Reaction with Fructose Detoxifies Fumonisin B_1 while Stimulating Liver-Associated Natural Killer Cell Activity in Rats

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Fumonisin B_1 (FB_1) was reacted with fructose in an attempt to detoxify this mycotoxin. Fischer 344/N rats were initiated with diethylnitrosamine (15 mg/kg body weight) and then fed 69.3 μmol FB_1/kg diet or 69.3 μmol FB_1 reacted with fructose (FB_1−fructose)/kg diet for 4 weeks. In comparison with the rats fed basal diet or FB_1−fructose, the FB_1-fed rats had significantly increased plasma cholesterol ($P < 0.01$), plasma alanine aminotransferase activity ($P < 0.05$), and endogenous hepatic prostaglandin production ($P < 0.05$). Placental glutathione S-transferase-positive and γ-glutamyl transferase-positive altered hepatic foci occurred only in the FB_1-fed rats. Liver-associated natural killer (NK) cell activity was significantly decreased in the FB_1-fed rats and increased in the group fed FB_1-fructose, as compared with the basal group ($P < 0.03$). Therefore, modifying FB_1 with fructose seems to prevent FB_1-induced hepatotoxicity and promotion of hepatocarcinogenesis while stimulating liver-associated NK cell activity in rats.

Keywords: *Fumonisin B_1; fructose; prostaglandins; NK cell; placental glutathione S-transferase*

INTRODUCTION

Fumonisin B_1 (FB_1), a mycotoxin produced by the commonly occurring corn fungi, *Fusarium moniliforme* and *Fusarium proliferatum*, was hepatocarcinogenic in rats when fed in amounts of 69.3 μmol/kg diet (50 ppm) for approximately 2 years (Gelderblom et al., 1991). Human esophageal cancer rate was high in areas where FB_1 concentration in corn reached approximately 11.1 μmol/kg (Sydenham et al., 1990a). Corn products for human and animal consumption were determined to contain 0.3−4.2 μmol FB_1/kg (Hopmans and Murphy, 1993; Murphy et al., 1993; Sydenham et al., 1990b).

The hepatocarcinogenic effect of FB_1 can be evaluated *in vivo* by measuring changes in placental glutathione *S*-transferase (PGST) (Lebepe-Mazur et al., 1995) and γ-glutamyl transferase (GGT) activity (Gelderblom et al., 1988), which are markers of altered hepatic foci (AHF) (Sato et al., 1984; Goldsworthy et al., 1985). Also, plasma alanine aminotransferase (ALT) activity was related to fumonisin hepatotoxicity (Voss et al., 1992) and hepatocarcinogenesis in rats (Hendrich et al., 1993). Increased plasma total cholesterol was also an indicator of FB_1 toxicity in vervet monkeys (Fincham et al., 1992) and in rats (Hendrich et al., 1993). Production of prostaglandin E_2 (PGE_2) and other eicosanoids from the cyclooxygenase pathway was stimulated in Yoshida hepatoma cells in rats and in human hepatocellular carcinomas (Trevisani et al., 1980; Hanai et al., 1993). Effects of FB_1 on PG production in rat liver have not been investigated yet.

Fumonisin B_1 can alter immunological functions mediating antitumor mechanisms. Macrophage structure and phagocytic function were down-regulated *in vitro* by FB_1 (Chatterjee and Mukherjee, 1994; Chatterjee et al., 1995) as was lymphocyte proliferation in response to lipopolysaccharides (LPS) (Dombrink-Kurtzman et al., 1994). The effects of *in vivo* administration of FB_1 produced both increases and decreases in plaque-forming cell response in BALB/C mice depending upon the timing of the fumonisin injections and the number of injections (Martinova and Merrill, 1995).

Efforts have been devoted to detoxifying FB_1 in several ways. Thermostability of FB_1 proved to be great, because nearly 85% of FB_1 was recovered after different heat treatments: 75 °C for 135 min, 100 °C for 45 min, and 125 °C for 5 min (Dupuy et al., 1993). Treatment of fumonisin-contaminated corn with 2% ammonia for 4 days, a process that detoxified aflatoxin B_1, led to slight reduction in the concentration of FB_1 without decreasing the toxicity in rats (Norred et al., 1991). Hydrolyzed FB_1, which was produced by boiling *F. proliferatum*-contaminated corn in 1.2% calcium hydroxide solution for 1 h, was similar in toxicity to FB_1 when the nutritional status of rats was adequate (Hendrich et al., 1993). *In vitro* toxicity studies on several analogs of FB_1 showed that the analogs containing FB_1's amine group and the tricarballylic side chains were more toxic than FB_1, whereas the analog containing only the tricarballylic side chains was not toxic (Kraus et al., 1992). The *N*-acetyl derivative of FB_1 at 1 mM was less toxic in primary rat hepatocytes than FB_1 at the same concentration. In addition, rats fed *N*-acetyl-FB_1 at about 1.3 mmol/kg diet did not exhibit hepatic neoplastic nodules or increased hepatic GGT activity (Gelderblom et al., 1993). Therefore, FB_1's amine group is likely to be critical for its toxicity. The *N*-acetyl-FB_1 may be produced during the isolation and purification of FB_1 from corn cultures of *F. moniliforme*. This paper describes a dedicated and more practical method to block FB_1's amine group by reacting the amine group with reducing sugars such as fructose in a Maillard reaction. It was hypothesized that modifying FB_1 with fructose would reduce or prevent promotion of hepatocarcinogenesis and hepatotoxicity in rats.

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MATERIALS AND METHODS

Preparation of Crude Fumonisin B_1. Fumonisin B_1 is a class 2B carcinogen (International Agency for Research on Cancer, 1993), and standard safety precautions were taken during its handling.

F. proliferatum strain M5991 (from Dr. Paul Nelson, Pennsylvania State University, College Station, PA) predominantly produces FB_1. Sterile, aflatoxin-free corn was inoculated with lyophilized cultures of *F. proliferatum* strain M5991 which had been reconstituted in pH 7.4 phosphate-buffered saline. This corn culture was incubated at 22 °C in the dark for 3 weeks. After incubation, freeze-dried corn culture was ground and extracted with 1:1 acetonitrile:water. The extract was partitioned with ethyl acetate, after which the water phase was loaded onto a 1 kg XAD-16 column (7.5 × 100 cm, Sigma Chemical Co., St. Louis, MO). The column was washed with water, and FB_1 was eluted with 4 L of methanol. The eluate was dried under vacuum, reconstituted in water, and loaded onto a Lobar LiChroprep RP-8 column (25 × 310 mm; EM Separations, Gibbstown, NJ). The column was washed with 20% acetonitrile:80% water containing 0.1% trifluoroacetic acid (TFA), and FB_1 was eluted with 50% acetonitrile:50% water containing 0.1% TFA. The purity of the isolated FB_1 was determined by analytical HPLC of the *o*-phthaldialdehyde (OPA) derivative (Hopmans and Murphy, 1993). The FB_1 standard curve was prepared with FB_1 generously donated by Dr. P. G. Thiel (Research Institute for Nutritional Diseases, South African Medical Research Council, South Africa). The isolated FB_1 was approximately 40% pure, containing 1.5 g of FB_1. The preparation also contained trace amounts of fumonisins B_2 and B_3.

Preparation of Highly Purified Fumonisin B_1. Liquid cultures were prepared as in Dantzer et al. (1996a) by inoculating capped baffled Erlenmeyer flasks containing 500 mL of modified Myro medium with a 4 day shake flask culture of *F. proliferatum* strain M5991. Fumonisin B_1 was isolated and purified by the procedure as described in Dantzer et al. (1996b). Briefly, harvested liquid cultures were filtered and run over a series of chromatographic steps, including Amberlite XAD-16, reverse phase Lobar C_8, ion exchange DEAE Sepharose, and reverse phase YMC C_{18}, until FB_1 purity >95% was obtained. Purified FB_1 solutions were freeze-dried, and a dry weight was determined. The purified FB_1 was dissolved volumetrically and assayed for FB_1 as described by Hopmans and Murphy (1993). The mass of FB_1 was calculated from the standard curve and compared with the weighed mass of freeze-dried FB_1. This ratio was taken as the percent purity of FB_1 (Dantzer et al., 1996b). The purity of the FB_1 was confirmed to be >95% by Dr. Ronald Plattner (USDA−ARS, National Center for Utilization Research, Peoria, IL).

Preparation of Fumonisin B_1−Fructose Adduct. Both crude and highly purified FB_1 were conjugated with fructose as described by Murphy et al. (1995). Briefly, 725 μM FB_1 in 50 mM potassium phosphate buffer, pH 7.0, containing 1 M fructose, was heated for 48 h at 80 °C. Less than 5% of the FB_1 was left unreacted with fructose. Fumonisin B_1−fructose was hydrolyzed by refluxing in 2 N KOH for 2 h at 100 °C, after which the pH was adjusted to 2.8 with 2 N HCl. All of the FB_1 could be recovered as hydrolyzed FB_1. In addition, FB_1 was heated under the same conditions without fructose and retained 100% reactivity with OPA.

Diets. Three experimental diets were fed to rats in each of the two studies. Basal diet supplying 40% of energy as fat was modified from AIN-76 (American Institute of Nutrition, 1977) in the study feeding crude FB_1, or from AIN-93G (American Institute of Nutrition, 1993) in the study feeding highly purified FB_1 (Table 1). Crude or highly purified FB_1-containing diets were prepared by incorporating 69.3 μmol FB_1/kg diet into the basal diets. Crude or highly purified FB_1 reacted with fructose were incorporated into the basal diets at a level equivalent to 69.3 μmol FB_1/kg diet. On the basis of the proposed reaction between FB_1 and a reducing sugar such as fructose (Murphy et al., 1996), it was estimated that approximately 17 g of unreacted fructose was added per kilogram of FB_1−fructose diet. All the diets were stored at 4 °C.

Table 1. Basal Diet Composition[a]

ingredient	crude FB_1 expt (g/kg)	purified FB_1 expt (g/kg)
beef tallow	139.6	139.6
corn oil	66.5	
soybean oil		66.5
casein	224.1	224.1
corn starch	228.5	228.5
dextrose	224.1	224.1
cellulose	56.0	56.0
vitamin mix (AIN-76)	11.2	
vitamin mix (AIN-93G-VX)		10.0
mineral mix (AIN-76)	39.2	
mineral mix (AIN-93G-MX)		39.2
$CaCO_3$	5.0	
choline chloride	2.2	
L-methionine	3.4	
ascorbate	0.1	
L-cystine		3.0
choline bitartrate		2.5
TBHQ		0.014

[a] Basal diet modified from AIN-76 was used in crude FB_1 experiment; basal diet modified from AIN-93G was used in highly purified FB_1 experiment.

Table 2. Differences in Body Weight but Not Feed Intake among Female Rats Fed Fumonisin B_1 (FB_1), FB_1 Reacted with Fructose (FB_1−Fructose), or a Basal Diet

	group		
	basal	FB_1−fructose	FB_1
total feed intake (g)	366 ± 37	354 ± 43	320 ± 25
average daily feed intake (g/d)	13 ± 1	13 ± 2	11 ± 1
final body weight (g)	191 ± 19	191 ± 10	171 ± 14[a]
body weight increase (g)	128 ± 15	128 ± 8	108 ± 15[a]

[a] Significantly different from the basal group, $P < 0.05$.

Animals. The use of animals and the experimental procedures were approved by the Iowa State University Animal Care Committee. In the experiment with crude FB_1, 20 10-day-old male F344/N rats obtained from Harlan Sprague−Dawley (Madison, WI) were injected intraperitoneally with diethylnitrosamine (DEN, 15 mg/kg body weight) in 0.1 mL of corn oil. At 3 weeks of age, the weaned rats were randomly assigned into one of the three treatment groups with six or seven rats each. In the experiment with highly purified FB_1, 39 10-day-old female F344/N rats obtained from Harlan Sprague−Dawley were initiated as described above, randomly assigned into the treatments with 12−15 rats each. In both experiments, rats were given free access to the experimental diets and water for 4 weeks in an animal facility with a 12-h light/dark cycle maintained at 22−25 °C and 50% humidity. Body weight and feed intake of the female rats were recorded weekly.

Plasma and Liver Sample Preparations. Part of the plasma obtained from heparinized blood was analyzed within 24 h for ALT activity. The remaining plasma was stored at −80 °C and later analyzed for plasma total cholesterol.

The liver was perfused with 40 mL of Hank's balanced salt solution containing 1% EDTA and 25 mM HEPES. The perfusate (10−12 mL) containing red blood cells, Pitt cells, and other leukocytes was used as the source of effectors in the NK cell activity assay.

Each of the three largest lobes of the liver 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, two 10-μm serial sections were cut with a Histostat Microtome (Model 855, Leica Inc., Deerfield, IL) for later stainings for GGT activity and PGST.

From each rat, 0.5 g of minced liver portions was immediately homogenized in an ice bath with 10 passes of a

Table 3. Increased Plasma Total Cholesterol Concentration, Plasma Alanine Aminotransferase (ALT) Activity, and Endogenous Hepatic Prostaglandin (PG) Production in Rats Fed Crude or Highly Purified Fumonisin B_1 (FB_1), Compared with Feeding FB_1 Reacted with Fructose (FB_1−Fructose) or a Basal Diet

	plasma cholesterol (mg/dL)	plasma ALT activity (units/L)	hepatic $PGF_{2\alpha}$ (ng/g)	hepatic PGE_2 (ng/g)
	Expt 1. Male Rats Fed Crude FB_1			
basal ($n = 6$)	143 ± 24	58 ± 13	49 ± 8	4 ± 1
FB_1 ($n = 7$)	261 ± 45[b]	105 ± 58[a]	77 ± 22[a]	7 ± 2
FB_1−fructose ($n = 7$)	145 ± 33	51 ± 8	53 ± 13	5 ± 3
	Expt 2. Female Rats Fed Highly Purified FB_1			
basal ($n = 15$)	113 ± 28	45 ± 11	5 ± 2	2 ± 1
FB_1 ($n = 12$)	261 ± 68[b]	75 ± 22[a]	10 ± 4[a]	4 ± 2[a]
FB_1−fructose ($n = 12$)	121 ± 27	50 ± 11	5 ± 3	2 ± 1

[a] Significantly different from the basal group in respective study, $P < 0.05$. [b] Significantly different from the basal group in respective study, $P < 0.01$.

Potter-Elvenhjem homogenizer in 5 mL, pH 7.4, 50 mM potassium phosphate buffer containing 4.2 mM acetylsalicylic acid. The liver homogenates were frozen on dry ice and stored at −80 °C for later analyses of endogenous hepatic $PGF_{2\alpha}$ and PGE_2 levels.

Plasma Total Cholesterol Concentration and Alanine Aminotransferase Activity. Plasma total cholesterol concentration was determined by using Sigma diagnostic kit, procedure 352-3 (Sigma). Plasma ALT activity was measured by using Sigma diagnostic kit for glutamate/pyruvate transaminase optimized ALT assay (Sigma).

Histochemical Staining and Computerized Stereology of AHF. One of the frozen serial sections was stained for the presence of PGST-positive altered hepatic foci (AHF). Placental glutathione S-transferase was detected by the peroxidase-anti-peroxidase (PAP) method using a Vectastain ABC avidin−biotin universal rabbit PAP kit (Vector Laboratories, Burlingame, CA). Placental glutathione S-transferase was purified by the method of Sato et al. (1984), and rabbit anti-rat PGST antiserum was prepared as described by Hendrich and Pitot (1987).

The second frozen serial section was stained for GGT activity as described by Rutenburg et al. (1968). The substrate for GGT was γ-glutamyl-4-naphthylamide (GMNA) (United States Biochemical Corp., Cleveland, OH).

Altered hepatic foci were quantified via computerized stereology by slight modification of a program which was developed by Dr. Harold Campbell at McArdle Laboratories, University of Wisconsin, Madison (Campbell et al., 1986). Two magnified images of the serial sections stained for GGT activity and PGST were projected onto a Summagraphics Microgrid II digitizer screen and automatically plotted on an HP 9872C plotter controlled by an HP9845B computer (Hewlett-Packard, Palo Alto, CA) connected to an LA120 printer (Digital Equipment Corp., Maynard, MA) to quantify AHF.

Radioimmunoassay for Endogenous Liver Prostaglandin $F_{2\alpha}$ and E_2 Levels. Endogenous liver $PGF_{2\alpha}$ and PGE_2 levels were determined by radioimmunoassay according to the method of McCosh et al. (1976). Anti-rat $PGF_{2\alpha}$ rabbit antiserum was purchased from Sigma. Anti-rat PGE_2 rabbit antiserum was a gift from Dr. Jaqueline Dupont, USDA−ARS, Beltsville, MD. Goat anti-rabbit gamma globulin (ARGG) was obtained from Western Chemical Research Corp., Fort Collins, CO. Concentrated [^{3}H]$PGF_{2\alpha}$ and [^{3}H]PGE_2 with specific activities of 168.0 and 154.0 Ci/mmol, respectively, were purchased from Dupont New England Nuclear, Boston, MA. Standards of PGF_{2a} and PGE_2 were obtained from Sigma. Scintiverse BD (Fisher Chemical, Fair Lawn, NJ) was added to each tube before counting. Sample-containing tubes were run in duplicate. Total count tubes and background tubes were counted in triplicate. Six replicates were made to determine total binding. Along with the samples, a standard curve was run in duplicate, one set at the beginning of the sample run and the other set at the end. Radioactivity was determined as cpm using a Packard liquid scintillation analyzer model 1900 TR (Packard Instrument Co., Downers Grove, IL).

Natural Tumor Cytotoxicity Assay. In the experiment with purified FB_1, tumor cytotoxicity of liver NK cells was assessed on the first seven animals sacrificed from each group due to time constraints in performing the assay. The liver perfusate containing Pitt cells was layered over 3 mL of Accupaque (Accurate Chemical Co., Westbury, NY). The cells were centrifuged for 15 min at 15 000 rpm in a swinging bucket centrifuge (Model CR312, Jouan, Winchester, VA). The cells at the interface of the density gradient media were collected, washed twice with complete media (RPMI-1640 supplemented with 50 μg/mL gentamicin, 25 mM HEPES, 2 mM L-glutamine (all from Life Technologies, Gaithersburg, MD) and 10% fetal bovine serum (FBS) (JRH Scientific, Lenexa, KS)), and enumerated on a Celltrack II (NOVA Biomedical, Waltham, MA). The cells from each sample were diluted to 5×10^6 and plated in 96-well plates (Model 3595, Costar, Cambridge, MA) to obtain 20, 10, 5, and 2.5×10^5 cells (effectors)/well in triplicate. The targets for the assay were YAC-1 cells, which had been labeled with 200 μCi of ^{51}Cr (401 mCi/mg, Dupont New England Nuclear) for 70 min, and maintained in complete media. The targets were washed three times prior to dilution, and 10^4 targets were plated in each well of the NK assay including control wells to determine spontaneous and maximum release. The plates were incubated for 4.5 h in a humidified CO_2 incubator (5% CO_2, 95% air) (Fisher Scientific). At the end of the incubation the plate was centrifuged at 500 rpm for 5 min, and 100 μL of supernatant was collected to determine the amount of ^{51}Cr released by dying cells using a Gamma Trac 1191 (TM Analytic, Inc., Elk Grove Village, IL). The cpm detected were used to calculate the lytic units (LU) of activity at 20% lysis in 10^7 effectors using a computer program.

Statistical Analysis. The liver $PGF_{2\alpha}$ and PGE_2 concentrations were determined by a computer program based on a logit transformation of the standard curve (Duddleson et al., 1972). One-way ANOVA was performed to analyze feed consumption, body weight, plasma total cholesterol level and ALT activity, endogenous hepatic PGF_{2a} and PGE_2 concentrations, and natural tumor cytotoxicity, using the Statistical Analysis System (Cary, NC). Student's *t*-test was performed to compare group differences after ANOVA. A P value of <0.05 was considered to be statistically significant. For the analysis of computerized stereology of AHF, only the means and standard deviations were given because the means equalled zero in basal and FB_1−fructose groups.

RESULTS

Experiment 1. Male Rats Fed Crude Fumonisin B_1. In comparison with the rats fed basal diet and the rats fed FB_1−fructose, the FB_1-fed rats had significantly increased plasma total cholesterol concentration and ALT activity by 85% ($P < 0.01$) and 100% ($P < 0.05$), respectively (Table 3).

The $PGF_{2\alpha}$ concentration was significantly increased by approximately 51% in the rats fed FB_1 as compared with those in the other groups ($P < 0.05$) (Table 3). There were no significant differences in PGE_2 levels among the groups (Table 3).

All FB_1-fed rats had both PGST- and GGT-positive AHF. The average PGST-positive AHF area percentage

Table 4. Placental Glutathione *S*-Transferase (PGST)- and γ-Glutamyl Transferase (GGT)-Positive Altered Hepatic Foci (AHF) Occurred Only in Rats Fed either Crude Fumonisin B_1 (FB_1) or Highly Purified FB_1

	no. of PGST lesions	no. of GGT lesions	% of PGST lesion area	% of GGT lesion area
Expt 1. Male Rats Fed Crude FB_1				
basal ($n = 6$)			0	0
FB_1 ($n = 7$)			5 ± 6	1 ± 1
FB_1−fructose ($n = 7$)			0	0
Expt 2. Female Rats Fed Highly Purified FB_1				
basal ($n = 15$)	0	0	0	0
pure FB_1 ($n = 12$)	50 ± 17	45 ± 13	68 ± 18	61 ± 13
FB_1−fructose ($n = 12$)	0	0	0	0

was 5.0 ± 6.3, and the average GGT-positive AHF area percentage was 1 ± 1 in the rats fed FB_1 at 69.3 μmol/kg diet (Table 4). There were no detectable PGST- or GGT-positive AHF in the group fed 69.3 μmol/kg FB_1 reacted with fructose or in the control group (Table 4).

Experiment 2. Female Rats Fed Highly Purified Fumonisin B_1. The average daily feed intake in FB_1 group, FB_1-fructose group and basal group was 11 ± 1, 13 ± 2, and 13 ± 1 g, respectively. There were no significant differences in the total feed intake or the average daily feed intake among the three groups (Table 2).

The rats in the FB_1-treated group had significant reductions in both body weight (by 12%, $P < 0.05$) and body weight gain (by 16%, $P < 0.05$) as compared with the rats in the groups fed FB_1−fructose and basal diet (Table 2).

In comparison with the basal group and the group fed FB_1-fructose, rats fed FB_1 at 69.3 μmol/kg diet had significantly increased plasma total cholesterol concentration and ALT activity by about 2.3-fold ($P < 0.01$) and 1.7-fold ($P < 0.05$), respectively (Table 3).

The $PGF_{2\alpha}$ and PGE_2 concentrations were both significantly increased by 2-fold in rats fed 69.3 μmol/kg FB_1-containing diet as compared with those in the other groups ($P < 0.05$) (Table 3).

All of the rats fed FB_1 developed PGST- and GGT-positive AHF. The average number of PGST-positive AHF and the average PGST-positive AHF area percentage were 50 ± 17 and 68 ± 18, respectively, and the average number of GGT-positive AHF and the average GGT-positive AHF area percentage were 45 ± 13 and 61 ± 13, respectively, in the rats fed FB_1 (Table 4). There were no detectable PGST- or GGT-positive AHF in either the group fed FB_1−fructose or basal diet (Table 4).

Compared with the control rats, tumor cytotoxicity was significantly suppressed in animals fed FB_1 ($P < 0.03$) and significantly elevated in animals fed FB_1−fructose diet ($P < 0.01$) (Figure 1). Thus, feeding highly purified FB_1 or FB_1−fructose induced significant and opposing changes in liver tumor cytotoxicity.

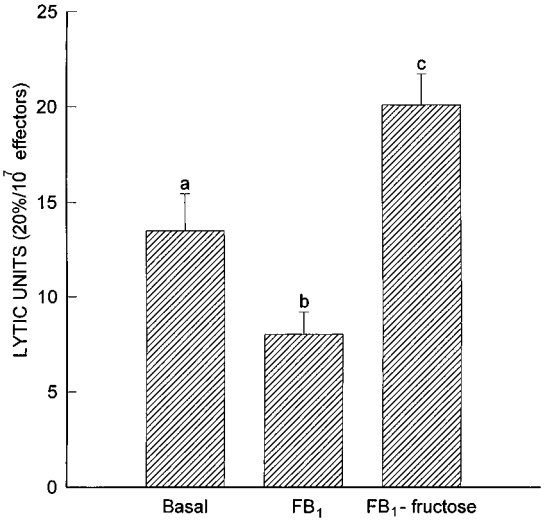

Figure 1. Tumor cytotoxicity of liver-associated NK cells against YAC-1 target cells, expressed as mean lytic units (LU) ± SE ($n = 7$/group), in female rats fed fumonisin B_1 (FB_1), FB_1 reacted with fructose (FB_1−fructose), or a basal diet. Treatments marked by different letters were significantly different from basal group: b, $P < 0.03$; C, $P < 0.01$.

DISCUSSION

These studies demonstrated that subjecting FB_1 to nonenzymatic browning conditions with fructose eliminated FB_1 toxicity as reflected in body weight, plasma total cholesterol concentration, and ALT activity, development of GGT- and PGST-positive AHF, and concentrations of endogenous hepatic $PGF_{2\alpha}$ and PGE_2. Loss of amine group reactivity toward OPA was taken as an indication that the amine had condensed with fructose (Murphy et al., 1995). Results with either crude or highly purified FB_1 were generally similar, showing detoxification of FB_1, because in both cases FB_1 reacted with fructose did not promote hepatocarcinogenesis in rats and did not increase plasma cholesterol concentration, plasma ALT activity, or hepatic $PGF_{2\alpha}$, whereas feeding crude or highly purified FB_1 significantly increased all of these parameters compared with rats fed a basal diet (Tables 3 and 4). The greater development of PGST- and GGT-positive AHF, by 5- and 13-fold, respectively, caused by highly purified FB_1 compared with crude FB_1 (Table 4), may be due simply to inherent variability of the two cohorts of rats in their responsiveness to the carcinogenesis protocol used. Rats are highly variable in individual response to such protocols. Other signs of FB_1 toxicity were quite similar whether crude or purified FB_1 was fed. This result may suggest that females are more susceptible to promotion of cancer by FB_1 than the males, but no other studies have examined this.

The chemical modification of FB_1 by fructose by a Maillard reaction probably caused the formation of a Schiff's base, FB_1−fructose adduct, in which the FB_1 amine group was combined with the fructose ketone. The addition of the bulky fructose did not cause FB_1 to be less absorbed in the intestine even though this may increase both water solubility and molecular size (Hopmans et al., submitted). At the molecular level, FB_1 toxicity is most likely due to its inhibition of ceramide synthase, a key enzyme in *de novo* sphingolipid biosynthesis (Wang et al., 1991). Perhaps the presence of fructose blocked the inhibitory binding of FB_1 to ceramide synthase.

The putative effectiveness of the detoxification of FB_1 by reaction with fructose suggested that the amine group of FB_1 was critical to FB_1 toxicity. This was in accordance with another study in which the *N*-acetyl

derivative of FB_1 showed no hepatotoxicity and no hepatocarcinogenicity (Gelderblom et al., 1993).

The significant reductions in body weight and body weight gain of female rats fed FB_1 compared with the other groups were accompanied by normal feed consumption (Table 2). This did not agree with another study in which both body weight and feed intake in male Sprague−Dawley rats were significantly decreased after intraperitoneal injection of FB_1 (Bondy et al., 1995). Neither body weight nor feed intake was significantly decreased after FB_1 consumption by male and female Fischer 344 rats (Voss et al., 1995). The difference between our results and the others cannot be readily explained.

Fumonisin B_1-induced hypercholesterolemia as observed in the present studies (Table 3) was reported in vervet monkeys (Fincham et al., 1992), as well as in rats fed FB_1 (Hendrich et al., 1993). Hypercholesterolemia is an observed effect of other tumor-promoting agents and toxicants, such as phenobarbital (Katayama et al., 1991). The hypercholesterolemic mechanism of FB_1 is unknown.

In agreement with the findings of Hendrich et al. (1993), FB_1 fed to rats at 69.3 μmol/kg diet for 4 weeks increased not only plasma total cholesterol but plasma ALT activity as well (Table 3). Elevated plasma ALT activity indicated hepatocyte membrane damage which led to the leakage of ALT into the blood. Such damage was associated with the development of AHF caused by FB_1 (Hendrich et al., 1993).

Placental glutathione *S*-transferase-positive AHF and GGT-positive AHF were found only in FB_1-fed rats in both studies (Table 4). Placental glutathione *S*-transferase and GGT are useful markers of FB_1 hepatocarcinogenesis. The induction of PGST could be demonstrated in single putatively initiated hepatocytes within 48 h after DEN treatment (Moore et al., 1987). Both PGST and GGT persisted during hepatocarcinogenesis (Hendrich and Pitot, 1987). In the present study, placental glutathione S-transferase- and GGT-positive AHF were detected after 4 weeks of FB_1 treatment. This indicated that both PGST and GGT were induced at an early stage of hepatocarcinogenesis and could serve as sensitive markers of FB_1-induced hepatocarcinogenesis. Because PGST- and GGT-positive AHF were virtually undetectable in DEN-initiated rats fed FB_1 reacted with fructose, FB_1 promotion of carcinogenesis may be blocked by modifying FB_1 with fructose.

Only FB_1-treated rats showed significantly greater amounts of endogenous $PGF_{2\alpha}$ and PGE_2 compared with the control group, and PGST- and GGT-positive AHF were only present in FB_1-fed rats. The elevation of hepatic $PGF_{2\alpha}$ and PGE_2 concentrations paralleled the induction of AHF in the liver. Therefore, increased PG production is related to promotion of rat hepatocarcinogenesis caused by FB_1. Both $PGF_{2\alpha}$ and PGE_2 may contribute to cell proliferation in the liver during FB_1 treatment. Prostaglandins of the F series stimulated the proliferation of neonatal rat hepatocytes (Armato and Andreis, 1983). Prostaglandins including $PGF_{2\alpha}$ and PGE_2 also played a role in proliferation of hepatoma cells (Trevisani et al., 1980). Direct evidence of stimulatory effect of PGs on the proliferation of hepatoma cells was shown by the reversal of decreased hepatoma cell numbers by PGE_2 in indomethacin-treated rats (Trevisani et al., 1980).

Prostaglandins may also exert indirect effects on proliferation of tumor cells by suppressing the local immune response. Prostaglandins were able to make macrophages and/or lymphocytes less sensitive to various stimuli (Pelus and Strausser, 1977; Schultz et al., 1978). Moreover, prostaglandin E_2 suppressed NK cells and lymphokine-activated killer (LAK) cells (Ohnishi et al., 1991; Roth and Golub, 1993; Baxevanis et al., 1993). The present studies showed that hepatic $PGF_{2\alpha}$ and PGE_2 concentrations were significantly increased in FB_1-induced promotion of rat hepatocarcinogenesis (Tables 3 and 4), and in the female rats fed purified FB_1, liver-associated antitumor response was decreased (Figure 1). However, the mechanism for enhancement of tumor cytotoxicity in the FB_1−fructose-fed group is not clear as this group did not differ from the basal diet group in any of the other measures. The tumor cytotoxic cells of the liver are Pitt cells, which are similar to blood NK cells, but with higher lytic activity. The possible immune-enhancing effect of FB_1−fructose deserves further study.

The mechanism of FB_1-induced carcinogenesis is still not fully understood. Fumonisin B_1 is an inhibitor of sphingolipid synthesis (Wang et al., 1991). Sphingolipids may be anticarcinogenic because they are downregulators of protein kinase C (Merrill, 1991), which could affect many enzyme activities after it is activated by carcinogens (Weinstein, 1987). It is not known whether FB_1 can stimulate phospholipase A_2 which hydrolyzes phospholipids to release the PG precursor, arachidonate, or activate cyclooxygenase to produce more PGs in the liver. Prostaglandin $F_{2\alpha}$ was shown to stimulate the formation of intracellular diacylglycerol in Swiss 3T3 cells (Macphee et al., 1984), which can in turn activate protein kinase C. Perhaps FB_1-induced alteration of sphingolipid metabolism stimulates PG production, by an unknown mechanism.

Possibly decreased inhibitory binding of FB_1 to ceramide synthase as a result of FB_1 reaction with fructose may be the mechanism of FB_1 detoxification in this experiment. Further study is needed to identify the structure of the putative FB_1−fructose conjugate and investigate its stability, possible processing occurrence, bioavailability and toxicity.

ABBREVIATIONS

Used FB_1, fumonisin B_1; DEN, diethylnitrosamine; ALT, alanine aminotransferase; PG, prostaglandin; PGST, placental glutathione *S*-transferase; GGT, γ-glutamyl transferase; AHF, altered hepatic foci; NK cell, natural killer cell; and OPA, *o*-phthaldialdehyde.

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