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Dose-dependent fumonisin B1 hepatotoxicity and hepatocarcinogenicity, detoxification of fumonisin B1, and suppression by isoflavones of fumonisin B1-promoted hepatocarcinogenesis in rats

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Dose-dependent fumonisin B_1 hepatotoxicity and hepatocarcinogenicity, detoxification of fumonisin B_1, and suppression by isoflavones of fumonisin B_1-promoted hepatocarcinogenesis in rats

by

Zhibin Lu

A dissertation submitted to the graduate faculty
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ABSTRACT

Three hypotheses were tested in present studies: 1. that a threshold dose of fumonisin B_1 (FB_1) hepatotoxicity and tumor promotion may be below 50 ppm in a short-term diethylnitrosamine (DEN) initiation/FB_1 promotion model; 2. that modifying FB_1 with fructose may reduce or prevent hepatotoxicity and tumor promotion in rats; 3. that isoflavones at 1 mmol/kg diet may at least partly inhibit FB_1-promoted rat hepatocarcinogenesis.

A short-term threshold dose of FB_1 hepatotoxicity and hepatocarcinogenicity was determined in rats initiated with DEN and promoted with 18, 35 and 70 μmol FB_1/kg diet (12.5, 25 and 50 ppm FB_1, respectively). Increases in plasma alanine aminotransferase (ALT) activity and plasma total cholesterol (TC) concentration were found in animals fed 35 and 70 μmol FB_1/kg diet, and greater hepatic prostaglandin (PG) production in the group fed 70 μmol FB_1/kg diet. Placental glutathione S-transferase (PGST)- and gamma-glutamyltransferase (GGT)-positive altered hepatic foci (AHF) developed with feeding 35 and 70 μmol FB_1/kg diet. The short-term threshold of FB_1 hepatotoxicity and hepatocarcinogenicity, therefore, was probably >18 μmol but ≤35 μmol FB_1/kg diet.

Fumonisin B_1 reacted with fructose (FB_1-fructose) was evaluated for detoxification in DEN-initiated rats. Feeding 70 μmol FB_1/kg diet increased plasma ALT activity, plasma TC concentration, and hepatic PG synthesis. Only FB_1-fed rats had PGST- and GGT-positive AHF. Liver-associated natural killer (NK) cell activity was significantly decreased in the FB_1-fed rats and increased in the group fed 70 μmol FB_1-fructose/kg diet. Therefore, reacting FB_1 with fructose eliminated tumor promotion by FB_1 while stimulating the NK cell activity.

To evaluate whether isoflavones suppress FB_1-promoted hepatocarcinogenesis, DEN-initiated rats were fed 1 mmol isoflavones/kg diet with 18, 35 and 70 µmol FB_1/kg diet. Development of PGST- and GGT-positive AHF and increases in plasma ALT activity, plasma TC concentration and hepatic PG production were found with feeding 70 µmol FB_1/kg diet with or without isoflavones. Significant increases in plasma ALT activity, plasma TC, and development of PGST- and GGT-positive AHF were shown with feeding 35 µmol FB_1/kg diet, but these effects were diminished by isoflavones. Therefore, isoflavones suppressed rat hepatocarcinogenesis promoted by 35 µmol but not 70 µmol FB_1/kg diet.

GENERAL INTRODUCTION

Results from three studies are presented in this dissertation. Fumonisin B_1 hepatotoxicity and hepatocarcinogenesis in rats are investigated in Study 1. Two different approaches to FB_1 detoxification are reported in Study 2 and Study 3.

Study 1. Threshold Dose for Short-Term Cancer-Promotion by Dietary Fumonisin-B_1 in Diethylnitrosamine-Initiated Rat Hepatocarcinogenesis

Fumonisins are recently discovered mycotoxins produced by the predominant corn fungi *Fusarium moniliforme* and *Fusarium proliferatum*. Fumonisin B_1 (FB_1), a major member of the fumonisin family, caused equine leukoencephalomalacia (Kellerman et al., 1990; Wilson et al., 1992) and porcine pulmonary edema (Harrison et al., 1990), and was hepatotoxic and hepatocarcinogenic in rats (Gelderblom et al., 1988 and 1991). The occurrence of FB_1 in *F. moniliforme*-contaminated corn was implicated in a relatively high rate of human esophageal cancer in southern Africa (Marasas et al., 1988; Sydenham et al., 1990).

Several biomarkers have been used to study FB_1 hepatotoxicity and hepatocarcinogenicity. Fumonisin B_1-promoted rat hepatocarcinogenesis was readily quantified by measuring placental glutathione S-transferase (PGST)-positive altered hepatic foci (AHF) (Lebepe-Mazur et al., 1995) and gamma-glutamyltransferase (GGT)-positive foci (Gelderblom et al., 1988). Because of the early occurrence of PGST induction, which was evident within 48 h in single hepatocytes after diethylnitrosamine (DEN) initiation (Moore et al., 1987), and because of the persistence of both PGST and GGT during hepatocarcinogenesis (Hendrich et al., 1987), these two enzymes can serve as sensitive biomarkers of early stages of hepatocarcinogenesis. Our previous study showed that both PGST- and GGT-positive AHF were detected after 4 weeks in rats fed 50 ppm crudely purified and highly purified FB_1 following DEN initiation (Lu et al., 1997). Increased plasma or serum total

cholesterol (TC) level and alanine aminotransferase (ALT) activity have been associated with fumonisin hepatotoxicity and hepatocarcinogenicity. *Fusarium proliferatum* strain 5991-fermented corn providing 50 ppm FB_1 to DEN-initiated rats significantly increased plasma TC and ALT while causing hepatocellular adenomas (Hendrich et al., 1993). Similar results were obtained using highly purified FB_1, and additionally, greater hepatic prostaglandin production was related to FB_1-promoted hepatocarcinogenesis in rats (Lu et al., 1997).

Fumonisin B_1 has recently been listed as a class 2B carcinogen (International Agency for Research on Cancer, 1993). Development of appropriate animal models of carcinogenesis is critical to understanding the probable FB_1 carcinogenicity in humans. Because nitrosamines often contaminate human diets (National Academy of Sciences, 1981), and FB_1 is almost always found in corn-based human foods, a 2-stage rodent model combining DEN to initiate and FB_1 to promote carcinogenesis may approach to some extent the reality of human carcinogenesis (Hendrich et al., 1997). One advantage of utilizing this model is that the cancer promoting potential of FB_1 can be readily quantified by PGST- and GGT-positive AHF. Our DEN initiation/FB_1 promotion model, i.e. initiation of rats with DEN (15 mg/kg body weight) followed by 50 ppm FB_1 promotion for 4 weeks, can induce rat hepatocarcinogenesis rapidly, requires relatively small amounts of FB_1, and may mimic the natural environment where humans are exposed to a combination of chemical carcinogens.

Although FB_1 was non-mutagenic in the *Salmonella* mutagenicity assay (Gelderblom and Snyman, 1991) and lacked genotoxicity based on unscheduled DNA synthesis assay in primary rat hepatocytes (Norred et al., 1992), it seemed to be a complete carcinogen either at 50 ppm long-term (18-24 months) or at 250 ppm short-term (3 weeks) (Gelderblom et al., 1991 and 1994). As a potent promoter in the DEN initiation/FB_1 promotion model, fumonisin B_1 was effective to promote hepatocarcinogenesis at a dietary

level of 50 ppm in 4-week studies (Hendrich et al., 1993; Lu et al., 1997). It was unknown, however, whether 50 ppm was the cancer-promoting threshold dose of FB_1 short-term. This exposure level to FB_1 may still be an order of magnitude higher than the natural occurrence of FB_1 in corn-based human foods and animal feeds, which commonly contained 0.2-3 ppm FB_1 (Hopmans and Murphy, 1993; Murphy et al., 1993; Sydenham et al., 1991).

In Study 1 using the short-term DEN initiation/FB_1 promotion model, it was hypothesized that FB_1 would still exert hepatotoxic and cancer-promoting effects at dietary concentrations lower than 50 ppm. The dietary levels of FB_1 were lowered to 25 and 12.5 ppm to approach more closely the natural occurrence of FB_1, to establish dose-response effects of FB_1 on hepatotoxicity and hepatocarcinogenesis in rats, and to ascertain a short-term cancer-promoting threshold for FB_1.

Study 2. Reaction with Fructose Detoxifies Fumonisin B_1 While Stimulating Liver-Associated Natural Killer Cell Activity in Rats

It was hypothesized in this study that modifying FB_1 with fructose would reduce or prevent FB_1 hepatotoxicity and promotion of hepatocarcinogenesis in rats. Efforts were 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 not toxic in primary rat hepatocytes. 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*. Study 2 employed 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. The study was designed to evaluate whether reaction with fructose would detoxify FB_1.

Study 3. Suppression of Fumonisin B_1-Promoted Rat Hepatocarcinogenesis by Genistein and Daidzein Depends upon Fumonisin B_1 Dose

Greater soybean consumption was associated with lower risk of certain hormone-dependent cancers in Asian countries (reviewed by Adlercreutz et al., 1995). The protective role that soybeans may play against development of human cancers was demonstrated in animals. Rats fed a diet containing 50% soybeans had a significantly lower incidence of X-ray irradiation-induced adenocarcinomas and fibroadenomas in mammary glands compared with chow-fed and casein-fed rats (Troll et al., 1980). Feeding soy protein isolate to rats decreased tumor incidence by 50% and increased latency in an N-methyl-N-nitrosourea (NMU) model of mammary cancer (Hawrylewicz et al., 1991). Feeding both autoclaved soybean powder and soy protein isolate to rats reduced the number of mammary tumors induced by carcinogens NMU and 7,12-dimethylbenz[a]anthracene (DMBA) (Barnes et al., 1990).

Isoflavones, mainly genistein, daidzein and their glucoside conjugates, are abundant in soybean foods (0.2-1.5 mg/g) (Wang and Murphy, 1994). Soybean isoflavones may contribute in large part to the anticarcinogenic effects associated with soybean consumption. Feeding genistein to rat pups in the neonatal period delayed the appearance and

reduced incidence of breast cancer after the rats were administered DMBA at 50 days of age (Lamartiniere et al., 1995). In DEN-initiated rats, soybean isoflavone extract providing 1 mmol or 2 mmol isoflavones/kg diet suppressed phenobarbital-promoted hepatocarcinogenesis after 3 months of feeding, as evidenced by the decreased the development of GGT- and PGST-positive AHF (Lee et al., 1995).

Most animal studies on the proposed anticarcinogenic ability of isoflavones utilized rodent mammary carcinogenesis models. Because FB_1 is almost always found in appreciable amounts in corn-based human foods and animal feeds, and it is potentially a human carcinogen, investigating whether isoflavones exert anticancer effect in a rodent model of FB_1 hepatocarcinogenesis is warranted. Using a 2-stage hepatocarcinogenesis model, i.e. DEN (15 mg/kg body weight) initiation of rats followed by FB_1 (70 μmol/kg diet) promotion for 4 weeks, our previous study demonstrated that feeding isoflavone extract providing 1 mmol isoflavones/kg diet suppressed FB_1-promoted rat hepatocarcinogenesis, as reflected in reduced hepatic prostaglandin $F_{2\alpha}$ production and plasma ALT activity, and suppressed development of PGST-positive AHF (Hendrich et al., 1997).

Study 3 was designed to determine whether our previous finding could be extended from soy isoflavone extract to isoflavones with the same carcinogenesis model. Fumonisin B_1 concentrations of 18, 35 μmol in addition to 70 μmol FB_1/kg diet were chosen to approximate the natural occurrence of FB_1 in corn-based human foods and animal feeds. Because isoflavone extract was able to suppress FB_1-promoted hepatocarcinogenesis, and isoflavones were considered to be responsible in large part for the effect, it was hypothesized that dietary isoflavones may at least partly inhibit rat hepatocarcinogenesis promoted by FB_1.

Dissertation Organization

This dissertation is composed of three manuscripts in addition to abstract, general introduction, literature review, general conclusions, and

acknowledgments. The manuscript of Study 1, "Threshold Dose for Short-Term Cancer-Promotion by Dietary Fumonisin-B_1 in Diethylnitrosamine-Initiated Rat Hepatocarcinogenesis", will be submitted to Natural Toxins. The manuscript of Study 2, "Reaction with Fructose Detoxifies Fumonisin B_1 While Stimulating Liver-Associated Natural Killer Cell Activity in Rats", was published in Journal of Agricultural and Food Chemistry. The manuscript of Study 3, "Suppression of Fumonisin B_1-Promoted Rat Hepatocarcinogenesis by Genistein and Daidzein Depends upon Fumonisin B_1 Dose", will be submitted to The Journal of Nutrition.

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LITERATURE REVIEW

Fumonisins are recently discovered mycotoxins produced by the predominant corn fungi *Fusarium moniliforme* and *Fusarium proliferatum*. Fumonisin B_1 (FB_1), a major member of the fumonisin family, caused equine leukoencephalomalacia (ELEM) (Kellerman et al., 1990; Wilson et al., 1992) and porcine pulmonary edema (PPE) (Harrison et al., 1990), and was hepatotoxic and hepatocarcinogenic in rats (Gelderblom et al., 1988 and 1991). The occurrence of *F. moniliforme* containing FB_1 was implicated in a relatively high rate of human esophageal cancer in southern Africa (Marasas et al., 1988; Sydenham et al., 1990).

Discovery of Fumonisins

The discovery of fumonisins began with observations that *F. moniliforme* was involved in widespread field outbreaks of animal diseases occurring in the United States in the early 1900s. The development of these diseases in cattle, horses, pigs and chickens was associated with ingestion of moldy corn. *Fusarium moniliforme* was the fungus most commonly found in moldy corn and was implicated as the cause of the disease "moldy corn toxicosis" (Peters, 1904). In the 1980s, the occurrence of *F. moniliforme* in corn was correlated with high rates of human esophageal cancer in Transkei, southern Africa and in China, where corn is a major dietary staple (Marasas et al., 1981; Yang, 1980). When culture material of several isolated strains of *F. moniliforme* from the contaminated corn was fed to animals, cirrhosis and nodular hyperplasia of the liver and intraventricular cardiac thrombosis were found in rats, leukoencephalomalacia and toxic hepatosis in horses, pulmonary edema in pigs, nephrosis and hepatosis in sheep, and acute congestive heart failure in baboons (Kriek et al., 1981). Strain MRC 826 of *F. moniliforme* grown on autoclaved corn was hepatocarcinogenic to rats (Marasas et al., 1984).

Corn-based animal feeds naturally contaminated with *F. moniliforme* and associated with outbreaks of ELEM caused hepatic nodules in F344 rats after 123 days of feeding. Histological examination revealed multiple hepatic neoplastic nodules, large areas of adenofibrosis and cholangiocarcinomas (Wilson et al., 1985).

The discovery of fumonisins results from the dedicated search for the etiology of the high incidence of human esophageal cancer in Transkei of southern Africa. Gelderblom et al. (1986) utilized a cancer initiation/promotion bioassay, which was based on a chemical carcinogenesis model established by Pitot et al. (1978), to evaluate the cancer promoting ability of strain MRC 826 of *F. moniliforme* isolated from Transkeian corn. Hepatectomized rats were initiated with diethylnitrosamine (DEN, 30 mg/kg body weight) and then fed a diet containing culture material of MRC 826 at a level of 2% for 14 weeks. Gamma-glutamyltransferase (GGT)-positive altered hepatic foci (AHF), which are preneoplastic, developed, indicating the cancer promoting activity of strain MRC 826. This carcinogenicity was also shown in non-hepatectomized rats when the culture material of strain MRC 826 (5% of the diet) was fed for 14 weeks following initiation with DEN (200 mg/kg body weight) (Gelderblom et al., 1986). A similar bioassay with shorter promotion was later used to screen MRC 826 and 10 other strains of *F. moniliforme* for their cancer promoting ability in rat liver. One week after initiation with DEN (200 mg/kg body weight), a diet containing culture material (5%) of each strain was fed to non-hepatectomized rats for 4 weeks. Based on the appearance of GGT-positive AHF, three out of 10 tested strains plus MRC 826 exhibited cancer promoting ability (Gelderblom et al., 1988a).

Compared with cancer initiation/promotion treatment, strain MRC 826 alone without DEN initiation caused far less pronounced development of GGT-positive AHF in rat liver (Gelderblom et al., 1986). *Fusarium moniliforme* corn culture fed to rats before a single dose of DEN caused an increase in

the number of placental glutathione S-transferase (PGST)-positive AHF in rat liver, another early indicator of hepatocarcinogenesis (Lebepe and Hendrich, 1991). These results suggest that in addition to cancer promoting activity, *F. moniliforme* has cancer initiating or co-initiating activity as well.

The short-term cancer initiation/promotion bioassay was further used as a monitoring system to isolate the carcinogenic fumonisins from the culture material of *F. moniliforme* strain MRC 826. The corn culture was extracted with ethyl acetate and methanol:water (3:1). The cancer promoting activity was recovered in the methanol:water extract and remained in the aqueous phase after partitioning with chloroform. This fraction was then run through Amberlite XAD-2 column, a silica gel column, and a C_{18} reverse phase column. Two compounds in the fraction were isolated and were named fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2). After being fed to rats for 4 weeks at a level of 1000 ppm (0.1%) in the diet, fumonisin B_1 induced hepatocarcinogenesis, as seen in the development of GGT-positive AHF (Gelderblom et al., 1988b).

In another experiment, a diet containing 50 ppm of isolated FB_1 was fed to 25 rats for 18-26 months. Ten of 15 FB_1-treated rats that died or were killed after 18 months of treatment developed primary hepatocellular carcinoma (Gelderblom et al., 1991). Taken together, fumonisin B_1 is a complete carcinogen to rats. Whether FB_1 causes human esophageal cancer needs further investigation.

Fumonisins are a family of structurally related mycotoxins. In addition to FB_1 and FB_2, four other fumonisins-B_3, B_4, A_1 and A_2, have been characterized. Fumonisin B_1 is a diester of 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyicosane with both C-14 and C-15 hydroxyl groups esterified with propane-1,2,3-tricarboxylic acids. Fumonisins B_2 and B_3 lack the hydroxyl groups at C-10 and C-5, respectively, whereas both

hydroxyl groups are replaced by hydrogens on fumonisin B_1. Fumonisins A_1 and A_2 are N-acetates of fumonisins B_1 and B_2, respectively. Most of the naturally produced fumonisins in corn cultures of *F. moniliforme* strain MRC 826 are fumonisins B_1, B_2 and B_3 (Bezuidenhout et al., 1988; Cawood et al., 1991).

Fusarium moniliforme is not the only fumonisins-producing *Fusarium* species. Ross et al. (1991) reported production of fumonisins by another *Fusarium* species, *Fusarium proliferatum*. *Fusarium moniliforme* and *F. proliferatum* were isolated from 9 feed samples, seven of which were associated with equine leukoencephalomalacia or porcine pulmonary edema. Production of FB_1 and FB_2 in corn cultures, incubated for 2 weeks at 27°C followed by two weeks at 15°C, was basically the same for the two related species. Testing the ability of *F. proliferatum* for FB_1 production, Nelson et al. (1992) found that 17 of 31 tested strains of *F. proliferatum* produced >500 ppm of FB_1. Those strains producing >500 ppm FB_1 were considered as high producers.

Fumonisin Levels in Corn-Based Human Foods and Animal Feeds

Murphy et al. (1993) conducted a survey on fumonisin B_1, B_2 and B_3 contents of corn from the 1988 to 1991 crop years and corn screenings from 1989. A total of 175 whole corn samples harvested from those years in Iowa and 160 corn screening samples were randomly selected. Fumonisin B_1, B_2 and B_3 levels in corn ranged from 0-37.9 ppm, 0-12.3 ppm and 0-4.0 ppm, respectively. Average FB_1 concentrations were similar (2.5-3.3 ppm) for the 4 crop years surveyed. Corn screenings contained about 10 times higher fumonisin levels than whole corn (Murphy et al., 1993). Feeds associated with animal health problems were assayed for FB_1 contents by Ross et al. (1991). Fumonisin B_1 concentrations ranging from <1 ppm to 126 ppm and from <1 ppm to 330 ppm were detected in feeds associated with ELEM and PPE,

respectively. The FB_1 concentrations in feeds not associated with ELEM or PPE were always <9 ppm, and 94% of the samples contained <6 ppm (Ross et al., 1991). In samples of poor quality corn associated with human esophageal cancer, fumonisin B_1 concentrations were generally higher than in good quality homegrown corn. Up to 140 ppm FB_1 were detected in naturally contaminated corn from some areas with high incidence of human esophageal cancer, whereas average FB_1 concentrations of 0.4 to 1.8 ppm were found in good quality corn (Rheeder et al., 1992). Several surveys to determine the occurrence of FB_1 in corn-based human foods and animal feeds showed that FB_1 levels commonly ranged from 0.2 to 3 ppm (Hopmans and Murphy, 1993; Murphy et al., 1993; Sydenham et al., 1991). Although human exposure to fumonisins was clearly indicated, further studies are needed to determine the potential risk associated with this exposure.

Fumonisin Toxicity and Carcinogenicity

Equine leukoencephalomalacia is a neurotoxic disease that affects horses, donkeys and mules. It is characterized by liquifactive necrosis of the white matter in one or both cerebral hemispheres (Marasas et al., 1988). Typical clinical signs of ELEM include uncoordination, aimless walking, blindness and head pressing (Wilson et al., 1990). The causative agent of ELEM was found by Wilson and Maronpot (1971) when they isolated *F. moniliforme* as the predominant contaminant of moldy corn that was associated with many cases of ELEM, and when they reproduced ELEM in 2 donkeys by feeding *F. moniliforme* corn culture material. Shortly after isolated FB_1 had been available, a horse was injected intravenously 7 times in 9 days with FB_1 at 0.125 mg/kg body weight. Clinical signs of ELEM appeared on day 8, and brain edema and focal necrosis in the medulla oblongata were found on day 10 (Marasas et al., 1988). Reproduction of ELEM in 2 horses by oral dosing of FB_1 was reported later. Typical lesions of ELEM were present in the brains of both horses (Kellerman et al., 1990).

Porcine pulmonary edema was associated with occurrence of *F. moniliforme* when 2 pigs developed the disease following feeding on bulk culture material of corn contaminated with *F. moniliforme* (Kriek et al., 1981). In another study, pulmonary edema and hydrothorax were found in 2 pigs and *F. moniliforme* was recovered from the cultured corn screenings. The concentration of FB_1 ranged from 105 ppm to 155 ppm in the feed samples associated with PPE (Harrison et al., 1990). Additional test was run on a pig with FB_1. The animal received daily intravenous injection of 0.4 mg FB_1/kg body weight and died on day 5. Necropsy showed the pig developed PPE (Harrison et al., 1990).

Fusarium moniliforme contamination of feed has been implicated in diseases of poultry (Engelhardt et al., 1989). Ducklings fed rations containing 100, 200 and 400 ppm FB_1 had decreased weight gain and increased weights of liver, heart, kidney and pancreas in a dose-dependent fashion. Mild to moderate hepatocellular hyperplasia was found in all ducklings fed FB_1 (Bermudez et al., 1995). Because the levels of fumonisins used in poultry studies were relatively high (75-644 ppm), poultry may be more resistant to the toxic effects of FB_1 than other animals.

Fumonisin B_1 is fetotoxic to some animals. Lebepe-Mazur et al. (1995) reported that rat fetuses from mothers fed with 60 mg FB_1/kg body weight on days 8-12 of gestation had significantly lower body weight by 21% and impaired bone development. Developmental toxicity of a water extract of *F. Moniliforme* culture material was also shown in hamsters (Floss et al., 1994). Up to 12 mg FB_1/kg body weight was administered by gavage to the pregnant hamsters from day 8 to day 12 of gestation. With increased FB_1 doses, more fetuses were lost per litter. At 12 mg FB_1/kg, all hamster fetuses were dead.

The liver is a target organ of fumonisin toxicity and carcinogenicity in rats. Culture material of strain MRC 826 of *F. moniliforme* fed to rats

at 8% dietary level caused cirrhosis, nodular hyperplasia and bile-duct proliferation in the liver, and was lethal to all rats. The culture material was also hepatocarcinogenic, causing hepatocellular carcinoma and ductular carcinoma (Marasas et al., 1984). Unlike most mycotoxins, fumonisins are water soluble. This feature was helpful to identify the causative hepatotoxins produced by *F. moniliforme* strain MRC 826. Diets containing water extract or chloroform:methanol (1:1) extract of MRC 826 culture material, or remaining culture material after the extraction, were fed to rats for 4 weeks. The animals fed water culture material extract or the remaining culture material after chloroform:methanol extraction had microscopic liver lesions, and elevated alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase activities. Fumonisins B_1 and B_2 were detected in the water extract but not chloroform:methanol extract, and were present in the toxic diets in concentrations of 93-139 and 82-147 ppm, respectively (Voss et al., 1990). In another experiment, a diet containing 50 ppm of isolated FB_1 was fed to 25 rats for 18-26 months. A group of control rats received no FB_1. Ten of 15 FB_1-treated rats that died or were killed after 18 months of treatment developed primary hepatocellular carcinoma (Gelderblom et al., 1991). Hepatotoxicity was evident after 6 months and progressed in severity with time. These results suggest that fumonisins are the main mycotoxins responsible for the hepatotoxic and hepatocarcinogenic effects in rats.

Biomarkers or End Points of Fumonisin Toxicity and Carcinogenicity

Increased plasma or serum total cholesterol (TC) level and alanine aminotransferase (ALT) activity have been associated with fumonisin hepatotoxicity and hepatocarcinogenicity in several studies. *Fusarium proliferatum* strain 5991-fermented corn providing 50 ppm FB_1 to DEN (15 mg/kg body weight)-initiated F344/N rats significantly increased plasma TC and ALT concentrations while causing hepatocellular adenomas (Hendrich et

al., 1993). Using the same chemical carcinogenesis model established in that study, i.e. initiation of rats with DEN at 15 mg/kg body weight followed by 50 ppm FB_1 promotion for 4 weeks, Lu et al. (1997) demonstrated that FB_1 caused significant increases in plasma TC concentration and ALT activity in rats showing development of PGST- and GGT-positive AHF, early indicators of FB_1 carcinogenesis.

Increase in low density lipoprotein cholesterol (LDL) probably accounted for the major part of the increase in plasma total cholesterol, because only LDL, but not high density lipoprotein cholesterol (HDL) or very low density lipoprotein cholesterol (VLDL), was significantly raised in vervet monkeys fed a low fat (12% of energy), high carbohydrate (72% of energy) diet containing 0.25-1% of *F. moniliforme* culture material (Fincham et al., 1992). In addition, there was no accumulation of cholesterol in the liver. It was therefore proposed that impaired cholesterol removal from the plasma rather than increased cholesterol synthesis in the liver probably occurred (Fincham et al., 1992). Whether LDL receptors in the hepatic membrane were affected by FB_1 hepatotoxicity was unknown because the receptors were not measured in that study. In addition, there was no direct evidence that the cholesterol synthesis was not stimulated by FB_1.

Increase in ALT activity generally indicates leakage of this enzyme from the hepatocytes as a result of membrane damage due to toxicants, such as FB_1.

Fumonisin B_1-induced rat hepatocarcinogenesis can be identified by changes in PGST expression (Lebepe-Mazur et al., 1995) and GGT activity (Gelderblom et al., 1988a), which are markers of AHF (Goldsworthy et al., 1985; Sato et al., 1984). Because of the early occurrence of PGST induction (within 48 h in single putatively initiated hepatocytes after DEN treatment (Moore et al., 1987), and because of the persistence of both PGST and GGT during hepatocarcinogenesis (Hendrich et al., 1987), these two enzymes can

serve as sensitive biomarkers of early stages of cancer development in the liver. In support of this view was a study on FB_1-induced rat hepatocarcinogenesis, in which PGST- and GGT-positive AHF were detected in all rats after 4 weeks of 50 ppm FB_1 treatment following DEN initiation (Lu et al., 1997).

Prostaglandins are a group of lipid molecules synthesized from arachidonic acid and released by various normal or cancerous cell types. It has been shown that production of prostaglandins 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). Increases in hepatic prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 (PGE_2) concentrations were associated with rat hepatocarcinogenesis promoted by FB_1 (Lu et al., 1997). Hepatic prostaglandins $F_{2\alpha}$ and E_2, therefore, can also serve as markers of FB_1 hepatocarcinogenicity.

Based on the finding that FB_1 inhibited sphingolipid biosynthesis in rat primary hepatocytes, it was proposed that exposure to FB_1 would elevate sphinganine to sphingosine ratio (Sa/So) in tissues and serum (Wang et al., 1991). An increase in serum Sa/So was demonstrated in horses given 44 ppm FB_1. The ratio was elevated before increases of serum enzymes indicative of cellular injury, and it returned to control level when the horses stopped eating the FB_1-containing feed. When the horses resumed consumption of the toxic feed, the Sa/So ratio became elevated again (Wang et al., 1992). Similar results were obtained in pigs fed fumonisins B_1 and B_2 at total levels of 0, 5, 23, 39, 101 and 175 ppm. The Sa/So ratio was significantly raised in liver, lung and kidney from pigs consuming feed containing as low as 23 ppm fumonisins. Elevation of the ratio in serum paralleled the increase in the tissues. Increases in serum enzymes indicative of tissue damages occurred only at 101 ppm and 175 ppm total fumonisins. Tissue lesions were observed histologically at >23 ppm in the liver and >175 ppm

in the lung, but not in the kidney at any doses of fumonisins (Riley et al., 1993). Dietary FB_1 also disrupted sphingolipid metabolism in Sprague-Dawley rats in a 4-week feeding study. The concentrations of FB_1 were 0, 15, 50 and 150 ppm. The ratio was significantly raised at 50-150 ppm FB_1 in the liver and 15 ppm in the kidney. In serum, a significantly greater Sa/So ratio was evident in all rats at 150 ppm FB_1. Light microscopic lesions were found at 50-150 ppm and 15-50 ppm FB_1 in the liver and the kidney, respectively. Ultrastructural lesions were found at 15 ppm in both the liver and the kidney (Riley et al., 1994). When the results from these animal studies were taken together, it was concluded that, first, increase in the Sa/So ratio was detected before or at the same time as tissue lesions; second, there was a dose-response relationship between dietary FB_1 levels and the degree of elevation of the ratio; and third, the increase in the ratio was detected prior to elevation of serum enzymes and at lower doses. Therefore, the Sa/So ratio was an early biomarker of general fumonisin toxicity, or an early indicator of FB_1 exposure. However, none of the studies linked this ratio to hepatocarcinogenesis.

Mechanisms of Fumonisin Toxicity and Carcinogenicity

Extensive research on mechanisms of fumonisin toxicity and carcinogenicity has been conducted since the discovery of fumonisins.

The structures of fumonisins resemble those of sphinganine and sphingosine, the long-chain backbone of sphingolipids. Wang et al.(1991) proposed that the mechanism of action of fumonisins might be via disruption of sphingolipid metabolism, i.e. fumonisins might interact with enzymes of sphingolipid biosynthesis and turnover.

In animal cells, the first steps of sphingolipid biosynthesis through formation of ceramide take place in the endoplasmic reticulum. One of the important reactions is the acylation of sphinganine to dihydroceramide, an immediate precursor of ceramide, by ceramide synthase. Subsequent

biosynthesis of complex sphingolipids, i.e. glycosphingolipids and sphingomyelin, occur in the Golgi apparatus and plasma membrane. Turnover of complex sphingolipids in cell membrane begins with internalization of sphingolipids, followed by their hydrolysis in acidic compartments (endosomes and lysosomes) to release ceramide, which is converted to sphingosine. Sphingosine is either reacylated by ceramide synthase to ceramide or phosphorylated (Merrill et al., 1993a).

Studies of effects of FB_1 on sphingolipid biosynthesis have shown that FB_1 inhibited ceramide synthase, the enzyme responsible for acylation of sphinganine in the biosynthetic pathway for sphingolipids as well as the reacylation of sphingosine that was released upon sphingolipid turnover. The consequences of the inhibition included accumulation of sphinganine, increase of Sa/So ratio, and depletion of complex sphingolipids (Merrill et al., 1993b; Wang et al., 1991; Yoo et al., 1992). In primary rat hepatocytes, fumonisin B_1 inhibited ceramide synthase with an IC_{50} of 0.1 µM. The concentration of sphinganine rose 110-fold over controls when rat hepatocytes were incubated with 1 µM FB_1 for 4 days (Wang et al., 1991). In cultured mouse cerebellar neurons, fumonisin B_1 inhibited sphingomyelin biosynthesis to a greater extent than it inhibited glycolipid biosynthesis (the IC_{50} for inhibition of sphingomyelin biosynthesis was 10-fold lower than for glycosphingolipids), suggesting that FB_1 preferentially inhibited complex sphingolipid biosynthesis (Merrill et al., 1993b). In pig renal epithelial cell line LLC-PK_1, the IC_{50} of FB_1 for inhibition of ceramide synthase was about 20 µM. Approximately 50-fold and 128-fold increases in sphinganine were measured after incubation with FB_1 (35 µM) for 6 hr and 24 hr, respectively (Yoo et al., 1992). Although sphingosine level did not increase in hepatocytes, it became significantly elevated in LLC-PK_1 cells (Yoo et al., 1992), suggesting that the reacylation of sphingosine derived from sphingolipid turnover was inhibited by FB_1. The increased sphingosine

may also come from breakdown of membrane lipids in dead cells. In LLC-PK$_1$ cells, however, the increase occurred before any evidence of cell death (Yoo et al., 1992). In spite of that, sphinganine accumulated to a much greater extent than sphingosine (Yoo et al., 1992), suggesting that the inhibition of sphinganine acylation was preferable to the inhibition of sphingosine reacylation. As a result of this differential inhibition, the ratio of sphinganine to sphingosine increased after exposure to FB$_1$ (Yoo et al., 1992).

The finding of *in vitro* inhibition of sphingolipid biosynthesis by FB$_1$ was extended to *in vivo* studies. The increases in sphinganine and the ratio of Sa/So, and reduction in the amount of complex sphingolipids were associated with FB$_1$ toxicity in horses (Wang et al., 1992), pigs (Riley et al., 1993), Sprague-Dawley rats (Riley et al., 1994) and other animals. It remains unclear, however, whether the disruption of sphingolipid biosynthesis after FB$_1$ exposure occurs in humans.

The disruption of sphingolipid biosynthesis can have adverse effects on cells because these molecules have important roles in cell membrane structure, cell-cell communication, interactions between cells and the extracellular matrix, regulation of growth factor receptors, and as second messengers for tumor necrosis factor, interleukin 1 and nerve growth factor (Merrill et al., 1993a).

However, the link between the disruption of sphingolipid biosynthesis and FB$_1$ toxicity and carcinogenicity has not been elucidated. In addition, what causes the species-specific and tissue-specific FB$_1$ toxicity and carcinogenicity is still unknown. Possible mechanisms of FB$_1$ toxicity and carcinogenicity via disruption of sphingolipid biosynthesis have been proposed such as inhibition of Na^+/K^+ ATPase, inhibition of protein kinase C, release of intracellular Ca^{2+}, induction of dephosphorylation of retinoblastoma protein (a key regulator of G to S phase transition in cell

cycle), induction of apoptosis, activation of epidermal growth factor receptor, activation of phospholipase D, activation of AP-1, loss of regulation of differentiation, etc. (Merrill et al., 1993a,c).

Another possible mechanism of FB_1 toxicity and carcinogenicity operates via prostaglandins. Prostaglandin production was stimulated in Yoshida hepatoma cells (Trevisani et al., 1980), in human hepatocellular carcinomas (Hanai et al., 1993), and in some other types of cancers (Turini et al., 1990). Elevated levels of prostaglandins in tumors were believed to facilitate tumor cell proliferation (Turini et al., 1990). Experiments *in vivo* indicated that prostaglandins were implicated in tumor growth in Wistar rats (Trevisani et al., 1980). Prostaglandins may also exert indirect effects on proliferation of tumor cells by suppressing the local immune response, because prostaglandins were able to make macrophages and/or lymphocytes less sensitive to various stimuli (Pelus and Strausser, 1977; Schultz et al., 1978), and PGE_2 suppressed NK cells and lymphokine-activated killer cells (Ohnishi et al., 1991; Roth and Golub, 1993; Baxevanis et al., 1993). Elevation of hepatic prostaglandins paralleled the induction of AHF in rat liver, indicating that increased prostaglandin production was related to promotion of rat hepatocarcinogenesis caused by FB_1 (Lu et al., 1997).

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 lipopolysaccarides (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 FB_1 infections and the number of injections (Martinova and Merrill, 1995).

Detoxification of Fumonisins

The wide spectrum of FB_1 toxicity and its potential carcinogenic effect on humans necessitates development of methods for FB_1 detoxification. Efforts have been devoted to detoxifying FB_1 in several ways. Fumonisin B_1 is heat stable, because nearly 85% of FB_1 was recovered after various heat treatments: 75°C for 135 min, 100°C for 45 min, and 125°C for 5 min. More than 90% of FB_1 was recovered after 16 h at 50°C (Dupuy et al., 1993).

Atmospheric ammoniation of FB_1-containing corn was not effective for detoxification. Treatment of the *F. moniliforme*-contaminated corn and culture material with 2% ammonia at low pressure for 4 days, a process that detoxifies aflatoxin B_1, led to slight reduction in the concentration in the concentration of FB_1 without decreasing the hepatotoxicity in rats (Norred et al., 1991). Ammoniation at high pressure (60 psi) and low temperature (20°C) reduced detectable FB_1 levels in corn by 79%, but the toxicity of the ammoniated corn was not evaluated (Park et al., 1992).

Ethanol distilled from the whole stillage after fermentation of moldy corn for ethanol production did not contain any FB_1, but most FB_1 was recovered in the distillers grain and thin stillage (Bothast et al., 1992), which are often used as animal feed.

Nixtamalization is a traditional processing of corn with $Ca(OH)_2$ and heat to produce masa (tortilla flour). The processing of corn is used to improve the nutritional value by increasing niacin bioavailability in the masa. When *F. proliferatum*-contaminated corn was boiled in 1.2% $Ca(OH)_2$ solution for 1 h, the concentration of FB_1 was lowered from 50 ppm to 0.4 ppm, and 7-10 ppm hydrolyzed FB_1 was produced. This hydrolyzed FB_1 was similar in toxicity to FB_1 when nutritional status of rats was adequate (Hendrich et al., 1993). In agreement with the finding, another study of effectiveness of nixtamalization for FB_1 detoxification showed that

hydrolyzed FB_1 at 58 ppm level was similar to 71 ppm FB_1 in causing hepatotoxicity and nephrotoxicity in rats (Voss et al., 1996).

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 1000 ppm 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 culture materials. The most successful FB_1 detoxification so far has been demonstrated in a study in which FB_1's amine group was blocked by reacting with reducing sugars such as fructose in a Maillard reaction. Subjecting FB_1 to nonenzymatic browning conditions with fructose eliminated FB_1 toxicity and carcinogenicity as reflected in body weight, plasma total cholesterol concentration, and ALT activity, development of PGST- and GGT-positive AHF, and hepatic $PGF_{2\alpha}$ and PGE_2 levels (Lu et al., 1997).

Another approach to inhibiting FB_1 toxicity and carcinogenicity is in close relation to naturally occurring plant constituents, such as soybean isoflavones, which possess potential health-protective activities including anticarcinogenicity.

Soybean Isoflavones and Their Contents in Soybean Foods

Soybeans contain 1-3 mg isoflavones/g (Wang and Murphy, 1994a), and are considered a rich and major source of isoflavones. Genistein, daidzein and their glycones (glucoside conjugates) are the major isoflavones in soybeans (Murphy, 1982) in which the glycones compose 97-98% of total

isoflavones (Wang and Murphy, 1994b). Twelve soybean isoflavone isomers have been identified, including glucoside conjugates genistin, daidzin, glycitin, 6"-O-acetylgenistin, 6"-O-acetyldaidzin, 6"-O-acetylglycitin, 6"-O-malonylgenistin, 6"-O-malonyldaidzin, 6"-O-malonylglycitin, and their corresponding aglycones, genistein, daidzein and glycitein (Kudou et al., 1991).

Soybean isoflavones are present in the range of 0.2-1.5 mg/g in commercial soybean foods (Wang and Murphy, 1994b). Similar to unprocessed soybeans (total isoflavone levels at 1.2-4.2 mg/g), high-protein soybean ingredients, such as soy flour and textured soy protein, contained 1.1-1.4 mg/g of total isoflavone concentrations. Soy concentrate, however, had very low isoflavone levels because alcohol washing removed most of the isoflavones from soy flakes. There was a major difference in the forms of isoflavones in traditional soybean foods. Nonfermented soybean foods, such as tofu and soymilk powder, had greater levels of glucoside conjugates of isoflavones, whereas in fermented soy foods (e.g. tempeh and miso), the isoflavone aglycones, genistein and daidzein, predominated because of the hydrolysis by bacterial glucosidases during fermentation. Second-generation soybean foods such as tofu yogurt and soy bacon contained only 6-20% of the isoflavones found in whole soybeans, because soy ingredients were minor components used as replacement for animal protein and fat (Wang and Murphy, 1994b).

Bioavailability and Metabolism of Soybean Isoflavones

Soybean isoflavones have been considered to possess cancer-protective potential. To understand bioavailability of isoflavones in humans, which is critical in determining the approximate amount of dietary isoflavones needed to maintain functional levels of isoflavones in the body, Hendrich and associates conducted a series of bioavailability studies of soybean isoflavones in adult women (Xu et al., 1994; Tew et al., 1996a,b). At all three doses of soymilk isoflavones (0.7, 1.3 and 2.0 mg/kg body weight),

average 24 h urinary excretion of daidzein and genistein was approximately 21% and 9%, respectively, of a single ingested dose. Approximately 1-2% of ingested isoflavones were excreted in feces. The average amount of urinary daidzein and genistein increased significantly in a dose-dependent fashion. The plasma total isoflavone concentration, i.e. 4.4 ± 2.5 μM at 6.5 h after a dose of 2.0 mg/kg, suggested that although 85% of soymilk isoflavones seemed to be degraded in the intestine, they might be present in sufficient amounts in blood to exert their health-protective effects (Xu et al., 1994). To understand whether the bioavailability of isoflavones would be affected by some dietary factors, Tew et al. (1996a,b) studied the effects of dietary fat and fiber content on soy isoflavone bioavailability in humans. In the first study, seven healthy women were given low or high fat diet (20% or 40% of energy from fat) containing a single dose of 0.9 mg isoflavones/kg body weight from tofu or texturized vegetable protein (TVP). The amount of dietary fat intake did not affect bioavailability as reflected on 24 h urinary isoflavone recovery (Tew et al., 1996a). In the second study, seven healthy women were fed a control diet or a wheat fiber supplemented diet (15 g and 40 g of dietary fiber, respectively) with a single dose of 0.9 mg isoflavones/kg body weight from tofu or TVP. The fiber-rich diet decreased plasma genistein at 24 h after dosing by 55% and reduced total urinary genistein by 20%. Therefore, dietary fiber content should be taken into account to determine the bioavailability of soybean isoflavones (Tew et al., 1996b).

Upon ingestion the glucoside conjugates of daidzein and genistein (daidzin and genistin) are hydrolyzed in the large intestine by intestinal bacteria to release the unconjugated daidzein and genistein (Setchell et al., 1984). The extent of absorption may be dependent on the chemical form of the isoflavones, with the aglycones more readily absorbed than their corresponding glucoside conjugates, because the latter are very polar.

Absorbed soybean isoflavones are transported via the hepatic portal vein to the liver, where they are rapidly conjugated with glucuronic acid and, to a much lesser extent, with sulfuric acid (Setchell et al., 1984). The glucuronides are excreted into either bile or urine, depending on the molecular weight and the polarity of the aglycones. The glucuronide conjugates of genistein and daidzein can be excreted into bile or urine in rats. Sulfate conjugates are readily excreted into urine. The glucuronide isoflavones which are excreted into bile enter the GI tract. Beta-glucuronidases in lower GI tract deconjugate isoflavones, so the isoflavone aglycones might undergo efficient enterohepatic circulation (Larsen, 1988). In both plasma and urine, isoflavones are primarily glucuronide conjugates, whereas in feces, isoflavones are predominantly found in the unconjugated form. The free and sulfate forms of isoflavones in plasma are biologically active (Adlercreutz et al., 1993a). It is still under investigation whether the glucuronide form of isoflavones is also biologically active.

Estrogenic and Antiestrogenic Activities of Isoflavones

Isoflavones are heterocyclic phenols resembling the structure of estrogen. The C-7 and C-4' hydroxyl groups of genistein and daidzein, which correspond to the C-3 and C-17 hydroxyl groups of 17 beta-estrodiol, are necessary for their estrogenicity via binding estrogen receptor; and the additional C-5 hydroxyl group of genistein may increase estrogenic activity (Miksicek, 1995). Isoflavones have very weak estrogenic effects, ranging from 10^{-3} to 10^{-5} that of diethylstilbestrol (DES) or estradiol (Farmakalidis et al., 1985; Santell et al., 1997). The growth of uterus, mammary glands and vagina of immature or ovariectomized animals is frequently used as a parameter to evaluate the weak estrogenicity of isoflavones (Farmakdlidis et al., 1985; Folman and Pope, 1966; Santell et al., 1997). Farmakalidis et al. (1985) compared the estrogenic potencies of genistin, genistein and daidzin with that of DES in the B6D2F1 strain of mouse. The compounds were administered by stomach intubation. The increase

in uterine weight over control was taken to rank their relative estrogenic potencies. When the relative potency was assigned as 1.00 for genistein, it was 0.66, 0.26 and 100,000 for genistin, daidzin and DES, respectively.

The weak estrogenic effects of isoflavones act through estrogen receptor. Relative binding affinity of genistein for the rat uterine estrogen receptor was about 10^{-2} that of estradiol (Santell et al., 1997). In addition, uterine expression of *c-fos*, which is an estrogen-responsive gene (Weisz and Rosales, 1990), was induced in ovariectomized Sprague-Dawley rats following the dietary administration of genistein (750 mg/kg diet for 14 days) or estradiol (1 mg/kg diet for 14 days). The induction of *c-fos* by genistein and the binding assay of the estrogen receptor suggested that isoflavones induced a weak estrogenic response by interacting with the estrogen receptor and were capable of initiating the biological response by forming an active complex with the receptor in uterine tissue (Santell et al., 1997). In support of this interpretation, genistein was also shown to stimulate estrogen-responsive pS2 mRNA expression in estrogen receptor positive human breast cancer cell line MCF-7 at concentrations as low as 10^{-8} M, and the stimulation of pS2 expression by genistein (10^{-7} M) was blocked by the addition of tamoxifen (10^{-5} M), an antiestrogen (Wang et al., 1996).

However, genistein injected subcutaneously at large dose (1.6 mg) was shown to inhibit DES-stimulated vaginal and uterine growth by approximately 40% in immature female albino mice of the BSVS strain. Therefore it was concluded that isoflavones might act as antiestrogens (Folman and Pope, 1966). A similar conclusion was drawn from another study in which injections of 0.3 and 0.9 mg of genistein, when administered simultaneously with estradiol, decreased mouse uterine estradiol uptake by 71% and 95%, respectively, and vaginal estradiol uptake by 70% and 89%, respectively (Folman and Pope, 1969).

The apparent antiestrogenicity of isoflavones may result from a combination of multiple effects. The primary one is likely to be the competitive binding to estrogen receptor. Isoflavones were shown to compete with estradiol for binding to the estrogen receptor with relative binding affinity of 10^{-2} to 10^{-4} that of estradiol (Shutt and Cox, 1972; Verdeal et al., 1980), but they were less effective than estradiol in translocating the cytoplasmic estrogen receptor to the nucleus (Martin et al., 1978), and they failed to initiate effective cytoplasmic receptor replenishment (Tang and Adams, 1980). Isoflavones may inhibit estrogen synthetase (aromatase), a cytochrome P-450 enzyme that catalyzes the conversion of androgen to estrogen in many tissues (Adlercreutz et al., 1993b). It was reported that daidzein and its metabolite equol inhibited human placental aromatase in microsomal preparation from human placenta, with IC_{50} of <1 mM and 150 μM, respectively (Adlercreutz et al., 1993b). Isoflavones may also decrease the amount of free and active estrogen in the blood by stimulating sex hormone binding globulin (SHBG) synthesis (Adlercreutz et al., 1990). Sex hormone binding globulin binds dihydrotestosterone, testosterone and estradiol in order of decreased affinity (Rosner, 1990). High SHBG level may reduce risk of hormone-related cancers, because SHBG regulates clearance and uptake of estrogen (Mousavi and Adlercreutz, 1993), which at physiological concentrations stimulates growth of human breast cancer cells (Horowitz and McGuire, 1978). In human hepatocarcinoma cells (Hep-G2), genistein at 5-30 μM significantly increased the production of SHBG without showing cytotoxicity (Mousavi and Adlercreutz, 1993). Therefore, isoflavones may exert antiestrogenic effects in animals through competing with estradiol for the occupancy of the estrogen receptor, inhibiting estradiol synthesis and decreasing availability of free estradiol in the blood.

Whether isoflavones are estrogenic or antiestrogenic probably depends on the amounts of exogenous isoflavones vs. the concentration of endogenous estradiol, and on the sensitivity of target tissues to these compounds.

After ingestion of a relatively large amount of soy foods, urinary excretion and plasma concentrations of isoflavones may well exceed endogenous estrogen levels. For example, urinary levels of estrone glucuronide, the principle estrogen in the urine, reached 2 to 27 µg/day during the follicular phase of menstrual cycle (Setchell et al., 1984), but urinary excretion of isoflavones was 0.35 to 7.49 mg/day after a 60 g soy protein diet providing 45 mg isoflavones (Cassidy et al., 1994), which was in agreement with 24 h urinary excretion levels in humans reported by Xu et al. (1994). In that isoflavone dose-response study, at a single dose of 0.7 mg soymilk isoflavones/kg body weight, representing an average intake of 44 mg total isoflavones, the average 24 h urinary excretion of isoflavones was 5.93 mg, and the excreted amounts increased with larger doses of soymilk isoflavones (Xu et al., 1994). At these levels of dietary intake, which are well within the range of average isoflavone consumption in Asian countries (50-100 mg isoflavones/day, Adlercreutz et al., 1991; Coward et al., 1993), urinary excretion and plasma concentrations of isoflavones may be several orders of magnitude higher than endogenous estrogen. Thus isoflavones seem to be antiestrogenic in humans. This is indirectly confirmed by the findings that daily consumption of soy protein containing 45 mg for 1 month by premenopausal women led to prolonged menstrual cycle and altered hormonal status, the responses that also occurred with tamoxifen, an antiestrogen for treating breast cancer (Cassidy et al., 1994). However, whether the antiestrogenicity of isoflavones is associated with health-beneficial effects such as cancer prevention in humans in uncertain.

Isoflavones as Antioxidants

Isoflavones are antioxidants. The antioxidative activity of isoflavones is obviously attributed to their hydroxyl groups. Inhibition by 10^{-3} M isoflavones of linoleate oxidation was 70%, 30%, 26% and 15% for genistein, genistin, daidzein and daidzin, respectively. Therefore, the antioxidative capacity was seemingly greatest for genistein followed by

genistin, daidzein and daidzin (Pratt and Birac, 1979). Wei et al. (1993) reported that genistein (1-150 μM) decreased tumor promoter-stimulated production of hydrogen peroxide (H_2O_2) in human polymorphonuclear leukocytes and HL-60 cells in a dose-dependent manner, with IC_{50} of 14.8 and 30.2 μM, respectively. In a subsequent study, effects of isoflavones and related flavones on H_2O_2 formation were compared in tumor promoter-activated HL-60 cells. Among the tested compounds at concentrations of 10, 50, 100 and 200 μM, genistein (IC_{50} = 25 μM) and daidzein (IC_{50} = 150 μM) were the most potent inhibitors (Wei et al., 1995). In addition, topically applied genistein (0.05 to 50 mM in acetone) inhibited H_2O_2 formation in CD-1 mouse skin by 31-117% (Wei et al., 1993), and feeding 250 ppm genistein to CD-1 mice for 30 days significantly increased the activities of antioxidant enzymes including catalase, superoxide dismutase, glutathione peroxidase in the skin and the small intestine by 10-30% (Wei at al., 1995). In rat peritoneal leukocytes stimulated by calcium ionophore A23187, certain flavonoids inhibited eicosanoid production via 5-lipoxygenase and cyclooxygenase pathways with IC_{50} of 1-180 μM. The presence of the hydroxyl groups seemed to be critical to the inhibitory effects (Moroney et al., 1988). Because isoflavones belong to flavonoids and are structurally similar to those tested compounds, genistein and daidzein may also suppress syntheses of eicosanoids including prostaglandins.

Anticarcinogenicity of Soybean Isoflavones

Epidemiological studies have shown that soybean consumption is greater in some Asian countries where risk of hormone-dependent cancers is relatively low (Reviewed by Adlercreutz et al., 1995a). The amounts of dietary intake of soybean products can be reflected accordingly in plasma, urine and feces. Urinary excretion of isoflavones is much greater in Japanese men and women consuming a traditional soy-rich diet compared with

that in women from Boston and Helsinki (Adlercreutz et al., 1986 and 1991). Plasma levels of biologically active isoflavones (free and sulfate forms) were 10-20 times higher in Japanese men than those in Finnish males (Adlercreutz et al., 1993c). Fecal excretion of isoflavones in Finnish women was 5-17 times greater in vegetarians than in omnivores (Adlercreutz et al., 1995b). The incidence of breast cancer in the oriental countries is 5-8 times lower than that in the U.S. (Nagasawa, 1980), where soybeans only contribute to a very small part of a conventional American diet. However, the incidence of breast cancer in Asian immigrants in the U.S. and their following generations approaches the typical incidence rates of U.S. women (Buell, 1973). These findings stimulated interest in the possibility that soybean consumption might prevent the development of breast cancer.

The speculated anticarcinogenic effects of soy feeding have been confirmed in animal studies. Feeding soy protein isolate to rats significantly decreased tumor incidence by 50% and noticeably increased latency in an N-methyl-N-nitrosouria (NMU) model of breast cancer (Hawrylewicz et al., 1991). Compared with chow-fed and casein-fed rats, those fed a diet containing 50% raw soybeans had a significantly lower incidence of X-ray irradiation-induced adenocarcinomas and fibroadenomas in mammary glands (Troll et al., 1980). Barnes et al. (1990) found that both autoclaved and nonautoclaved whole soybeans (10% and 20%) and soy protein isolate reduced the number of mammary tumors induced by both direct-acting (NMU) and indirect-acting (7,12-dimethylbenz[a]anthracene, DMBA) carcinogens. In addition to breast cancer, hepatocarcinogenesis is also inhibited by soy consumption in animals. Feeding a diet containing 30% autoclaved soybeans significantly decreased ultrastructural changes induced by dibutylamine and nitrite in rat liver (Fitzsimons et al., 1989).

Soybean isoflavones may contribute in large part to the anticarcinogenic effects associated with soybean feeding. In F344/N rats initiated with DEN (15 mg/kg body weight) and promoted by phenobarbital

(500 mg/kg diet), soybean isoflavone extract providing 1 mmol or 2 mmol isoflavones/kg diet suppressed phenobarbital-promoted hepatocarcinogenesis as evidenced by the decreased volume percentage of GGT- and PGST-positive AHF after 3 months of feeding (Lee et al., 1995). Using a DEN initiation/FB_1 promotion carcinogenesis model, Hendrich et al. (1997) demonstrated that feeding isoflavone extract (1 mmol isoflavones/kg diet) suppressed PGST-positive AHF by over 50%. In that study, the isoflavone extract was prepared from roasted and defatted soy flakes by acetone extraction. These steps of preparation could have removed other proposed anticarcinogenic soy constituents such as saponins and Bowman-Birk protease inhibitor. Therefore, soybean isoflavones were very likely responsible for the protective effect against cancer. This speculation was supported by an observation that feeding genistein to rats in the perinatal period delayed the appearance of breast cancer after the pups were administered DMBA at 50 days of age (Lamartiniere et al., 1995).

Several mechanisms have been proposed to explain the anticarcinogenicity of isoflavones in animals. Since estrogen has been shown to stimulate human breast cancer cells at physiological concentrations (Horowitz and McGuire, 1978), isoflavones may be anticarcinogenic by acting as antiestrogens (Setchell et al., 1984). Originally, the antiestrogenicity of isoflavones was only thought to be related to the estrogen receptor, but now this activity may be considered to be due to several effects: competitive binding to the estrogen receptor (Shutt and Cox, 1972; Verdeal et al., 1980), inhibition of estrogen synthetase (aromatase) (Adlercreutz et al., 1993b) and stimulation of SHBG synthesis (Mousavi and Adlercreutz, 1993).

The antiestrogenic effect of isoflavones through competition with estradiol for binding to the estrogen receptor, however, did not seem to be associated with the antiproliferative effect of isoflavones *in vitro*. Wang et al. (1996) reported that genistein exerted a concentration-dependent

biphasic effect on the proliferation of an estrogen receptor-positive human breast cancer cell line MCF-7: at lower concentrations (10^{-9} to 10^{-6} M), genistein stimulated growth, but at higher concentrations ($>10^{-5}$ M), genistein was inhibitory to growth. The cell proliferative effect of isoflavones was mediated through the estrogen receptor, but their antiproliferative effect was independent of the estrogen receptor, because in the estrogen receptor-negative MDA-MB-23 cells, genistein did not stimulate cell growth at lower concentrations ($<10^{-7}$ M), whereas it was still inhibitory to cell growth at higher concentrations ranging from 10^{-7} to 10^{-4} M. In support of this finding, another study showed that genistein was inhibitory to the proliferation of both estrogen receptor-positive (MCF-7) and -negative (MDA-468) breast cancer cell lines at similar IC_{50}: 24 to 44 µM (Peterson and Barnes, 1991).

At high concentrations ($>10^{-5}$ M for genistein), the estrogen receptor-independent antiproliferative effect of isoflavones may be attributed to other mechanisms, which involve protein tyrosine kinases. Some peptide growth factor receptors have intrinsic tyrosine kinase activity. For example, protein tyrosine kinase activity is associated with receptors for epidermal growth factor (EGF), insulin-like growth factor I, platelet-derived growth factor and mononuclear phagocyte growth factor. Tyrosine kinases are important for cell proliferation, in that activation of certain peptide growth factor receptors requires autophosphorylation at tyrosine residues (Akiyama et al., 1987). Genistein is a specific inhibitor of protein tyrosine kinases. It was shown that genistein specifically inhibited the autophosphorylation of purified EGF in membrane preparations with an IC_{50} of 2.6 µM (Akiyama et al., 1987). Therefore, genistein may elicit antiproliferative effect on cells via inhibition of protein tyrosine activity associated with EGF receptor (Peterson and Barnes, 1993).

To test this hypothesis, the effects of genistein on tumor cell growth and EGF receptor protein tyrosine kinase activity were evaluated in tumor cell lines. Genistein, however, did not inhibit the activation of the growth factor receptor in tumor cells, because genistein did not block EGF receptor autophosphorylation in DU-145 prostate cancer cells (Peterson and Barnes, 1993). In contrast, tyrphostin, a synthetic tyrosine kinase inhibitor which suppressed EGF-stimulated prostate cancer cell growth with a similar IC_{50} to that of genistein, fully blocked EGF receptor tyrosine autophosphorylation (Peterson and Barnes, 1993). These results suggested that the cellular target of genistein's antiproliferative activity was located down-stream from EGF receptor activation in related signal transduction pathways. This view was substantiated by a more recent study showing that genistein inhibited fetal bovine serum-, estradiol-, and EGF-stimulated cell growth in 5 different breast cancer cell lines (estrogen receptor-positive or -negative) with IC_{50} ranging from 9.6 to over 74 uM. The antiproliferative effect of genistein was not accompanied by inhibition of EGF-induced tyrosine autophosphorylation. It was therefore concluded that the genistein-elicited cell growth inhibition did not depend on estrogen receptor or inhibition of EGF receptor protein tyrosine kinase activity (Peterson and Barnes, 1996).

But genistein may block cancer cell growth by interfering with estradiol- or growth factors-stimulated signal transduction pathways leading to cell proliferation. In human colon carcinoma-derived Caco-2 cell line, which was estradiol responsive, estradiol stimulated cell growth and activated 4 intermediates in a signal transduction pathway known to trigger cell proliferation: 2 tyrosine kinases, *c-src* and *c-yes*, which belong to a *c-src*-related tyrosine kinase family; and 2 serine/threonine kinases, erk-1 and erk-2, which are mitogen-activated protein kinases (MAP). The tyrosine kinases *c-src* and *c-yes* were up-stream from MAP kinases and cell

proliferation, because genistein (80 μM) blocked the stimulatory effects of estradiol (10 nM) on both erk-2 activity and cell proliferation through inhibition of the tyrosine kinases. It was considered that estradiol activated the c-src, c-yes/MAP kinase signal transduction pathway which was also a target of various peptide growth factors (Di Domenico et al., 1996). If both estradiol and EGF can activate this pathway to exert their cell proliferative effects, and genistein can selectively inhibit the tyrosine kinase intermediates in the pathway, the estradiol receptor- and EGF receptor-independent antiproliferative effect of genistein could be explained. In support of this view, the IC_{50} values for estradiol-stimulated breast cancer cell growth (2.3-2.7 μM) were similar to those for EGF-stimulated growth (2.8-5.4 μM) (Peterson and Barnes, 1996).

Genistein was also an effective inhibitor of eukaryotic DNA topoisomerase II, an enzyme involved in cellular replication (Markovits et al., 1989). The inhibition was accomplished by stabilizing DNA-topoisomerase II complex, and the stabilization of the complex in tumor cells caused double- and single-strand breaks in DNA, leading to growth inhibition and differentiation (Constantinou et al., 1995). In human promyelocytic HL-60 and erythroid K-562 leukemia cells and in human SK-MEL-131 melanoma cell lines, an IC_{50} of approximately 37 to 56 μM was observed for genistein to inhibit cell growth; and at the same concentrations, genistein induced cell differentiation identified by appearance of characteristic markers; both single- and double-strand DNA breaks were evident after 24 h treatment with the same concentrations required to inhibit cell growth and induce cell differentiation (Constantinou et al., 1995).

Genistein was shown to inhibit basic fibroblast growth factor-stimulated proliferation of bovine brain-derived capillary endothelial cells and *in vitro* angiogenesis at IC_{50} of 5 and 150 μM, respectively (Fotsis et al., 1993). This is an interesting finding in regard to the

anticancer activity of isoflavones, because unregulated angiogenesis or neovascularization was associated with tumor growth and metastasis (Weidner et al., 1991).

Isoflavones may elicit their anticancer effects through an antioxidant mechanism. Isoflavones are antioxidants because of the presence of their hydroxyl groups. Isoflavones may prevent oxidative damage by inhibiting the formation of free radicals (Wei et al., 1993) and thus may contribute to their anticancer activity. The ability of some flavonoids to inhibit prostaglandin synthesis may be one of mechanisms by which isoflavones exert anticarcinogenic effects. In rat peritoneal leukocytes stimulated by calcium ionophore A23187, certain flavonoids inhibited eicosanoid production via 5-lipoxygenase and cyclooxygenase pathways with IC_{50} of 1-180 μM. The presence of the hydroxyl groups seemed to be critical to the inhibitory effect (Moroney et al., 1988). Because isoflavones belong to flavonoids and their structures are similar to those of the tested compounds, genistein and daidzein may also suppress prostaglandin production. Prostaglandins of the F series stimulated the proliferation of neonatal rat hepatocytes (Armato and Andreis, 1983). Inhibition of prostaglandin synthesis by indomethacin, a cyclooxygenase inhibitor, played a role in suppressing tumor growth *in vivo* and prolonging survival time of hepatoma-bearing rats by approximately 50% (Trevisani et al., 1980).

Isoflavones may also diminish growth-stimulatory effect of prostaglandins. Macphee et al. (1984) have reported that $PGF_{2\alpha}$ at 300 ng/ml stimulated phosphatidylinositol turnover which led to increase in 1,2 diacylglycerol (DAG) in resting Swiss 3T3 cells, and this event was associated with the stimulatory effect of $PGF_{2\alpha}$ (250 ng/ml) on DNA synthesis. A study of mechanism underlying growth-stimulatory effect of prostaglandins in primary hepatocytes shows that prostaglandins stimulated DNA synthesis and increased intracellular inositol-1,4,5-triphosphate (IP_3) at concentrations ranging from 10^{-6} to 10^{-4} μM, and elicited elevation of

free cytosolic Ca^{2+} at 10^{-5} uM. The prostaglandin-stimulated DNA synthesis was independent of cAMP, but was at least partly dependent on phospholipase C (Refsnes et al., 1995). Isoflavones may suppress prostaglandin-stimulated DNA synthesis and thus cell proliferation, because genistein and daidzein suppressed phospholipase C activation, resulting in decreased formation of inositol phosphates in 3T3 cells (IC_{50} = 2.2 and 7.9 µM for genistein and daidzein, respectively) (Higashi and Ogawara, 1992). The inhibition of phosphatidyl inositol 4,5-bisphosphate (PIP_2) hydrolysis via inactivation of phospholipase C by genistein and daidzein may decrease prostaglandin production as well. This is because arachidonic acid, which is the prostaglandin precursor and occupies the 2-position on the glycerol backbone of PIP_2, can be released from the sequential hydrolysis of PIP_2 by phospholipase C and diacylglycerol lipase. Therefore, isoflavones may decrease prostaglandin production and diminish prostaglandin-stimulated DNA synthesis by inhibiting phospholipase C.

There has been increasing emphasis on the importance of using physiologically achievable isoflavone concentrations in cancer prevention studies. Some studies on the mechanisms by which soybean isoflavones inhibit carcinogenesis may have overlooked physiological relevance with respect to the reported isoflavone concentrations. Some proposed anticancer mechanisms of isoflavones may not operate in humans, because the required isoflavone concentrations seem to well exceed the physiologically achievable levels in the blood. For example, the IC_{50} required for genistein to inhibit angiogenesis *in vitro* was 150 µM, or 1.5 x 10^{-4} M (Fotsis et al., 1993), whereas the plasma isoflavone concentrations reported in women (19-41 years of age) consuming soymilk isoflavones only ranged from 1 to 5 µM at 6.5 h after single doses of 0.7, 0.9, 1.3 and 2.0 mg isoflavones/kg body weight (Tew et al., 1996a; Xu et al., 1994), and from 0.55 to 0.86 uM after two-week feeding of isoflavones (Barnes et al.,

1996). It was suggested that only those mechanisms requiring isoflavone concentrations below 18 μM were likely to be physiologically relevant (Barnes and Peterson, 1995). This concentration, 18 μM, however, may still not be physiologically achievable. Some researchers believed that even with increased intake of soy products, the circulating levels of genistein did not exceed 1 μM (Wang et al., 1996).

The probable anticarcinogenic effect of isoflavones is usually represented by their antiproliferative activity in a variety of cell lines. A concentration of $>10^{-6}$ M seemed to be required for isoflavones (genistein) to be antiproliferative (Constantinou et al., 1995; Fotsis et al., 1993; Mousavi and Adlercreutz, 1993; Peterson and Barnes, 1991 and 1996; Wang et al., 1996). This isoflavone concentration is probably at the high end of the physiologically achievable concentration range in humans. Since there is large variation in isoflavone content of biological samples among human subjects (Xu et al., 1994), in other words, the difference in isoflavone bioavailability is substantial among humans, those with greater circulating isoflavone concentrations after soy consumption may benefit more from the potential anticarcinogenic effects of isoflavones. To maintain greater plasma concentration of isoflavones, frequent (daily) soy consumption may be needed, because there is little evidence for retention of isoflavones in the human body. One study showed that 24 h after a single dose of soymilk isoflavones, isoflavones were nearly undetectable in both the blood and the urine (Xu et al., 1994).

The growth inhibition of cells by isoflavones is cytostatic and reversible at their IC_{50}. At higher concentrations, however, genistein is cytotoxic (Fotsis et al., 1993; Mousavi and Adlercreutz, 1993; Peterson and Barnes, 1996). Although the therapeutic index of genistein cannot be calculated due to lack of information on toxic dosage causing 50% cell death in those studies, the ratio of minimum toxic concentration to IC_{50} is 2-5.

The relevance of the *in vitro* studies in understanding the potential cancer-preventive effects of isoflavones is unknown. As mentioned above, many of the experiments are conducted using high concentrations of isoflavones and few of the proposed anticarcinogenic mechanisms of isoflavones in cancer cells may operate at physiological plasma concentrations.

Human cancer cell lines such as MCF-7 have been extensively used in the majority of the *in vitro* experiments with isoflavones. To understand the physiological effects of isoflavones on normal cell proliferation, Traganos et al. (1992) compared growth inhibition by genistein of normal human lymphocytes, human leukemic MOLT-4 and HL-60 cells. Genistein showed greater antiproliferation in the leukemic cells (IC_{50} = 31 and 48 μM for HL-60 and MOLT-4, respectively) than in the normal lymphocytes (IC_{50} = 741 μM). Thus, the sensitivity of normal cells and cancer cells to the growth-inhibitory effect of isoflavones seemed to be different. The same interpretation was given by a recent study examining the differential sensitivity of normal human mammary epithelial cells and human breast cancer cells MCF-7 to growth inhibition by genistein and biochanin A, a genistein precursor (Peterson et al., 1996). In the normal cells genistein was 5-fold more potent as a growth inhibitor than biochanin A, but in MCF-7 cells biochanin A was as potent as genistein to be antiproliferative. Biotransformation of both isoflavones differed in the two kinds of cells. The MCF-7 cells produced 2 genistein metabolites and 3 biochanin A metabolites. Two of the biochanin A metabolites were the same as the genistein metabolites, and the third one was genistein. In contrast, significant genistein or biochanin A biotransformation was not found in the normal cells. The difference in biotransformation could be responsible for the difference in growth inhibition: the MCF-7 cells were less sensitive to genistein inhibition than the normal cells; in contrast, the MCF-7 cells were more sensitive to biochanin A inhibition than the normal cells

(Peterson et al., 1996). The significance of these findings is yet to be determined. On one hand, cancer cells inactivate genistein to a less potent growth inhibitor via biotransformation. On the other hand, cancer cells activate biochanin A to a more potent growth inhibitor through biotransformation. Maybe the self-protective role that cancer cells play against genistein inhibition is more significant for themselves, because genistein is the major isoflavone in soybeans.

The Link Between Isoflavones and FB_1 Detoxification

The FB_1 toxicity and carcinogenicity may be diminished by isoflavones. It has been shown that FB_1 caused hypercholesterolemia in vervet monkeys (Fincham et al., 1992), but feeding soy isolate containing approximately 340 mg isoflavones/kg diet, however, lowered plasma TC and LDL + VLDL concentrations by 22-27% and 30-40%, respectively, in rhesus monkeys as compared with feeding soy isolate with isoflavones removed by ethanol extraction (Anthony et al., 1996). Both the FB_1-caused hypercholesterolemia and the isoflavone feeding-induced hypocholesterolemia seem to be primarily due to changes in plasma LDL, because plasma HDL and VLDL remained basically unchanged. This suggests that LDL receptor activity may be decreased by FB_1, but increased by isoflavones, so that the LDL removal from the blood is affected. Since the increased plasma TC is associated with FB_1-promoted hepatocarcinogenesis, it is possible that soy isoflavones counteract FB_1 hepatotoxicity and hepatocarcinogenicity by suppressing plasma TC.

Isoflavones may inhibit FB_1 carcinogenicity by diminishing the FB_1-stimulated DNA synthesis via inositol phosphate pathway, and probably by suppressing the prostaglandin formation as well. As mentioned above, phospholipase C is likely to be a target of isoflavones for blocking the release of arachidonic acid from PIP_2. The arachidonate release may be an important control point of prostaglandin synthesis (Smith, 1989). The major sources of arachidonate, however, are probably the most abundant

glycerophospholipids, phosphatidylcholine and phosphatidylethanolamine, which are hydrolyzed by phospholipase A_2 to release arachidonic acid (Smith, 1989). Certain flavonoids produced dose-dependent inhibition of snake venom phospholipase A_2 in the concentration range of 5 to 500 μM (Alcaraz and Hoult, 1985). Since isoflavones are flavonoids, they may decrease prostaglandin production by suppressing phospholipase A_2. In addition, isoflavones may produce inhibition on cyclooxygenase to block prostaglandin synthesis, because some flavonoids were shown to exert inhibitory effect on cyclooxygenase and prostanoid production (Moroney et al., 1988).

As protein tyrosine kinase inhibitors, isoflavones may also inhibit FB_1-promoted hepatocarcinogenesis by suppressing DNA synthesis in hepatocytes. Genistein was shown to inhibit DNA synthesis elicited by human hepatocyte growth factor (HGF) in rat primary hepatocytes (Arakaki et al., 1992). The early events of the HGF-mediated signal transduction pathways leading to increased DNA synthesis included elevation of IP_3 and intracellular Ca^{2+} concentrations, and were dependent on protein tyrosine kinase activity (Baffy et al., 1992). The stimulation of DNA synthesis by HGF, however, was potentiated by 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (Arakaki et al., 1992), an inhibitor of protein kinase C (PKC) which is a serine/threonine kinase. This is intriguing, because FB_1 also seems to be a PKC inhibitor. Fumonisin B_1 increased sphingosine concentration *in vitro* and *in vivo* (Riley et al., 1994; Yoo et al., 1992), and disruption of sphingolipid metabolism was related to stimulation of DNA synthesis by FB_1 in Swiss 3T3 cells (IC_{50} = 10 μM) (Schroeder et al., 1994). Since FB_1 is structurally similar to sphingosine, a potent PKC inhibitor (Wang et al., 1991), it was hypothesized that FB_1 might suppress PKC activity as well (Huang et al., 1995). The hypothesis was supported by the finding that FB_1 inhibited PKC activity in a dose-dependent manner (IC_{50} = 1

μM) in a monkey kidney cell line CV-1 (Huang et al., 1995). Based on the fact that the concentration required for 50% PKC inhibition by FB_1 (1 μM) is an order of magnitude lower than the concentration required for 50% stimulation by FB_1 of DNA synthesis (10 μM), it is likely that PKC is implicated in the FB_1-elicited signal transduction pathways leading to carcinogenesis. At present, it is difficult to evaluate the physiological relevance of these findings, because the information on the circulating concentrations of FB_1 in the body is currently unavailable.

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THRESHOLD DOSE FOR SHORT-TERM CANCER-PROMOTION BY DIETARY FUMONISIN B_1 IN DIETHYLNITROSAMINE-INITIATED RAT HEPATOCARCINOGENESIS

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ABSTRACT

To establish a short-term threshold dose of fumonisin B_1 (FB_1) hepatotoxicity and hepatic tumor promotion in rats, female F344/N rats were initiated with 15 mg/kg diethylnitrosamine at 10 days of age, and were fed 12.5, 25 and 50 ppm highly purified FB_1 for 4 weeks after weaning. Significant increases in plasma alanine aminotransferase activities by 86% and 132% were found in animals fed 25 and 50 ppm FB_1, respectively. Plasma total cholesterol concentrations were significantly raised by 91% and 145% with feeding 25 and 50 ppm FB_1, respectively. The rats fed 50 ppm FB_1 had significantly greater hepatic prostaglandin production by 34-61%. Placental glutathione S-transferase (PGST)- and gamma-glutamyltransferase (GGT)-positive altered hepatic foci (AHF) developed in 25 and 50 ppm FB_1 groups, but not 12.5 ppm. Feeding 50 ppm FB_1 significantly increased the percent areas of PGST- and GGT-positive AHF by about 2.1- and 2.5-fold, respectively, compared with feeding 25 ppm FB_1. Fumonisin B_1, as low as 25 ppm, was hepatotoxic and tumor-promoting in rats. The short-term threshold of FB_1 hepatotoxicity and hepatic tumor promotion was probably >12.5 ppm and ≤25 ppm.

INTRODUCTION

Fumonisin B_1 (FB_1) is a mycotoxin produced primarily by the commonly occurring corn fungi *Fusarium moniliforme* and *Fusarium proliferatum*.

Fumonisin B_1 caused equine leukoencephalomalacia (Kellerman et al., 1990; Wilson et al., 1992) and porcine pulmonary edema (Harrison et al., 1990), and was hepatotoxic and hepatocarcinogenic in rats (Gelderblom et al., 1988 and 1991). The occurrence of FB_1 in *F. moniliforme*-contaminated corn was associated with a relatively high rate of human esophageal cancer in southern Africa (Marasas et al., 1988; Sydenham et al., 1990).

Several biomarkers have been used to study FB_1 hepatotoxicity and hepatic tumor promotion. Fumonisin B_1-promoted rat hepatocarcinogenesis was readily quantified by measuring placental glutathione S-transferase (PGST)-positive altered hepatic foci (AHF) (Lebepe-Mazur et al., 1995) and gamma-glutamyltransferase (GGT)-positive foci (Gelderblom et al., 1988). Because of the early occurrence of PGST induction, which was evident within 48 h in single hepatocytes after diethylnitrosamine (DEN) initiation (Moore et al., 1987), and because of the persistence of both PGST and GGT during hepatocarcinogenesis (Hendrich et al., 1987), these two enzymes can serve as sensitive biomarkers of early stages of cancer development in the liver. Our previous study showed both PGST- and GGT-positive AHF were detected after 4 weeks in rats fed 50 ppm FB_1 following DEN initiation (Lu et al., 1997). Increased plasma or serum total cholesterol (TC) level and alanine aminotransferase (ALT) activity have also been associated with fumonisin hepatotoxicity and tumor promotion. *Fusarium proliferatum* strain 5991-fermented corn providing 50 ppm FB_1 to DEN-initiated rats significantly increased plasma TC and ALT while causing hepatocellular adenomas (Hendrich et al., 1993). In addition, greater hepatic prostaglandin production was related to FB_1-promoted hepatocarcinogenesis in rats (Lu et al., 1997).

Fumonisin B_1 has recently been listed as a class 2B carcinogen (International Agency for Research on Cancer, 1993). Development of appropriate animal models of carcinogenesis is critical to understanding the probable FB_1 carcinogenicity in humans. Because nitrosamines often contaminate human diets (National Academy of Sciences, 1981), and FB_1 is

almost always found in corn-based human foods (Hopmans and Murphy, 1993; Sydenham et al., 1991), a 2-stage rodent model combining DEN to initiate and FB_1 to promote carcinogenesis may approach the reality of human carcinogenesis (Hendrich et al., 1997). One advantage of utilizing this model is that the cancer promoting potential of FB_1 can be readily quantified by PGST- and GGT-positive AHF. Our DEN initiation/FB_1 promotion model, i.e. initiation of rats with DEN (15 mg/kg body weight) followed by 50 ppm FB_1 promotion for 4 weeks, can induce rat hepatocarcinogenesis rapidly, requires relatively small amounts of FB_1, and may mimic the natural environment where humans are exposed to a combination of chemical carcinogens.

Although FB_1 was non-mutagenic in the *Salmonella* mutagenicity assay (Gelderblom and Snyman, 1991) and lacked genotoxicity based on unscheduled DNA synthesis assay in primary rat hepatocytes (Norred et al., 1992), it seemed to be a complete carcinogen either at 50 ppm long-term (18-24 months) or at 250 ppm short-term (3 weeks) (Gelderblom et al., 1991 and 1994). As a potent promoter in the DEN initiation/FB_1 promotion model, fumonisin B_1 was effective to promote hepatocarcinogenesis at a dietary level of 50 ppm in 4-week studies (Hendrich et al., 1993; Lu et al., 1997). It was unknown, however, whether 50 ppm was the cancer-promoting threshold dose of FB_1 short-term. This exposure level to FB_1 may still be greater than 10-fold higher than the natural occurrence of FB_1 in corn-based human foods and animal feeds, which commonly contained 0.2-3 ppm FB_1 (Hopmans and Murphy, 1993; Murphy et al., 1993; Sydenham et al., 1991).

The present study tested our hypothesis that the threshold dose of FB_1 hepatotoxicity and tumor promotion may be below 50 ppm in the short-term DEN initiation/FB_1 promotion model. The dietary FB_1 levels of 12.5 and 25 ppm in addition to 50 ppm were selected to approach the natural occurrence of FB_1 in food, to establish dose-response effects of FB_1 on

hepatotoxicity and hepatocarcinogenesis in rats, and to ascertain a short-term cancer-promoting threshold for FB_1.

MATERIALS AND METHODS

Preparation of Purified 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.

Liquid cultures of *F. proliferatum* strain M5991 were prepared as described by Dantzer et al. (1996a). *Fusarium proliferatum* strain M5991, which predominantly produces FB_1, was a gift from Dr. Paul Nelson (Pennsylvania State University, State College, PA). Fumonisin B_1 was purified by the same procedure as described by Dantzer et al. (1996b). Purified FB_1 solutions were freeze-dried, and a dry weight was determined. The purified FB_1 was 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) and Dr. Chet Mirocha (University of Minnesota, St. Paul, MN).

Diets

All dietary ingredients except FB_1 were obtained from Harlan Teklad (Madison, WI). Four experimental diets including basal diet were fed to DEN-initiated rats for 4 weeks. The basal diet supplying 40% of energy as fat was modified from AIN-93G (American Institute of Nutrition, 1993). The ingredients of the basal diet were beef tallow 140 g/kg; soybean oil 67 g/kg; casein 224 g/kg; corn starch 228 g/kg; dextrose 224 g/kg; cellulose 56 g/kg; vitamin mix (AIN-93G-VX), 10 g/kg; mineral mix (AIN-93G-MX), 39 g/kg; L-cystine, 3 g/kg; choline bitartrate, 2.5 g/kg; and TBHQ, 0.014

g/kg. Highly purified FB_1-containing diets at 3 different FB_1 concentrations (12.5, 25 and 50 ppm) were prepared by incorporating FB_1 into the basal diet. All the experimental diets were stored at 4°C before use.

Animals

The use of animals and the experimental procedure were approved by the Iowa State University Animal Care Center Committee. Forty-eight 10-day-old female F344/N rats obtained from Harlan Sprague-Dawley (Madison, WI) were injected intraperitoneally with DEN (15 mg/kg body weight) in 0.1 mL of corn oil. At 3 weeks of age, the weaned rats were randomly assigned to one of the 4 treatment groups with 12 rats each. The 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 rats were recorded weekly.

Plasma and Liver Sample Preparation

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 TC concentration.

The liver was excised and 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 staining for GGT and PGST.

From each rat, 0.5 g of minced liver portions 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 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 TC concentration was determined by using Sigma diagnostic kit, procedure 352-3 (Sigma Chemical Co., St. Louis, MO). Plasma ALT activity was measured by using Sigma diagnostic kit for the glutamate/pyruvate transaminase optimized ALT assay (Sigma Chemical Co., St. Louis, MO).

Staining and Quantification of Altered Hepatic Foci

One of the frozen serial sections was stained for the presence of PGST-positive AHF. Placental glutathione S-transferase was detected by using a Vectastain ABC Elite anti-rabbit IgG 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 gamma-glutamyl-4-naphthylamide (GMNA) (United States Biochemical Corp., Cleveland, OH).

Altered hepatic foci were quantified via computer-assisted image analysis. Images of the serial sections stained for PGST and GGT were taken by a Sony 3-chip color video camera DXC-3000A, digitally transferred from the camera to a SiliconGraphics Indigo 2 XZ computer work station (SiliconGraphics, Mountain View, CA), and analyzed with Visilog image analysis software (Version 5.0.2, Noesis, St. Laurent, Quebec, Canada). The images were broken down to red, blue and green colors, followed by contrast enhancement. The whole tissue was outlined to obtain its total area after erosion was processed to delete any edge effect (artifactual darkening around the tissue edge as a result of staining procedure). A threshold of pixel values was selected for the lesions, so that only lesions were counted for calculation of lesion area. Calibration for the image analysis was accurate to mm.

Radioimmunoassay for Hepatic 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 Chemical Co., St. Louis, MO. Anti-rat PGE_2 rabbit antiserum was a gift from Dr. Jaqueline Dupont, USDA-ARS, Beltsville, MD. Goat anti-rabbit gamma globulin 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 $PGF_{2\alpha}$ and PGE_2 were obtained from Sigma Chemical Co., St. Louis, MO. 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 beginning of the sample run and the other set at the end. Radioactivity was determined as cpm by using a Packard liquid scintillation analyzer model 1900 TR (Packard Instrument Co., Downers Grove, IL).

Statistical Analysis

The hepatic $PGF_{2\alpha}$ and PGE_2 concentrations were determined by a computer program based on a logit transformation of the standard curve (Duddleson et al., 1972). General Linear Models procedure (GLM) was performed on feed consumption, body weight, liver to body weight ratio, plasma TC level and ALT activity, and hepatic concentrations of $PGF_{2\alpha}$ and PGE_2, using the Statistical Analysis System (SAS, Release 6.06, Cary, NC). Contrasts were done to compare treatment differences. For the analysis of percent volume of PGST- and GGT-positive AHF, Student's *t*-test was performed to compare the groups showing lesions. Treatment differences were considered significant at $P<0.05$.

RESULTS

Mean daily feed intake among the groups of rats were the same for all groups (Table 1). Overall mean daily feed intake was approximately 10 g. Fumonisin B_1 caused a significant reduction in final body weight. A weight reduction by 15% was found in the rats fed 50 ppm FB_1 ($P<0.01$), and a 7% weight reduction in the rats fed 25 ppm FB_1 was significant ($P<0.05$). In addition, feeding 50 ppm FB_1 significantly decreased body weight by 9% as compared with feeding 25 ppm FB_1 ($P<0.01$). Fumonisin B_1 at 12.5 ppm did not exert a toxic effect on body weight (Table 1). The liver weight to body weight ratio was significantly increased by 11% as a result of 50 ppm FB_1 feeding ($P<0.01$). Feeding 50 ppm FB_1 significantly increased the liver weight to body weight ratio by 9% compared with feeding 25 ppm FB_1 ($P<0.01$). Neither 25 nor 12.5 ppm FB_1 feeding had a significant effect on the ratio as compared with that of the control feeding (Table 1).

Significant increases in plasma ALT activity compared to control rats by 86% and 132% were found in the animals fed 25 and 50 ppm FB_1, respectively ($P<0.01$). There was no significant difference in the plasma ALT activity between the group fed 12.5 ppm FB_1 and the control group. The 25% difference in the plasma ALT activity between the rats fed 50 ppm FB_1 and those fed 25 ppm FB_1 was not significant (Table 2).

Fumonisin B_1 had a dose-dependent effect on plasma TC concentration. Feeding 25 and 50 ppm FB_1 significantly raised the plasma TC by 91% and 145%, respectively, over the control group ($P<0.01$). The plasma TC level was elevated significantly by 28% with the increasing FB_1 concentrations from 25 ppm to 50 ppm ($P<0.05$) (Table 2).

Compared with the control rats, the animals fed 50 ppm FB_1 had significantly greater hepatic $PGF_{2\alpha}$ and PGE_2 concentrations by 61% and 34%, respectively ($P<0.05$). No significant difference from the control group was found in prostaglandin production in the other two groups (Table 2).

All of the rats fed 50 ppm FB_1 developed both PGST- and GGT-positive AHF, which also appeared in 11 of 12 rats fed 25 ppm FB_1. In addition, feeding 50 ppm FB_1 significantly increased the percent areas of PGST- and GGT-positive AHF, expressed as the percentage of the area occupied by PGST- or GGT-positive AHF over the total area of the liver section, by about 2.1- and 2.5-fold, respectively, compared with feeding 25 ppm FB_1 ($P<0.05$). There were no PGST- or GGT-positive AHF in either the 12.5 ppm FB_1 group or the control group (Table 3). More and larger GGT-positive AHF with sharper edge developed in livers of rats fed 50 ppm FB_1 than those in livers of rats fed 25 ppm FB_1, and no GGT-positive AHF developed in the group fed 12.5 ppm FB_1 (Figure 1).

DISCUSSION

This study demonstrated that FB_1 was able to exert hepatotoxic and tumor-promoting effects at as low as 25 ppm in a short-term DEN initiation/FB_1 promotion rodent model, and greater effects were produced by feeding 50 ppm FB_1 than by feeding 25 ppm FB_1 to rats during the promotion phase. The dose-dependent FB_1 toxicity and tumor promotion were reflected in reduced body weight, increased liver weight to body weight ratio, elevated plasma TC concentration and ALT activity, increased hepatic prostaglandin production, and development of PGST- and GGT-positive AHF.

Fumonisin B_1 caused significant reduction in final body weight, apparently without affecting feed intake (Table 1). The toxic FB_1 doses would approximate 2.5-5 mg FB_1/kg body weight per day. The body weight and feed intake data were in agreement with our results reported previously (Hendrich et al., 1993; Lu et al., 1997), but not with those of two other studies using different animal models. Bondy et al. (1995) reported that both body weight and feed consumption of Sprague-Dawley rats were significantly decreased within 4 days, with daily intraperitoneal injection of 7.5 or 10 mg FB_1/kg body weight. Voss et al. (1995) demonstrated that

neither body weight nor feed intake was significantly decreased in F344 rats fed 1, 3, 9, 27 and 81 ppm FB_1 for 90 days. The reduction in feed intake in the 4-day study may be temporary because it was reported that FB_1 at dietary levels of 250, 500 and 750 ppm induced feed refusal in rats during the first days of a 21-day study, but the feed intake of the FB_1-treated rats was increased after 14 days and reached control level by 21 days (Gelderblom et al., 1994). The lack of toxic effect of FB_1 on body weight in the 90-day feeding study may be explained by the fact that hepatotoxicity and hepatocarcinogenesis were not found in the rats fed up to 81 ppm FB_1.

The dose-dependent increase in the relative liver weight, expressed as the liver weight to body weight ratio, was not only a sign of FB_1 hepatotoxicity, but was related to tumor promotion by FB_1 as well, because the group with the largest relative liver weight had the most PGST- and GGT-positive AHF (Tables 2 and 3). This relative hepatomegaly has been observed in other studies using FB_1 as a tumor promoter (Hendrich et al., 1993; Lebepe-Mazur et al., 1995).

Plasma ALT activity, plasma TC concentration and hepatic prostaglandin production were also associated with both the toxicity and the tumor-promoting ability of FB_1 in the present study. Fumonisin B_1 exerted dose-dependent effects on the increases in the concentrations or the activity of these biomarkers, with the greatest effects in the group (50 ppm FB_1) showing relatively most advanced tumor promotion which was represented by the greatest percent areas of PGST- and GGT-positive AHF (Tables 2 and 3). The alterations of these biomarkers, therefore, were closely related to the FB_1-promoted hepatocarcinogenesis in addition to hepatotoxicity.

This study supported previous findings that elevated plasma ALT activity and cholesterol concentration in FB_1-treated rats were associated with hepatotoxicity (Bondy et al., 1996; Voss et al., 1993), and

hepatocarcinogenesis as well (Hendrich et al., 1993; Lu et al., 1997). Increased plasma ALT activity generally indicates release of this enzyme from damaged hepatocytes with altered plasma membrane permeability. Exposure of hepatocytes to hepatotoxicants is likely to cause oxidative stress, which in turn may induce plasma membrane damage (Halliwell, 1994). Fumonisin B_1, as a hepatotoxicant, could subject hepatocytes to increased lipid peroxidation, based on a reported accumulation of polyunsaturated fatty acids in primary hepatocytes treated with 150 and 500 μM FB_1 (Gelderblom et al., 1996).

The mechanism underlying the effect of FB_1 on plasma total cholesterol is unknown. The increase in plasma TC might result from stimulated cholesterol synthesis in hepatocytes, or impaired cholesterol removal by the liver. Indirect evidence supports both viewpoints. Activity of 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase, a rate-limiting enzyme of cholesterol biosynthesis, was 2-fold greater in primary hepatocellular carcinomas induced by feeding 0.04% 2-acetylaminofluorene for 20 weeks than in normal livers from rats (Mitchell et al., 1978). Maybe HMG CoA reductase activity can be stimulated during FB_1-promoted hepatocarcinogenesis as well. Based on elevation of plasma LDL, but not HDL or VLDL, and lack of accumulation of cholesterol in livers of vervet monkeys fed a low fat diet containing fumonisins, it was proposed that the removal of cholesterol from plasma via hepatic LDL receptor could be impaired (Fincham et al., 1992). The increase in plasma TC seemed to be secondary to FB_1 hepatotoxic and tumor-promoting effects. Whether cholesterol-lowering agents can counteract the effects of FB_1 has not been investigated.

Changes in hepatic prostaglandin production mirrored those observed in our previous study showing greater hepatic concentrations of $PGF_{2\alpha}$ and PGE_2 in rats fed 50 ppm FB_1 than in rats fed basal diet (Lu et al., 1997). Our data suggested that the elevation of prostaglandin levels paralleled

the induction of PGST- and GGT-positive AHF, but prostaglandin status was extremely variable among individuals, making this biomarker less sensitive than the other biomarkers studied. Increased prostaglandin production may have been caused by hepatocarcinogenesis promoted by FB_1. Similar to plasma TC, the increase in prostaglandin production was also likely to be secondary to hepatotoxicity and hepatocarcinogenesis. Prostaglandins may contribute to cell proliferation in the liver at certain stages during FB_1 treatment. Increased prostaglandin concentrations were associated with preneoplastic cell proliferation (Delescluse et al., 1982). Inhibition of prostaglandin synthesis by indomethacin played a role in suppressing tumor growth *in vivo* and prolonging survival time of hepatoma-bearing rats by approximately 50% (Trevisani et al., 1980). The possible suppressive effect of prostaglandin synthesis inhibitors on FB_1 tumor promotion has yet to be studied.

Placental glutathione S-transferase and GGT are markers of AHF (Goldsworthy and Pitot, 1985; Sato et al., 1984). These enzymes effectively scored many types of chemical hepatocarcinogenesis (Hendrich et al., 1987). The development of GGT- and/or PGST-positive AHF was also demonstrated in FB_1-induced hepatocarcinogenesis (Gelderblom et al., 1988; Lebepe-Mazur et al., 1995; Lu et al., 1997). In the present study all the rats fed 50 ppm FB_1 developed both PGST- and GGT-positive AHF, which also appeared in 11 of 12 rats fed 25 ppm FB_1. The dose-dependent increase in the percent areas of PGST- and GGT-positive AHF suggested that the promotion in the rats fed 25 ppm FB_1 was not as advanced as in the ones fed 50 ppm FB_1, although at both concentrations, the carcinogenesis was still at early stages as indicated by PGST- and GGT-positive AHF, which were preneoplastic lesions.

This study confirmed our previous finding that feeding 50 ppm FB_1 for 4 weeks elicited hepatotoxicity and tumor promotion in DEN-initiated rats (Hendrich et al., 1993; Lu et al., 1997). The consistency of the results indicated that our short-term DEN initiation/FB_1 promotion model

was proficient in quantifying FB_1-promoted carcinogenesis. This model was adapted from Peraino's (Peraino et al., 1984), in which effects of tumor promoters generally can be detected at 3 months after weaning. However, tumor promotion by FB_1 at 25 and 50 ppm can be detected at only 1 month after weaning. The rapidity of FB_1 tumor promotion in this model might facilitate studies of FB_1 promotion mechanisms and inhibitors of FB_1 promotion. This model may also be used to screen potential tumor promoters in a relatively short period of time. The relevance of this model to human carcinogenesis may be attributed to the combination of 2 carcinogens, DEN and FB_1, which commonly contaminate human foods in appreciable amounts. The combination of a tumor initiator and a tumor promoter in the bioassay may serve as a simplified model of the natural environment in which humans are often exposed to multiple carcinogens. The presence of FB_1 and nitrosamines in human foods raises a possibility that populations in high risk areas of human esophageal cancer are exposed to both carcinogens concomitantly.

The present study extended our previous finding to a lower dose of FB_1, i.e. feeding 25 ppm FB_1 for 4 weeks elicited hepatotoxicity and tumor promotion in DEN-initiated rats. Fumonisin B_1 at 12.5 ppm, however, was unable to produce any adverse effects as evidenced by lack of significant changes in plasma TC concentration, plasma ALT activity and hepatic prostaglandin production, and by complete absence of PGST- and GGT-positive AHF (Table 3). Therefore, using the short-term DEN initiation/FB_1 promotion model, an approximate short-term threshold dose of FB_1 was determined. Although it was impossible to ascertain its exact value, the threshold, at which FB_1 should evoke an all-or-none response, was very probably >12.5 ppm but ≤25 ppm. The threshold dose of FB_1 obtained in this study was well within the range of fumonisin concentrations associated with a relatively high risk of human esophageal cancer (3-47 ppm, Sydenham et al., 1991) and field outbreaks of animal diseases (<1-330 ppm, Ross et al., 1990), and this threshold dose was approximately an order of magnitude greater than

the FB_1 levels found in corn-based human foods and animal feeds (0.2-3 ppm).

Using different animal models, some investigators have reported various minimum doses required for FB_1 to produce toxic and carcinogenic effects. Feeding 1000 ppm FB_1 to DEN (200 mg/kg body weight)-initiated rats for 4 weeks induced GGT-positive foci in the liver (Gelderblom et al., 1988). When corn cultures of *F. proliferatum* providing 20 ppm FB_1 were fed to DEN (30 mg/kg body weight)-initiated rats for 6 months, induction of PGST-positive AHF was exhibited (Lebepe-Mazur et al., 1995). Rats fed 50 ppm FB_1 for 18-26 months developed primary hepatocellular carcinoma and cholangiocarcinoma (Gelderblom et al., 1991). The lowest dietary dose of FB_1 required for cancer initiation in the rat liver over 21 days was 250 ppm (Gelderblom et al., 1994). Therefore, longer-term exposure seemed to lower FB_1's minimum toxic dose. Apart from the liver, the kidney was also a target organ of fumonisin toxicity in rats. Voss et al. (1993) reported that FB_1 was hepatotoxic to rats fed 150 ppm for 4 weeks and was nephrotoxic at 15-50 ppm. In a subchronic feeding study, hepatotoxicity was not found in rats fed up to 81 ppm FB_1 for 90 days, but nephrotoxicity occurred at 9-81 ppm (Voss et al., 1995). Comparing the studies of Voss et al. (1993 and 1995) with their own 28-day feeding study on fumonisin hepato- and nephrotoxicity in rats, Tolleson et al. (1996) estimated that the lowest hepatotoxic and the lowest nephrotoxic doses of FB_1 in rats were 99-250 ppm and 9-100 ppm, respectively. Taken together, with respect to FB_1 hepatotoxicity and hepatocarcinogenicity, the threshold of FB_1 determined in our present study was apparently among the lowest.

CONCLUSION

This study demonstrated that feeding 25 ppm FB_1 for 4 weeks induced tumor promotion in DEN-initiated rats. Different threshold doses of FB_1, however, probably exist, depending on the selection of animal models. Based

on the results from the other studies discussed above, a longer-term feeding could further lower the threshold of FB_1 in a DEN initiation/FB_1 promotion model, thus approaching more closely the naturally occurring dose of FB_1.

It is noticeable that esophageal cancer occurring in humans has not been experimentally reproduced with FB_1 in any animal models, and there has been no evidence associating FB_1 with human hepatocarcinogenesis. Moreover, information on the mechanisms underlying the species- and organ-specificity of toxic and/or carcinogenic effects of FB_1 is currently very limited. Future studies are greatly needed to understand the mechanisms of fumonisin toxicity and carcinogenicity, to determine whether there is a causative relationship between fumonisins and human esophageal cancer, and to establish tolerance levels for fumonisins in corn-based human foods and animal feeds.

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Table 1. Fumonisin B_1 (FB_1) Decreased Body Weight and Increased Liver Weight to Body Weight Ratio (LW/BW Ratio), but Did Not Affect Feed Intake*

	Body Weight(g)	LW/BW($\times 10^{-2}$)	Feed Intake(g/d)
Control	119 ± 9	4.4 ± 0.3	10 ± 1
12.5 ppm FB_1	112 ± 10	4.2 ± 0.2	10 ± 2
25 ppm FB_1	110 ± 7[a]	4.5 ± 0.5	10 ± 2
50 ppm FB_1	100 ± 9[bc]	4.9 ± 0.5[bc]	10 ± 2

*The values are given as group means ± standard deviations, n=12.

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

[b]Significantly different from the control group, $P<0.01$.

[c]Significantly different from the 25 ppm FB_1 group, $P<0.01$.

Table 2. Fumonisin B_1 (FB_1) Increased Plasma Total Cholesterol (TC) Concentration, Plasma Alanine Aminotransferase (ALT) Activity, and Hepatic Prostaglandin (PG) Production*

	Plasma TC (mg/dL)	Plasma ALT act. (u/L)	Hepatic $PGF_{2\alpha}$ (ng/g)	Hepatic PGE_2 (ng/g)
Control	89 ± 15	28 ± 4	59 ± 34	50 ± 16
12.5 ppm FB_1	103 ± 28	27 ± 6	61 ± 47	56 ± 26
25 ppm FB_1	170 ± 52[a]	52 ± 25[a]	85 ± 50	60 ± 23
50 ppm FB_1	218 ± 73[ac]	65 ± 24[a]	95 ± 48[c]	68 ± 19[c]

*The values are given as group means ± standard deviations, n=12.

[a]Significantly different from the control group, $P<0.01$.

[c]Significantly different from the control group, $P<0.05$.

[b]Significantly different from the 25 ppm FB_1 group, $P<0.05$.

**Table 3. Placental Glutathione S-Transferase (PGST)-Positive (PGST$^+$) and Gamma Glutamyltransferase (GGT)-Positive (GGT$^+$) Altered Hepatic Foci (AHF) Occurred in Rats Fed 25 and 50 ppm Fumonisin B_1 (FB_1), and the Percent Areas of PGST$^+$ and GGT$^+$ AHF (% PGST Area and % GGT Area) Increased with the Higher FB_1 Level*

	# of Rats with PGST$^+$ AHF	% PGST Area	# of Rats with GGT$^+$ AHF	% GGT Area
Control	0	0	0	0
12.5 ppm FB_1	0	0	0	0
25 ppm FB_1	11	12 ± 13	11	6 ± 5
50 ppm FB_1	12	24 ± 16[a]	12	14 ± 8[a]

*The percent areas of PGST$^+$ and GGT$^+$ AHF are expressed as the percentage of the area occupied by PGST$^+$ or GGT$^+$ AHF over the total area of the liver section. The values are given as group means ± standard deviations, n=12.

[a]Significantly different from the 25 ppm FB_1 group, $P<0.05$.

Figure 1. More and larger gamma-glutamyltransferase-positive altered hepatic foci with sharper edge developed in livers of rats fed 50 ppm FB_1 (FB1-50) than those in livers of rats fed 25 ppm FB_1 (FB1-25), and no gamma-glutamyltransferase-positive altered hepatic foci occurred in the 12.5 ppm FB_1 (FB1-12.5) group.

FB1-12.5

FB1-25

REACTION WITH FRUCTOSE DETOXIFIES FUMONISIN B_1 WHILE STIMULATING LIVER-ASSOCIATED NATURAL KILLER CELL ACTIVITY IN RATS

A paper published in the Journal of Agricultural and Food Chemistry

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ABSTRACT

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-glutamyltransferase-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.

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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., 1990). 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., 1991).

The hepatocarcinogenic effect of FB_1 can be evaluated *in vivo* by measuring changes in placental glutathione S-transferase (PGST) (Lebepe-Mazur et al., 1994) 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 anti-tumor 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 hr, was similar in toxicity to FB_1 when 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.

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.

Fusarium 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 x 100 cm, Sigma Chemical Co., St. Louis, MO). The column was washed with water, and FB_1 was eluted with 4 L methanol. The eluate was dried under vacuum, reconstituted in water and loaded onto a Lobar LiChroprep RP-8 column (25 x 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 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 % 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 hours 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. Based on the proposed reaction between FB_1 and a reducing sugar such as fructose (Murphy et al., 1996), it was estimated that approximately 17 g unreacted fructose was added per kg of FB_1-fructose diet. All the diets were stored at 4°C.

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 corn oil. At 3 weeks of age, the weaned rats were randomly assigned into one of the 3 treatment groups with 6-7 rats each. In the experiment with highly purified FB_1, 39 10-day old female F344/N rats obtained from Harlan Sprague-Dawley (Madison, Wisconsin) 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-hr 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 hours 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 3 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 minced liver portions were 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. 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 Chemical Co., St. Louis, MO). Plasma ALT activity was measured by using Sigma diagnostic kit for glutamate/pyruvate transaminase optimized ALT assay (Sigma Chemical Co., St. Louis, MO).

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 gamma-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 Chemical Co., St. Louis, MO. 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 Ci/mmol and 154.0 Ci/mmol, respectively, were purchased from Dupont New England Nuclear, Boston, MA. Standards of $PGF_{2\alpha}$ and PGE_2 were obtained from Sigma Chemical Co., St. Louis, MO. 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 7 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, Boston, MA) for 70 min, and maintained in complete media. The targets were washed 3 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 hours in a humidified CO_2 incubator (5% CO_2, 95% air) (Fisher Scientific, Chicago, IL). 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 $PGF_{2\alpha}$ 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 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 g, 13 ± 2 g 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 twofold 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 ($P<0.01$) (Fig. 1). Thus, feeding highly purified FB_1 or FB_1-fructose induced significant and opposing changes in

liver tumor cytotoxicity.

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 towards 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, 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). The greater development of PGST- and GGT-positive AHF, by 5-fold and 13-fold, respectively, caused by highly purified FB_1 compared with crude FB_1 (Table 4), maybe 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 hours 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 3,4), and in the female rats fed purified FB_1 liver-associated anti-tumor 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 were down-regulators of protein kinase C (Merrill, 1991), which could affect many enzyme activities after it was 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.

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Table 1. Basal Diet Composition[1]

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

[1]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-fru.) or an Basal Diet

	Group		
	Basal	FB_1-fru.	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*
Body weight increase (g)	128 ± 15	128 ± 8	108 ± 15*

*Significantly different from the basal group, $P<0.05$.

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-fru.) or a Basal Diet

	Plasma Cholesterol (mg/dl)	Plasma ALT activity (u/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**	105 ± 58*	77 ± 22*	7 ± 2
FB_1-fru. (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 9n=12)	261 ± 68**	75 ± 22*	10 ± 4*	4 ± 2*
FB_1-fru. (n=12)	121 ± 27	50 ± 11	5 ± 3	2 ± 1

*Significantly different from the basal group in respective study, $P<0.05$.

**Significantly different from the basal group in respective study, $P<0.01$.

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

	# of PGST lesions	# 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-fru. (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-fru. (n=12)	0	0	0	0

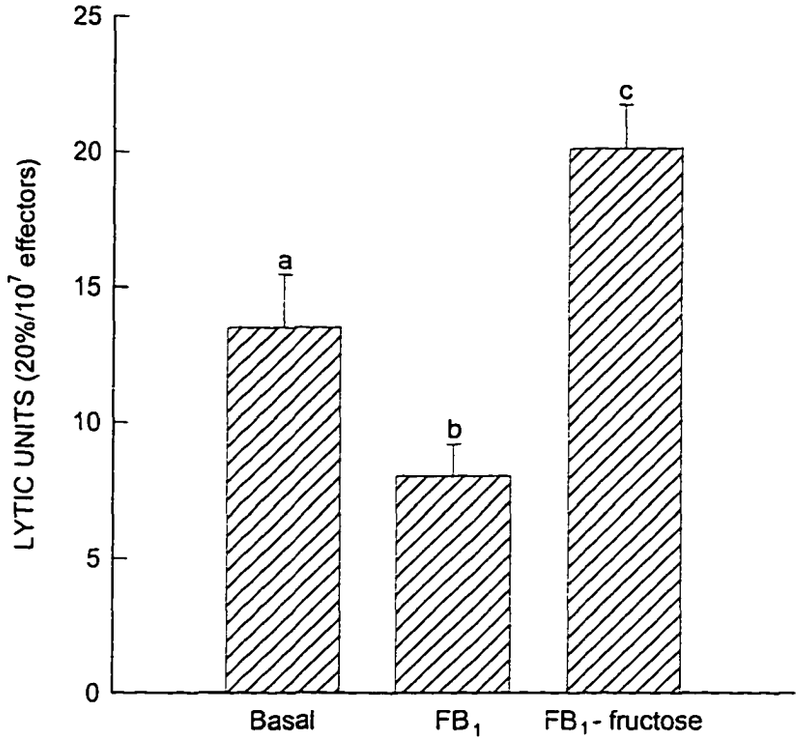

Figure 1. Tumor cytotoxicity of liver-associated NK cells against YAC-1 target cells, expressed as mean lytic units (LU) ± SE (n = 7/group), in female rats fed fumonisin B_1 (FB_1), FB_1 reacted with fructose (FB_1-fructose) or a basal diet. Treatments marked by different letters were significantly different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

SUPPRESSION OF FUMONISIN B_1-PROMOTED RAT HEPATOCARCINOGENESIS BY GENISTEIN AND DAIDZEIN DEPENDS UPON FUMONISIN B_1 DOSE

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ABSTRACT

To test our hypothesis that isoflavones may suppress tumor promotion by fumonisin B_1, female F344/N rats initiated with diethylnitrosamine were fed 1 mmol isoflavones with 18, 35 and 70 μmol FB_1 per kg diet for 4 weeks. Plasma total cholesterol (TC) concentration, alanine aminotransferase (ALT) activity, and hepatic prostaglandin production were significantly greater in the rats fed FB_1 (70 μmol/kg diet)-containing diets with and without isoflavones than in control rats. Placental glutathione S-transferase (PGST)-positive and gamma-glutamyltransferase (GGT)-positive altered hepatic foci (AHF) developed in the rats fed diets containing 70 μmol FB_1/kg regardless of isoflavone content. Increased plasma TC (91% over that of the control) was found in the animals fed 35 μmol FB_1/kg diet, and the increase in plasma TC was diminished by isoflavones. Plasma ALT activity of the rats fed 35 μmol FB_1/kg diet was significantly raised by 86%. Isoflavones normalized plasma ALT activity to the control level when co-administered with 35 μmol FB_1/kg diet. Fumonisin B_1 at 18 and 35 μmol/kg diet did not significantly affect hepatic prostaglandin concentrations compared with the control diet, neither did isoflavones at 1 mmol/kg diet. Rats fed the diet containing 35 μmol/kg FB_1 developed both PGST- and GGT-positive AHF, but adding isoflavones to this FB_1-containing diet significantly suppressed the development of PGST- and GGT-positive AHF by

75% and 80%, respectively. These results showed that isoflavones at 1 mmol/kg diet suppressed FB_1 tumor promotion only at an FB_1 dose of 35 µmol/kg diet and not when 70 µmol FB_1/kg was fed.

INTRODUCTION

Greater soybean consumption was associated with lower risk of breast cancer in Asian countries (reviewed by Adlercreutz et al., 1995). Animal studies showed that soybeans played a protective role against carcinogenesis (Barnes et al., 1990; Hawrylewicz et al., 1991; Troll et al., 1980). Isoflavones, mainly genistein, daidzein and their glucoside conjugates, are abundant in soybean foods (0.2-1.5 mg/g) (Wang and Murphy, 1994). Soybean isoflavones may contribute in large part to the anticarcinogenic effects associated with soybean consumption (Lamartiniere et al., 1995; Lee et al., 1995).

Fumonisin B_1 (FB_1) is a mycotoxin produced primarily by the commonly occurring corn fungi *Fusarium moniliforme* and *Fusarium proliferatum*. Fumonisin B_1 has recently been listed as a class 2B carcinogen (International Agency for Research on Cancer, 1993). In addition, fumonisin B_1 is a potent rat hepatocarcinogen (Gelderblom et al., 1991). Our previous studies showed that FB_1 at 70 µmol/kg diet rapidly promoted diethylnitrosamine (DEN)-initiated rats in 4 weeks (Hendrich et al., 1993; Lu et al., 1997a). The natural occurrence of FB_1 in corn-based human foods and animal feeds commonly ranged from 0.3-4.2 µmol/kg (Hopmans and Murphy, 1993; Murphy et al., 1993; Sydenham et al., 1991).

Several biomarkers have been used to study FB_1 hepatocarcinogenicity. Placental glutathione S-transferase (PGST) and gamma-glutamyltransferase (GGT) were markers of altered hepatic foci (AHF) (Goldsworthy and Pitot, 1985; Sato et al., 1984), and effectively scored certain types of chemical hepatocarcinogenesis (Hendrich et al., 1987). The development of GGT- or PGST-positive AHF, or both, was also demonstrated in FB_1-promoted

hepatocarcinogenesis in rats (Gelderblom et al., 1988; Lebepe-Mazur et al., 1995; Lu et al., 1997a). Because of the early occurrence of PGST induction, which was evident within 48 h in single hepatocytes after DEN initiation (Moore et al., 1987), and because of the persistence of both PGST and GGT during hepatocarcinogenesis (Hendrich et al., 1987), these two enzymes can serve as sensitive biomarkers of early stages of carcinogenesis in the liver. Increased plasma or serum total cholesterol (TC) level and alanine aminotransferase (ALT) activity have been associated with FB_1-promoted hepatocarcinogenesis. *Fusarium proliferatum*-fermented corn providing FB_1 (70 μmol/kg diet) to DEN-initiated rats significantly increased plasma TC level and ALT activity while causing hepatocellular adenomas (Hendrich et al., 1993). In addition, greater hepatic prostaglandin production was related to FB_1 tumor promotion in rat liver (Lu et al., 1997a). These biomarkers of FB_1 tumor promotion can be readily used to study the anticarcinogenic effects of isoflavones.

Most animal studies on the anticarcinogenic ability of isoflavones utilized rodent mammary carcinogenesis models. Because FB_1 is almost always found in appreciable amounts in corn-based human foods and animal feeds, and it is potentially a human carcinogen, investigating whether isoflavones exert anticancer effects on FB_1-promoted carcinogenesis is warranted. Using a 2-stage hepatocarcinogenesis model, i.e. DEN (15 mg/kg body weight) initiation of rats followed by FB_1 (70 μmol/kg diet) promotion for 4 weeks, our previous study demonstrated that feeding isoflavone extract providing 1 mmol isoflavones/kg diet suppressed FB_1 tumor promotion, as reflected in reduced hepatic prostaglandin $F_{2\alpha}$ production and plasma ALT activity, and suppressed development of PGST-positive AHF (Hendrich et al., 1997).

The present studies were designed to determine whether our previous finding can be extended from soy isoflavone extract to isoflavones with the same carcinogenesis model. On selection of FB_1 concentrations, 35 and 18 μmol in addition to 70 μmol FB_1/kg diet were chosen to approach more

closely the natural occurrence of FB_1 in corn-based human foods and animal feeds. Because isoflavone extract was able to suppress FB_1-promoted hepatocarcinogenesis, and isoflavones were considered to be responsible in large part for the effect, it was hypothesized that dietary isoflavones may at least partly inhibit rat hepatocarcinogenesis promoted by FB_1.

MATERIALS AND METHODS

Syntheses of Daidzein and Genistein

Daidzein was synthesized in high yield (82%) via cyclization of 2,4,4'-trihydroxydeoxy benzoin (THB) which was produced as reported (Chang et al., 1994). Two hundred mg of THB were dissolved in 8 mL N,N-dimethyl formamide in a 500 mL beaker. Following addition of 4 mL boron trifluoride-diethyl ether, the reaction mixture was heated in a Kenmore U88-332 microwave oven for 21 s at medium (40%) power output. Then 4 mL methanesulfonyl chloride was added and the mixture was heated in the microwave oven for 70 s at the same power level. A light-yellow precipitate appeared after 400 mL cold water was added to the hot reaction mixture. The precipitate was collected by centrifugation at 170,000 rpm (Beckman J2-21) for 10 min., washed with water, dissolved in methanol, and recrysallized by adding water. The purity (>98%) of daidzein was determined by retention time on HPLC, spectral analysis and melting point. The HPLC system consisted of a YMC-pack ODS-AM-303 column (5 μm, 25 cm x 4.6 cm), a Beckman Model 126 pump, a Beckman Model 168 photodiode array detector monitoring 200-300 nm, an IBM computer with Beckman Gold system HPLC data processing software, and a Beckman Model 507 autosampler. The spectral analysis of daidzein was performed with a Beckman DU 7400 spectrophotometer, and the melting point of daidzein was measured on a Perkin Elmer 7 series/Unix differential scanning calorimeter.

Genistein was synthesized as reported (Chang et al., 1994). The purity (>98%) was determined by the same methods used for daidzein.

Preparation of Purified 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.

Liquid cultures of *F. Proliferatum* strain M5991 were prepared as in Dantzer et al. (1996a). *Fusarium Proliferatum* strain M5991, a predominant producer of FB_1, was a gift from Dr. Paul Nelson (Pennsylvania State University, State College, PA). Fumonisin B_1 was purified by the same procedure as described by Dantzer et al. (1996b). 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) and Dr. Chet Mirocha (University of Minnesota, St. Paul, MN).

Diets

All dietary ingredients except isoflavones and FB_1 were obtained from Harlan Teklad (Madison, WI). In both experiments, a basal diet without incorporation of isoflavones and FB_1 was used as a control. The basal diet supplying 40% of energy as fat was modified from AIN-93G (American Institute of Nutrition, 1993). The ingredients of the basal diet were beef tallow 140 g/kg; soybean oil 67 g/kg; casein 224 g/kg; corn starch 228 g/kg; dextrose 224 g/kg; cellulose 56 g/kg; vitamin mix (AIN-93G-VX), 10 g/kg; mineral mix (AIN-93G-MX), 39 g/kg; L-cystine, 3 g/kg; choline bitartrate, 2.5 g/kg; and TBHQ, 0.014 g/kg. In Experiment 1, eight experimental diets including the basal diet were fed to DEN-initiated rats for 4 weeks (Table 1). Diets containing genistein (1 mmol/kg diet), daidzein (1 mmol/kg diet) or their equimolar combination (1 mmol total

isoflavones/kg diet) were prepared by incorporating isoflavones into the basal diet. Fumonisin B_1 (69.3 μmol/kg diet) was incorporated into the basal diet alone, with each isoflavone, or with isoflavone combination. In Experiment 2, six experimental diets including the basal diet were fed to DEN-initiated-rats for 4 weeks (Table 1). Equimolar combination of genistein and daidzein (1 mmol total isoflavones/kg diet), which approximated soy isoflavone composition, was incorporated into the basal diet alone, with 18 μmol FB_1/kg diet, or with 35 μmol FB_1/kg diet. In addition, fumonisin B_1 alone was incorporated into the basal diet at 18 and 35 μmol/kg diet. All the experimental diets were stored at 4°C before use.

Animals

The use of animals and the experimental procedure were approved by the Iowa State University Animal Care Center Committee. In both experiments, ten-day-old female F344/N rats obtained from Harlan Sprague-Dawley (Madison, WI) were injected intraperitoneally with DEN (15 mg/kg body weight) in 0.1 mL corn oil. At 3 weeks of age, the weaned rats were randomly assigned into one of the 8 (Experiment 1) or 6 (Experiment 2) treatment groups with 12 rats each. The 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 rats were recorded weekly.

Plasma and Liver Sample Preparation

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 TC concentration.

The liver was excised and 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 staining for GGT and PGST.

From each rat, 0.5 g of minced liver portions 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 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 TC concentration was determined by using Sigma diagnostic kit, procedure 352-3 (Sigma Chemical Co., St. Louis, MO). Plasma ALT activity was measured by using Sigma diagnostic kit for glutamate/pyruvate transaminase optimized ALT assay (Sigma Chemical Co., St. Louis, MO).

Staining and Quantification of Altered Hepatic Foci

One of the frozen serial sections was stained for the presence of PGST-positive AHF. Placental glutathione S-transferase was detected by using a Vectastain ABC Elite anti-rabbit IgG 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 gamma-glutamyl-4-naphthylamide (GMNA) (United States Biochemical Corp., Cleveland, OH).

Altered hepatic foci were quantified via computerized stereology or computer-assisted image analysis. The computerized stereology used a slightly modified program 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 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 LA 120 printer (Digital Equipment Corp., Maryland, MA) to quantify AHF. In computer-assisted image analysis, images of the serial sections stained for PGST and GGT were taken by a Sony 3-chip color video camera DXC-3000A, digitally transferred from the camera to a SiliconGraphics Indigo 2 XZ computer work station (SiliconGraphics, Mountain View, CA), and analyzed with Visilog image analysis software (Version 5.0.2, Noesis, St. Laurent, Quebec, Canada). The images were broken down to red, blue and green colors, followed by contrast enhancement. The whole tissue was outlined to obtain its total area after erosion was processed to delete any edge effect (artifactual darkening around the tissue edge as a result of staining procedure). A threshold of pixel values was selected for the lesions, so that only lesions were counted for calculation of lesion area. Calibration for the image analysis was accurate to mm.

Radioimmunoassay for Hepatic Prostaglandin $F_{2\alpha}$ and E_2 Levels

Endogenous liver $PGF_{2\alpha}$ and prostaglandin E_2 (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 Chemical Co., St. Louis, MO. Anti-rat PGE_2 rabbit antiserum was a gift from Dr. Jaqueline Dupont, USDA-ARS, Beltsville, MD. Goat anti-rabbit gamma globulin 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 $PGF_{2\alpha}$ and PGE_2 were obtained from Sigma Chemical Co., St. Louis, MO. Scintiverse BD (Fisher Chemical, Fair Lawn, NJ) was added to each tube before counting. Sample-containing tubes were run in duplicate, one set at beginning of the sample run and the other set

at the end. Radioactivity was determined as cpm by using a Packard liquid scintillation analyzer model 1900 TR (Packard Instrument Co., Downers Grove, IL).

Statistical Analysis

The hepatic $PGF_{2\alpha}$ and PGE_2 concentrations were determined by a computer program based on a logit transformation of the standard curve (Duddleson et al., 1972). General Linear Models procedure (GLM) was performed on feed consumption, body weight, liver to body weight ratio, plasma TC level and ALT activity, and hepatic concentrations of $PGF_{2\alpha}$ and PGE_2, using the Statistical Analysis System (SAS, Release 6.06, Cary, NC). Contrasts were done to compare treatment differences. For the analysis of percent areas of PGST- and GGT-positive AHF, Student's *t*-test was performed to compare the groups showing lesions. Treatment differences were considered significant at $P<0.05$.

RESULTS

Experiment 1. Feeding Rats 1 mmol Isoflavones with 70 μmol FB_1/kg Diet

There was no significant difference in mean daily feed intake among the groups of rats (Table 2). Compared with the control rats, a significant reduction in final body weight was found in the rats fed FB_1 with and without isoflavones ($P<0.05$). Feeding isoflavones without FB_1 did not affect the final body weight as compared with feeding the basal diet (Table 2).

After 4 weeks of feeding, the rats fed isoflavones alone did not differ significantly from the rats fed the basal diet in plasma TC concentration, ALT activity, and hepatic $PGF_{2\alpha}$ and PGE_2 production, whereas all the measured parameters were significantly greater in the rats fed FB_1-containing diets with and without isoflavones as compared with the control rats ($P<0.05$) (Table 3).

Placental glutathione S-transferase-positive and GGT-positive AHF developed in all rats fed 70 µmol FB_1/kg diet regardless of dietary isoflavone content, but no AHF occurred in the rats fed the basal diet or the isoflavones alone (Table 4).

Experiment 2. Feeding Rats 1 mmol Isoflavones with 18 and 35 µmol FB_1/kg Diet

As in Experiment 1, there was no significant difference in mean daily feed intake among the groups of rats. Overall mean daily feed intake was approximately 11 g (Table 2). Compared with the control rats, all the other groups of rats showed an average of 7% lesser final body weight ($P<0.05$-0.01). Adding genistein and daidzein at a total isoflavone concentration of 1 mmol/kg diet to either FB_1-containing diet (18 or 35 µmol FB_1/kg diet) did not normalize the body weight (Table 2). With respect to liver weight to body weight ratio, there was no significant difference in the ratio among all the treatment groups (Table 2).

Significant increase by 91% in plasma TC concentration over that of the control rats was found in the animals fed 35 µmol/kg FB_1 ($P<0.01$). This effect of FB_1 was counteracted by isoflavones, as reflected in that the plasma TC concentration of the rats fed FB_1 (35 µmol/kg diet) + isoflavones was significantly less than that of the rats fed FB_1 without isoflavones ($P<0.01$). Feeding isoflavones alone or 18 µmol FB_1/kg diet did not affect plasma TC as compared with feeding the basal diet. There was no significant difference in plasma TC between the rats fed 18 µmol/kg FB_1 and the ones fed FB_1 + isoflavones (Table 3).

Similar to the plasma TC concentration, plasma ALT activity of the rats fed 35 µmol FB_1/kg diet was significantly raised by 86% over that of the control rats ($P<0.01$). Isoflavones at 1 mmol/kg diet normalized plasma ALT activity to the control level when added to the diet containing 35 µmol FB_1/kg diet ($P<0.01$). Neither isoflavones alone nor 18 µmol FB_1/kg diet affected plasma ALT activity. There was no significant difference in plasma

ALT activity between feeding 18 μmol/kg FB_1 with and without isoflavones (Table 3).

Fumonisin B_1 at both dietary levels did not significantly affect either hepatic $PGF_{2\alpha}$ or hepatic PGE_2 concentration over the control, neither did isoflavones at 1 mmol/kg diet. Although adding isoflavones to the diet containing 35 μmol/kg FB_1 lowered hepatic $PGF_{2\alpha}$ concentration by 34%, the difference was not significant ($P = 0.06$) (Table 3).

Rats fed the diet containing 35 μmol FB_1/kg developed both PGST- and GGT-positive AHF, but adding 1 mmol/kg isoflavones to this FB_1-containing diet significantly diminished the development of PGST- and GGT-positive AHF by 75% and 80%, respectively ($P<0.05$). There were fewer and smaller GGT-positive AHF in livers of rats fed 1 mmol isoflavones/kg diet with 35 μmol FB_1/kg diet, and the AHF seemed to be less evenly stained than those in livers of rats fed 35 μmol FB_1/kg diet (Figure 1). There were no AHF developed in the rats fed 18 μmol FB_1/kg diet (Table 4).

DISCUSSION

The ability of a soy isoflavone extract to inhibit FB_1-promoted hepatocarcinogenesis was previously investigated in our laboratory. An isoflavone extract supplying 1 mmol isoflavones/kg diet was fed with 70 μmol crudely purified FB_1 (40% pure)/kg diet to DEN-initiated female rats for 4 weeks. The soy isoflavone extract was anticarcinogenic, because it blocked development of PGST-positive AHF by over 50%, and decreased plasma ALT activity and hepatic $PGF_{2\alpha}$ production which were increased by FB_1 feeding (Hendrich et al., 1997). The effectiveness of a similar isoflavone extract to inhibit multistage chemical carcinogenesis was evident in another previous study, showing that rat hepatocarcinogenesis promoted by a different tumor promoter, phenobarbital, was suppressed by the soy isoflavone extract providing 1 mmol isoflavones/kg diet, as indicated by

the reduced development of PGST- and GGT-positive AHF (Lee et al., 1995). In both studies, the isoflavone extract was prepared from roasted and defatted soy flakes by ethanol and acetone extraction. Those steps of preparation could have removed s majority of other proposed anticarcinogenic soy constituents such as saponins and Bowman-Birk protease inhibitor, so that remaining isoflavones were considered to be responsible in large part for the observed anticarcinogenic effect.

The present studies were designed to prove the proposed anticancer ability of isoflavones. Experiment 1, however, showed isoflavones at 1 mmol/kg diet were unable to suppress hepatocarcinogenesis promoted by 70 µmol/kg FB_1, because the changes in plasma TC concentration, plasma ALT activity and hepatic prostaglandin production in the rats fed isoflavones + FB_1 were similar to those in the rats fed FB_1 alone, and PGST- and GGT-positive AHF developed in all rats fed FB_1 regardless of isoflavone content (Tables 3 and 4). In contrast, Experiment 2 demonstrated the ability of isoflavones at the same dietary level to suppress hepatocarcinogenesis promoted by 35 µmol/kg FB_1, as evidenced by decreased plasma TC level and ALT activity which were increased by FB_1, and significantly reduced development of PGST- and GGT-positive AHF in rats fed isoflavones + 35 µmol FB_1/kg (Tables 3 and 4). Taken together, these results suggested that suppression of FB_1-promoted rat hepatocarcinogenesis by isoflavones may depend upon FB_1 dose. The hepatocarcinogenesis promoted by 70 µmol/kg FB_1 may be developing more rapidly, so that it could be more resilient to suppression by isoflavones. Comparing hepatocarcinogenesis promoted by different dietary levels of FB_1 in the established DEN initiation/FB_1 promotion model, fumonisin B_1 at increasing concentrations caused relatively more advanced hepatocarcinogenesis in 4 weeks, as indicated by that greater percent areas of PGST- and GGT-positive AHF were found in the rats fed 70 µmol FB_1/kg diet vs. 35 µmol FB_1/kg diet (23% vs. 12% for PGST, 17% vs. 5% for GGT) (Table 4). A more convincing result was obtained in our

recent FB_1 dose-response study showing that FB_1 at increasing concentrations caused relatively more advanced hepatocarcinogenesis in 4 weeks. The severity of FB_1-elicited hepatocarcinogenic effects in rats was dose-dependent, as quantified by the mentioned biomarkers associated with hepatocarcinogenesis (Lu et al., 1997b). Therefore, isoflavones at 1 mmol/kg diet suppressed very early stages of hepatocarcinogenesis promoted by 35 μmol/kg FB_1, but they were ineffective against very rapidly developing neoplasia promoted by 70 μmol/kg FB_1.

In the present studies, PGST- and GGT-positive AHF developed in rats fed 70 μmol/kg FB_1, and in 92-100% of rats fed 35 μmol/kg FB_1. Our FB_1 dose-response study also showed greater percent areas of PGST- and GGT-positive AHF with increasing FB_1 concentrations (Lu et al., 1997b). The dose-dependent increase in the percent areas of PGST- and GGT-positive AHF suggested that these 2 enzymes were closely associated with FB_1-promoted hepatocarcinogenesis, and they did serve as early and sensitive biomarkers of FB_1 hepatotoxicity and hepatocarcinogenicity. Therefore, the anticarcinogenic effects of isoflavones on FB_1-promoted hepatocarcinogenesis can be readily quantified by the development of PGST- and GGT-positive AHF.

The inhibition of AHF formation by isoflavones was accompanied by suppression of both plasma TC concentration and plasma ALT activity (Table 3), biomarkers associated with FB_1-promoted hepatocarcinogenesis (Hendrich et al., 1993; Lu et al., 1997a). Increased plasma ALT activity generally indicates release of the enzyme from damaged hepatocytes with altered plasma membrane permeability. Exposure of hepatocytes to hepatotoxicants is likely to lead to oxidative stress, which in turn may induce plasma membrane damage (Halliwell, 1994). Fumonisin B_1 as a hepatotoxicant might subject hepatocytes to increased lipid peroxidation, based on a reported accumulation of polyunsaturated fatty acids in primary hepatocytes treated with FB_1 at 150 and 500 μmol/L (Gelderblom et al., 1996). Isoflavones as

antioxidants may alleviate FB_1-induced oxidative stress on hepatocyte plasma membrane, so that the leakage of ALT from hepatocytes can be reduced or prevented.

The mechanism underlying the stimulatory effect of FB_1 on plasma cholesterol is unknown. Whether cholesterol-lowering agents can counteract the increase in plasma cholesterol stimulated by FB_1 has not been investigated. Therefore, the observed cholesterol-lowering effect of isoflavones in Experiment 2 cannot be readily explained. In an experiment showing an association of elevated plasma cholesterol with rat hepatocarcinogenesis promoted by another tumor promoter (phenobarbital, 500 mg/kg diet), an isoflavone extract providing approximately 1 mmol isoflavones/kg diet was ineffective against hypercholesterolemia (Lee et al., 1995). Lack of effect of isoflavones on hypercholesterolemia during hepatocarcinogenesis was also demonstrated when an isoflavone extract and crudely purified FB_1 were co-administered to rats for 4 weeks. In that study, the isoflavone extract supplied 1 mmol isoflavones/kg diet and the concentration of FB_1 was 70 μmol/kg diet (Hendrich et al., 1997). Perhaps isoflavones can be effective against hypercholesterolemia during early stages of hepatocarcinogenesis promoted by low doses of FB_1, but not during very rapidly developing neoplasia promoted by high doses of FB_1. In other words, effect of isoflavones on plasma cholesterol may depend upon doses of the tumor promoter. This seemingly dose-dependent effect can only be explained when the mechanisms of the development of hypercholesterolemia associated with hepatotoxicity and hepatocarcinogenesis is elucidated. Mitchell et al. (1978) reported that activity of 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase, a rate-limiting enzyme of cholesterol biosynthesis, was 2-fold greater in primary hepatocellular carcinomas induced by feeding 0.04% 2-acetylaminofluorene for 20 weeks than in normal livers from rats. Maybe HMG CoA reductase activity can be stimulated during FB_1-promoted hepatocarcinogenesis as well. Based on elevation of plasma

LDL, but not HDL or VLDL, and lack of accumulation of cholesterol in livers of vervet monkeys fed a low fat diet containing fumonisins, it was proposed that the removal of cholesterol from plasma via hepatic LDL receptor could be impaired (Fincham et al., 1992). If both mechanisms of hypercholesterolemia during FB_1 hepatocarcinogenesis exist, it would be more difficult for isoflavones to counteract this effect.

Hepatic prostaglandin production was only significantly stimulated in rats fed FB_1 at 70 μmol, but not 35 μmol/kg diet. In contrast, development of PGST- and GGT-positive AHF, plasma ALT activity and plasma cholesterol concentration were increased at both FB_1 concentrations. This suggested that hepatic prostaglandin production may not be as a sensitive biomarker as the others.

Experiment 1 showed that feeding isoflavones with FB_1 did not suppress hepatic prostaglandin synthesis which was increased by 70 μmol FB_1/kg diet, and Experiment 2 demonstrated that effect of feeding isoflavones on hepatic prostaglandin concentrations was marginal compared with feeding FB_1 at 35 μmol/kg diet ($P=0.06$). Our previous study, however, showed a lowering effect of the isoflavone extract on hepatic $PGF_{2\alpha}$ concentration (Hendrich et al., 1997). These results suggested that in addition to genistein and daidzein, other components in the isoflavone extract might also contribute to the prostaglandin-suppressive effect.

In conclusion, isoflavones suppressed rat hepatocarcinogenesis promoted by 35 μmol FB_1/kg diet. They were ineffective against very rapidly developing neoplasia promoted by 70 μmol FB_1/kg diet. Suppression of FB_1-promoted hepatocarcinogenesis in rats depended upon FB_1 dose.

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Table 1. Concentrations of Genistein (G), Daidzein (D) and Fumonisin B_1 (FB_1) in Experimental Diets*

	Genistein	Daidzein	Fumonisin B_1
Experiment 1			
Diets			
Basal	0	0	0
G	1 mmol/kg	0	0
D	0	1 mmol/kg	0
G + D	0.5 mmol/kg	0.5 mmol/kg	0
FB_1-70	0	0	70 µmol/kg
G + FB_1-70	1 mmol/kg	0	70 µmol/kg
D + FB_1-70	0	1 mmol/kg	70 µmol/kg
G + D + FB_1-70	0.5 mmol/kg	0.5 mmol/kg	70 µmol/kg
Experiment 2			
Diets			
Basal	0	0	0
G + D	0.5 mmol/kg	0.5 mmol/kg	0
FB_1-35	0	0	35 µmol/kg
FB_1-18	0	0	18 µmol/kg
G + D + FB_1-35	0.5 mmol/kg	0.5 mmol/kg	35 µmol/kg
G + D + FB_1-18	0.5 mmol/kg	0.5 mmol/kg	18 µmol/kg

*In the experimental diets, FB_1-70 = 70 µmol FB_1/kg diet, FB_1-35 = 35 µmol FB_1/kg diet, and FB_1-18 = 18 µmol FB_1/kg diet.

Table 2. Average Daily Feed Intake, Final Body Weight and Liver Weight to Body Weight Ratio (LW:BW Ratio) of Rats Initiated with Diethylnitrosamine and Promoted with Fumonisin B_1 (FB_1)*

	Feed Intake (g/d)	Body Weight (g)	LW:BW Ratio ($\times 10^{-2}$)
Experiment 1			
Diets			
Basal	10 ± 2	112 ± 17[a]	
G	10 ± 1	118 ± 10[a]	
D	10 ± 1	113 ± 12[a]	
G + D	10 ± 1	115 ± 10[a]	
FB_1-70	9 ± 2	106 ± 8[b]	
G + FB_1-70	9 ± 2	106 ± 12[b]	
D + FB_1-70	9 ± 2	102 ± 12[b]	
G + D + FB_1-70	10 ± 1	105 ± 11[b]	
Experiment 2			
Diets			
Basal	10 ± 1	119 ± 9[a]	4.4 ± 0.3
G + D	11 ± 2	111 ± 7[b]	4.3 ± 0.3
FB_1-35	10 ± 2	110 ± 7[b]	4.5 ± 0.5
FB_1-18	10 ± 2	112 ± 10[b]	4.2 ± 0.2
G + D + FB_1-35	12 ± 2	110 ± 6[b]	4.3 ± 0.3
G + D + FB_1-18	10 ± 2	113 ± 8[b]	4.4 ± 0.3

*In the experimental diets, G = Genistein, D = Daidzein, FB_1-70 = 70 µmol FB_1/kg diet, FB_1-35 = 35 µmol FB_1/kg diet, and FB_1-18 = 18 µmol FB_1/kg diet. Values are given as group means ± standard deviations, n=12. Groups with different letters were significantly different ($P<0.05$-0.01).

Table 3. Plasma Total Cholesterol (TC) Concentration, Alanine Aminotransferase (ALT) Activity, and Hepatic Prostaglandin (PG) $F_{2\alpha}$ and E_2 Levels in Rats Initiated with Diethylnitrosamine and Promoted with Fumonisin B_1 (FB_1)*

	Plasma TC (mg/dL)	Plasma ALT (u/L)	Hepatic $PGF_{2\alpha}$ (ng/g)	Hepatic PGE_2 (ng/g)
Experiment 1				
Diets				
Basal	85 ± 36[a]	33 ± 9[a]	10 ± 3[a]	3 ± 1[a]
G	89 ± 32[a]	31 ± 8[a]	11 ± 5[a]	4 ± 1[a]
D	92 ± 24[a]	32 ± 10[a]	8 ± 4[a]	3 ± 1[a]
G + D	88 ± 26[a]	29 ± 10[a]	9 ± 5[a]	4 ± 2[a]
FB_1-70	143 ± 53[b]	50 ± 16[b]	19 ± 9[b]	8 ± 4[b]
G + FB_1-70	146 ± 62[b]	47 ± 15[b]	18 ± 9[b]	7 ± 3[b]
D + FB_1-70	141 ± 57[b]	49 ± 13[b]	22 ± 9[b]	8 ± 3[b]
G + D + FB_1-70	149 ± 43[b]	47 ± 12[b]	20 ± 7[b]	9 ± 4[b]
Experiment 2				
Diets				
Basal	89 ± 15	28 ± 4	59 ± 34	50 ± 16
G + D	82 ± 10	25 ± 3	58 ± 32	49 ± 26
FB_1-35	170 ± 52[c]	52 ± 25[c]	85 ± 50	60 ± 23
FB_1-18	103 ± 28	27 ± 6	61 ± 37	56 ± 26
G + D + FB_1-35	134 ± 43[ce]	30 ± 6[d]	56 ± 31	58 ± 21
G + D + FB_1-18	86 ± 11	29 ± 6	59 ± 26	55 ± 15

*In the experimental diets, G = Genistein, D = Daidzein, FB_1-70 = 70 µmol FB_1/kg diet, FB_1-35 = 35 µmol FB_1/kg diet, and FB_1-18 = 18 µmol FB_1/kg diet. Values are given as group means ± standard deviations, n=12. Groups with different letters (a, b) were significantly different in Experiment 1 ($P<0.05$).

[c]Significantly different from the control group in Experiment 2 ($P<0.01$).

[d]Significantly different from the FB_1-35 group in Experiment 2 ($P<0.01$).

[e]Significantly different from the control group in Experiment 2 ($P<0.01$).

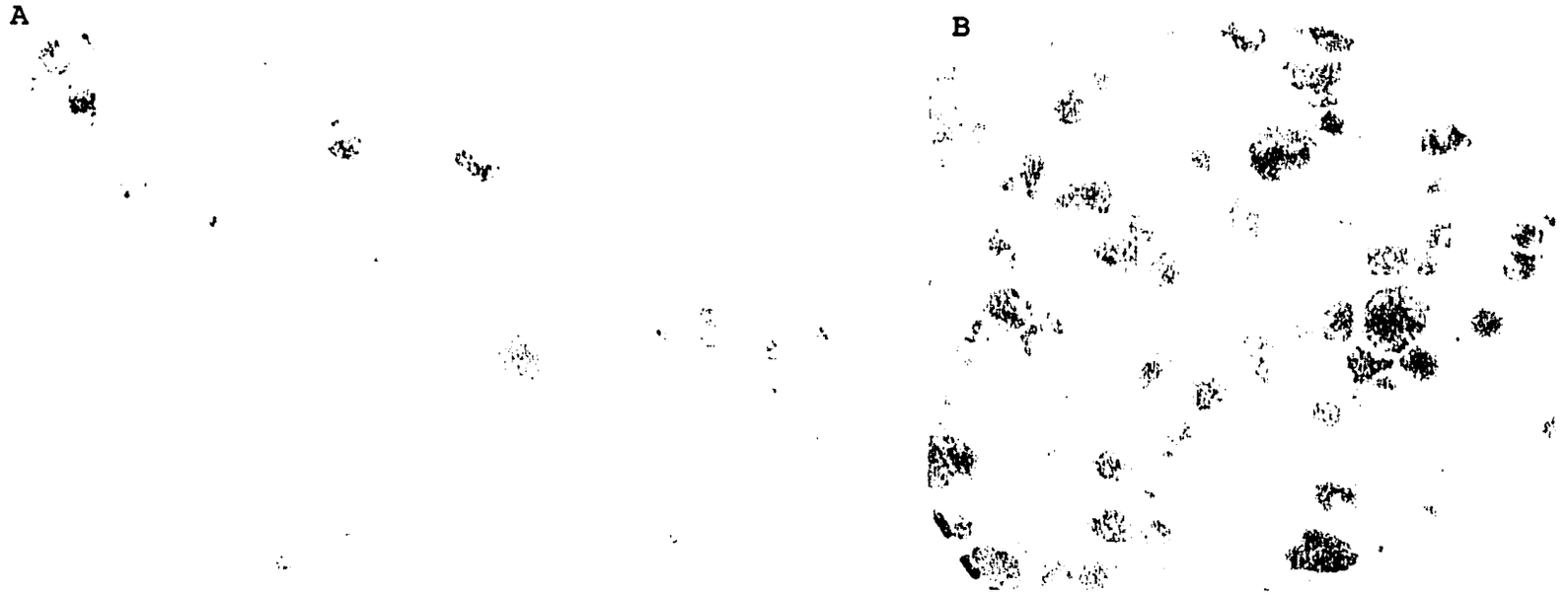
Table 4. Percent Areas of Placental Glutathione S-Transferase-Positive (PGST$^+$) and Gamma-Glutamyltransferase-Positive (GGT$^+$) Altered Hepatic Foci (AHF) in Rats Initiated with Diethylnitrosamine and Promoted with Fumonisin B_1 (FB_1)*

	% of Rats Showing PGST$^-$ AHF	% Area of PGST$^-$ AHF	% of Rats Showing GGT$^-$ AHF	% Area of GGT$^-$ AHF
Experiment 1				
Diets				
Basal	0	0	0	0
G	0	0	0	0
D	0	0	0	0
G + D	0	0	0	0
FB_1-70	100	23 ± 19	100	18 ± 17
G + FB_1-70	100	21 ± 18	100	16 ± 16
D + FB_1-70	100	23 ± 15	100	19 ± 17
G + D + FB_1-70	100	23 ± 19	100	15 ± 13
Experiment 2				
Diets				
Basal	0	0	0	0
G + D	0	0	0	0
FB_1-35	92	12 ± 13	92	5 ± 5
FB_1-18	0	0	0	0
G + D + FB_1-35	92	3 ± 4[a]	75	1 ± 2[a]
G + D + FB_1-18	0	0	0	0

*In the experimental diets, G = Genistein, D = Daidzein, FB_1-70 = 70 µmol FB_1/kg diet, FB_1-35 = 35 µmol FB_1/kg diet, and FB_1-18 = 18 µmol FB_1/kg diet. Values are given as group means ± standard deviations, n=12.

[a]Significantly different from the FB_1-35 group ($P<0.05$).

Figure 1. Fewer and smaller gamma-glutamyltransferase-positive altered hepatic foci developed in livers of rats fed 1 mmol isoflavones with 35 μmol FB_1/kg diet (A), and the AHF seemed to be less evenly stained than those in livers of rats fed 35 μmol FB_1/kg diet (B).


A
B

GENERAL CONCLUSIONS

Placental glutathione S-transferase- and GGT-positive AHF were closely associated with FB_1-promoted hepatocarcinogenesis, and they did serve as early and sensitive biomarkers of FB_1 hepatotoxicity and hepatocarcinogenicity. Development of PGST- and GGT-positive AHF was also accompanied by increased plasma ALT activity and cholesterol concentration at both 50 ppm (70 μmol/kg diet) and 25 ppm (35 μmol/kg diet) FB_1. Hepatic prostaglandin production was only significantly stimulated in rats fed FB_1 at 70 μmol but not 35 μmol/kg diet. Therefore hepatic PG synthesis may not be as a sensitive biomarker as are PGST- and GGT-positive AHF, plasma ALT activity, and TC concentration to quantify FB_1 hepatotoxicity and hepatocarcinogenicity.

Feeding as low as 25 ppm FB_1 for 4 weeks induced hepatocarcinogenesis in DEN-initiated rats. The short-term cancer-promoting threshold dose of FB_1 was probably >12.5 ppm (18 μmol/kg diet) but ≤25 ppm.

The amine group of FB_1 was critical to FB_1 hepatotoxicity and hepatocarcinogenicity. Subjecting FB_1 to nonenzymatic browning conditions with fructose blocked the amine group and thus eliminated FB_1 hepatotoxicity and hepatocarcinogenicity.

Isoflavones as antioxidants may alleviate FB_1-induced oxidative stress on hepatocyte plasma membrane, so that the leakage of ALT from hepatocytes can be reduced or prevented. Effect of isoflavones on hypercholesterolemia associated with FB_1-promoted hepatocarcinogenesis may depend upon doses of the tumor promoter.

Isoflavones suppressed rat hepatocarcinogenesis promoted by 35 μmol FB_1/kg diet, but they were ineffective against very rapidly developing neoplasia promoted by 70 μmol FB_1/kg diet. Suppression of FB_1-promoted hepatocarcinogenesis in rats depended upon FB_1 dose.

Future studies are greatly needed to understand the mechanisms of FB_1 toxicity and carcinogenicity, to determine whether there is a causative relationship between FB_1 and human esophageal cancer, to establish tolerance levels for FB_1 in corn-based human foods and animal feeds, to identify the structure of the putative FB_1-fructose conjugate, to investigate its stability, possible processing occurrence, bioavailability and toxicity in other organs, and to elucidate the mechanisms of the observed anticarcinogenic effects of isoflavones.

These findings should help establish an action level for FB_1, develop effective FB_1 detoxification through processing of corn-based food, and further explore anticarcinogenic ability of isoflavones.

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