 9 and 20 weeks of age ( $P < 0.001$ ), greater endogenous hepatic prostaglandin E<sub>2</sub> production at 20 weeks of age ( $P < 0.05$ ), and significantly lower plasma cholesterol at 20 weeks of age ( $P < 0.01$ ). Placental glutathione *S*-transferase (PGST)-positive and  $\gamma$ -glutamyltransferase (GGT)-positive altered hepatic foci (AHF) occurred only in rats fed 25 ppm of FB<sub>1</sub> at 20 weeks of age. Hepatic natural killer (NK) cell activities were similar among the four groups, but the percentage of total liver-associated mononuclear cells exhibiting the NKR-P1<sup>bright</sup> marker was significantly greater in rats fed FB<sub>1</sub>-glucose, FB<sub>1</sub> (8 ppm) and FB<sub>1</sub> (25 ppm) than in control rats at 9 weeks of age, and FB<sub>1</sub>-glucose-treated rats had significantly lower NKR-P1<sup>bright</sup> cells as a percentage of total liver-associated mononuclear cells than rats fed 25 ppm of FB<sub>1</sub> at 20 weeks of age ( $P < 0.05$ ). PGST- or GGT-positive AHF were not detected in any treatment group at 9 or 12 weeks of age. At 20 weeks of age, half of the rats fed 25 ppm of FB<sub>1</sub> had PGST- and GGT-positive AHF. The sphinganine (Sa) concentration and the Sa/sphingosine (So) ratio were significantly greater in the rats fed 25 ppm of FB<sub>1</sub> diet as compared with the control groups at, respectively, 12 or 20 weeks of age. Therefore, modifying FB<sub>1</sub> with glucose seems to prevent FB<sub>1</sub>-induced hepatotoxicity and promotion of hepatocarcinogenesis. The Sa/So ratio was not the most sensitive biomarker of FB<sub>1</sub> toxicity.

**Keywords:** Glucose; hepatic; rats; fumonisin; carcinogenesis

## INTRODUCTION

The carcinogenic and toxic effects of fumonisin B<sub>1</sub> (FB<sub>1</sub>), a mycotoxin produced by the commonly occurring corn fungi, *Fusarium moniliforme* and *Fusarium proliferatum*, have been studied intensively. Fumonisin B<sub>1</sub> (69.3  $\mu$ mol/kg, 50 ppm) was hepatocarcinogenic in rats fed the toxicant for  $\sim$ 2 years (1). The incidence of *F. moniliforme* in corn for human consumption has been correlated with the incidence of esophageal cancer in Transkei, southern Africa (2), and in China (3). The concentration of FB<sub>1</sub> in corn reached  $\sim$ 11.1  $\mu$ mol/kg in areas of southern Africa where the human esophageal cancer rate was high (4). Corn products for human and animal consumption were determined to contain 0.3–4.2  $\mu$ mol of FB<sub>1</sub>/kg in the United States (5–7).

Several biomarkers have been used to study FB<sub>1</sub> hepatocarcinogenicity. FB<sub>1</sub>-promoted rat hepatocarcinogenesis was readily quantified by measuring placental glutathione *S*-transferase (PGST)-positive altered hepatic foci (AHF) (8) and  $\gamma$ -glutamyltransferase (GGT)-positive AHF (9). Plasma alanine aminotransferase (ALT) activity was increased during fumonisin hepatotoxicity (10) and hepatocarcinogenesis in rats (11). Increased plasma total cholesterol was observed in FB<sub>1</sub>-treated vervet monkeys (12) and in rats (11) in short-term studies. Greater hepatic prostaglandin F<sub>2 $\alpha$</sub>  production was also observed in FB<sub>1</sub> tumor promotion in rat liver (13). In vivo administration of 50 ppm of FB<sub>1</sub> significantly suppressed hepatic natural killer (NK) cell activity while stimulating hepatic preneoplasia (13). NK cell activity suppression by FB<sub>1</sub> during tumor promotion may be mechanistically significant, but this remains to be determined. Thus, numerous possible biomarkers of FB<sub>1</sub> toxicity and tumor promotion may be used to probe the mechanism of action of this mycotoxin.

Fumonisin selectively inhibit ceramide synthase, a key enzyme in the sphingolipid biosynthetic pathway

\* Address correspondence to this author at the Department of Food Science and Human Nutrition, 1127 Human Nutrition Science Building, Iowa State University, Ames, IA 50010 [telephone (515) 294-4272; e-mail shendric@iastate.edu].

<sup>†</sup> Department of Food Science and Human Nutrition.

<sup>‡</sup> Department of Veterinary Pathology.

<sup>§</sup> Department of Microbiology.

(14). The subsequent accumulation of the sphingoid bases, sphinganine (Sa) and sphingosine (So), could have an important role in the toxicological effects of fumonisin in the kidney and liver of rats (15, 16). Because the sphingoid bases are important regulators of cellular growth and differentiation (17), disruption of sphingolipid biosynthesis has been implicated in the hepatocarcinogenicity of fumonisin (18).

Many strategies have been examined for the reduction of human and animal exposure to these fungal toxins. Biological, chemical, and physical processes have been explored to salvage fumonisin-contaminated corn. The thermostability of FB<sub>1</sub> proved to be great. When dry corn was heated at 50, 75, 100, and 125 °C for 40 min, only a small amount of FB<sub>1</sub> was lost (19). Treatment of fumonisin-contaminated corn with 2% ammonia for 4 days, a process that detoxified aflatoxin B<sub>1</sub>, led to a slight reduction in the concentration of FB<sub>1</sub> without decreasing its toxicity in rats (15). Nixtamalization, the traditional process to produce masa or tortilla flour, reduced the amount of FB<sub>1</sub> by hydrolyzing FB<sub>1</sub> to hydrolyzed FB<sub>1</sub> (HFB<sub>1</sub>), but HFB<sub>1</sub> was similar in toxicity to FB<sub>1</sub> when the nutritional status of rats was adequate (11). In vitro toxicity studies of several FB<sub>1</sub> analogues showed that the analogues containing FB<sub>1</sub> amine groups and the tricarballylic side chains were more toxic than analogues containing only the tricarballylic side chains (20), and naturally occurring *N*-acetyl-FB<sub>1</sub> was not toxic (21). Therefore, the primary amine of FB<sub>1</sub> is likely to be critical for its toxicity. Murphy et al. (22) reported a method to detoxify FB<sub>1</sub> by derivatizing the amine group with a reducing sugar, fructose, in a nonenzymatic browning reaction. Diethylnitrosamine-initiated (15 mg/kg of body weight) male F344/N rats were fed for 4 weeks either 69.3 μmol of FB<sub>1</sub>/kg or 69.3 μmol of FB<sub>1</sub>/kg reacted with fructose (FB<sub>1</sub>-fructose). Rats fed FB<sub>1</sub> had significantly greater biomarkers of hepatocarcinogenicity, whereas rats fed FB<sub>1</sub>-fructose showed no signs of hepatocarcinogenicity or hepatotoxicity (13). A more practical and efficient method to block FB<sub>1</sub>'s amine group by reacting the amine group with glucose had been developed in Dr. Murphy's laboratory (23). The FB<sub>1</sub>-glucose reaction was more complete than the reaction with fructose, and the reaction products were more easily isolated than FB<sub>1</sub>-fructose products. It was hypothesized that modifying FB<sub>1</sub> with glucose would prevent promotion of hepatocarcinogenicity by FB<sub>1</sub>. Our experiment was designed to test the effectiveness of this detoxification method by examining the effects of FB<sub>1</sub>-glucose on several markers of FB<sub>1</sub> promotion of hepatocarcinogenesis.

## MATERIALS AND METHODS

**Preparation of Fumonisin B<sub>1</sub>—Glucose Adduct.** FB<sub>1</sub>-glucose was prepared by heating 1.39 mM FB<sub>1</sub> (total = 8.1 mmol) with 0.1 M D-glucose in 50 mM potassium phosphate buffer, pH 7.0, at 80 °C. After 48 h, the pH of the reaction mixture was adjusted to pH 2.7 to stop the reaction. Reversed-phase C<sub>18</sub> SPE cartridges (Supelco, Bellefonte, PA) were preconditioned with 50 mL of 100% methanol at an apparent pH of 2.7 followed by 100 mL of deionized water at an apparent pH of 2.7. An aliquot of 50 mL of the 1.39 mM FB<sub>1</sub>/0.1 M D-glucose reaction mixture was loaded onto the cartridge. The cartridge was washed with 100 mL of deionized water and 100 mL of 30% methanol at an apparent pH of 2.7. The D-glucose was washed out at this step. The FB<sub>1</sub>-glucose was eluted with 50 mL of 40% methanol and 100 mL of 100% methanol at an

apparent pH of 2.7. The eluent was evaporated to dryness with a Brinkmann rotavapor R110 (Westbury, NY) at 35 °C. The residue was redissolved in distilled deionized water and brought to 10.0 mL in a volumetric flask. The unreacted free FB<sub>1</sub> was detected by HPLC after derivatization with *o*-phthalaldehyde (5). The solution was freeze-dried and the total mass determined on an analytical balance. The amount of FB<sub>1</sub>-glucose was determined by subtracting the free FB<sub>1</sub> mass from the total mass.

**Diets.** Four experimental diets were fed to rats. The control group was fed basal diet AIN-93G (24). The FB<sub>1</sub> group was fed a diet containing highly purified FB<sub>1</sub> that was prepared by incorporating 25 ppm of FB<sub>1</sub> into basal diets. The FB<sub>1</sub>-glucose group was fed a diet in which purified FB<sub>1</sub> reacted with glucose was incorporated into the basal diet at a level equivalent to 25 ppm of FB<sub>1</sub> diet. Analysis of the FB<sub>1</sub>-glucose products showed that ~8 ppm of unreacted FB<sub>1</sub> remained in the FB<sub>1</sub>-glucose-containing diet. Thus, another control group was fed 8 ppm of FB<sub>1</sub>.

**Animals.** The experimental procedures were approved by the Iowa State University Animal Care Committee. Eighty 10-day-old female F344/N rats obtained from Harlan Sprague-Dawley (Madison, WI) were injected intraperitoneally with diethylnitrosamine (DEN; 15 mg/kg of body weight) in 0.1 mL of corn oil. At 4 weeks of age, the weaned rats were randomly assigned to one of the four treatment groups with 20 rats each. At 9 weeks of age, four rats from each treatment group were killed. At 12 weeks, another five rats from each group were killed. At 20 weeks of age, the remaining rats were killed. The carcasses of the rats were stored at -20 °C in a freezer. Before scanning by dual-energy X-ray absorptiometry (QDR 2000, Hologic Inc., Waltham, MA), the carcasses were thawed at 0 °C for ~2 h. The amounts of lean and fat tissues in the body were expressed as a percentage of the body weight (25). Rats were given free access to the experimental diets and were exposed to a 12-h light/dark cycle maintained at 22–25 °C and 50% humidity. Body weight and feed intake were recorded weekly.

**Plasma and Liver Sample Preparations.** Before the liver was perfused, 1 mL of 0.85% sodium chloride solution (containing 100 units of heparin) was injected into the abdominal vein, and ~3 mL blood was removed. Part of the plasma obtained from the heparinized blood was analyzed within 24 h for ALT activity. The remaining plasma was stored at -80 °C for later plasma total cholesterol analysis.

Rat livers were perfused with 40 mL of Hank's balanced salt solution (HBSS, supplemented with 25 mM Hepes and 0.1% EDTA). Approximately 12 mL of perfusate was concentrated to 3 mL, laid on 3 mL of Accupaque density gradient medium (Accurate Chemical Co., Westbury, NY), and then centrifuged at 1500 rpm for 10 min. The mononuclear cells at the interface were collected and washed two times, once with HBSS (with 25 mM Hepes) and once with complete medium [RPMI-1640, supplemented with 50 μg/mL gentamicin, 25 mM Hepes, 2 mM L-glutamine, and 10% fetal bovine serum (FBS)]. Cells were enumerated on a Celltrack II (Nova Biomedical, Waltham, MA), in preparation for the NK cell activity and cell surface immunofluorescence analysis.

Each of the left, median, and right lateral lobes of the livers was sliced into 1 cm slices. Three slices, one from each lobe, were immediately frozen as a block on dry ice and stored at -80 °C. From each of the frozen liver blocks were cut five 10-μm serial sections with a Histostat microtome (model 855, Leica Inc., Deerfield, IL) for later staining of GGT and PGST.

For each rat, a portion of 0.5 g of minced liver was immediately homogenized in an ice bath with 10 passes of a Potter-Elvehjem homogenizer in 5 mL, pH 7.4, 50 mM potassium phosphate buffer containing 4.2 mM acetyl salicylic acid (Sigma Chemical Co., St. Louis, MO). The liver homogenates were frozen at -80 °C for later analysis of endogenous hepatic PGE<sub>2</sub> and PGF<sub>2α</sub>.

**Plasma Total Cholesterol Concentration and ALT Activity.** Plasma total cholesterol concentration was determined by using a Sigma diagnostic kit, procedure 352-3 (Sigma Chemical Co.). Plasma ALT activity was measured by using a



Sigma diagnostic kit for glutamate/pyruvate transaminase optimized for the ALT assay (Sigma Chemical Co.).

**Determination of Sphingosine (So) and Sphinganine (Sa).** Thawed liver tissues were homogenized in 4 volumes 0.05 M potassium phosphate buffer. Homogenate (0.1 mL) was transferred to a cold (13 × 100 mm) glass tube with a Teflon-lined screw cap. The extraction method was performed as described by Riley et al. (26). Sphingosine and sphinganine were quantified by HPLC as described by Riley et al. (26) but using C17-phytosphingosine (Sigma Chemical Co.) as the internal standard. The C17-phytosphingosine, Sa, and So standard (Sigma Chemical Co.) mixture at different concentrations (1, 3, 5, 7, and 9 nmol) were prepared for standard curves.

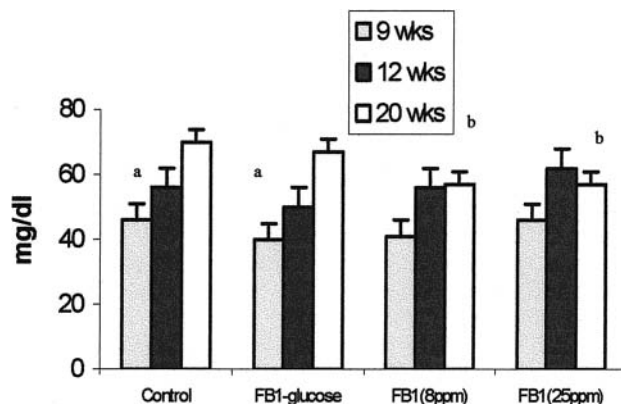
**Prostaglandin Assays.** PGE<sub>2</sub> and PGF<sub>2α</sub> were determined in a radioimmunoassay as described by McCosh et al. (27). Anti-PGE<sub>2</sub> antiserum (0.2 mL/per assay tube) and anti-PGF<sub>2α</sub> antiserum (0.2 mL/per assay tube) were obtained from Sigma Chemical Co. H<sup>3</sup>-PGE<sub>2</sub> (10<sup>-6</sup> μCi/aliquot) and H-PGF<sub>2α</sub> (10<sup>-6</sup> μCi/aliquot) were obtained from NEN Products (Boston, MA). PGE<sub>2</sub> and PGF<sub>2α</sub> were quantified by using a computer program based on a logit transformation of the standard curve (28).

**NK Cell Assays.** NK cell assays were performed as previously described (29). Cells were plated in triplicate at the following effector to target ratios in 96 well plates: 25:1, 12.5:1, 6.25:1, and 3:1. The target cells for the assay were YAC-1 cells (ATCC Co., Rockville, MD) (8 × 10<sup>3</sup>/well), which had been labeled with 200 μCi of <sup>51</sup>Cr. The amount of <sup>51</sup>Cr released by dying cells was counted using a Gamma Trac 1191 (TM Analytic, Inc., Elk Grove Village, IL). Lytic units were calculated using a computer program based on the equation of Pross and Maroun (30).

**Fluorescent Staining of Lymphoid Cells.** Leukocyte suspensions were diluted with an equal volume of PBS/0.1% azide (cold) and incubated at 4 °C (5 min). Separate aliquots were stained with 0.2 μg/2 × 10<sup>5</sup> (mononuclear cell) of anti-rat NKR-P1A-Biotin (mAb 3.2.3) or an equivalent amount of isotype control (murine IgG1-biotin). A second step of 0.1 μg/2 × 10<sup>5</sup> (mononuclear cell) Strep-AvidinCychrome was used (all from Pharmingen, San Diego, CA). All incubations were performed at 4 °C in the dark for 30 min and washed with PBS/0.1% azide. The contaminating red blood cells were lysed using 10% ammonium chloride buffer (pH 7.4). Cells were fixed with PBS/1% paraformaldehyde prior to analysis using an EPICS-XL-MCI flow cytometer (Coulter, Miami, FL). According to the density of fluorescence, three cell populations were distinguished, NKR-P1<sup>bright</sup>, NKR-P1<sup>dim</sup>, and negative, and gates were set to help measure the percentages of different populations (31) (Figure 1).

**Immunohistochemical Staining.** One of the frozen serial sections was stained for the presence of PGST-positive AHF. PGST was detected by the peroxidase-anti-peroxidase (PAP) method using a Vectastain ABC avidin-biotin universal rabbit PAP kit (Vector Laboratories, Burlingame, CA). Anti-PGST antiserum was prepared as described previously (32). The second frozen serial section was stained for GGT activity as described by Rutenburg et al. (33). The substrate for GGT was glutamyl-4-naphthylamide (GMNA) (United States Biochemical Corp., Cleveland, OH). AHF were quantified via computerized stereology. A Sony three-chip color video camera DXC-3000A took the images of the liver section stained for GGT and PGST, which were digitally transferred from the camera to an Apple Power Mac G3 computer (Apple Computer, Inc., Cupertino, CA) and analyzed with IP Lab image analysis software (version 3.2.3, Scanalytics, Fairfax, VA). Lung, kidney, brain, tibia, and additional liver samples were processed by routine histopathological methods for hematoxylin-eosin staining (34).

**Statistical Analysis.** Multivariate regression analysis was used to compare the four growth curves. One-way ANOVA was performed to analyze plasma total cholesterol, ALT activity, and the percentage of leukocytes that carry NKR-P1<sup>bright</sup> and NKR-P1<sup>dim</sup> population markers as well as the amounts and ratio of Sa/So. The hepatic NK activity was compared using one-way ANOVA combined with covariance for the percentage of NKR-P1<sup>bright</sup> population. Two-way ANOVA was performed



**Figure 1.** Comparison of the total plasma cholesterol concentration at three time points. At 9 weeks of age, no difference was found among four treatment groups. At 12 weeks, the rats fed 25 ppm of FB<sub>1</sub> exhibited significantly greater cholesterol concentration as compared with the FB<sub>1</sub>-glucose group. At 20 weeks of age, both the rats fed with 8 ppm of FB<sub>1</sub> and the rats fed with 25 ppm of FB<sub>1</sub> exhibited significantly lower cholesterol concentration as compared with control group. The error bar represents the standard error of the mean (SEM). "a" indicates significant difference as compared with the FB<sub>1</sub> (25 ppm) group at 12 weeks of age (*N* = 5/group). "b" indicates significant difference as compared with the control group at 20 weeks of age (*N* = 10/group).

**Table 1. Comparison of the Final Body Weights, Body Mass Compositions, Relative Liver Weights, and Food Intakes among Four Treatment F344/N Female Rats<sup>a</sup>**

	body wt (g)	liver wt/body wt (%)	body composition (% fat)	food intake (g/day)
control	204 ± 12	4.1 ± 0.5	32.8 ± 3.0	13.5 ± 3.0
FB <sub>1</sub> -glucose	205 ± 12	3.9 ± 0.6	34.2 ± 5.0	14.5 ± 2.0
FB <sub>1</sub> (8 ppm)	198 ± 15	3.9 ± 0.4	33.4 ± 3.0	12.8 ± 2.0
FB <sub>1</sub> (25 ppm)	197 ± 16	3.7 ± 0.5	31.5 ± 2.0	11.5 ± 2.0

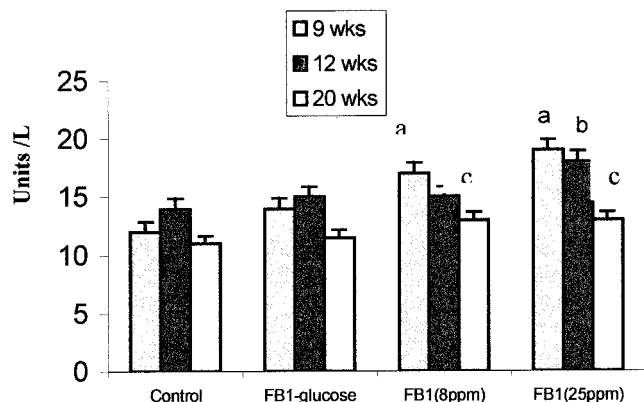
<sup>a</sup> No difference was found in these four indicators among these groups under each treatment over 20 weeks (*N* = 10/group). The data entries in the table are expressed as mean ± standard error of mean (SEM).

to analyze endogenous PGE<sub>2</sub> and PGF<sub>2α</sub> at different ages. Student's *t* test was performed to compare all possible group differences after ANOVA. A *P* value of <0.05 was considered to be statistically significant.

## RESULTS

**Effect on Body Weight Gains, Relative Liver Weight, and Body Mass Composition.** The body weights and food intakes did not differ among treatments. Relative liver weights were not different among the four treatment groups at any time point, and body compositions (percent fat) were similar among all groups at 20 weeks of age. Body weights and food intakes did not differ among treatments (Table 1). The growth curves of four treatment groups were similar over a period of 20 weeks (data not shown).

**Plasma Total Cholesterol Levels and ALT Activities.** At 9 weeks of age, the total plasma cholesterol concentrations were not different among the four treatment groups. At 12 weeks of age, in comparison with the rats fed basal diet and the rats fed FB<sub>1</sub>-glucose, the rats fed 25 ppm FB<sub>1</sub> diet had significantly greater plasma total cholesterol concentration (*P* < 0.05). At 20 weeks of age, in comparison with the rats fed basal or FB<sub>1</sub>-glucose diets, the rats fed 8 or 25 ppm FB<sub>1</sub> diets had significantly decreased plasma total cholesterol (*P* < 0.05) (Figure 1). The ALT activity of the rats fed 8 or

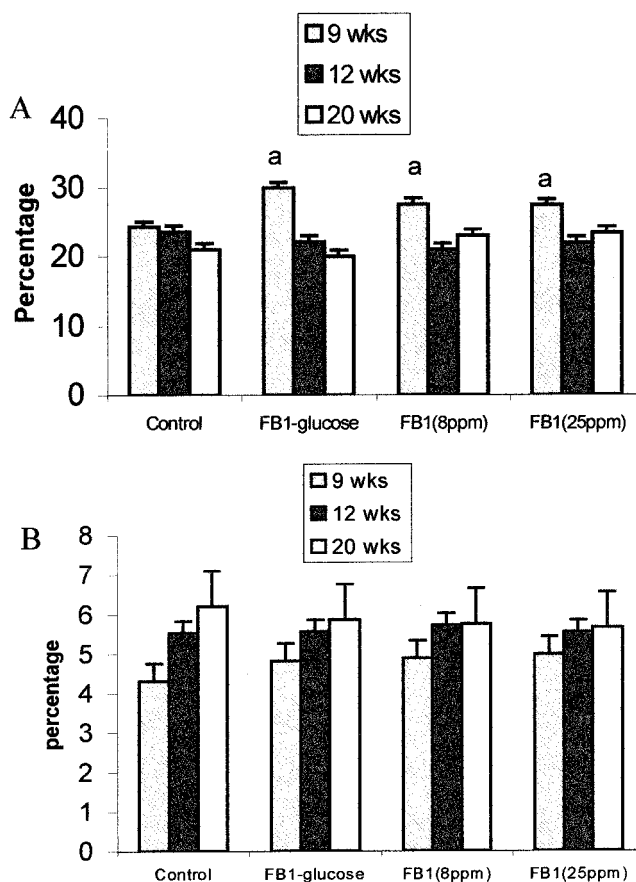


**Figure 2.** Comparison of the ALT activities at three age points. At 9 weeks of age, both the rats fed with 8 ppm of FB<sub>1</sub> and the rats with 25 ppm with FB<sub>1</sub> showed significantly greater ALT activity as compared with the control group. At 12 weeks of age, the rats fed with 25 ppm of FB<sub>1</sub> showed significantly greater ALT activity as compared with the control group. At 20 weeks of age, both the rats fed with 8 ppm of FB<sub>1</sub> and the rats fed with 25 ppm of FB<sub>1</sub> showed significantly greater ALT activity as compared with the control group. The error bar represents the SEM. "a" indicates significantly greater than the control group at 9 weeks of age ( $N = 4/\text{group}$ ). "b" indicates significantly greater than the control group at 12 weeks of age ( $N = 5/\text{group}$ ). "c" indicates significantly greater than the control group at 20 weeks of age ( $N = 10/\text{group}$ ).

25 ppm diet was significantly greater than that of the rats fed basal or FB<sub>1</sub>-glucose diet at 9 weeks age. At 12 weeks of age, only the rats fed 25 ppm of FB<sub>1</sub> exhibited significantly greater ALT activity than did other groups. At 20 weeks of age, the rats fed 8 or 25 ppm of FB<sub>1</sub> exhibited greater ALT activity compared with rats fed basal or FB<sub>1</sub>-glucose diet at 20 weeks of age (Figure 2).

**Hepatic NKR-P1<sup>bright</sup> and NKR-P1<sup>dim</sup> Populations.** Flow cytometric analysis using mAb 3.2.3 [NK cell receptor protein 1 (NKR-P1)] revealed two distinct subsets of hepatic mononuclear cells expressing variable levels of NKR-P1. The NKR-P1<sup>bright</sup> population, which expresses a high level of NKR-P1, has been identified as the population causing NK-associated lytic activity (35). The NKR-P1<sup>dim</sup> population, which expresses a low level (2–10-fold lower) of NKR-P1, has been linked to a subset of T lymphocytes that have an NK-like cytolytic function under activation by interleukin 2 (36). The results showed that all three treatment groups had greater percentages of NKR-P1<sup>bright</sup> population at 9 weeks of age than did the control group. FB<sub>1</sub>-glucose lowered the percentage of NKR-P1<sup>bright</sup> mononuclear cells as compared with the group fed the 25 ppm diet at 20 weeks of age (Figure 3A). The NKR-P1<sup>dim</sup> populations were not different among each group at three time points, but with increasing age, each group had a greater percentage of NKR-P1<sup>dim</sup> mononuclear cells as compared with the same group at 9 weeks of age (Figure 3B).

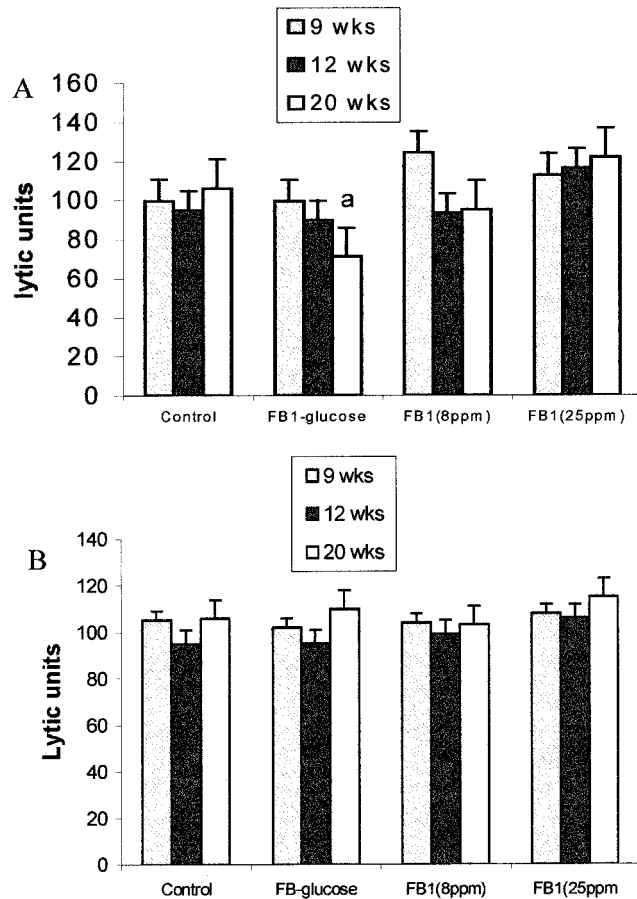
**Hepatic NK Cell Activity.** Before covariation with the percentage of NKR-P1<sup>bright</sup> population, all groups of rats exhibited similar NK lytic activities at 9 and 12 weeks of age. The rats fed FB<sub>1</sub>-glucose exhibited significantly lower NK lytic activity as compared with the rats fed the 25 ppm FB<sub>1</sub> diet at 20 weeks of age (Figure 4A). After covariation with the percentage of NKR-P1<sup>bright</sup> mononuclear cells, all treatment groups exhibited similar NK lytic activities at each age (Figure 4B).



**Figure 3.** (A) Comparison of the percentage of hepatic NKR-P1<sup>bright</sup> mononuclear cells at three time points. At 9 weeks of age, all of the rats fed FB<sub>1</sub>-glucose, 8 ppm of FB<sub>1</sub>, or 25 ppm of FB<sub>1</sub> exhibited greater NKR-P1<sup>bright</sup> percentage as compared with the control group. No difference was found among treatment groups at 12 or 20 weeks of age. The error bar represents the SEM. (B) Comparison of the percentage of hepatic NKR-P1<sup>dim</sup> cells at three time points. All of the rats showed a similar percentage among treatments within the same time point. "a" indicates significantly greater than the control group at 9 week age ( $N = 4/\text{group}$ ). The error bar represents the SEM.

**Hepatic Concentration of PGE<sub>2</sub> and PGE<sub>2α</sub>.** The hepatic PGE<sub>2</sub> concentrations were similar among all groups at both 9 and 12 weeks of age, but hepatic PGE<sub>2</sub> concentration in rats fed both 8 and 25 ppm diets was significantly increased by ~50% as compared with the control group at 20 weeks of age. With the age increase, PGE<sub>2</sub> concentrations in rats at 12 or 20 weeks of age were significantly greater than that of rats fed the same diets at 9 weeks of age (Figure 5). The PGE<sub>2α</sub> concentrations were not different among treatments at any time. Hepatic PGE<sub>2α</sub> was greater in all treatments at 12 and 20 weeks of age than at 9 weeks of age (Figure 6).

**AHF Indicated by PGST or GGT Staining.** There were no detectable PGST- or GGT-positive AHF in any treatment group at 9 or 12 weeks of age. At 20 weeks of age, half of the rats fed 25 ppm of FB<sub>1</sub> had PGST- and GGT-positive AHF. The average PGST-positive AHF area percentage was  $0.4 \pm 0.7$ , and the average GGT-positive AHF area was  $0.6 \pm 0.8$ . There were no detectable PGST- or GGT-positive AHF foci in the groups fed 25 ppm of FB<sub>1</sub> reacted with glucose, 8 ppm of FB<sub>1</sub>, or basal diet at 20 weeks of age (Table 2). Histopathology studies showed that all of the groups exhibited similar mild fatty changes in periportal hepatocytes



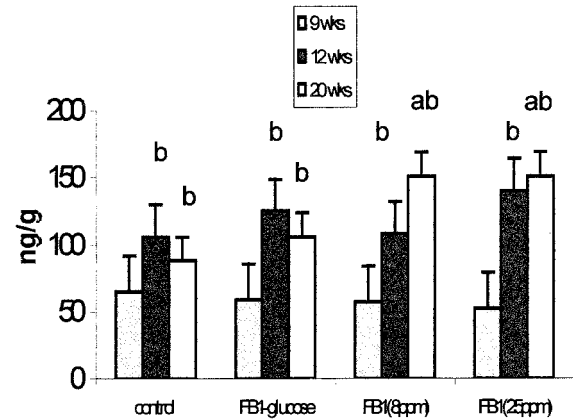
**Figure 4.** (A) Comparison of hepatic NK activity at three time points prior to covariation with the percentage of NKR-P1<sup>bright</sup> mononuclear cells. Activity was expressed as lytic units, calculated from the specific lysis curve. At 9 and 12 weeks of age, all of the rats exhibited similar NK lytic activity. At 20 weeks of age, the rats fed FB<sub>1</sub>-glucose exhibited significantly lower NK activity as compared with the rats fed 25 ppm of FB<sub>1</sub>. The error bar represents the SEM. (B) Comparison of hepatic NK activity at three age stages after covariation with the percentage of NKR-P1<sup>bright</sup> population. At three age stages, no difference was found among treatments within each age stage. The error bar represents the SEM. "a" indicates significant difference as compared with the group fed 25 ppm of FB<sub>1</sub> ( $N = 10$ /group).

and mild increases of lipofuscin in the epithelium of proximal convoluted tubules. No lesions were found in lung, brain, or tibia.

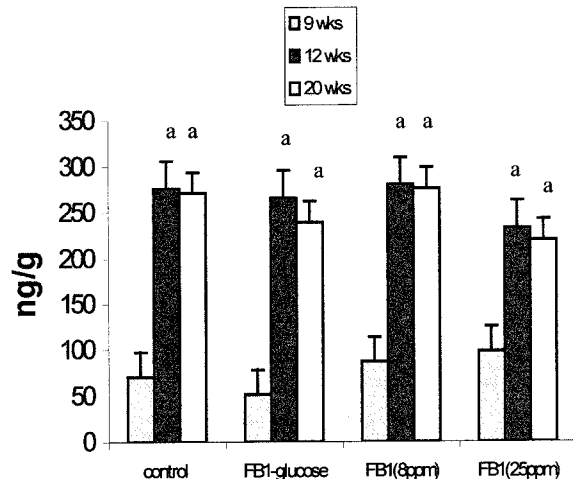
**Sphingolipid Analysis.** The levels of Sa, So, and the ratio Sa/So were not affected in any treatment group at 9 weeks of age. The Sa concentration as well as the ratios of Sa/So were significantly greater in the rats fed the 25 ppm FB<sub>1</sub> diet as compared with the control group at 12 or 20 weeks of age. The So concentration was unaffected among all treatment groups at 12 or 20 weeks of age (Table 3).

## DISCUSSION

This study demonstrated that subjecting FB<sub>1</sub> to a nonenzymatic browning reaction with glucose avoided FB<sub>1</sub> toxicity as reflected in plasma total cholesterol concentration, ALT activity, development of GGT- and PGST-positive AHF, concentration of endogenous hepatic PGE<sub>2</sub>, accumulation of Sa, or the Sa/So ratio. These results agreed with the findings of Lu et al. (13)



**Figure 5.** Comparison of the hepatic PGE<sub>2</sub> concentration in four treatment groups at three time points. The concentration of PGE<sub>2</sub> is expressed as nanograms per gram of liver tissue. At 9 weeks of age, there is no difference among the treatment groups. At 12 weeks of age, no difference was found among the treatment groups. At 20 weeks of age, the rats fed 8 or 25 ppm of FB<sub>1</sub> exhibited significantly greater concentration of PGE<sub>2</sub> as compared with the control group. Rats at both 12 and 20 weeks of age had greater PGE<sub>2</sub> concentration as compared with the same treatment at 9 weeks of age. The error bar represents the SEM. "a" indicates significantly greater than the control group at 20 weeks of age ( $N = 10$ /group). "b" indicates significantly greater than the 9 weeks of age group of the same treatment.



**Figure 6.** Comparison of the hepatic PGF<sub>2 $\alpha$</sub>  concentration in four groups at three time points. The concentration of hepatic PGF<sub>2 $\alpha$</sub>  was expressed as nanograms per gram of liver tissue. There is no difference among treatment groups at three time points. The rats at both 12 and 20 weeks of age had greater hepatic PGF<sub>2 $\alpha$</sub>  concentration as compared with the same treatment at 9 weeks of age. The error bar represents the SEM. "a" indicates significant difference as compared with rats of 9 weeks of age within the same treatment.

in which FB<sub>1</sub> was detoxified by reaction with fructose. Because the FB<sub>1</sub>-fructose and FB<sub>1</sub>-glucose products are likely to be similar, both reactions probably had similar detoxification effects. The present study showed a lack of toxicity of FB<sub>1</sub>-glucose over 15 weeks of treatment, compared with a lack of toxicity over 4 weeks in an earlier FB<sub>1</sub>-fructose detoxification study (13).

The effectiveness of the detoxification of FB<sub>1</sub> by reaction with glucose is probably not explained by diminished bioavailability. FB<sub>1</sub> when reacted with fructose had much greater absorption than did FB<sub>1</sub> (37). Because FB<sub>1</sub>-fructose and FB<sub>1</sub>-glucose products are likely to be similar, it is likely that FB<sub>1</sub>-glucose products



**Table 2. Placental Glutathione S-Transferase (PGST)- and  $\gamma$ -Glutamyl Transferase (GGT)-Positive Altered Hepatic Foci (AHF)<sup>a</sup>**

	no. of rats with PGST AHF	% PGST area	no. of rats with GGT AHF	% GGT area
control	0	0	0	0
FB <sub>1</sub> -glucose	0	0	0	0
FB <sub>1</sub> (8 ppm)	0	0	0	0
FB <sub>1</sub> (25 ppm)	5	0.4 ± 0.7	5	0.6 ± 0.8

<sup>a</sup> The AHF occurred only in rats fed 25 ppm of FB<sub>1</sub> at 20 weeks of age. The data entries in the table are expressed as mean ± SEM.

were also absorbed. The addition of glucose to FB<sub>1</sub> may have prevented the inhibitory binding of FB<sub>1</sub> to ceramide synthase, thought to be a pathway of FB<sub>1</sub> toxicity (14). The observation that sphingolipid ratios remained at control values in the rats exposed to FB<sub>1</sub>-glucose in this study further supports this assumption.

The rats fed the 25 ppm FB<sub>1</sub> diet had noticeably greater plasma total cholesterol concentration as compared with the control group and with the rats fed FB<sub>1</sub>-glucose at 12 weeks of age in the present study. The observation of FB<sub>1</sub>-induced hypercholesterolemia was reported in vervet monkeys (12), as well as in rats (11, 13). The reason that the increase of cholesterol in our experiment was not as great as that reported in earlier experiments might be that basal diets contained only 7% fat, which was much less than the fat content (20%) used by Lu et al. (13). The mechanism underlying the effect of FB<sub>1</sub> on plasma cholesterol is unknown. The increase in plasma total cholesterol by FB<sub>1</sub> might result from stimulation of cholesterol synthesis in hepatocytes or impaired cholesterol removal by liver. We also observed that the rats fed the 8 or 25 ppm FB<sub>1</sub> diet had significantly lower plasma total cholesterol level as compared with controls or rats fed FB<sub>1</sub>-glucose at 20 weeks of age. The cholesterol levels in primary rat hepatocytes were decreased under the effect of 500  $\mu$ M FB<sub>1</sub> (38). The mechanism of the decrease in the levels of cholesterol is not clear, but it could be the result of a decreased level of sphingomyelin (SM) in cell membranes that influenced cholesterol synthesis and/or metabolism.

The plasma ALT activity was significantly increased in rats fed 8 and 25 ppm of FB<sub>1</sub> as compared with the control group at 9 weeks of age. At 12 weeks of age, only the rats fed 25 ppm of FB<sub>1</sub> exhibited greater ALT activity as compared with the control group. This result is partially in agreement with the finding of Lu (37), who found that ALT activity in rats fed 25 ppm of FB<sub>1</sub> for 4 weeks increased significantly compared with controls but that ALT did not increase in rats fed a 12.5

ppm of FB<sub>1</sub> diet. This difference between our results and the results obtained by Lu may be explained by the greater time of FB<sub>1</sub> exposure in the present study, which might have caused more hepatocellular damage.

Groups fed 8 or 25 ppm of FB<sub>1</sub> but not the group fed FB<sub>1</sub>-glucose showed significantly greater endogenous hepatic PGE<sub>2</sub> as compared with the control group at 20 weeks of age. This result differed from the findings of Lu (39), in which rats fed 50 ppm of FB<sub>1</sub> showed greater amounts of PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub> , but no significant increase of PGE<sub>2</sub> or PGF<sub>2 $\alpha$</sub>  was observed in rats fed 25 or 12.5 ppm of FB<sub>1</sub>. As with ALT, the longer period of FB<sub>1</sub> exposure in the present study may have permitted a lower dose of FB<sub>1</sub> to increase PG levels. Also, as with ALT, we have previously proposed that increased PG production was a hallmark of promotion of rat hepatocarcinogenesis caused by FB<sub>1</sub> (13). However, in our experiment, only rats fed 25 ppm of FB<sub>1</sub> showed induction of AHF in the liver, yet rats fed 8 ppm of FB<sub>1</sub> also exhibited greater amounts of PGE<sub>2</sub> but had no induction of AHF. This suggests that the increase of PG production may precede the occurrence of preneoplasia.

The NKR-P1<sup>bright</sup> population in rats fed FB<sub>1</sub> or FB<sub>1</sub>-glucose was significantly greater than in the control group at 9 weeks of age. This might reflect a general immune response of the host to exogenous antigen. In vitro experiments have shown that the NK percentage significantly increased under the effect of antigen (40). At 12 weeks of age, all four groups had similar NKR-P1<sup>bright</sup> percentages. At 20 weeks of age, both FB<sub>1</sub>-fed groups had similar percentages of NKR-P1 cells compared with the control group, but the percentage of NKR-P1<sup>bright</sup> mononuclear cells in FB<sub>1</sub>-glucose-treated rats was significantly decreased as compared with the 25 ppm FB<sub>1</sub>-treated group. Before covariation with the percentage of NKR-P1<sup>bright</sup> population, all groups exhibited similar lytic activities at 9 or 12 weeks of age, but FB<sub>1</sub>-glucose-treated rats had significantly decreased NK lytic activity as compared with 25 ppm FB<sub>1</sub>-treated rats. After covariation with the percentage of NKR-P1<sup>bright</sup> population, all four groups had similar NK lytic activities per NK cell. Thus, FB<sub>1</sub>-glucose was not metabolically inert. It remains to be seen whether the suppression of NK cells by FB<sub>1</sub>-glucose is physiologically significant, and if so, by what mechanism this occurs. The inhibition of NK lytic activity in rats fed FB<sub>1</sub>-glucose at 20 weeks of age probably resulted from the decreased percentage of NKR-P1<sup>bright</sup> population.

Our results do not agree with the findings of Lu et al. (13), who showed that hepatic NK lytic activity was significantly inhibited when rats were fed 50 ppm of FB<sub>1</sub> as compared with control group. Lu et al. (13) proposed that the inhibition of NK activity might be through the

**Table 3. Comparison of Sphingosine (So), Sphinganine (Sa), and the Ratio of Sa/So at Three Time Points<sup>a</sup>**

	9 weeks of age (nmol/mL)			12 weeks of age (nmol/mL)			20 weeks of age (nmol/mL)		
	So	Sa	Sa/So	So	Sa	Sa/So	So	Sa	Sa/So
control	5.6 ± 1.2	0.7 ± 0.2	0.1 ± 0.1	6.5 ± 2.2	0.9 ± 0.3	0.12 ± 0.1	5.9 ± 2.5	1 ± 0.5	0.15 ± 0.1
FB <sub>1</sub> -glucose	6.5 ± 1.5	0.6 ± 0.2	0.1 ± 0.1	5.7 ± 2.5	0.8 ± 0.2	0.12 ± 0.1	5.8 ± 1.5	0.8 ± 0.5	0.12 ± 0.1
FB <sub>1</sub> (8 ppm)	5.4 ± 2.3	0.8 ± 0.2	0.15 ± 0.1	6.3 ± 3.0	1.3 ± 0.5	0.18 ± 0.2	6.5 ± 2.9	1.2 ± 0.8	0.2 ± 0.1
FB <sub>1</sub> (25 ppm)	5.5 ± 2.8	1.1 ± 0.3	0.2 ± 0.1	7.5 ± 3.5	3.5 ± 1.5 <sup>a</sup>	0.5 ± 0.2 <sup>a</sup>	5.8 ± 1.6	4.5 ± 1.5 <sup>b</sup>	0.8 ± 0.3 <sup>b</sup>

<sup>a</sup> The concentration of the sphingolipids is expressed as nanomoles per gram of liver tissue. At 9 weeks of age Sa, So, and the ratio were similar among treatment groups. At 12 weeks of age the rats fed 8 or 25 ppm of FB<sub>1</sub> diet exhibited greater Sa concentration and increased ratio of Sa/So as compared with the control group. At 20 weeks of age, the rats fed 8 and 25 ppm of FB<sub>1</sub> diet exhibited greater Sa concentration and increased ratio of Sa/So as compared with the control group. The data entries in the table are expressed as mean ± SEM. "a" indicates significant difference as compared with the control group at 12 weeks of age (N = 5/group), "b" indicates significant difference as compared with the control group at 20 weeks of age (N = 10/group).

effect of increased PG production (41, 42). A dose-response inhibition by PGE<sub>2</sub> of NK activity had been reported by Liu et al. (43) in cocultures of hepatic NK cells with PGE<sub>2</sub> in vitro. In our experiment, although FB<sub>1</sub> feeding increased production of PGE<sub>2</sub>, NK activity was not affected. The reason that we did not observe the inhibition of NK lytic activity might be that less (25 ppm) FB<sub>1</sub> as well as less fat (7%) was fed to the rats as compared with the experiment of Lu et al. (13; 50 ppm of FB<sub>1</sub> and 20% fat in the diet). A possible explanation for the lack of effect of FB<sub>1</sub> on NK activity in our study may lie in interactions between carcinogenesis and NK activity. Lee et al. (44) showed that, in male F344/rats given 40 ppm of DEN in drinking water for 10 weeks, as PGST-positive AHF developed, splenic NK activity changed. After 5 weeks, DEN-treated and control rat spleen NK activities were similar, but at 10 weeks, NK activity was significantly greater in DEN-treated rats compared with control rats. At 20 weeks, DEN-treated rats had significantly lower NK activity than did control rats. Lower NK activity was accompanied by PGST-positive AHF. Lu et al. (13) showed a 20-fold greater area of PGST-positive AHF as compared with our study, which implies that with greater extent of carcinogenesis, NK lytic activity was affected adversely.

In the present study, only half of the rats fed 25 ppm of FB<sub>1</sub> showed PGST- and GGT-positive AHF, in comparison with Lu (39) in which 11 of 12 animals fed 25 ppm of FB<sub>1</sub> showed PGST- and GGT-positive AHF. The area occupied by AHF was much greater than in our study. The fat content of the diet was 20% (39), which was much greater than the 7% fat content in our experimental diet. High levels of dietary fat markedly shorten the time between exposure to ultraviolet light and its induction of skin tumors (45). Many studies have shown that dietary fat is a tumor promoter (46, 47). In our study, although the feeding time was longer than in the study by Lu (39), the lesser dietary fat in the present study might have produced fewer and smaller GGT- and PGST-positive AHF.

Increases in free sphingosine and sphinganine in animal tissue, serum, and urine have been used extensively as an experimental biomarker for fumonisin exposure (48). In our study, the accumulation of Sa and the increase of the Sa/So ratio only appeared in rats fed 25 ppm of FB<sub>1</sub> at 12 and 20 weeks of age. This did not agree with previous observations that animals exposed to FB<sub>1</sub> developed altered Sa/So ratios before other signs of toxicity were observed (14, 49). In the present study, we observed increased ALT in both FB<sub>1</sub>-treated groups at 9 weeks of age and in rats fed 25 ppm of FB<sub>1</sub> at 12 weeks of age, but we did not observe the Sa/So ratio change at 9 weeks of age. Our study agreed with the findings of Gelderblom et al. (50), who indicated that no significant change in Sa/So ratio was observed at the lowest dietary level of FB<sub>1</sub> that induced cancer promotion and inhibition of cell proliferation. Further studies are needed to determine the role of the Sa/So ratio in fumonisin toxicity and how the effect of FB<sub>1</sub> on this ratio interacts with other dietary components.

Whereas both FB<sub>1</sub>-glucose and FB<sub>1</sub> (8 ppm) diets contained the same amount (8 ppm) of free FB<sub>1</sub>, only rats fed FB<sub>1</sub> (8 ppm) exhibited adverse effects, which was reflected in increased ALT activity, total cholesterol, and PGE<sub>2</sub> concentrations. Rats fed FB<sub>1</sub>-glucose, which contained 8 ppm of free FB<sub>1</sub>, showed the same lack of toxic effects as exhibited by control rats. It seemed that

FB<sub>1</sub>-glucose prevented adverse effects of FB<sub>1</sub> when low concentrations of FB<sub>1</sub> and FB<sub>1</sub>-glucose were fed together. Perhaps the FB<sub>1</sub>-glucose prevented the free FB<sub>1</sub> from interacting with its site of action.

In conclusion, reaction of FB<sub>1</sub> with glucose detoxified FB<sub>1</sub> in a rat hepatocarcinogenesis model. The mechanism might be that the binding ability of FB<sub>1</sub>-glucose to ceramide synthase was inhibited, as was reflected in the lack of accumulation of Sa when FB<sub>1</sub>-glucose was fed. The sphinganine/sphingosine ratio increased when the same amount of FB<sub>1</sub> was fed (25 ppm). Further study is needed to determine the bioavailability of FB<sub>1</sub>-glucose and to determine its effects in other animal models.

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