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Role and modulation factors of liver-associated natural killer cell during fumonisin B1 hepatocarcinogenesis in rats

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Role and modulation factors of liver-associated natural killer cell during fumonisin B_1 hepatocarcinogenesis in rats

by

Hongjun Liu

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ABSTRACT

Three hypotheses were tested in the present studies: 1. Prostaglandin E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) produced during tumor promotion are key factors suppressing liver-associated natural killer cell activity, a proposed biomarker of carcinogenesis. 2. Reaction of fumonisin B_1 (FB_1) with glucose may prevent FB_1 hepatotoxicity, tumor development and changes in biomarkers associated with carcinogenesis, such as natural killer cell activity, in rats. 3. Increased fat and energy intake promote FB_1 hepatocarcinogenesis and inhibit the liver-associated natural killer (NK) cell activity in association with increased PGE_2 and $PGF_{2\alpha}$. Before testing these two hypotheses, immune function as well as concentrations of PGE_2 and $PGF_{2\alpha}$ were compared between Fischer 344/N (F344/N) and Spague-Dawley (SD) rats to determine which strain was more appropriate for testing the hypotheses.

Hepatic NK cell activity was compared in 9 week old male and female F344/N and Sprague Dawley(SD) rats. Natural killer cells were stained using an anti-NKR-P1 monoclonal antibody and quantitated by flow cytometry. SD rats exhibited significantly greater total hepatic NK activity($p<0.001$) than F344/N rats, and male rats had significantly greater total hepatic NK activity than female rats. There were no strain or gender differences in the concentration of hepatic PGE_2. No strain difference was found in the concentration of hepatic PGF_{2a}, but the hepatic PGF_{2a} concentration in female rats was two-fold of that in male rat (130 vs 60ng/g). Prostaglandin E_2 (10ng/ml and 25ng/ml) significantly inhibited hepatic natural killer cell(NK) activity in vitro compared with untreated cells from both genders and strains($p<0.05$). In contrast, 50ng $PGF_{2\alpha}$/ml and 100ng $PGF_{2\alpha}$/ml significantly stimulated hepatic NK activity compared with untreated hepatic cells from both F344/N and SD rats. Prostaglandin E_2 and $F_{2\alpha}$ had opposite effect on liver-associated NK cell activity.

The reaction of the primary amine of fumonisin B_1 (FB_1) with glucose was hypothesized to detoxify this mycotoxin. Eighty 10-day old female F344/N rats were injected intraperitoneally with diethylnitrosamine (DEN, 15mg/kg body weight). At 4 weeks of age, the weaned rats were randomly assigned to one of the 4 treatment groups (20 rats/group): the group fed basal diet, the group fed FB_1-glucose (containing the reaction mixture of 25ppm FB_1 with glucose), the group fed 8ppm (residual amount of free FB_1 in the FB_1-glucose mixture) or the group fed 25 ppm FB_1. The group fed FB_1-glucose did not showed increased plasma total cholesterol concentration, alanine aminotrasferase (ALT) activity compared with control group. No development of Gamma-glutamyltrasferase (GGT)- and placental glutathione trasferase (PGST)-positive altered hepatic foci (AHF) in FB_1-glucose group. The concentration of endogenous hepatic PGE_2, $PGE_{2\alpha}$, sphinganine (Sa), sphingosine (So) and Sa/So were not different for the control group. In comparison with the rats fed basal diet or FB_1-glucose (the products of reacting 25ppm FB_1 with glucose), the rats fed 8ppm FB_1 and 7% fat or 25ppm FB_1 and 7% fat showed greater ALT activity and endogenous production of PGE_2 at 20 weeks of age. But only half the rats fed 25ppm FB_1 and 7% fat developed GGT- and PGST-positive AHF, and the area of AHF were less than 1%. And the tumor developed in all rats stayed at preneoplasia stage. The hepatic natural killer cell activity did not differ among all the groups at any time point, although increased PGE_2 accompanied fumonisin promotion of carcinogenesis.

Prostaglandin E_2 and $F_{2\alpha}$ did not seem to be modulators of hepatic NK activity in this study.

We hypothesized that greater dietary fat and energy intake promote FB_1 carcinogenesis, and the inhibition of NK cell activity paralleled the development of preneoplasia. Twenty four 10-day old female F344/N rats were injected intraperitoneally with diethylnitrosamine (DEN, 15mg/kg body weight). At 4 weeks of age, the weaned rats were randomly assigned to one of 4 treatment groups with 6 rats each.Greater PGST- but not GGT- positive foci were observed in the rats consuming high fat, high energy and FB_1 than in rats fed FB_1 and low fat diet. The greater inhibition of total NK activity was also observed in the rats fed high fat, high energy and FB_1 compared with the rats fed low fat, low energy and FB_1. Increased PGE_2 paralleled the extent of preneoplasia and inhibition of NK activity. Prostaglandin E_2 seemed to be a modulation factor of NK activity in the present study. But, in our previous study, we observed the increase of PGE_2, but we did not find the inhibition of NK activity. These findings suggested that some other factors, such as glycosphinglipid, than PGE_2 produced by preneoplasms seemed to be more important down-regulators of NK activity as they occur during progression of carcinogenesis.

GENERAL INTRODUCTION

Results from four studies are presented in this dissertation. The immune function and the physical concentration of prostaglandin E_2 and prostaglandin $F_{2\alpha}$ were compared between F344/N and SD rats in the first study. The effects of prostaglandins on liver-associated natural killer cells were investigated by co-culture hepatic natural killer cell with different concentration of prostaglandins in the second study. The detoxification effect by reacting FB_1 with D-glucose was evaluated in the third study. The effect of increased fat and energy intake on FB_1 hepatocarcinogenesis was investigated in the fourth study.

Study 1. Sprague –Dawley rats have greater liver-associated natural killer cell activity than do F344/N rats, although a greater proportion of lymphocytes are natural killer cells in F344/N rats

The use of an animal carcinogenicity bioassay in assessing the oncogenic risk involved with chemical exposure is an important and necessary process, but presents many difficulties in interpretation when extrapolating to humans (Gergory., 1988). There are large inter-species, and inter-strain and gender variations in the incidence of some tumors. Variable tumor formation may be related to factors, including: organ/strain specific oncoviruses, hereditary disorders (Drinkwater et al., 1989), or differing immune capabilities, such as hepatic natural killer cell(NK) activity (Lee et al., 1999). F344/N and SD rats were included in this study as these are two rodent strains predominantly used in carcinogenicity bioassays, and there is a large database on cancer development in these strains. In one study, newborn F344/N and SD rats were irradiated with whole body single doses of 3Gy gamma rays with or without intraperitoneally-injected diethylnitrosamine (DEN) (15 mg/kg body weight) within 1 h of irradiation. Tumor development was promoted with 0.05% phenobarbital. In groups treated with radiation alone or radiation combined with DEN, F344/N rats had threefold greater development of placental S-glutathione transferase-positive (GST-P^+) altered hepatic foci (AHF, biomarkes of preneoplasia and neoplasia) than did SD rats. In SD rats, females had 1.5 greater induction of GST-P^+ AHF than did males (Lee et al., 1998). In another study in which hexachlorobenzene was fed to male and female F344/N rats for 15 weeks, 100% of surviving females had multiple liver tumors which were strongly γ-glutamyl transferase (GGT) positive and histologically classified as neoplastic nodules or hepatocellular carcinomas. In contrast, only 16% of males developed tumors which were smaller and fewer in number than those in females (Smith et al., 1985).

The capacity to mediate MHC-unrestricted cytotoxicity against certain tumor cells without apparent prior sensitization has by definition been primarily ascribed to NK cells (Trinchieri et al., 1989). NK cells are phenotypically and functionally distinct population of lymphocytes with morphology characteristic of large granular lymphocytes Trinchieri et al., 1989; Whiteside et al., 1990). In rats, high density cell surface expression of NKR-P1 antigen(i.e. NKR-P1^{bright}) is an exclusive property of all mature NK cells (Chambers et al., 1989; Brissette-Storkus et al., 1994). NKR-P1 is also expressed on a subset of T cells, but with a 2- to 10- fold lower density(i.e., NKR-P1^{dim}) than on NK cells (Chambers et al., 1989; Brissette-Storkus et al., 1994). Some subsets of NKR-P1^{dim} lymophocytes are capable of mediating NK-like cytotoxicity, particularly after incubation in 1000U/ml interleukin-2(IL-2) for five days (Brissette-Storkus et al., 1994).

Attention has focused on the role of the liver as a tumor killing organ. Hepatic NK activity is much higher than in peripheral blood and spleen preparations (Vanderkerken et al., 1993). The liver also harbors the largest population of fixed macrophages (Malter et al., 1986). These tumoricidal cells may affect metastasizing tumors in addition to resident hepatic tumors (Lukomaska et al., 1987). However, the role of impaired natural immunity in chemically-induced hepatocarcinogenesis is still unclear. Neonatal B6C3F1 mice were given a single carcinogenic dose of diethylnitrosamine (DEN) and the time-response kinetics for the early (altered foci) and late (adenomas/carcinomas) phases of hepatocellular carcinogenesis were compared to changes in hematopoiesis and immune functions associated with immune surveillance and natural resistance (Germolec et al., 1988). Increases in hematopoiesis occurred just prior to or concurrent with the appearance of hepatocarcinomas, while increased macrophage and natural killer cell cytotoxicity and suppression of cell-mediated immunity occurred following tumor appearance and progressed with increasing tumor burden. Neither immunological nor hematopoietic changes were associated with early phases of hepatocarcinogenesis, as monitored by the appearance of AHF. Although changes in hematopoiesis may represent an early indicator for hepatocarcinogenesis in the mouse tumor model, the data suggest that altered immune surveillance and natural resistance are not factors in the development of chemically induced hepatocellular tumors, and the changes in immune function are probably secondary to tumor development. Neither immunological nor hematopoietic changes were associated with early phases of hepatocarcinogenesis, as monitored by the appearance of AHF (Germolec et al., 1988). In male F344/N rats given 40ppm DEN in drinking water for 10 weeks, as GST-P^+ foci developed, splenic NK activity changed. After 5 weeks, DEN-treated and control rat spleen NK activity was similar, but at 10 weeks, NK activity was significantly greater in DEN treated rats compared with control rats. At 20 weeks, DEN-treated rats had significantly lower NK activity than did controls (Lee et al., 1998). This suggests an interaction between chemical carcinogenesis and NK activity. Lu et al. (1997) also showed that chemical carcinogenesis (initiation by DEN, 15mg/kg at 10 days of age, and promotion by fumonisin B1 (50mg/kg diet)) caused significantly decreased NK activity after 4 weeks of development of AHF (Lu et al., 1997).

We propose that liver-associated NK activity will be greater in SD than in F344/N rats and in males than in females, due to greater liver associated NK cell numbers, even after covariation for body weight. This proposed difference in NK activity might partly explain some previous findings of gender and strain difference in susceptibility to carcinogenesis.

Study 2. Opposing effects of prostaglandin E_2 and $F2\alpha$ on rat liver-associated natural killer cell activity in vitro

The suggestion that prostaglandins may play a role in immune response/tumor cell interactions is based upon several observation. First, a variety of prostaglandins are produced both by cells that are themselves active in the expression and regulation of immune response activity (Tomar et al., 1981) as well as by a number of tumor targets

(Karmali et al., 1980; Goodwin et al., 1981). Carcinogenesis may be associated with increased prostaglandin production by neoplastic organs, such as during promotion of rat hepatocarcinogenesis by fumonisin B (Lu et al., 1997). Second, the production of prostaglandins has been found to increase as a result of direct contact between effector lymphocytes and tumor targets (Owen et al., 1980). Third, prostaglandins at levels produced during these interactions have been shown to influence the ultimate expression in vitro of lymphocyte and macrophage cytotoxicity against tumor targets (MaCarthy et al., 1981; Koren et al., 1981).

Prostaglandins mediate inter- and intracellular communication, as may stimulate hepatocyte proliferation (Miura et al., 1979; Andreis et al., 1981). The concentration of PGE equivalents in rat liver *in vivo* was increased during liver regeneration. This stimulation of prostaglandin synthesis was confirmed in vitro by the ability of homogenates of regenerating liver tissue to synthesize PGE_2 and $PGF_{2\alpha}$ from arachidonate. Indomethacin prevented these prostaglandin changes, and the subsequent increase in DNA synthesis (MacManus et al., 1976). During the regeneration of mammalian liver after a 70% partial hepatectomy (PHx), Kupfer cells produced significantly elevated PGE_2, and *in vivo* Kupfer cell PGE_2 blockade by indomethacin (5 mg/kg) significantly ($P < 0.05$) inhibited hepatic regeneration (Goss et al., 1982). The association of neoplastic tumors with increased levels of prostaglandins (Robertson et al., 1986; Bennett et al., 1975) provided the rationale for investigating their role in turmorigenesis. Animal and human tumors contain high levels of prostaglandins, particularly those of the E series that have been shown to significantly affect cell proliferation and tumor growth and suppress immune responsiveness. DNA synthesis of hepatocytes in primary culture was significantly enhanced by addition of PGE_2 (2-200 nmol/L). Intracellular cAMP level in the hepatocytes increased during culture, and cAMP increase was enhanced by PGE_2. Prostaglandin E_2 production in the liver increases hepatic regeneration and PGE_2 enhances the proliferation of hepatocytes by a seemingly cAMP-dependent specific receptor-mediated process (Tsujii et al., 1993). At concentrations of 10^{-12}-10^{-9} mol/L, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ very intensely stimulated both the DNA-synthetic and mitotic activities of hepatocytes in 4-day-old primary cultures of neonatal rat liver. DNA replication was more intensely enhanced by $PGF_{2\alpha}$ than $PGF_{1\alpha}$, whereas mitotic activity was nearly equally affected by the two prostaglandins (Armato et al., 1983). Thus enhanced PGE_2 and $PGF_{2\alpha}$ may promote hepatocarcinogenesis by stimulating DNA synthesis and proliferation of hepatocytes.

A high level of PGE_2 in the portal vein suppresses liver-associated immunity and promotes liver metastases (Okuno et al., 1995). Some *in vitro* experiments showed a similar phenomenon. The ability of Syrian hamster tumor cells of the same origin but with different degrees of malignancy to secrete prostaglandin E was studied following their in vitro contact with Syrian hamster natural killer cells (NK cells). Syrian hamster NK cells were shown to lose cytotoxic activity significantly after their contact with malignant tumor cells. Short-term in vitro contact of malignant tumor cells with human and Syrian hamster NK cells resulted in a rapid PGE secretion into the culturing medium. Therefore, PGE_2 may promote tumor progression by inhibiting immune function. The effect of $PGF_{2\alpha}$ on NK cells is still not clear. The regulatory effects of prostaglandins on immune response appear to be mediated by the production of cyclic AMP (Robison et al., 1971). PGE_2 activates adenylate cyclase with a subsequent rise in cyclic AMP (Smith et al., 1971), which acts as a "second messenger". Cyclic AMP itself is an inhibitor of lymphocyte activation (Melmon et al., 1974). The presence of receptors for PGE_1 and PGE_2 on the lymphocyte surface had been demonstrated, while there were no binding sites for PGA, $PGF_{1\alpha}$ or $PGF_{2\alpha}$. Henney and Lichtenstein, using splenic lymphocytes from mice immunized with an allogeneic mast cell tumor (Henney et al., 1971), suggested that elevated cyclic AMP content of cytolytic lymphocyte might inhibited their ability to kill target cells. As a test of this hypothesis, prostaglandins were shown to inhibit lymphocyte cytolytic activity (Henney et al., 1972). The relative potency of seven prostaglandins in inhibiting cytolytic activity correlated very well with their potency in stimulating cyclic AMP accumulation in lymphocytes: $E_1=E_2>A_1=A_2>F_{1\alpha}=F_{2\alpha}\approx 0$ (Lichtenstein et al., 1972). But effects of PGs on NK cells could be mediated in other ways.

We suggest that prostaglandin regulation of immune response might partly explain some previous findings of gender and strain difference in susceptibility to carcinogenesis. Effects of $PGF_{2\alpha}$ on NK activity have not been well characterized. To that end, we hypothesize that high levels of $PGF_{2\alpha}$, as found in female rat liver, may inhibit liver associated NK activity, an effect similar to that of PGE_2.

Study 3. Reacting of fumonisin with glucose prevents promotion of hepatocarcinogenesis in female F344/N rats while maintaining normal hepatic sphinganine: sphingosine

The carcinogenic and toxic effects of fumonisin B_1 (FB_1) , a mycotoxin produced by the commonly occurring corn fungi, *Fusarium moniliforme* and *Fusarium proliferatum*, have been studied intensively. Fumonisin B_1 (69.3μM/kg, 50ppm) was hepatocarcinogenic in rats fed the toxicant for approximately two years (Gelderblom et al., 1991). The incidence of *F. moniliforme* in corn for human consumption has been correlated with the incidence of esophageal cancer in Transkei, Southern Africa (Marass et al., 1981) and in China (Yang et al., 1980). The concentration of FB_1 in corn reached approximately 11.1 μmol/kg in areas of southern Africa where human esophageal cancer rate was high(Sydenham et al., 1990). Corn products for human and animal consumption were determined to have FB_1 concentration between 0.3-4.2 μmol/kg in the U.S. (Hopmans et al., 1993; Murphy et al., 1991; Syndenham et al., 1991).

Several biomarkers have been used to study FB_1 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 γ-glutamyltransferase (GGT)-positive AHF (Gelderblom et al., 1988). Plasma alanine aminotransferase (ALT) activity was increased during fumonisin hepatotoxicity (Voss et al., 1993), and hepatocarcinogenesis in rats (Hendrich et al., 1993). Increased plasma total cholesterol was observed in FB_1-treated vervet monkeys (Fincham et al., 1992), and in rats (Hendrich et al., 1993) in short term studies. Greater hepatic prostaglandin $F_{2\alpha}$ production was also observed in FB_1 tumor promotion in rat liver (Lu et al., 1997). *In vivo* administration of 50ppm FB_1 significantly suppressed hepatic natural killer (NK) cell activity while stimulating hepatic preneoplasia (Lu et al., 1997). Natural killer cell activity suppression by FB_1 during tumor promotion may be mechanistically significant, but this remains to be determined. Thus numerous possible biomarker of FB_1 toxicity and tumor promotion may be used to probe mechanism of action of mycotoxin.

Recent studies regarding the biological effects of fumonisins indicated that they selectively inhibit ceramide synthase, a key enzyme in the sphingolipid biosynthetic pathway (Wang et al., 1991). It was suggested that the subsequent accumulation of the sphingoid bases, sphinganine (Sa) and sphingosine (So), could have an important role in the toxicological effects of fumonisin in the kidney and the liver of rats (Norred et al., 1991; Yoo et al., 1992). In addition, as the sphingoid bases are important regulators of cellular growth and differentiation (Merrill et al., 1991), the continued disruption of sphingolipid biosynthesis has been implicated in the hepatocarcinogenicity of fumonisin (Schroeder et al., 1994).

Currently, there has been increased attention directed at reducing the human and animal exposure to these fungal toxins. Biological, chemical, and physical processes have been explored to salvage fumonisin-contaminated corn. Thermostability of FB_1 proved to be great. When dry corn was heated at 50, 75, 100 and 125°C for 40 minutes, only a small amount of FB_1 was lost (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 its toxicity in rats (Norred et al., 1991).

Nixtamalization, the traditional process to produce masa or tortilla flour, reduced the amount of FB_1 by hydrolyzing FB_1 to hydrolyzed FB_1 (HFB_1), but HFB_1 was similar in toxicity to FB_1 when the nutritional status of rats was adequate (Hendrich et al., 1993). In vitro toxicity studies of several FB_1 analogs showed that the analogs containing FB_1 amine groups and the tricarballylic side chains were more toxic than analogs containing only the tricarballylic side chains (Kraus et al., 1992), and naturally occurring N-acetyl –FB_1 was not toxic (Gelderblom et al., 1993). Therefore, the primary amine of FB_1 is likely to be critical for its toxicity. Murphy et al. (1995) reported a method to detoxify FB_1 by derivatizing the amine group with a reducing sugar, fructose, in a nonenzymatic browning reaction. Diethylnitrosamine-initiated (15 mg/kg body weight) male F344/N rats were fed for 4 weeks either 69.3 µM FB_1 or 69.3 µM FB_1 reacted with fructose (FB_1 –fructose). Rats fed FB_1 had significantly increased levels of several markers of hepatocarcinogenicity, while rats receiving FB_1 – fructose showed no signs of hepatocarcinogenicity or hepatotoxicity(Lu et al., 1997). A more practical and efficient method to block FB_1's amine group by reacting the amine group with glucose had been developed in Dr. Murphy's lab (Lu., 2000) .The FB_1-glucose reaction was more complete than the reaction with fructose, and the reaction products were more easily isolated than FB_1-fructose products. It was hypothesized that modifying FB_1 with glucose would prevent promotion of hepatocarcinogenicity by FB_1 . Our experiment was designed to test the effectiveness of this detoxification method by examining effects of FB_1 –glucose on several markers of FB_1 promotion of hepatocarcinogenesis.

Study 4. Increased dietary fat and energy intake during fumonisin promoted hepatocarcinogenesis increase hepatic prostaglandins, sphinganine, and development of placental glutathione trasferase (+) foci, while inhibiting natural killer cell number

FB_1 toxicity and carcinogenicity were evaluated in female F344/N rats (Lu et al., 1997), initiated by diethylnitrosamine (DEN, 15mg/kg body weight) at 10 days of age, and given free access to the control diet (AIN93, 7% soybean oil + 13% beef tallow) or treatment diet (AIN93, 7% corn oil +13% beef tallow + 69.3μmol FB_1/kg diet) for 5 weeks, FB_1 - fed rats developed altered hepatic foci (AHF), and hepatic NK activity in FB_1 –fed rats was significantly inhibited as compared with the control group. When female F344/N rats were initiated by DEN (15mg/kg bw), and given free access to control diet (AIN93, 7% soybean oil) or the same diet containing 25ppm FB_1 for 16 weeks, only half of the animals of the FB_1 –fed rats developed GGT- and PGST-positive foci (Liu et al., 2001), and no difference in hepatic NK cell activity was observed between control and FB_1 -fed rats. These two experiments suggested that there was an interaction between dietary fat and FB_1 carcinogenesis, as reflected in the altered hepatic foci and hepatic NK activity.

Evidence from experimental animal models strongly suggests that liver-associated NK cells and Kupffer cells are the first line of defense against blood-borne metastasizing solid tumor cells invading the liver. Thus NK cells protect the parenchyma. The primary role of NK cells in neoplasia is directed against blood-borne tumor cells during the intravascular phase of tumor metastasis(Winnock et al., 1993). In male F344/N rats given 40ppm DEN in drinking water for 10 weeks, as GST-P^+ foci developed, splenic NK activity changed. After 5 weeks, DEN-treated and control rat spleen NK activity was similar, but at 10 weeks, NK activity was significantly greater in DEN treated rats compared with controls. At 20 weeks, DEN-treated rats had significantly lower NK activity than did controls (Lee et al., 1998). This suggests an interaction between chemical carcinogenesis and NK activity, at the very early stage of carcinogenesis, the NK cell activity may not change or even increase, with the progression of neoplasia, the inhibition of NK cell activity occurs, and may parallel the development of neoplasia.

Increased dietary fat increased the development of mammary tumors induced by chemical carcinogens in rats (Aksoy, et al., 1987; Aylsworth et al., 1986). Hopkins and Carrol (1979) reported that in rats initiated with 7,12-dimethylbenz[a]anthracene one week before dietary treatment, rats fed 3% sunflower seed oil and 17% of either tallow or coconut oil developed twice as many tumors as those fed 3% sunflower seed oil. Rats were first intubated with diethylnitrosamine (DEN, 10 mg/kg) 20 hr after partial hepatectomy; 1 week later, rats were fed one of three purified diets (a low-fat diet similar to the AIN-76 diet, a high saturated fat diet, or a high polyunsaturated fat diet) with or without 0.05% phenobarbital in the diet for 10 months. Increasing the fat level of the diet did not increase the number of GGT-positive foci arising spontaneously or induced by DEN alone. When phenobarbital was present in the diet, both high-fat diets enhanced the induction of GGT-positive foci. Increasing the dietary fat level, may enhance promotion of liver foci by phenobarbital (Glauert et al., 1986). We hypothesized that greater dietary fat and energy intake promotes FB_1 carcinogenesis, the inhibition of NK cell activity parallels the development of preneoplasia, and the inhibition of NK activity may be modulated by prostaglandins and/or sphingolipids which had been observed to accumulate in previous experiments of FB_1 carcinogenesis (Lu et al., 1997; Liu et al., in press).

Dissertation Organization

This dissertation is composed of four manuscripts in addition to abstract, general introduction, literature review, general conclusions, and acknowledgements. The manuscript of Study 1, "Sprague –Dawley rats have greater liver-associated natural killer cell activity than do F344/N rats, although a greater proportion of lymphocytes are natural killer cells in F344/N rats", was submitted to Comparative Immunology, Microbiology and Infectious Diseases. The manuscript of Study 2, " Opposing effects of prostaglandin E_2 and $F2\alpha$ on rat liver-associated natural killer cell activity in vitro", was published in Prostaglandins, Leukotrienes and Essential Fatty Acids. The manuscript of Study 3, " Reacting of fumonisin with glucose prevents promotion of hepatocarcinogenesis in female F344/N rats while maintaining normal hepatic sphinganine: sphingosine ", was accepted by Journal of Agricultural and Food Chemistry. The manuscript of Study 4, " Increased dietary fat and energy intake during fumonisin promoted hepatocarcinogenesis increase hepatic prostaglandins, sphinganine, and development of placental glutathione trasferase (+) foci, while inhibiting natural killer cell number", will be submitted to Carcinogenesis.

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LITERATURE REVIEW

Fumonisins, a group of structurally related mycotoxins which were first identified in 1988 (Gelderblom et al., 1988), are produced by the corn fungi *Fusarium verticillioides* 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). Fumonisins have been categorized as Class 2B probable human carcinogens (IARC 1993). The occurrence of *F.verticillioides* containing FB_1 was associated with a relatively high rate of human esophageal cancer in southern Africa (Sydenham et al., 1990). Although more than ten years have passed since the discovery of fumonisins, the molecular mechanisms of their toxicity remain unclear (Wang et al., 1996).

Fumonisins: Discovery and Distribution

The fungus *Fusarium verticillioides* Sheldon is one of the most prevalent seedborne fungi associated with corn intended for human and animal consumption throughout the world (Marasas et al., 1984a). The fumonisins, food-borne carcinogenic mycotoxins ,were first isolated from cultures of *F.verticillioides* strain MRC 826 by Gelderblom et al. (1988a). According to the taxonomic system of Nelson, Marasas and coworkers, at least some strains of six additional *Fusarium* species also produce fumonisins: *F. anthophilum, F.dlamini, F.Napiforme, F.nygamai,* and *F.subglutinans.* In addition to fumonisins, strains of *F.verticillioides* and closely related species also produce several other mycotoxins and phytotoxins at high levels in laboratory culture. The toxicity of these other secondary metabolites has had much less study and their role in animal toxicoses and human exposure risk, if any, remains unclear. These components include the fusarins, potent mutagens; moniliformin, a potent avian toxin; the naphtazarine pigment complex, a group of linear aromatics known to be phytotoxins, but untested for animal toxicity; fusaric acid, 1- carboxy-4-butene-pyridine, a known phytotoxin of unknown animal toxicity. Toxicity data and reports on the concentrations of these compounds in naturally contaminated corn samples are much less available than data on fumonisins (Plattner et al., 1996).

The discovery of fumonisins began with observation that *F. verticillioides* was involved in widespread field outbreaks of animal diseases occurring in the United States in the early 1900s. *Fusarium verticillioides* was the fungus most commonly found in moldy corn and was implicated as the cause of the disease "moldy corn toxicosis" (Peters, 1904). Butler (1902) reproduced leukoencephalomalacia (LEM) in horses with naturally contaminated moldy corn in United States. In the 1980s, the occurrence of *F.verticillioides* in corn was correlated with high rates of human esophageal cancer in Transkei, South Africa and in China, where corn is a major dietary staple (Marass et al., 1981; Yang, 1980).

Gelderblom et al. (1986) utilized a cancer initiation/promotion bioasssay, 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.verticillioides* isolated from Transkeian corn. Hepatectomized rats were initiated with diethylnitrosamine (DEN, 30mg/kg body weight) and then fed a diet containing culture material of *F.verticillioides* strain MRC826 at a level of 2% for 14 weeks. Gamma-glutamyltransferase (GGT)-positive preneoplastic altered hepatic foci (AHF), developed , indicating the cancer promotion activity of strain MRC 826. A similar assay with shorter promotion was later used to screen 10 toxic strains of *F.verticillioides*, isolated from corn from a high-risk area for esophageal cancer in the Transkei, for their cancer promoting activity in rats Gelderblom et al. (1988a). After initiation with diethylnitrosamine (DEN, 200mg/kg body weight), a diet containing 5% culture material was fed for four weeks. The presence of γ-glutamyltranspeptidase (GGT) - positive foci in the liver was taken as an indication of tumor-promoting activity. Three out of the ten tested *F. verticillioides* strains showed such activity, which was correlated with toxicity expressed as reduction of body weight gain. This observation, the development of progressive toxic hepatitis and the induction of GGT-positive foci was also observed in the rats without DEN initiation, but with far less pronounced development of GGT-positive foci in rat liver (Gelderblom et al., 1986). *Fusarium verticillioides* 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 indicator of hepatocarcinogenesis (Lebepe et al., 1991). These results suggest that in addition to cancer promoting activity, *F. verticillioides* has cancer co-initiating activity as well. Gelderblom et al. (1988b) used this short-term cancer bioassay mentioned above as a monitoring system to isolate the mycotoxins fumonisin B_1 and B_2. Cultured material of *F. verticillioides* MRC826, grown on corn, was extracted with ethyl acetate and methanol:water (3:1). The cancer-promoting activity was observed in the methanol:water extract and remained in the aqueous phase after partition with chloroform. This fraction was further fractionated using an Amberlite XAD-2 column, silica gel column and finally a C18 reverse phase column. Purified FB_1

had cancer promoting effect in rats when fed for four weeks at level of 0.1% in the diet which was associated with a toxic effect as shown by a significant reduction in weight gain.

Bezuidenhout et al. (1988) chemically characterized the fumonisins, a family of structurally related mycotoxins. The molecules, determined by ^1H and ^{13}C NMR, were diesters of propane-1,2,3-tricarboxylic acid and either 2-amine- or 2-acetylamino-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. Fumonisin B_1 has hydroxyl groups at both C-10 and C-5. Fumonisins B_2 and B_3 lacked hydroxyl groups at C-10 and C-5, respectively, whereas both hydroxyl groups were replaced by hydrogens on fumonisin B_4. Fumonisin A_1 and A_2 were N-acetates of fumonisin B_1 and B_2, respectively. Most of the naturally produced fumonisins in corn cultures of *F.verticillioides* strain MRC 826 were fumonisins B_1, B_2, and B_3 (Bezuidenhout et al., 1988; Cawood et al., 1991). Besides *Fusarium verticillioides,* a closely related species, *Fusarium proliferatum*, was also frequently isolated from shelled corn, which was capable of producing fumonisins B_1, B_2, and B_3 too (Ross et al., 1991).

Sydenham et al. (1991) assessed the occurrence of FB_1 and FB_2 in corn foods from the U.S., South Africa, Canada, Egypt, and Peru. Cornmeal samples were found to contain FB_1 in ranges of 1-2790ng/g for the U.S., 0-475ng/g from South Africa, 0-660ng/g for Peru, 1780-2980ng/g from Egypt, and 0-50ng/g from Canada. Shepard et al.(1996) surveyed corn and corn-based products to determine the extent of possible FB_1 exposure. Compiled data from Brazil, Italy, South Africa, and the U.S. for FB_1 levels in feeds implicated in equine leukoencephalomalacia ranged from 0.2 to 130 µg/g. Levels of FB_1 for porcine pulmonary edema in Brazil and U.S. ranged from 3-330 µg/g. Food samples from areas of the Transkei region of South Africa with high rates of esophageal cancer ranged from 0-117.5 µg/g. Food samples from high esophageal cancer regions of China ranged in FB_1 contents from < 0.10 to 154.9µg/g. An extensive summary of corn products meant for human consumption from around the world from corn meal, grits, flour, breakfast cereal, to milk demonstrated a range of FB_1 levels from 0 to 16µg/g (Shephard et al., 1996).

Fumonisins exist throughout the world food supply, varying in contamination severity, regional location, and in food items produced from contaminated corn. Classified as a Class 2B probable human carcinogen (IARC, 1993), fumonisins are of potential concern to human health. It is important to monitor fumonisin contamination. Detoxification of mycotoxin-contaminated grain is of particular interest to prevent harm to humans or animals and economic loss.

Fumonisin Toxicity and Carcinogenesis

Equine leukoencephalomalacia (ELEM)

Fumonisins have been shown to be toxic to a variety of species in a range of concentrations. Equine leukoencephalomalacia is a neurotoxic disease that affects horses, donkeys and mules. Typical 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. verticillioides* as the predominant contaminant of moldy corn that was associated with many cases of ELEM, and reproduced ELEM in donkeys by feeding *F.verticillioides* corn culture material (Wilson and Maronpot 1971). Marasas et al. (1988) conducted neurotoxicity tests with FB_1 to determine the effects of feeding FB_1–containing culture material to horses. Effects of intravenous dosing of horses with purified FB_1 were studied. Two horses were dosed by stomach tube with FB_1–containing *F.verticillioides* culture material six times over seven days at either 2.5g culture material/kg/day or 1.25g culture material/kg/day. The horse dosed with 2.5g culture material/kg/day developed severe hepatosis and mild edema of the brain stem, and the horse dosed with 1.25g culture material/kg/day developed moderate edema of the brain stem and mild hepatosis. An additional horse dosed intravenously seven times with 0.125mg FB_1 over nine days developed leukoencephalomalacia in the brain stem. Marasas et al.(1988) concluded that low FB_1 doses caused mild liver injury and severe brain lesions while high FB_1 doses caused severe liver injury and mild brain lesions. Reproduction of ELEM in ponies fed fumonisin-contaminated corn screenings associated with a previous outbreak of ELEM was reported. Typical lesions of ELEM were present in the brains of ponies which exposed to FB_1 for different phases (Ross et al. 1993).

Porcine pulmonary edema (PPE)

Porcine pulmonary edema was associated with occurrence of *F.verticillioides* when 2 pigs developed the disease following feeding on bulk culture material of corn contaminated with *F.verticillioides* (Kriek et al., 1981). Harrison et al. (1990) induced PPE and hydrothorax in three pigs which were intravenously adminstered FB_1 or FB_2 and compared to one control pig adminstered saline. One pig each was administered 0.4mg FB_1 /kg for four days, 0.174mg FB_1 /kg body weight for seven days, and 0.3 mg FB_2 /kg for five days. The high FB_1 dose caused pulmonary edema as previously noted in field cases, whereas the lower FB_1 dose and the FB_2 dose did not. Harrison et al. (1990) identified FB_1 as the agent responsible for causing pulmonary edema/hydrothorax in swine . Haschek et al.(1992) analyzed the toxicity of FB_1 in intravenously dosed pigs and total fumonisin toxicity of FB_1 + FB_2, in pigs fed contaminated corn screenings. Two female, weanling pigs were dosed with FB_1 intravenously at either 0.88 mg/kg/day for nine days or 1.15 mg/kg/day for four days. Three pigs were fed corn screenings contaminated with FB_1 and FB_1 at total doses of 5.5 mg/kg/day for five days, 4.5 mg/kg/day for six days, or 6.6 mg/kg/day for 15 days. Both pigs dosed intravenously developed liver and pancreatic lesions, but only the pig dosed intravenously with 0.88 mg FB_1 /kg/day developed PPE. All pigs fed contaminated corn meal demonstrated liver and pancreatic changes, and pigs fed doses of 5.5 mg/kg/day and 4.5 mg/kg/day developed pulmonary edema. Haschek et al. identified the liver, lung, pancreas as primary target organs in pigs.

Ruminant toxicity of FBs

Osweiler et al. (1993) investigated the effects of corn screenings, naturally contaminated with fumonisins, on calves. Cattle appeared to be much less susceptible to fumonisins than horses and swine. Some change in liver function and immune function were found in calves receiving the highest dose of FB_1 (148 mg/kg diet) but they all appeared healthy. Fumonisin containing corn culture material was found to be acutely toxic to sheep by Edrington et al. (1995) when dosed intraruminally. Liver and kidney function were affected in all lambs and deaths occurred in the medium and high dose groups. However, the doses used in this study were extremely high (11, 22 and 45 mg FB_1 /kg BW).

Toxicity of FBs in poultry

Several reports have been made on fumonisin toxicity in poultry. Weibking et al.(1993a) reported histological liver lesions when young broiler chicks were fed diets containing >225mg FB_1 / kg diet from *F.verticillioides* corn culture material for 21 days. Dietary levels of 10mg purified FB_1 / kg diet fed for 6 days, or 30mg FB_1 /kg diet from *F.verticillioides* culture material fed for 2 weeks, resulted in diarrhea, deceased body weight, and change in serum chemistry (Espada et al., 1994). One hundred mg FB_1 /kg diet from culture material, fed for 3 weeks., caused liver damage and altered serum chemistry in female turkey pouts (Weibking et al., 1993b). Ducklings fed rations containing 100, 200, and 400 mg FB_1 /kg diet 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 were relatively high, poultry may be more resistant to the toxic effects of FB_1 than other animals.

Developmental toxicity of FBs

Fumonisin B_1 is fetotoxic to rats. 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. Golden syrian hamster orally gavaged with culture material containing fumonisins (0.25-18mg FB_1 /kg BW) or pure FB_1 (12mg/kg BW and 18mg/kg BW) did not exhibit maternal toxicity based on weight gain, serum aspartate aminotrasferase activity or total bilirubin (Floss et al., 1994). With increased FB_1 doses, more fetuses were lost per litter. At 12 mg FB_1/kg, all hamsters fetuses were died. This suggested that prenatal exposure to aqueous culture extracts containing fumonisin or pure FB_1 were detrimental to fetal hamster survivability in the absence of maternal toxicity.

Organ toxicity of FBs

Fumonisin B_1 is nephrotoxic to rats and rabbits. FB_1 toxicity was examined using gavage administration of purified toxin to female Sprague-Dawley rats. For 11 consecutive days each rat received a single dose of FB_1 at the following concentrations: control (saline), 1, 5, 15, 35, or 75 mg FB_1/kg body weight/d. Kidneys and bone marrow were most sensitive to FB_1 exposure. Changes in renal morphology were observed from 5 to 75 mg FB_1/kg/d, accompanied by transient changes in urine osmolality and urine enzyme levels. Increased cellular vacuolation was the primary change associated with bone-marrow toxicity, starting at doses of 5 mg FB_1/kg/d. Hepatotoxicity was indicated by reduced liver weight, elevated serum ALT, and mild histopathological changes occurring at doses of 15 mg FB_1/kg/d and higher (Bondy et al., 1998). Fumonisin B_1 was shown to inhibit renal proximal tubule cell (RPTC) regeneration in cell cultures of rabbit RPTC following mechanical injury (Counts et al., 1995). Cultures were treated with treated with 1μM and 2 μM FB_1 after a Teflon policeman were used to swipe RPTC monolayers, removing ~24% of the area. 1μM FB_1 (1μM) significantly inhibited the regeneration of RPTC as compared with control group on day 7, and exposure to 2 μM FB_1 was cytolytic and resulted in degeneration of the culture monolayer after 3 days (Counts et al., 1995).

The fumonisins are not very toxic to primary rat hepatocytes in culture with a CD_{50} dosage of 1000μM and 500μM for FB_1 and FB_2 respectively (Gelderblom et al., 1993). The water solubility of fumonisins appears to be the reason for the low cytoxicity as the more polar FB_1 is less cytotoxic than the less polar FB_2. The irreversible nature of the interaction of fumonisins with cellular membranes (Cawood et al., 1994) suggested that a slow accumulation of fumonisins in the cell precipitates hepatoxicity and nephrotoxicity.

Immunotoxicity of FBs

There have been very few studies that address directly the potential for fumonisins to modify immune response *in vivo*. Nonetheless, there are many studies with fumonisins or fumonisin-containing diets that either altered function of blood cells *in vitro* or changes in hematological parameters *in vivo*. Macrophage phagocytic function was down-regulated *in vitro* by FB_1 (500 μM/ L) (Qureshi et al., 1992) as was lymphocyte proliferation in response to lipopolysaccharides (LPS) (Dombrink-Kurtzman et al., 1994). Immunosuppression in chickens was produced in birds fed maize cultured with *F.verticillioides* (MRC826) (Marijanovic et al., 1991). The broiler chicks fed diets containing 10mg FB_1/kg diet had reduced spleen and or/ bursa weights and altered hematological parameters (Espada et al., 1994). *In vivo* administration of 50ppm FB_1 significantly suppressed hepatic natural killer (NK) cell activity while stimulating hepatic preneoplasia (Lu et al., 1997; Liu et al., 2001), and the production of hepatic PGE_2 increased in both studies. Prostaglandin E_2 was observed to inhibit hepatic NK lytic activity when hepatic NK cell was co-cultured with PGE_2 (10ng/mL or 25ng/ml) (Liu et al., 2000). This suggested that PGE_2 might be one of the modulator of NK activity., Ceramide is an important signalling molecule and recognition site in the cellular immune response (Merrill et al., 1997a). Ceramide is the activator of the transcription factors, NF-κB. Activation of NF-κB occurs in mature T and B cells in response to antigen stimulation, in macrophages exposed to cytokines, as well as in many non-lymphoid cell types exposed to cytokines (Ballou et al., 1996). Fumonisins are inhibitors of ceramide synthase and can reduce the production of ceramide (Merrill et al., 1993a; 1993c). Fumonisin may inhibit the immune function through the inhibition of ceramide production. Tumor cells can produce a large amount of glycosphingolipids, and glycosphingolipids had been observed to suppress the proliferation of a variety of T and B lymphocytes induced by lectins, antigens, and interleukin-2 (Jackson et al., 1987), as well as inhibition of T helper cells and cytotoxic effector function (Offner et al., 1987), and the production of glycosphingolipids may increase during the FB carcinogenesis. The change of prostaglandins (Lu et al., 1997; Liu et al., 2000), as well as other factors which may change during fumonisin carcinogenesis, such as ceramide, glycosphingolipids, might be important modulation factors of immune function.

Carcinogenicity of FBs

Fumonisin B_1 is hepatotoxic and hepatocarcinogenic in rats. Culture material of strain MRC 826 of *F.verticillioides* fed to rats at 8% by weight of diet 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). Gelderblom et al (1988) identified fumonisins as cancer promoting agents in rats. Male BD IX rats initiated with an intraperitoneal injection of DEN at 200 mg/kg body weight were fed for four weeks with a diet of 5% lyophilized *F.verticillioides* culture material in rat mash or 0.1% FB_1. Control rats were administered dimethyl sulfoxide (DMSO) instead of DEN following by promoting treatment. Rats fed 0.1% FB_1 developed GGT positive AHF, whether initiated with DEN or given DMSO vehicle, with significantly greater GGT positive AHF in DEN-initiated rats. Using the same chemical model established in the above study, initiation of rats with DEN at 15 mg/kg body weight followed by 34.7 µmol FB_1 /kg diet promotion for 15 weeks, Liu et al. (2001) demonstrated that FB_1 caused the development of PGST – and GGT-positive AHF, early indicators of FB_1 carcinogenesis. In another experiment, a diet containing 50 µmol FB_1 /kg diet 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). Howard et al.(2001) fed FB_1 to female and male F344/N rats and B6C3F1 mice for two years. Female rats were fed 0, 5, 15, 50, and 100 ppm FB_1; male rats were fed 0, 5, 15, 50, and 150 ppm FB_1; female mice were fed 0, 5, 15, 50, and 80 ppm FB_1; male mice were fed 0, 5, 15, 80, and 150 ppm FB_1. Fumonisin B_1 was not tumorigenic in female F344 rats with doses as high as 100 ppm. Including FB_1 in the diets of male rats induced renal tubule adenomas and carcinomas in 0/48, 0/40, 9/48, and 15/48 rats at 0, 5, 15, 50, and150 ppm, respectively. Including up to 150 ppm FB_1 in the diet of male mice did not affect tumor incidence. Hepatocellular adenomas and carcinomas were induced by FB_1 in the female mice, occurring in 5/47, 3/48, 1/48, 19/47, and 39/45 female mice that consumed diets containing 0, 5, 15, 50, and 80 ppm FB_1, respectively. This study demonstrates that FB_1 is a rodent carcinogen that induced renal tubule tumors in male F344 rats and hepatic tumors in female B6C3F1 mice.

Detoxification of Fumonisin

Biological, chemical, and physical processes have been explored to salvage fumonisin-contaminated corn. Thermostability of FB_1 proved to be great. When dry corn was heated at 50, 75, 100 and 125°C for 40 minutes, only a small amount of FB_1 was not detected, more than 90% of FB_1 was recovered after 16 h at 50°C (Dupuy et al., 1993). Dupuy et al. (1993) concluded that additional detoxification procedures would be required to reduce or eliminate FB_1 as no heat treatments completely eliminated FB_1 .

Co-contamination of corn with aflatoxin and fumonisins prompted research into possible detoxification of fumonisins by ammoniation, the standard detoxification method used for aflatoxin-contaminated corn (Norred et al., 1991). Treatment of fumonisin-contaminated corn with 2% ammonia at low pressure for 4 days, a process that detoxified aflatoxin B_1 , led to slight reduction in the concentration of FB_1 without decreasing its toxicity in rats (Norred et al., 1991). Voss et al.(1992) examined the effects of ammoniation on FB_1 in corn and F.verticillioides corn culture material. Corn and culture material were treated with 2% ammonia and incubated at 50°C. Male SD rats were fed diets 10% in corn, ammoniated corn, culture material, or ammoniated corn culture material for four weeks. FB_1 content of culture material was not significantly reduced by ammoniation. Serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and γ-glutamyl transpeptidase levels were significantly greater in rats fed either the ammoniated or non-ammoniated *F.verticillioides* culture material. Voss et al.

(1992) concluded that the ammoniation procedure used to detoxify aflatoxin-contaminated corn did not significantly reduce FB_1 content or toxicity of *F.verticillioides* culture material.

Bothast et al.(1992) examined the possibility of ethanol fermentation to utilize contaminated grains. No FB_1 was found in distilled ethanol. Bothast et al.(1992) suggested that ethanol fermentation of FB_1 –contaminated grain would allow use of contaminated corn, but concern remained with spent grains as they are used in animal feeds. The detection of FB_1 in spent grains after fermentation, but detection of very little FB_1 in corn used for ethanol fermentation, suggested that FB_1 was conjugated through the amine group, preventing detection prior to fermentation. Increased fumonisin contents after fermentation indicates a potential human and animal hazard that needs to be addressed for undistilled, fermented products.

Nixtamalization, the traditional process to produce masa or tortilla flour, reduced the amount of FB_1 by hydrolyzing FB_1 to hydrolyzed FB_1 (HFB_1). To evaluate if this traditional process decreased the toxicity of *F.proliferatum* fermented corn, male F344/N rats were initiated with 15mg/kg DEN 10 days of age. At weaning, animal were randomly assigned to 8 groups of 6 each to evaluate the effect of *F. proliferatum* fermented corn, with or without nixtamalization and nutrient supplementation. After 4 weeks feeding, the animals fed nixtamalized *F. proliferatum* corn containing 10 mg HFB_1/kg had decreased body weight, increased relative liver weight, total cholesterol and ALT activity. However, these changes were less than those observed in animals fed *F. proliferatum* corn containing 50mg FB_1 /kg. The animals fed the nixtamalized diet with or without nutrient supplements developed adenoma. These data suggested that HFB_1 was similar in toxicity to FB_1 when the nutritional status of rats was adequate (Hendrich et al., 1993).

In vitro toxicity studies of several FB_1 analogs showed that the analogs containing FB_1 amine groups and the tricarballylic side chains were more toxic than analogs containing only the tricarballylic side chains (Kraus et al., 1992), and naturally occurring N-acetyl –FB_1 was not toxic (Gelderblom et al., 1993). Therefore, the primary amine of FB_1 is likely to be critical for its toxicity. Murphy et al. (1995) reported a method to detoxify FB_1 by derivatizing the amine group with a reducing sugar, fructose, in a nonenzymatic browning reaction. Diethylnitrosamine-initiated (15 mg/kg body weight) male F344/N rats were fed for 4 weeks either 69.3 µM FB_1 or 69.3 µM FB_1 reacted with fructose (FB_1 –fructose). Rats fed FB_1 had significantly increased levels of several markers of hepatocarcinogenicity, whereas rats receiving FB_1 – fructose showed no signs of hepatocarcinogenicity or hepatotoxicity (Lu , Thesis). A more practical and efficient method to block FB_1's amine group by reacting the amine group with glucose had been developed in Dr. Murphy's lab (Lu, Thesis) .The FB_1-glucose reaction was more complete than the reaction with fructose, and the reaction products were more easily isolated than FB_1-fructose products.

Fumonisin Bioavailability

The metabolism of fumonisin has been studied by using ^{14}C-FB_1 , which permits rapid analysis of the biological disposition of this compound. In fasted rats, Norred et al. (1993) detected 80% and 2.3% of FB_1 administrated by gavage (1.4 µmol of ^{14}C-FB_1 /kg bw) in feces and urine, respectively. Liver, kidney, and blood retained a total of 0.6% of the dose 96h after treatment. In fed rats at 24h after a dose administered by gavage (10.4μmol of ^{14}C-FB_1 /bw), Shephard et al. (1992) detected 100% and trace levels of FB_1 in feces and urine, respectively. Trace levels of ^{14}C-FB_1 were detected in liver, kidney, and blood of these rats. Biliary excretion of FB has been suggested by observations of fecal recovery of ^{14}C-FB given by intravenous or intraperitoneal routes (Norred et al., 1993; Shephard et al., 1992). Shephard et al. recovered 67% of an intraperitoneal dose (10.4μmol of ^{14}C-FB_1 /bw) after 24 h in bile whereas 0.2% of a 0.4 μml dose administered by gavage was detected in bile of fed rats (Shephard et al., 1994).

The excretion of FB_1, hydrolyzed FB_1 (HFB_1), and FB_1 -fructose adducts was determined in male F344/N rats by Hopmans et al. (1997; Dantzer et al., 1999). Rats were dosed by gavage with 0.69, 6.93 or 69.3μmol/kg of body weight FB_1, H FB_1, or FB_1 – fructose. Average total FB_1 backbone excretion in feces was 101, 76, and 50% of the dose for FB_1, HFB_1, and FB_1 –fructose, respectively. Average total FB_1 backbone excretion in urine was 2.7%, 5.0%, and 5.3% of the dose for FB_1, HFB_1, or FB_1 - fructose. In another experiment, the excretion of FB_1, HFB_1, and FB_1 –fructose were determined in both male and female F344/N rats (Dantzer et al., 1999).Urinary excretion of FB_1, and FB_1 –fructose was 0.5% and 4.4% of the total dose, respectively, and was similar between male and female rats. Urinary excretion of HFB_1 was significantly greater in female rats as compared with male rats (17.3% vs 12.8% of the total dose, respectively). The three fumonisin compounds had a similar biliary excretion with a mean of 1.4% of the dose excreted at 4 h after dosing. The HFB_1 was absorbed to a greater extent than FB_1 suggested that tricarballylic acids limited FB_1 absorption by increasing polarity. The loss of two tricaballylic acids would render HFB_1 a considerably less polar molecule with the GI tract environment, possibly facilitating its absorption. Although absorption of the FB_1 – fructose was greater than FB_1, the formation of the adduct may detoxify FB_1 by masking the primary amine group, leaving the molecule unable to exert its toxic effects.

Mechanism of Fumonisin Toxicity and Carcinogenicity

The fumonisins are non-mutagenic when tested in the Salmonella mutagenicity test (Gelderblom et al., 1991) and lack genotoxicity in *in vitro* DNA repair assays in primary rat hepatocytes (Gelderblom et al., 1992; Norred et al., 1992). The lack of mutagenesis by the fumonisins was confirmed when different concentrations, ranging from 0.7 to 500ug per plate, were tested against strains TA100 and TA98 in the presence and absence of Aroclor 1245 induced S9 enzyme fraction. Fumonisin B_1 also failed to induce micronuclei and did not alter the mitotic activity of primary rat hepatocytes. Despite the fact the fumonisins were negative in various genotoxicity and mutagenicity assays. Howard et al. (2001) showed that FB_1 is a rodent carcinogen that induced renal tubule tumors in male F344/N rats and hepatic tumors in female B6C3F1 mice in a two-year cancer bioassay study. Gelderblom et al. (1992; 1994) hypthesized that FB_1 mimics genotoxic carcinogens with respect to the induction of preneoplastic hepatocytes in rat liver. This was substantiated by the observation that FB_1 induces two important enzymes, GGT and PGST (Lu et al., 1997), which are accepted histogical markers for putative preneoplastic lesions initiated by genotoxic carcinogens. Feeding experiments with fumonisins in rats indicated that an increase in cell proliferation is also likely to play a critical role in the induction of the preneoplastic phenotype as hepatoxicity, and the resultant regenerative cell proliferation, is a prerequisite for initiation (Gelderblom et al., 1994). The only difference noticed thus far in the induction of resistant phenotype between the fumonsins and other genotoxic carcinogens lies in the kinetics of the cancer initiation step. It is know that, with genotoxic carcinogens, cancer initiation is normally completed within a matter of hours or a few days (Farber et al., 1989). However, single and/or multiple dosages of fumonisins in the presence of a stimulus for regenerative cell proliferation failed to effect initiation (Gelderblom et al., 1992). A recent study showed that prolonged exposure of rats to fumonisin FB_1 or even a single gavage dosage, effectively inhibits compensatory hepatocyte proliferation following partial hepatectomy (Gelderblom et al., 1994). In this regard, fumonisin B_1 resembles many other carcinogens which are all potent inhibitors of normal cell proliferation (Farber 1990). Since regenerative cell proliferation is sometimes a prerequisite for cancer initiation (Cayama et al., 1978), it was suggested that inhibitory effect of FB_1 could explain the fact that FB_1 is a slow cancer initiator (Gelderblom et al., 1994). All these suggested that the cancer initiating potential of fumonisins is that the rate limiting step for cancer induction may be the induction of cell death and the resultant compensatory cell proliferation, and the inhibitory effect of fumonisin on cell proliferation could delay and /or inhibit cancer initiation of this mycotoxin.

Contribution of sphingolipid metabolites to FB_1 toxicity and carcinogenesis

The fumonisins bear a remarkable structural similarity to sphingosine and sphinganine, which led many to hypothesize that the mechanism of action of fumonisins may be via disruption of sphingolipid metabolism. The cellular target of fumonisins has been found to be the enzyme ceramide synthase, which catalyzes the addition of a fatty acid to sphinganine in the *de novo* biosynthesis of sphingolipids, and in the reacylation of long-chain bases that arise from sphingolipid turnover (Wang et al., 1991; Merrrill et al., 1993a and 1993b). The inhibition is competitive with both the long-chain (sphingoid) base and fatty-acyl-CoA (Merrill et al., 1993), which indicates that fumonisins may inhibit ceramide synthase by interacting with both the binding site for sphinganine(sphingosine) and the site for the fatty-acyl-CoA. It is possible that fumonisins also inhibit other enzymes that interact with either long-chain bases or fatty acyl-CoA, To test this possibility, fumonisin B_1 has been tested with sphingosine kinase and serine palmitoyltransferase (Wang et al., 1991), and neither enzyme was inhibited by the levels(1μM FB_1) that result in complete inhibition of ceramide synthase.

The disruption of sphingolipid biosynthesis would be predicted to have profound effects on cells because these compounds have important roles in membrane and lipoprotein structure, cell-cell communication, interaction between cells and the extracellular matrix, and as second messengers for a wide range of factors (Merrill et al., 1993a and 1993c). Two likely explanations for the toxicity and carcinogenicity of FB_1 after inhibition of sphingolipid biosynthesis by this mycotoxins have been proposed. First, the accumulation of free sphinganine(and possibly its metabolites, such as the 1-phosphate)is growth inhibitory and cytotoxic for the cells. Long-chain (sphingoid) bases are well known to be growth inhibitory and cytotoxic (Merrill et al., 1983); therefore, the accumulation of sphinganine (and sometimes sphingosine) might lead to cell death. The cellular target that accounts for these effects of long-chains is unknown, but there are a number of candidates. Long-chain bases have been shown to inhibit protein kinase C, to activate phospholipase

D, and to activate or inhibit other enzymes of lipid signaling pathways, to inhibitthe Na+/K+ ATPase, to induce dephosphorylation of retinoblastoma protein(a key regulator of the G to S transition of the cell cycle), to induce release of Ca^{2+} from intracellular stores (apparently via sphingosine 1 –phosphate), and to affect a large number of other cell regulatory systems. In addition, sphingosine has been observed to indue apoptosis in thymocytes (Bai et al., 1990) and neutrophils (Ohta et al., 1994). Second, the loss of complex sphingolipid biosynthesis would be expected to alter cell behavior, and could lead to cell death based on findings with mutants in serine palmitoyltransferase, the initial enzyme of sphingolipid biosynthesis (Hanada et al., 1990). At low dosages, fumonisins appear to be mainly tumor promoters (Gelderblom et al., 1988). Tumor promoters are often mitogens, it has been found that sphingosine and sphingosine 1-phosphate can induce DNA synthesis in growth-arrested Swiss 3T3 cells (Zhang et al., 1990 and 1993). Therefore, it was hypothesized that fumonisins might induce DNA synthesis via the accumulation of sphinganine (Schroeder et al., 1994). Merrill et al (1995) demonstrated that addition of fumonisin B_1 to Swiss 3T3 cells elevates sphinganine and induces an increase in [^{3}H]thymidine incorporation into DNA. Furthermore, both were blocked by addition of an inhibitor of serine palmitoyltransferase (β-fluoro-L-alanine), which established that this effect of fumonisins is due to sphinganine accumulation, not the depletion of complex sphingolipids. *In vivo*, fumonisin acting as a mitogen could increase the probability of , or irreversibly lock in , a spontaneous genomic error. It has been proposed that mitogenesis increases the cancer risk by increasing the probability of DNA damage being converted to mutations, making DNA more sensitive to being damaged, increasing gross chromosomal alterations, and increasing expression of oncogenes(Ames et al., 1990).

The free sphingosine and sphinganine accumulation in lymphocytes was shown to inhibit DNA synthesis and to disrupt lymphocyte proliferation in response to mitogens and T-dependent anitgens (Martinova 1996; Martinova et al., 1991; 1995). Sphingosine has been found to inhibit IL-8-induced and integrin-dependent lymphocyte migration. Sphingosine also prevents both the MHC-restricted and non-restricted cell adhesion, and it inhibits cytotoxic lymphocyte proliferation in response to IL-2 and in mixed lymphocyte culture (Borchardt et al., 1994). In normal T lymphocytes and T helper cell clones, the selective activation of cAMP-dependent knase type I was found to block the signals transducing through TCR/CD3 complex. In transformed Swiss 3T3 fibroblasts, cAMP accumulation may activate the MAPK kinase cascade, G1/S-phase transition and cell differentiation (Spiegel et al., 1994). Sphingolipid balance defines the proliferation of murine cytotoxic lymphocytes (Yatomi et al., 1996). The mitogenic or suppressive effect of sphingolipids on lymphocyte proliferation is dependent on the cell cycle and additional signal pathways in the cell. Sphingolipid cross-talk with the cAMP system in lymphocytes (that suppresses the TCR/CD3 signaling in normal cells and up-regulates this in transformed cell lines) may explain both the mitogenic and inhibitory effect of sphingolipids on immune cells.

The precise mechanism by which disrupted sphingolipid metabolism contribute to the increased organ toxicity and neoplasia in rodents is unclear. The balance between the intracellular concentration of sphingolipid effectors that protect cells from apoptosis (decreased ceramide, increased sphingosine 1-phosphate) and the effectors that induce apoptosis (increased ceramide, increased free sphingoid bases) will determine the observed cellular response, and the balance between the rates of apoptosis and proliferation might be one of the critical determinants in the process of tumorigenesis.

The contribution of prostaglandins to fumonisin B_1 toxicity and carcinogenesis

The idea that prostaglandins may play a role in immune response/tumor cell interactions is based upon several observations. First, a variety of prostaglandins are produced both by cells that are themselves active in the expression and regulation of immune response activity (Tomar et al.,1981; Sinder et al., 1982) as well as by a number of tumor targets (Karmali et al., 1980; Goodwin et al., 1981). Carcinogenesis may be associated with increased prostaglandin production by neoplastic organs, such as during promotion of rat hepatocarcinogenesis by fumonisin B_1 (Lu et al., 1997). Second, the production of prostaglandins has been found to increase as a result of direct contact between effector lymphocytes and tumor targets (Owen et al., 1980). Third, prostaglandins at levels produced during these interactions have been shown to influence the ultimate expression in vitro of lymphocyte and macrophage cytotoxicity against tumor targets (MacCarthy et al., 1981; Koren et al., 1981).

Prostaglandins mediate inter- and intracellular communication, as may stimulate hepatocyte proliferation (Miura et al., 1979; Andreis et al., 1981). The concentration of PGE equivalents in rat liver *in vivo* was increased during liver regeneration. This stimulation of prostaglandin synthesis was confirmed in vitro by the ability of homogenates of regenerating liver tissue to synthesize PGE_2 and $PGF_{2\alpha}$ from arachidonate.

Indomethacin prevented these prostaglandin changes, and the subsequent increase in DNA synthesis (MacManus et al., 1976). During the regeneration of mammalian liver after a 70% partial hepatectomy (PHx), Kupfer cells produced significantly elevated PGE_2, and *in vivo* Kupfer cell PGE_2 blockade by indomethacin (5 mg/kg) significantly ($P < 0.05$) inhibited hepatic regeneration (Goss et al., 1993). The association of neoplastic tumors with increased levels of prostaglandins (Robertson et al., 1986; Bennett et al., 1975) provided the rationale for investigating their role in tumorigenesis. Animal and human tumors contain high levels of prostaglandins, particularly those of the E series that have been shown to significantly affect cell proliferation and tumor growth and suppress immune responsiveness. DNA synthesis of hepatocytes in primary culture was significantly enhanced by addition of PGE_2 (2-200 nmol/L). Intracellular cAMP level in the hepatocytes increased during culture, and cAMP increase was enhanced by PGE_2. Prostaglandin E_2 production in the liver increases hepatic regeneration and PGE_2 enhances the proliferation of hepatocytes by a seemingly cAMP-dependent specific receptor-mediated process (Tsujii et al., 1993) At concentrations of 10^{-9} -10^{-12}mol/L, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ very intensely stimulated both the DNA-synthetic and mitotic activities of hepatocytes in 4-day-old primary cultures of neonatal rat liver. DNA replication was more intensely enhanced by $PGF_{2\alpha}$ than $PGF_{1\alpha}$, whereas mitotic activity was nearly equally affected by the two prostaglandins (Armato et al., 1983). Thus enhanced PGE_2 and $PGF_{2\alpha}$ may promote hepatocarcinogenesis by stimulating DNA synthesis and proliferation of hepatocytes.

A high level of PGE_2 in the portal vein suppresses liver-associated immunity and promotes liver metastases (Okuno et al., 1995). Some *in vitro* experiments showed a similar phenomena. The ability of Syrian hamster tumor cells of the same origin but with different degrees of malignancy to secrete prostaglandin E was studied following their in vitro contact with Syrian hamster natural killer cells (NK cells). Syrian hamster NK cells were shown to lose cytotoxic activity significantly after their contact with malignant tumor cells. Short-term in vitro contact of malignant tumor cells with human and Syrian hamster NK cells resulted in a rapid PGE secretion into the culturing medium. Therefore, PGE_2 may promote tumor progression by inhibiting immune function. The effect of $PGF_{2\alpha}$ on NK cells is still not clear. The regulatory effects of prostaglandins on immune response appear to be mediated by the production of cyclic AMP (Robison et al., 1971). PGE_2 activates adenylate cyclase with a subsequent rise in cyclic AMP (Smith et al., 1971), which acts as a "second messenger". Cyclic AMP itself is an inhibitor of lymphocyte activation (Smith et al., 1971; Melmon et al 1974). The presence of receptors for PGE_1 and PGE_2 on the lymphocyte (B & T cell) surface had been demonstrated, while there were no binding sites for PGA, $PGF_{1\alpha}$ or $PGF_{2\alpha}$. Henney and Lichtenstein, using splenic lymphocytes from mice immunized with an allogeneic mast cell tumor (Henney et al., 1971), suggested that elevated cyclic AMP content of cytolytic lymphocytes might inhibited their ability to kill target cells. As a test of this hypothesis, prostaglandins were shown to inhibit lymphocyte cytolytic activity (Henney et al., 1972). The relative potency of seven prostaglandins in inhibiting cytolytic activity correlated very well with their potency in stimulating cyclic AMP accumulation in lymphocytes: $E_1=E_2>A_1=A_2>F_{1\alpha}=F_{2\alpha}$

≈ 0 (Lichtenstein et al., 1972). By co-culturing the liver associated natural killer cells of 9 week-old rats of both genders of F344/N and SD rats with prostaglandins, Liu et al. (2000) showed that prostaglandin E_2 significantly inhibited hepatic natural killer cell(NK) activity *in vitro* compared with untreated cells from both genders and strains, and 25ng PGE_2/ml inhibited NK activity significantly more than did 10ng PGE_2/ml. In contrast, 50ng $PGF_{2\alpha}$/ml and 100ng $PGF_{2\alpha}$/ml significantly stimulated hepatic NK activity compared with untreated hepatic cells from both F344/N and SD rats.

Lu et al.(1997) showed that elevation of hepatic prostaglandin paralleled the induction of AHF in rat liver, indicating that increased prostaglandin production was related to promotion of rat hepatocarcinogenesis caused by FB_1. At the same time, the inhibition of hepatic natural killer cell activity was observed with the increasing of hepatic prostaglandins and the induction AHF in rat liver under effect of FB_1. The inhibition of FB_1 hepatocarcinogenesis was observed when the female SD rats were supplemented with 14% dietary menhaden oil, which can inhibit the production of prostaglandins. Lee (2000) showed that the induction of hepatic AHF in female SD rats fed with 14% menhaden oil and 50ppm FB_1 was significantly lower as compared with the group fed with 14% lard and 50ppm FB_1. Hepatic PGE_2 and $PGF_{2\alpha}$ in first group was significantly lower than in the second group.

These studies suggested that elevation of hepatic prostaglandins faciliates tumor cell proliferation, and promotes the development of neoplasia. 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 (Schultz et al., 1978), and PGE_2 suppressed NK cells and lymphokine-activated killer cells.

The effect of total dietary fat on carcinogenesis

The amount of dietary fat has profound effects on the development of mammary tumors in mice and rats. Increased dietary fat was reported to increase the development of mammary tumors in experimental animal models. Many studies reported accelerated mammary tumorigenesis with increases in the quantity of ingested fat used unsaturated fatty acids derived from vegetable products, such as, corn oil, sunflower seed oil, etc. (Branden et al., 1986). Studies also reported enhanced mammary tumorgenesis with increased quantities of saturated fatty acids such as lard and beef tallow. Aksoy et al.(1987) reported the influence of isocalorically fed diets (containing different amounts of fat) on tumor incidence and parameters of fat metabolism in female Sprague-Dawley rats. Comparisons were made between rats induced with methylnitrosourea (25 mg/kg body wt) and untreated controls (Group I). The animals received either control diets (3.9% fat by weight, Groups I and II) or fat-enriched diets (10.7%, Group III; 15.6%, Group IV; 21.4%, Group V) over a period of 180 days. At the termination of the experiment, intake of the diet containing 10.7% fat by weight (24% fat per total calories) was associated with the highest tumor incidence. This study suggested increased fat intake increased neoplasia independent of the effect of energy intake. Another experiment studied weanling 21-day-old female Sprague-Dawley rats divided into different dietary treatment groups and allowed to feed *ad libitum* on one of the following diets: 5% (normal fat) corn oil; 20% (high fat) corn oil; 20% palm oil; 20% beef tallow; or 20% lard. At 52 days of age, all rats were given p.o. 7.5 mg 7,12-

dimethylbenz(a)anthracene (DMBA). One week following DMBA administration, all rats were switched to the 5% corn oil control diet and were maintained on this diet for the duration of the experiment. Rats fed a 20% lard diet during the treatment period showed a significant increase in mammary tumor incidence and number 19 weeks after DMBA administration, when compared to all other dietary treatment groups. Rats fed a 20% beef tallow diet during this same time period also demonstrated enhanced mammary tumor development, during the 10- to 19-week time period after DMBA. Mammary tumor development in rats fed 20% corn oil or palm oil diets during this treatment period was similar to that of control fed 5% corn oil. In conclusion, high dietary intake of lard and beef tallow, but not vegetable fat, fed from weaning until only 1 week after DMBA administration, significantly enhances mammary tumorigenesis in rats. The mechanism(s) by which animal fat induces this stimulation is not clear, but it did not appear to result from endogenous or exogenous endocrine stimulation, because estrogens, which are potent stimulators of mammary tumor growth and development in rats, were not different among the groups (Sylvester et al., 1986). Birt et al. (1990) reported that an enhancement of pancreatic carcinogenesis induced by N-nitrosobis(2-oxopropyl)amine (BOP) in hamsters fed diets containing high levels of beef tallow. They compared diets high in corn oil with those high in beef tallow in the enhancement of pancreatic carcinogenesis. Pancreatic cancer was induced with 20 mg BOP/kg body wt, s.c. administered at 8 weeks of age. One week later, hamsters were assigned to one of five diet treatments: (i) 4.3% corn oil (control); (ii) 20.5% corn oil (high corn oil); (iii) 0.5% corn oil + 3.8% beef tallow (low beef tallow); (iv) 0.6% corn oil + 19.9% beef tallow (high beef tallow); and (v) 5.1% corn oil + 15.4% beef tallow (high fat mixture). These diets were fed until the study ended 84 weeks after BOP treatment. Hamsters were pair fed to consume the same calorie allotment as the control corn oil group. By the end of the experiment, BOP-treated hamsters that were fed diets containing beef tallow were consistently heavier than those fed corn oil. Pancreatic adenoma incidence and multiplicity were higher in hamsters fed beef tallow than those fed corn oil diets. Carcinoma *in situ* multiplicity was elevated in hamsters fed high-fat diets irrespective of the nature of fat fed. Pancreatic adenocarcinoma multiplicity was elevated in hamsters fed the low- or high-beef tallow diets compared with the low- or high-corn oil diets. This experiment suggested that greater total amount of dietary fat promote the carcinogenesis when animal were fed isocalorically. Rats were first intubated with diethylnitrosamine (DEN, 10 mg/kg) 20 hr after partial hepatectomy; 1 week later, rats were fed one of three purified diets (a low-fat diet similar to the AIN-76 diet, a high saturated fat diet, or a high polyunsaturated fat diet) with or without 0.05% phenobarbital in the diet for 10 months. Increasing the fat level of the diet did not increase the number of GGT-positive foci arising spontaneously or induced by DEN alone. When phenobarbital was present in the diet, both high-fat diets enhanced the induction of GGT-positive foci. Increasing the dietary fat level, may enhance promotion of liver foci by phenobarbital (Glauert et al., 1986).

How increased quantities of dietary fat enhance the tumorigenic process is not clear. But virtually all studies reporting a significant enhancing effect of dietary fat on experimental mammary tumorigenesis compared very low levels of fat with high levels, i.e., 0.5-5% fat versus 20-30% fat, and the animals consuming the low levels of fat may not have received adequate quantities of an essential lipid such as linoleic acid. Intensely proliferating mammary tumors may need greater quantities of this nutrient compared with normal tissue (Clifford, 1992).

In order to determine the degree of energy restriction necessary to achieve significant inhibition of mammary tumor promotion in rats treated with 7,12-dimethylbenz[a]anthracene (DMBA). A control group of rats was fed a diet containing 5% corn oil *ad libitum*. Four other groups were pair-fed to the controls; these rats were subjected to energy restriction of 10, 20, 30, or 40%. Weight gains among the groups were proportional to energy intake. The differences in weight were due primarily to reductions in body fat stores. Tumor incidence was reduced slightly by 20% calorie restriction and significantly by 30 and 40% restriction. There were also reductions in number of tumors per tumor-bearing rat and in mean tumor weight. The groups subjected to 30 and 40% energy restriction had significantly reduced serum levels of insulin in the fasting state. These data suggest that body weight, body fat, and fasting serum insulin correlate with susceptibility to mammary tumor promotion and that insulin may be a growth factor for DMBA-induced tumors (Klurfeld et al., 1989). In order to study if moderate degree of caloric restriction, 25%, would inhibit tumor growth in rats fed the equivalent of 20% dietary fat which approximates human consumption in affluent countries, rats were fed diets *ad libitum* that contained 5, 15 or 20% corn oil. Groups of rats were pair-fed to the last 2 groups, but subjected to a 25% caloric restriction. These groups were fed 20 or 26.7% corn oil so that absolute fat intake in the paired groups was identical. Significant inhibition of tumor incidence, tumor weight, tumor burden, body fat deposition, and fasting serum insulin were observed in the 2 calorically restricted groups. This experiment concluded that moderate caloric restriction is significantly more effective in inhibiting tumor growth than is the promoting effect of diets high in fat. Total body weight, body fat and serum insulin concentrations may be better correlates of risk of developing mammary tumors than is dietary fat (Klurfeld 1989).

Lu et al. (1997) reported that feeding 7% soybean oil and 14% beef tallow to DEN (15mg/kg bw)-intiated and 50ppm FB_1 –promoted female F344/N rats for 5 weeks caused all animals developed GGT- and PGST- positive altered hepatic foci, and the hepatic NK activity was inhibited by 50ppm FB_1. Liu et al. (2001) reported that feeding 7% soybean oil only as dietary fat to DEN (15mg/kg)-intiated and 25 ppm FB_1-promoted female F344/N rats for 16 weeks caused only half of the animals to develop PGST- and GGT-positive foci, and the NK activity was not different among groups. These two experiments suggested that there was an interaction between dietary fat and FB_1 carcinogenesis, as reflected in their effects on the altered hepatic foci, and hepatic NK activity.

The relationship between dietary fat and calories in mammary tumorigenesis in rodents was examined in lots of studies. Caloric restriction has a significant and consistent inhibitory activity on the development of rodent mammary tumors. The restriction of caloric consumption suppressed the development of mammary tumors in experimental animals and appeared to block the difference in mammary tumorigenesis between rats fed low- and high-fat diets. In carcinogen-treated rats, reducing energy consumption by 25% (Cohen et al., 1988) or as little as 12% (Welsch et al., 1990) of that consumed by *ad libitum* –fed controls negated the significant mammary tumor stimulation of a high fat diet.

Furthermore, carcinogen-treated rats fed low- and high-fat diets and restricted in food consumption to a level equivalent to that consumed by the animals consuming the least amount of food showed no differences in mammary tumor development (Thompson et al., 1985). It appeared that enhancement of mammary tumorigenesis in rodents by low- or high-fat depended on an *ad libitum* feeding protocol. In summary, high caloric density appears to contribute more than the high dietary fat to the development of cancer.

Natural killer cells and carcinogenesis

Natural killer cells comprise a heterogeneous population of large granular lymphocytes, appoximately 5-10% of peripheral blood mononuclear cells, that are known to participate in both homeostatic and inflammatory host defense functions (Herberman et al., 1981; Robertson et al., 1990; Trinchieri et al., 1989) . These cells spontaneously lyse a variety of parasites, fungi, bacteria, virally infected cells, and certain transformed cell populations, and are considered to play a role in the early protection against microbial infections and tumor cell development in a host before the development of specific immunity (Herberman et al., 1981; Whiteside et al., 1994). NK cells are mostly found circulating in the blood; however, under certain inflammatory conditions or in response to the administration of a biologic response modifier such as interleukin-2 (IL-2), interferon-γ (INF-γ), these cells preferentially traffic to several organ sites, including the liver, spleen, and the peritoneal cavity (Pilaro et al., 1994; Allavena et al., 1997). This organ-specific trafficking is believed to be owing to the local release of various cytokines and other inflammatory mediators. Moreover, considerable evidence has accumulated to demonstrate that the cytokine-induced augmentation of organ-associated NK cell function can contribute to nonspecific anti-metastatic response observed in a number of immunotherapy studies(Allavena et al., 1997). Moreover, the ability of NK cells to secrete various cytokines including IFN-γ, tumor necrosis factor (TNF), and granulocyte –macrophage colony-stimulating factor (GM-CSF) is believed to play a central role in the regulation of both the immune response and hematopoiesis (Holmberg et al., 1981). Indeed, studies within several experimental immunodeficient animal models have clearly demonstrate the importance of INF-γ production by infiltrating NK cells in preventing overwhelming infection from several obligate intracellular pathogens (Glimpel et al., 1988). Additionally, the production of both IFN-γ and TNF-α by NK cells appear to play a role in the pathogenesis of septic shock.

The capacity to mediate Major Histocompatibility (MHC)-unrestricted cytotoxicity against certain tumor cells without apparent prior sensitization has by definition been primarily ascribed to NK cells (Trinchieri 1989). NK cells are phenotypically and functionally distinct population of lymphocytes with characteristic morphology of large granular lymphocytes (Trinchieri 1989; Whiteside et al., 1990). In rats, high density cell surface expression of NKR-P1 antigen(i.e. NKR-P1$^{\text{bright}}$) is an exclusive property of all mature NK cells (Chambers et al., 1989; Brissette-Storkus et al. 1994). NKR-P1 is also expressed on a subset of T cells, but with a 2- to 10- fold lower density (i.e., NKR-P1 $^{\text{dim}}$) than on NK cells (Chambers et al., 1989; Brissette-Storkus et al. 1994). Some subsets of NKR-P1$^{\text{dim}}$ are capable of mediating NK-like cytotoxicity, particularly after incubation with high concentration of IL-2 (Brissette-Storkus et al. 1994)

Wisse and Daem (1970) described the presence of cells with a lymphoid morphology and characteristic cytoplasmic granules in the sinusoids of perfused rat liver. They called these cells "pit cell". In 1983, Kaneda et al (1983) proposed that pit cells may represent NK cells. Independent studies demonstrated that NK cells can indeed be isolated from rodent liver (Wiltrout et al., 1984; Cohen et al., 1985). Natural killer cells can be isolated from normal liver by two methods, namely enzymatic digestion of the liver, and "sinusoidal lavage"(Bouwens et al., 1987). The sinusoidal lavage method to isolate hepatic NK cells has several advantages to the enzymatic dissociation method: 1. It is faster and does not require the equipment for centrifugal elutriation. 2. Its reproducibility is high and does not depend on the activity of the batch of enzymes. 3. It does not damage the cells due to the proteolytic activity of enzymes. With this sinusoidal lavage method, an average of about 30% of the isolated mononuclear cells were identified as NK cells. This result demonstrated that an NK cell-enriched lymphocyte population is present in normal rat liver, because in peripheral blood less than 8% of the mononuclear cells are NK cells, and in the spleen this frequency is even lower. The total number of NK cells in the liver is also high in comparison to other anatomical locations(Bouwens et al., 1987). In athymic nude rats, a significantly higher number of NK cells can be isolated from the liver as compared to normal rats (Bouwens et al., 1987).

Foreign antigens, damaged cells, viruses and gut-derived microbial products such as endotoxins are cleared and detoxified by cells lining the liver sinusoids (Nolan 1989). Since the liver is a major organ site for metastasis of tumor cells (Vidal-Vanaclocha 1993), attention has been recently directed to the role of cells from the liver sinusoids in the defense against invading tumor cells (Tzung 1992). Rappaport et al. (1973) defined different zones in the liver according to certain anatomical properties. Zone 1 is the region surrounding the portal vein while zone 3 encompasses the central vein. The nonparenchymal liver cells, such as liver-associated NK cells and Kupffer cells are more abundant in zone 1 than in zone 3 (Barbera-Guillem et al., 1991). Hepatocyte regeneration following injury and adult reactive hematopoiesis both begin in zone 1. More importantly, metastatic tumor cells developed colonies only in zone 1 (Vidal-Vanaclocha et al., 1990). Consequently, liver associated-NK cells and other nonparenchymal liver cells in zone 1 of the liver may play a critical role in control of implantation and the growth of metastatic solid tumor cells.

Evidence from experimental animal models strongly suggests that liver-associated NK cells and Kupffer cells are the first -line defense against blood-borne metastasizing solid tumor cells invading the liver and thus protect the parenchyma. The primary role of NK cells in neoplasia is directed against blood-borne tumor cells during the intravascular phase of tumor metastasis(Winnock et al., 1993). The number of metastatic foci in the liver increases dramatically after tumor cell inoculation when in vivo liver associated NK activity is ablated by anti-asialoGM1 antiserum [anti-AsGm1] (Wiltrout et al., 1985). On the other hand , reconstitution of NK cells restores both tumor cell clearance and antimetastatic efficiency(Barlozzari et al., 1985).

Purified rat liver-associated NK cells but not splenic NK cells lyse syngeneic mammary and colon tumor cells *in vitro* (Bouwens et al., 1988). Vanderkerken et al (1990) have further divided rat liver-associated NK cells into high and low density NK cells. High density NK cells from rat liver which resemble NK cells from peripheral blood are less cytotoxic in vitro than low density NK cells from rat liver against YAC-1cells and CC531 colon carcinoma cells. However, only the low density rat liver NK cells lyse P815 tumor cells. According to these investigation (Vanderkerken et al., 1993), the immature high density NK cells migrate to liver from the blood, interact with Kupffer cells and other NPC in vivo, differentiate and become mature, highly cytotoxic low density liver NK cells.

Attention has focused on the role of the liver as a tumor killing organ. The hepatic NK activity is much higher than in peripheral blood and spleen preparation(Vanderkerken 1993). The liver also harbors the largest population of fixed macrophages(Malter et al., 1986). These tumoricidal cells may affect metastasizing tumors in addition to resident hepatic tumors(Lukomska et al., 1987). However, the role of impaired natural immunity in chemically-induced hepatocarcinogenesis is still unclear.

Lu et al. (1997) reported that feeding 7% soybean oil and 14% beef tallow to DEN (15mg/kg bw)-initiated and 50ppm FB_1 –promoted female F344/N rats for 5 weeks caused all animals to develop GGT- and PGST- positive altered hepatic foci. The hepatic NK activity in the treatment group was significantly inhibited as compared with control group, and hepatic PGE_2 and $PGF_{2\alpha}$ were significantly increased in FB_1 –fed rats compared with controls. Liu et al. (2001) reported that feeding a diet containing 7% soybean oil (AIN 93) to DEN (15mg/kg)-initiated female F344/N rat promoted with 25ppm FB_1 for 16 weeks caused only half of the animals to develop PGST- and GGT-positive foci, and no difference of hepatic NK cell activity was observed between control and FB_1 fed rats. These two experiments suggested that there was an interaction between dietary fat and FB_1

carcinogenesis, as reflected in their effects on the altered hepatic foci, and hepatic NK activity. It seemed that increased preneoplasia and high levels of PGE_2 and may signal to decrease NK cell activity. We hypothesized that NK activity decreases with the development of FB_1 carcinogenesis and the increase of prostaglandins , sphingolipids as well as other factors produced during FB_1 carcinogenesis may down-regulate hepatic NK activity during FB_1 carcinogenesis.

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SPRAGUE-DAWLEY RATS HAVE GREATER LIVER-ASSOCIATED NATURAL KILLER CELL ACTIVITY THAN DO F344/N RATS, ALOTHOUGH A GREATER PROPORTION OF LYMPHOCYTES ARE NATURAL KILLER CELLS IN F344/N RATS

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ABSTRACT

Strain and gender differences in cancer incidence are proposed to be due partly to difference in immune function. In this study, the percentage of liver-associated total lymphocytes composed of natural killer cells and their activity were compared in 9 week old male and female F344/N and Sprague Dawley(SD) rats. Natural killer cells were stained using an anti-NKR-P1 monoclonal antibody and quantitated by flow cytometry. Two populations were found to stain positively: the NKR-P1^{bright} population, which represents natural killer cells with a high density of staining for NKR-P1, and an NKR-P1^{dim} population, which is a subset of T cells that express a low density staining for NKR-P1. F344/N rats had a greater hepatic NKR-P1^{bright} percentage of total leukocytes than did SD rats (P<0.001). No gender difference was found in the NKR-P1^{bright} percentage. The NKR-P1^{dim} population was not significantly different between F344/N and SD rats, but the F344/N male rats exhibited a significantly higher NKR-P1^{dim} population than F344/N females. After covariance of liver NK activity with the percentage of NKR-P1^{bright} Leukocytes, F344/N and SD rats exhibited similar lytic activity per NK cell. There was no gender difference in liver-associated NK activity. After covariance with body weights, SD rats exhibited significantly greater total hepatic NK activity($p<0.001$) than F344/N rats, and male rats had significantly greater total hepatic NK activity than female rats. Thus, SD rats would experience greater total liver-associated NK activity which may partly explain their lesser cancer susceptibility than do F344/N rats under some conditions.

Key words: F344/N, Sprague-Dawley, Natural killer cell, NKR-P1^{bright}, NKR-P1^{dim},

INTRODUCTION

The use of an animal carcinogenicity bioassay in assessing the oncogenic risk involved with chemical exposure is an important and necessary process, but presents many difficulties in interpretation when extrapolating to humans [1]. There are large inter-species, and inter-strain and gender variations in the incidence of some tumors. Variable tumor formation may be related to factors, including: organ/strain specific oncoviruses, hereditary disorders [2], or differing immune capabilities, such as hepatic natural killer cell(NK) activity [3]. F344/N and SD rats were included in this study as these are two rodent strains predominantly used in carcinogenicity bioassays, and there is a large database on cancer development in these strains. In one study, newborn F344/N and SD rats were irradiated with whole body single doses of 3Gy gamma rays with or without intraperitoneally-injected diethylnitrosamine(DEN) (15 mg/kg body weight) within 1 h of irradiation. Tumor development was promoted with 0.05% phenobarbital. In groups treated with radiation alone or radiation combined with DEN, F344/N rats had threefold greater development of placental S-glutathione transferase-positive (GST-P^{+}) altered hepatic foci (AHF, biomarkes of preneoplasia and neoplasia) than did SD rats. In SD rats, females had 1.5 greater induction of GST-P^+ AHF than did males [4]. In another study in which hexachlorobenzene was fed to male and female F344/N rats for 15 weeks, 100% of surviving females had multiple liver tumors which were strongly γ-glutamyl transferase (GGT) positive and histologically classified as neoplastic nodules or hepatocellular carcinomas. In contrast, only 16% of males developed tumors which were smaller and fewer in number than those in females [5].

The capacity to mediate MHC-unrestricted cytotoxicity against certain tumor cells without apparent prior sensitization has by definition been primarily ascribed to NK cells [6]. NK cells are phenotypically and functionally distinct population of lymphocytes with morphology characteristic of large granular lymphocytes [7,8]. In rats, high density cell surface expression of NKR-P1 antigen(i.e. NKR-$P1^{bright}$) is an exclusive property of all mature NK cells [9, 10]. NKR-P1 is also expressed on a subset of T cells, but with a 2- to 10- fold lower density(i.e., NKR-$P1^{dim}$) than on NK cells [9, 10]. Some subsets of NKR-$P1^{dim}$ lymophocytes are capable of mediating NK-like cytotoxicity, particularly after incubation in 1000U/ml interleukin-2(IL-2) for five days [10].

Attention has focused on the role of the liver as a tumor killing organ. Hepatic NK activity is much higher than in peripheral blood and spleen preparations [11]. The liver also harbors the largest population of fixed macrophages [12]. These tumoricidal cells may affect metastasizing tumors in addition to resident hepatic tumors [13]. However, the role of impaired natural immunity in chemically-induced hepatocarcinogenesis is still unclear. Neonatal B6C3F1 mice were given a single carcinogenic dose of diethylnitrosamine (DEN) and the time-response kinetics for the early (altered foci) and late (adenomas/carcinomas) phases of hepatocellular carcinogenesis were compared to changes in hematopoiesis and immune functions associated with immune surveillance and natural resistance [14]. Increases in hematopoiesis occurred just prior to or concurrent with the appearance of hepatocarcinomas, while increased macrophage and natural killer cell cytotoxicity and suppression of cell-mediated immunity occurred following tumor appearance and progressed with increasing tumor burden. Neither immunological nor hematopoietic changes were associated with early phases of hepatocarcinogenesis, as monitored by the appearance of AHF. Although changes in hematopoiesis may represent an early indicator for hepatocarcinogenesis in the mouse tumor model, the data suggest that altered immune surveillance and natural resistance are not factors in the development of chemically induced hepatocellular tumors, and the changes in immune function are probably secondary to tumor development. Neither immunological nor hematopoietic changes were associated with early phases of hepatocarcinogenesis, as monitored by the appearance of AHF [14]. In male F344/N rats given 40ppm DEN in drinking water for 10 weeks, as GST-P^+ foci developed, splenic NK activity changed. After 5 weeks, DEN-treated and control rat spleen NK activity was similar, but at 10 weeks, NK activity was significantly greater in DEN treated rats compared with control rats. At 20 weeks, DEN-treated rats had significantly lower NK activity than did controls [15]. This suggests an interaction between chemical carcinogenesis and NK activity. Lu et al. [16] also showed that chemical carcinogenesis (initiation by DEN, 15mg/kg at 10 days of age, and promotion by fumonisin B1 (50mg/kg diet)) caused significantly decreased NK activity after 4 weeks of development of AHF [16].

We propose that liver-associated NK activity will be greater in SD than in F344/N rats and in males than in females, due to greater liver associated NK cell numbers, even after covariation for body weight. This proposed difference in NK activity might partly explain some previous findings of gender and strain difference in susceptibility to carcinogenesis.

MATERIALS AND METHODS

Animals

The experimental procedures were approved by the Iowa State University Animal Care Committee. In 3 replicate experiments, six week old male and female F344/N and Sprague Dawley rats (n= 4 of each strain /gender) were given free access to diets (AIN-93G) and water for 3 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 were recorded weekly.

Liver perfusion

Rat livers were perfused with 40ml of Hank's Balanced Salt Solution (HBSS, supplemented with 25mM Hepes and 0.1% EDTA). Approximately 12 ml of perfusate was concentrated to 3 ml and laid on 3 ml Accupaque density gradient media (Accurate Chemical Co., Westbury, NY), then centrifuged at 1500rpm for 10 minutes. The mononuclear cells at the interface were collected, and washed two times, once with HBSS(with 25mM Hepes) and once with complete medium (RPMI-1640, supplemented with 50μg/ml gentamicin, 25 mM Hepes, 2mM L-glutamine, and 10% fetal bovine serum (FBS)). Cells were enumerated on a Celltrack II (Nova Biomedical, Waltham, MA).

Natural killer cell assays

Natural killer cell assays were performed as previously described [17]. Cells were plated in triplicate at the following effector to target ratios in 96 well plates: 25:1, 12.5:1, 6.25:1, 3 : 1. The target cells for the assay were YAC-1 cells (ATCC Co, Rockville, MD) (8×10^3/well) which had been labeled with 200μCi ^{51}Cr. The amount of ^{51}Cr released by dying cells was counted using a Gamma Trac 1191(TM Analytic, Inc., Elk Grove Village, IL). Lytic units were calculated using a computer program based on the equation of Pross and Maroun [18].

Fluorescent staining of lymphoid cells

Leukocyte suspensions were diluted with an equal volume of cold PBS/0.1% azide and incubated at 4°C (5min). Separate aliquots were stained with 0.2μg/2×10^5 cells of anti-rat NKR-P1A-Biotin(mAb 3.2.3), or an equivalent amount of isotype control antibody (murine IgG1-biotin). A second step of 0.1μg strep-AvidinCychrome was used(All from Pharmingen, San Diego,CA). All incubations were performed at 4°C in the dark for 30mins, and washed with PBS/0.1%azide. The contaminating red blood cells were lysed using ammonium chloride buffer(PH=7.4).Cells were fixed with PBS/1% paraformaldehyde prior to analysis using an EPICS-XL-MCI flow cytometer (Coulter, Miami,FL).Gates were defined to distinguish three cell populations: NKR-P1^{bright} , NKR-P1^{dim} and negative [17]

Statistical analysis

Data from three replicate experiments were combined for analysis. A two-way ANOVA was used to assess strain and gender relationships for the percentage of Leukocytes that carry NKR-P1^{bright} and NKR-P1 ^{dim} population markers. The hepatic NK exhibited by F344/N and SD rats was compared using ANCOVA by adjusting for the percentage of NKR-P1^{bright} population. Total hepatic NK numbers were compared between strain and gender using a two-way ANOVA combined with covariance for the body weight.

RESULTS

NKR-P1^{bright} and NKR-P1^{dim} population in F344/N and SD rats

The flow cytometric analysis using mAb 3.2.3 (NKR-P1) revealed two distinct subsets of rat hepatic mononuclear cells expressing variable levels of NKR-P1 in both F344/N and SD rats. A representative flow cytometric histogram chart illustrates that NKR-P1 was expressed at high levels (NKR-P1^{bright}) (Gate C) and at low levels (NKR-P1^{dim}) (Gate B) in a clearly defined populations of cells(Figure 1). The NKR-P1^{bright} population has been identified as the population causing NK associated lytic activity [19]. The NKR-P1^{dim} population have been linked to NK-T cells and can have NK-like cytolytic function under activation by IL-2 [10]. The percentage of hepatic NKR-P1^{bright} was significantly greater in F344/N than in SD rats($p<0.001$). Both F344/N male and female rats exhibited significantly greater hepatic NKR-P1^{bright} percentage than male and female SD rats ($p<0.001$)(Fig 2). There was no significant difference between male and female rats, nor was there a strain by gender interaction. The percentage of NKR-P1^{dim} population was not significantly different between F344/N and SD rats(Fig 3). But F344/N male rats exhibited a significantly greater NKR-P1^{dim} population than the F344/N female rats ($P<0.01$). Based on the percentage of NKR-P1^{bright} in leukocytes and the total leukocytes in the hepatic perfusate, we calculated the total hepatic NK cells in F344/N and SD rats. After covariance of the total hepatic NK cell number with body weight, Sprague-Dawley rats had significantly greater total number of hepatic NK cells than F344/N rats($p<0.001$) (Fig 4). The male rats of both strain exhibited significantly greater total number of hepatic NK cells than female rats after covariance for body weight ($p<0.01$).

Liver-associated NK activity in F344/N and SD rats

F344/N rats exhibited significantly greater levels of liver NK activity than SD rats before covariance with the percentage of the NK^{bright} population ($p<0.001$)(Fig 5A). But the NK activity was not significantly different between male and female rats, After covariance of the liver NK activity with the percentage of $NKR\text{-}P1^{bright}$ in leukocytes, F344/N rats and SD rats exhibited similar lytic activity per NK cell (Figure 5B). Again there was not a difference in NK activity between male and female rats, nor gender by strain interaction.

DISCUSSION

In this study, the percentage of two subsets of normal hepatic mononuclear cells which express NKR-P1 on the cell surface, $NKR\text{-}P1^{bright}$ and $NKR\text{-}P1^{dim}$ were compared between F344/N and SD rats. Virtually all basal NK cell lytic activity is associated with $NKR\text{-}P1^{bright}$ cells [20]. $NKR1\text{-}P1^{dim}$ cells lack lytic activity against NK targets. However, after incubation with IL-2 and subsequent removal of contaminating $NKR\text{-}P1^{bright}$ cells, the $NKR\text{-}P1^{dim}$ cells can demonstrate the ability to lyse YAC-1 target cells and mediate reverse antibody-dependent cellular cytotoxicity (rADDC) via NKR-P1 [10] F344/N rats exhibited a significantly greater percentage of $NKR\text{-}P1^{bright}$ cells than did SD rats (Fig.2), F344/N also exhibited significantly greater hepatic NK lytic activity than SD rats before covariance with the percentage of $NKR\text{-}P1^{bright}$ population (Fig 5A). After covariance with the percentage of the NKR-P1^{bright} population, the hepatic NK cells exhibited similar lytic activity on a per cell basis in F344/N and SD rats (Fig 5B). We also observed that male F344/N rats had a significantly greater percentage of NKR-P1^{dim} lymphocytes than female F344/N rats (Fig 3). A sexual dimorphism in natural killer cell activity has been observed during tumor metastasis in F344/N rats [21]. Syngeneic mammary tumors (MADB106) were used to assess the host anti-metastatic activity. Prepubescent (36 days of age) female rats exhibited greater splenic NK lytic activity than prepubescent males. After the onset of puberty (63 days of age), the males surpassed the females in NK activity, and at maturity (140 days of age) displayed greater splenic NK activity than females. Both NKR-P1^{bright} and NKR-P1^{dim} populations may contribute to the gender difference in NK activity during tumor metastasis observed in F344/N rats [21], as we observed greater NKR-P1^{bright} and NKR-P1^{dim} populations in male than in female F344/N rats. Activated NKR-P1^{dim} cells can lyse tumor cells in vitro [10, 19, 22]. With the progression of tumors, there is a production of endogenous IL-2 cytokine which can stimulate the activity of the NKR-P1^{dim} cells. Intraportal inoculation of CC531 adenocarcinoma cells into syngeneic rats caused an increase of liver macrophage cell number [23], and macrophages can stimulate T-cell proliferation and IL-2 production [24]. Once activated, the NKR-P1^{dim} lymphocytes can perform tumor cytotoxic activity and secrete cytokines. It would be expected that male F344/N rats may exhibit higher liver NK lytic activity than female F344/N rats during carcinogenesis, which may explain a lower incidence of tumor formation or lower growth rate of tumors.

Our data also suggested that males exhibited significantly greater total hepatic NK activity than females of both SD and F344/N strains. These data may support some findings that females are more susceptible to liver tumors than males under the effect of some hepatocarcinogens in both SD and F344/N strains [4, 25]. Sprague-Dawley rats initiated by DEN, and additional weekly application of polychlorinated biphenyl (50 or 100 mg/kg body wt./week, for 7 weeks) demonstrated an enhanced number of ATPase-deficient islands in males 3-fold and in females9-fold. The total area occupied by AHF increased 4-fold in males and 15-fold in females. The Number and area of GGT-positive AHF were similarly enhanced [26]. It has been suggested that tumor development and immunocompetence are affected by the estrous cycle, and sex hormones have been shown to modulate lymphokine production, neuroendocrine activity and immunity. IN female rats inoculated intravenously with MADB106 tumor cells, a syngeneic mammary adenocarcinoma cell line that metastasizes only to the lungs, susceptibility to metastatic development was found to be significantly higher during pro-estrus and estrus than during metestrus and diestrus, when estrogn levels are low [27]. The number and activity levels of circulating blood NK cells (NKR-P1^{bright}) indicated the estrous-dependent alterations in the number of NK cells and suggested a diminished NK activity per NK cell during pro-estrus/estrus [27], the same phases that were characterized by greater susceptibility to metastatic development. These findings support a causal relationship between a short-term exposure to elevated estradiol/low progesterone levels and decreased resistance to tumor metastasis, and it is hypothesized that an alteration in large granular lymphocyte (LGL) /NK cell activity underlies these effects [27]. Thus, sex hormones may play a role in affecting NK activity in male and female rats.

Experiments suggest that NK cells are important effector cells against bone marrow grafts, bacterial infections and viral hepatitis [28]. NK cells in neoplasia are primarily directed against blood-borne tumor cells during the intravascular phase of tumor development [29]. The number of metastatic foci in the liver of mice increased dramatically after tumor cell inoculation when *in vivo* liver-NK activity is ablated by anti-asialoGM1 antiserum [30]. Newborn females SD rats initially given a single intraperitoneal injection of 15 mg DEN/kg and phenobarbital (PB) administered in drinking water, demonstrated that GST-P+ hepatocytes increased with age in DEN-treated rats. The NK activity of DEN-treated rats did not significantly differ from that of control rats until week 12, but it progressively decreased from week 15 to 30. These results indicate that changes of NK activity are inversely correlated with the induction of preneoplastic hepatic foci. This strong correlation of decreased NK activity with enhanced induction of GST-P^+ foci suggests that NK activity is important in the early progression of hepatocarcinogenesis in rats [3]. This strong inverse relationship between NK activity and the induction of preneoplastic hepatic foci was also observed in rat carcinogenesis initiated by DEN and promoted by fumonisin B1 [16]. On the other hand, reconstitution of NK-depleted rats with purified NK cells restores both tumor cell clearance and anti-metastatic efficiency. Wistar Furth rats pretreated with rabbit anti-asialo GM1 serum exhibited a diminished ability to destroy circulating MADB106 mammary adenocarcinoma cells, which in turn caused an increased incidence of experimental pulmonary metastasis. When large granular lymphocytes(LGL), highly enriched in NK activity, were transferred into NK-depressed rats, the ability of these rats to inhibit the development of pulmonary metastases was partly or fully restored [31]. There is wide variation in the tumor incidence between F344/N and SD rats. Sprague Dawley rats have greater forestomach cancer incidence than F344/N rats under the effect of butylated hydroxyanisole [32]. Sprague Dawley rats also showed greater susceptibility to urinary bladder tumors than F344/N rat under the effect of N-butyl-N-(4-hydroxybutyl) nitrosamine [33]. But F344/N rats exhibited greater susceptibility to hepatocellular tumors than did SD rats when treated with DEN [34]. F344/N rats exhibited relatively high susceptibility to promotion by the liver carcinogens 2-acetylaminofluorene (2-AAF) and phenobarbital [35], with a hundred fold increase in lesion area being observed after 2-AAF treatment compared with Lewis and SD cases. Our data showed that there is no difference in hepatic NK lytic activity between F344/N and SD or male and female rats per NK cell. But SD rats had significantly greater total hepatic NK cells than F344/N rats after covariance with their body weight. This suggested that SD rats had significantly greater total hepatic NK activity than F344/N rats. We propose that the difference of total hepatic NK activity between SD and F344/N rats may play an important role in explaining the strain difference in hepatocarcinogenesis between these strains.

In summary, we examined hepatic NK populations for function and receptor density in both F344/N and SD rats. The decreased total NKR-P1^{bright} and NKR-P1^{dim} populations may partially explain the greater hepatocarcinogenesis in F344/N female than male rats. The significantly greater total NK activity in SD rats may contribute to the decreased susceptibility to hepatocarcinogenesis of SD rats compared with F344/N rats.

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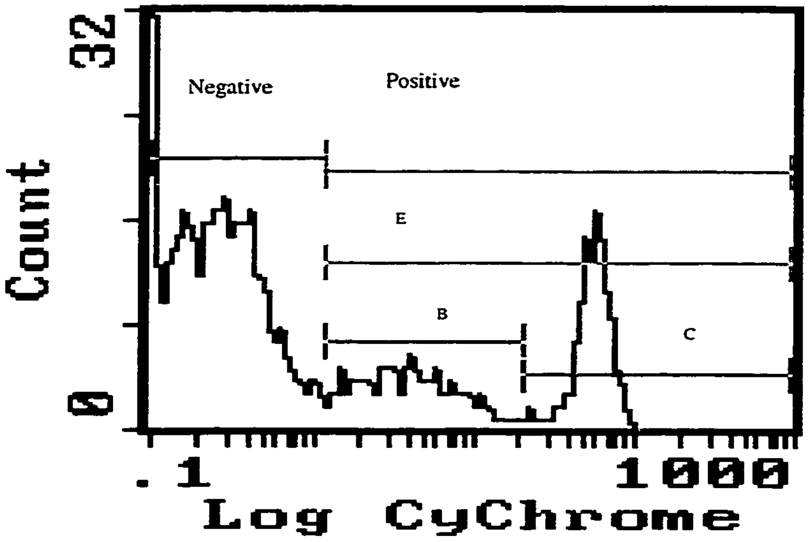


Figure 1: Representative graph of flow cytometry of liver-associated mononuclear cells that express NKR-P1 antigen. Gate B represents the NKR-P1$^{\text{dim}}$ population, Gate C represents NKR-P1$^{\text{bright}}$ population. Gate E represents the total population that express NKR-P1 antigen.

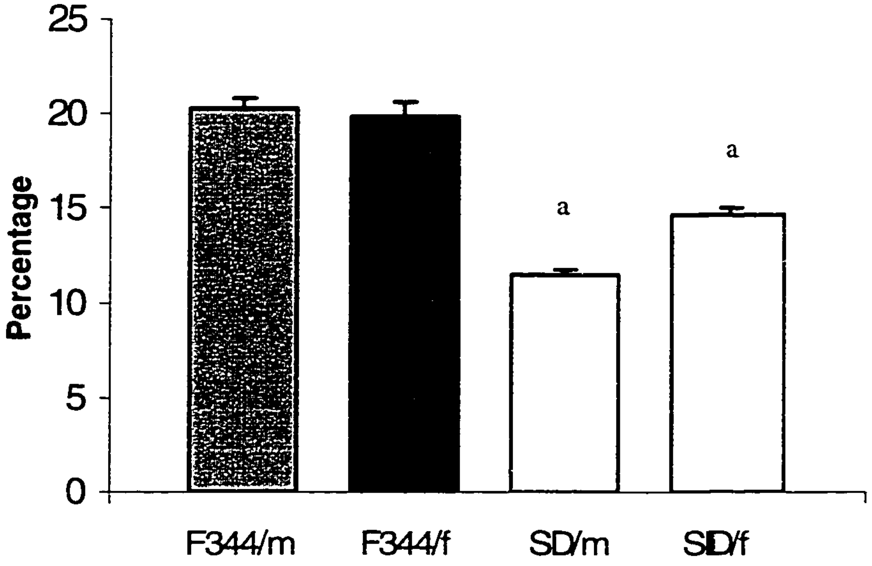


Figure 2: Comparison of the percentage of hepatic NKR-P1^{bright} cells in F344/N and SD rats . /m = male, /f = female. (N=12/group) .

[a] Significantly different compared with SD/m and SD/f groups, ($P<0.05$)

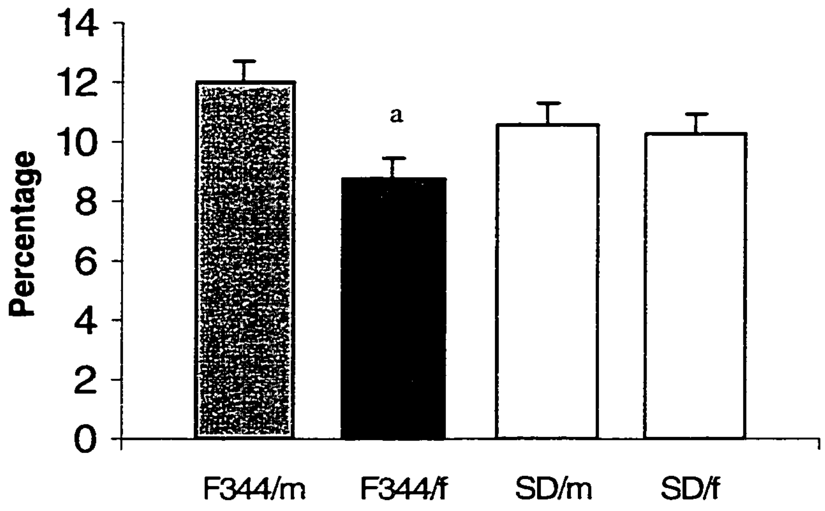


Figure 3 : Comparison of the percentage of hepatic NKR-P1^{dim} cells in F344/N and SD rats (N=12/group). /m=male , /f=female

[a] Significantly different compared with F344/m group, (P<0.05)

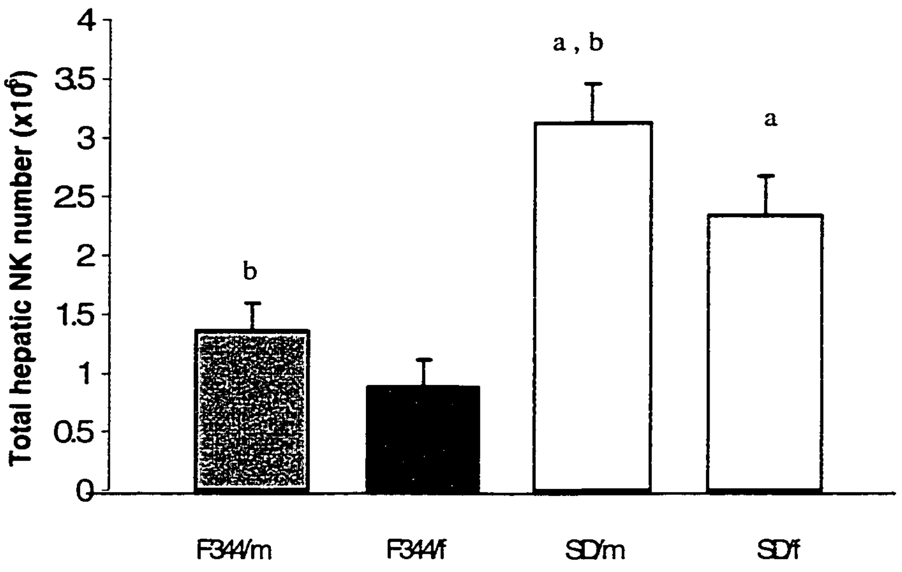


Figure 4: Comparison of the total hepatic NKR-P1^{bright} population in F344/N and SD rats (N=12/group). /m=male, /f=female.

[a] Significantly different compared with F344/f and F344/m groups , ($p<0.01$)

[b] Significantly different compared with females of same strain.

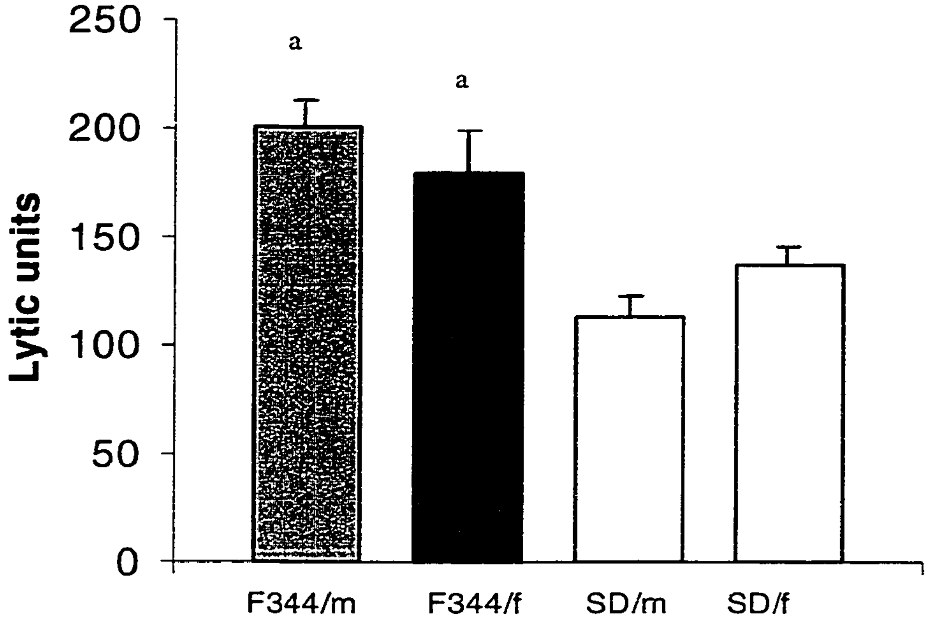
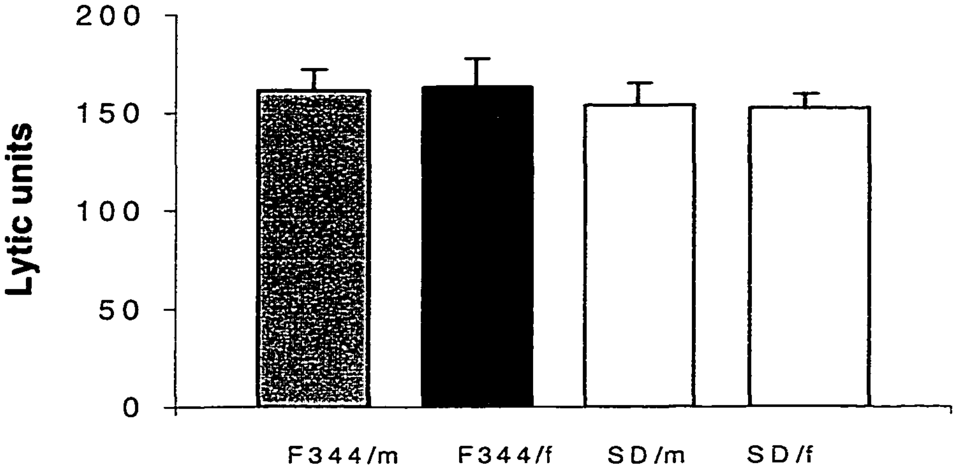


Figure 5A: Hepatic NK activity in F344/N and SD rats at 9 wks of age prior to covariance with the percentage of NKR-P1^{bright} cells (N=8 per/group) . Activity is expressed as lytic units, calculated from the specific lysis curve(/m = male, /f = female).

[a] Significantly different compared with SD/m and SD/f groups , ($P<0.05$)



5B: Hepatic NK activity of F344/N and SD rats at 9 wks of age after covariance with the percentage of NKR-P1^{bright} population (N=8/group). Activity is expressed as lytic units.

OPPOSING EFFECTS OF PROSTAGLANDINS E2 AND F2α ON RAT LIVER-ASSOCIATED NATURAL KILLER CELL ACTIVITY IN VITRO

A paper published in *Prostaglandins, Leukotrienes and Essential Fatty Acids*

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ABSTRACT:

Strain differences in cancer incidence are proposed to be due partly to difference in immune function. As potential cancer associated immunological regulators, the concentration of hepatic prostaglandins E_2(PGE_2) and $F_{2\alpha}$($PGF_{2\alpha}$) were compared in 9 week old male and female F344/N and Sprague Dawley(SD) rats . There were no strain or gender differences in the concentration of hepatic PGE_2. No strain difference was found in the concentration of hepatic $PGF_{2\alpha}$, but the hepatic $PGF_{2\alpha}$ concentration in female rats was two-fold of that in male rat(130 vs 60ng/g). Prostaglandin E_2 significantly inhibited hepatic natural killer cell(NK) activity in vitro compared with untreated cells from both genders and strains($p<0.05$), and 25ng PGE_2/ml inhibited NK activity significantly more than did 10ng PGE_2/ml ($P<0.05$). In contrast, 50ng $PGF_{2\alpha}$/ml and 100ng $PGF_{2\alpha}$/ml significantly stimulated

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hepatic NK activity compared with untreated hepatic cells from both F344/N and SD rats. This study suggests that prostaglandins may have negligible net effect on NK activity associated with rat liver, and may be unlikely to mediate cancer-related immune function.

INTRODUCTION

The use of an animal carcinogenicity bioassay in assessing the oncogenic risk involved with chemical exposure is an important and necessary process, but presents many difficulties in interpretation when extrapolating to humans[1]. There are large inter-species, inter-strain and gender variations in the incidence of some tumors. Variable tumor formation may be related to factors, including: organ/strain specific oncoviruses, hereditary disorders[2], or differences in some possible regulatory factors, such as prostaglandins, as well as their effects on immune capabilities, such as hepatic natural killer cell(NK) activity. F344/N and SD rats were included in this study because these are two rodent strains predominantly used in carcinogenicity bioassays, and there is a large database on cancer development in these strains. In one study, newborn F344/N and SD rats were irradiated with whole body single doses of 3Gy gamma rays with or without intraperitoneally-injected diethylnitrosamine (DEN) (15 mg/kg body weight) within 1 h of irradiation. Tumor development was promoted with 0.05% phenobarbital. In groups treated with radiation alone or radiation combined with DEN, F344/N rats had threefold greater development of placental S-glutathione transferase (GST-P^+) hepatic foci than did SD rats. In SD rats, female rats had significantly greater induction of GST-P^+ hepatic foci than did males[3]. In another study in which hexachlorobenzene was fed to male and female F344/N rats for 15 weeks, 100% of surviving females had multiple liver tumors which were strongly gamma-glutamyl transpeptidase (GGT) positive and histologically classified as neoplastic nodules or hepatocellular carcinomas. In contrast, only 16% of males developed tumors which were smaller and fewer in number than those in females[4]. Females had greater hepatic stearate, arachidonate and $PGF_{2\alpha}$ than did males when F344/N rats were fed diets with 9% of energy (en%) from linoleate and 15.5, 20, 30 or 40 en% fat[5]. It is hypothesized that greater tumor promotion in females may be related to their greater levels of $PGF_{2\alpha}$, a cell proliferative factor[6].

The suggestion that prostaglandins may play a role in immune response/tumor cell interactions is based upon several observation. First, a variety of prostaglandins are produced both by cells that are themselves active in the expression and regulation of immune response activity[7-8] as well as by a number of tumor targets[9-10]. Carcinogenesis may be associated with increased prostaglandin production by neoplastic organs, such as during promotion of rat hepatocarcinogenesis by fumonisin B[11]. Second, the production of prostaglandins has been found to increase as a result of direct contact between effector lymphocytes and tumor targets[12]. Third, prostaglandins at levels produced during these interactions have been shown to influence the ultimate expression in vitro of lymphocyte and macrophage cytotoxicity against tumor targets[13-14].

Prostaglandins mediate inter- and intracellular communication, as may stimulate hepatocyte proliferation[15-16]. The concentration of PGE equivalents in rat liver *in vivo* was increased during liver regeneration. This stimulation of prostaglandin synthesis was confirmed in vitro by the ability of homogenates of regenerating liver tissue to synthesize PGE_2 and $PGF_{2\alpha}$ from arachidonate. Indomethacin prevented these prostaglandin changes, and the subsequent increase in DNA synthesis[17]. During the regeneration of mammalian liver after a 70% partial hepatectomy (PHx), Kupfer cells produced significantly elevated PGE_2, and *in vivo* Kupfer cell PGE_2 blockade by indomethacin (5 mg/kg) significantly ($P < 0.05$) inhibited hepatic regeneration[18]. The association of neoplastic tumors with increased levels of prostaglandins[19-20] provided the rationale for investigating their role in turmorigenesis. Animal and human tumors contain high levels of prostaglandins, particularly those of the E series that have been shown to significantly affect cell proliferation and tumor growth and suppress immune responsiveness. DNA synthesis of hepatocytes in primary culture was significantly enhanced by addition of PGE_2 (2-200 nmol/L). Intracellular cAMP level in the hepatocytes increased during culture, and cAMP increase was enhanced by PGE_2. Prostaglandin E_2 production in the liver increases hepatic regeneration and PGE_2 enhances the proliferation of hepatocytes by a seemingly cAMP-dependent specific receptor-mediated process[21]. At concentrations of 10^{-12}-10^{-9} mol/L, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ very intensely stimulated both the DNA-synthetic and mitotic activities of hepatocytes in 4-day-old primary cultures of neonatal rat liver. DNA replication was more intensely enhanced by $PGF_{2\alpha}$ than $PGF_{1\alpha}$, whereas mitotic activity was nearly equally affected by the two prostaglandins[6]. Thus enhanced PGE_2 and $PGF_{2\alpha}$ may promote hepatocarcinogenesis by stimulating DNA synthesis and proliferation of hepatocytes.

A high level of PGE_2 in the portal vein suppresses liver-associated immunity and promotes liver metastases[22]. Some *in vitro* experiments showed a similar phenomena. The ability of Syrian hamster tumor cells of the same origin but with different degrees of malignancy to secrete prostaglandin E was studied following their in vitro contact with

Syrian hamster natural killer cells (NK cells). Syrian hamster NK cells were shown to lose cytotoxic activity significantly after their contact with malignant tumor cells. Short-term in vitro contact of malignant tumor cells with human and Syrian hamster NK cells resulted in a rapid PGE secretion into the culturing medium. Therefore, PGE_2 may promote tumor progression by inhibiting immune function. The effect of $PGF_{2\alpha}$ on NK cells is still not clear. The regulatory effects of prostaglandins on immune response appear to be mediated by the production of cyclic AMP[23]. PGE_2 activates adenylate cyclase with a subsequent rise in cyclic AMP[24], which acts as a "second messenger". Cyclic AMP itself is an inhibitor of lymphocyte activation[24-25]. The presence of receptors for PGE_1 and PGE_2 on the lymphocyte surface had been demonstrated, while there were no binding sites for PGA, $PGF_{1\alpha}$ or $PGF_{2\alpha}$. Henney and Lichtenstein, using splenic lymphocytes from mice immunized with an allogeneic mast cell tumor[26], suggested that elevated cyclic AMP content of cytolytic lymphocyte might inhibited their ability to kill target cells. As a test of this hypothesis, prostaglandins were shown to inhibit lymphocyte cytolytic activity[27]. The relative potency of seven prostaglandins in inhibiting cytolytic activity correlated very well with their potency in stimulating cyclic AMP accumulation in lymphocytes: $E_1=E_2>A_1=A_2>F_{1\alpha}=F_{2\alpha}\approx 0$[28]. But effects of PGs on NK cells could be mediated in other ways.

We suggest that prostaglandin regulation of immune response might partly explain some previous findings of gender and strain difference in susceptibility to carcinogenesis. Effects of $PGF_{2\alpha}$ on NK activity have not been well characterized. To that end, we hypothesize that high levels of $PGF_{2\alpha}$, as found in female rat liver, may inhibit liver associated NK activity, an effect similar to that of PGE_2.

MATERIALS AND METHODS

Animals

Experimental procedures were approved by the Iowa State University Animal Care Committee. Six week old male and female F344/N and Sprague Dawley rats were given free access to diets (AIN-93G) and water for 3 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 were recorded weekly.

Preparation of liver-associated mononuclear cells

Rat livers were perfused with 40ml of Hank's Balanced Salt Solution (HBSS, supplemented with 25mM Hepes and 0.1% EDTA). Approximately 12 ml of perfusate was concentrated to 3 ml and laid on 3 ml Accupaque (Accurate Chemical Co., Westbury, NY), then centrifuged at 1500rpm for 10 minutes. The mononuclear cells at the interface were collected, and washed two times, once with HBSS(with 25mM Hepes) and once with complete medium (RPMI-1640, supplemented with 50μg/ml gentamicin, 25 mM Hepes, 2mM L-glutamine, and 10% fetal bovine serum (FBS)). Cells were enumerated on a Celltrack II (Nova Biomedical, Waltham, MA).

Natural killer cell assays

Natural killer cell assays were performed as previously described using liver-associated mononuclear cells from both rat strains and genders[29]. Cells were plated in triplicate at the following effector to target ratios in 96 well plates: 25:1, 12.5:1, 6.25:1, 3 : 1. The target cells for the assay were YAC-1 cells(ATCC Co, Rockville, MD). Each well contained 6×10^6 YAC-1 cells which had been labeled with 200μCi ^{51}Cr. The amount of ^{51}Cr released by dying cells was counted using a Gamma Trac 1191(TM Analytic, Inc., Elk Grove village, IL). Lytic units were calculated using a computer program based on the equation of Pross and Maroun[30].

Effects of prostaglandins on natural killer cell activity

Prostaglandin E_2 and prostaglandin F_{2a} were obtained from Sigma Chemical Company(St.Louis, MO) and stored in sealed desiccated vials at -20°C until use. For each assay PGE_2 and $PGF_{2\alpha}$ was diluted to 2.5 mg/ml in absolute ethanol and subsequently diluted in RPMI-1640 complete medium for use in culture. PGE_2 or PGF_{2a} were added at the start of the NK assay in 50 μl aliquots to wells containing each effector to target ratio of cells, and cocultured with NK and YAC-1 cells for 4.5 hours. The final concentration are 10, 25ng/ml for PGE_2 and 50, 100 for $PGF_{2\alpha}$. Cells incubated with PGs were processed in parallel with baseline NK activity.

Prostaglandin assays

Prostaglandin E_2 and $PGF_{2\alpha}$ were determined in a radioimmunoassay as described by McCosh et al[31]. Anti-PGE_2 antiserum (0.2ml /per assay tube) and anti-$PGF_{2\alpha}$ antiserum (0.2ml / per assay tube) were obtained from Sigma Co.(St Louis, MO). H^3-PGE_2 (10^{-6}μCi/ per aliquot) and H^3- $PGF_{2\alpha}$ (10^{-6}μCi/ per aliquot) was obtained from NEN Products (Boston, MA). PGE_2 and $PGF_{2\alpha}$ were quantified by using a computer program based on a logit transformation of the standard curve[32].

Statistical analysis

A two-way ANOVA was used to assess strain and gender relationships for hepatic PGE_2 or PGF_{2a}. Repeated-measures ANOVA was used to analyze the effects of PGE_2 or PGF_{2a} on the in vitro hepatic NK activity from F344/N and SD rats. For all statistical tests of significance, α was set at $P \leq 0.05$.

RESULTS

The concentration of hepatic PGE_2 and $PGF_{2\alpha}$ in F344/N and SD rats

The average concentration of hepatic PGE_2 for F344/N rats was 34 ± 10 ng/g, and 42 ± 11ng/g for SD rats, which was not significantly different between the strains. Also, the average concentration of hepatic PGE_2 was 43 ± 11ng/g for males and 33 ± 12ng/g for females, with no significant difference between male and female rats (Figure 1). There was no gender by stain interaction. F344/N and SD rats exhibited similar hepatic $PGF_{2\alpha}$ concentration, which were 106 ± 9 ng/g and 81 ± 8ng/g respectively. But the concentration of $PGF_{2\alpha}$ in females was twofold greater than in males in both strain ($P<0.01$) (Figure 2). There was no strain by gender interaction.

In vitro effects of PGE2 and PGF2a on hepatic NK activity of F344/N and SD rats

Prostaglandin E_2 significantly decreased hepatic NK activity in a dose-dependent manner in cells from both F344/N and SD rats ($p<0.05$) (Figure 3). Prostaglandin E_2 (10ng/ml) significantly inhibited hepatic NK activity compared with hepatic NK activity from both rat strains($p<0.05$). Hepatic NK activity at 25ng/ml PGE_2 was significantly inhibited compared with liver-associated mononuclear cells from both rat strains treated with 10ng/ml PGE_2($p<0.05$). There was no significant strain by concentration or gender by concentration interaction. $PGF_{2\alpha}$ has been reported to have no effect on NK lytic activity of rabbit peripheral blood at a concentration of 50 ng/ml[33]. Our results showed that $PGF_{2\alpha}$ significantly increased the hepatic NK activity of F344/N and SD rats of both genders at both 50 and 100 ng/ml in comparison with the control hepatic NK activity ($p<0.05$)(figure 4), but the hepatic NK activity was not significantly different between 50 and 100ng/ml $PGF_{2\alpha}$ in both F344/N and SD rats of either genders. No strain by gender, strain by concentration or gender by concentration interaction was observed.

DISCUSION

There was no strain or gender difference in hepatic PGE_2. Thus, difference in PGE_2 is very unlikey to be responsible for strain or gender difference in hepatic cancer promotion. Significantly increased $PGF_{2\alpha}$ was observed in females compared with males in both F344/N and SD rats. This has been reported previously[5]. The growth of hepatic preneoplastic lesions has been shown to be greater in females than in males. Under the effect of some hepatic carcinogens, females are more susceptible to liver tumors than males in both SD and F344/N strains[5, 34]. Sprague-Dawley rats were initiated by DEN, and additional weekly application of ploychlorinated biphenyl (50 or 100 mg/kg body wt./week, for 7 weeks) enhanced the number of ATPase-deficient islands 3-fold in males and 9-fold in females. The total area was increased 4-fold in males and 15-fold in females. Number and area of GGT-positive islands were similarly enhanced[35]. The volume of GGT-positive AHF in livers of F344/N males initiated with DEN and promoted with phenobarbital(PB) was significantly less than in females[36]. It may be that the greater promotability of preneoplasia in females is related to the greater prostaglandin F_{2a} production. Several other studies have demonstrated that tumor promoters, such as phenobarbital stimulated hepatic PGF_{2a} production[37] and greater PGE_2 and PGF_{2a} levels and prostaglandin synthetase activity have been reported in 7, 12-dimethylbenzanthracene or methylnitrosourea induced rat mammary tumors[38]. $PGF_{2\alpha}$ had been demonstrated to stimulate very intensely both the DNA-synthetic and mitotic activities of hepatocytes in 4-day-old primary cultures of neonatal rat liver[6]. It might be expected that greater $PGF_{2\alpha}$ concentration in females may contribute to the greater incidence of tumor formation or greater growth rate of tumors in females compared with males because of the cell proliferative effects of PGF_{2a}.

Several studies have revealed that natural cellular defenses in mice bearing spontaneous or transplanted tumors is severely compromised by the immunosuppressive effects of prostaglandins of the E series secreted by host macrophages appearing in lymphoid organs as well as at the site of a tumor[39]. Certain tumor cells can also produce high levels of prostaglandins *in vivo*. When a choline deficient (CD) diet, an efficient liver tumor promoting regimen, was fed to SD rats, PGE_2 levels were increased 2-2.5 fold, and the GGT positive hepatocyte foci in the liver of rats initiated with a single dose of diethylnitrosamine after 8 weeks of the dietary promotion significantly increased[40]. Fumonisin B1, a tumor promoter, has been shown to increase hepatic $PGF_{2\alpha}$ concentration compared with control groups[11]. High levels of NK, and lymphokine-activated cell activity can not be generated in the presence of PGE_2[41]. To elucidate the correlation between hepatic NK cell activity and prostaglandin, cells were cultured with PGE_2 or $PGF_{2\alpha}$. With increased PGE_2 concentration, hepatic NK activity in both F344/N and SD and male and female rats was inhibited in a dose-dependent relationship. But we also observed that increased exogenous $PGF_{2\alpha}$ can increase the hepatic NK activity in both F344/N and SD and male and female rats. This result seems different from our previous *in vivo* study which showed the coexistence of increased $PGF_{2\alpha}$ and decreased NK activity[11].We assume that one of the reasons for the difference between *in vivo* and *in vitro* conditions might be that the rats were exposed to both PGE_2 and $PGF_{2\alpha}$ simultaneously during *in vivo* study, and the two PGs counteracted effect of each other. The experimental results also suggested that the decreased NK activity previously observed by Lu et al[11]. may not be directly mediated by prostaglandins. Further study of *in vivo* function of $PGF_{2\alpha}$ and the combined effect of PGE_2 and $PGF_{2\alpha}$ on NK activity *in vitro* might be helpful to explain this phenomena. Further investigation of the role of prostaglandin and NK cell activity in development of hepatocellular carcinogenesis, as well as the direct determination of prostaglandin production at a tumor site, are required .

In summary, we examined the hepatic concentration of PGE_2 and $PGF_{2\alpha}$ as well as the effect of these PGs *in vitro* on hepatic NK cell in both F344/N and SD rats. Our results suggested that PGE_2 is not responsible for strain and gender differences in hepatic tumor promotion. The greater $PGF_{2\alpha}$ may partially explain greater hepatocarcinogenesis in females than male rats under some condition.

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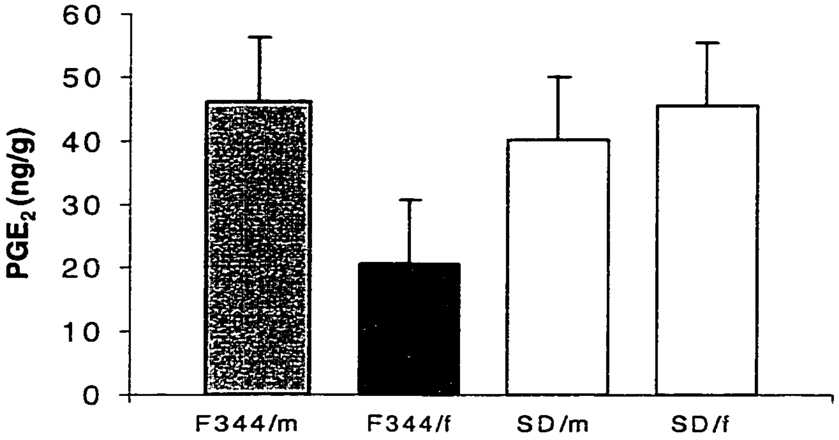

Figure 1: The comparison of PGE_2 concentration in liver tissue in F344/N and SD rats (N=8/group). The concentration was expressed as ng PGE_2 per gram liver tissue, /m=male, /f=female.

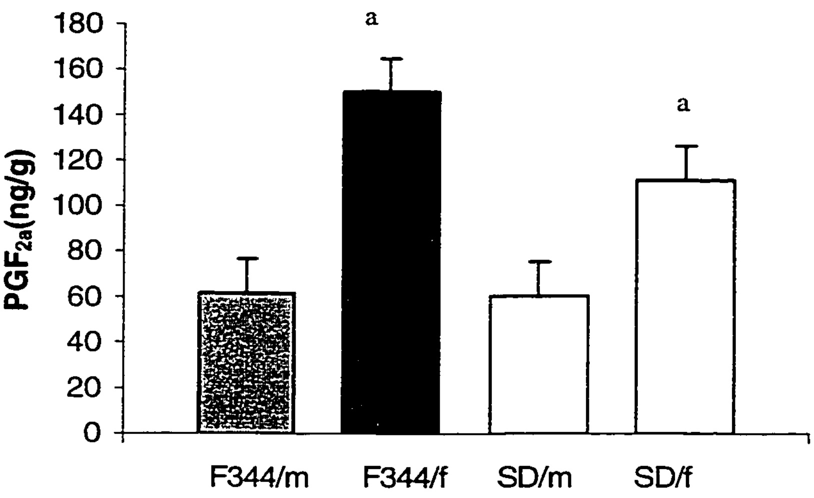

Figure 2: The hepatic PGF_{2a} concentration in F344/N and SD rats (N= 8/ group). The concentration of PGF_{2a} is expressed as ng per gram liver tissue, /m=male, /f=female. ^{a}significantly different compared with F344/m and SD/m groups , (P<0.01)

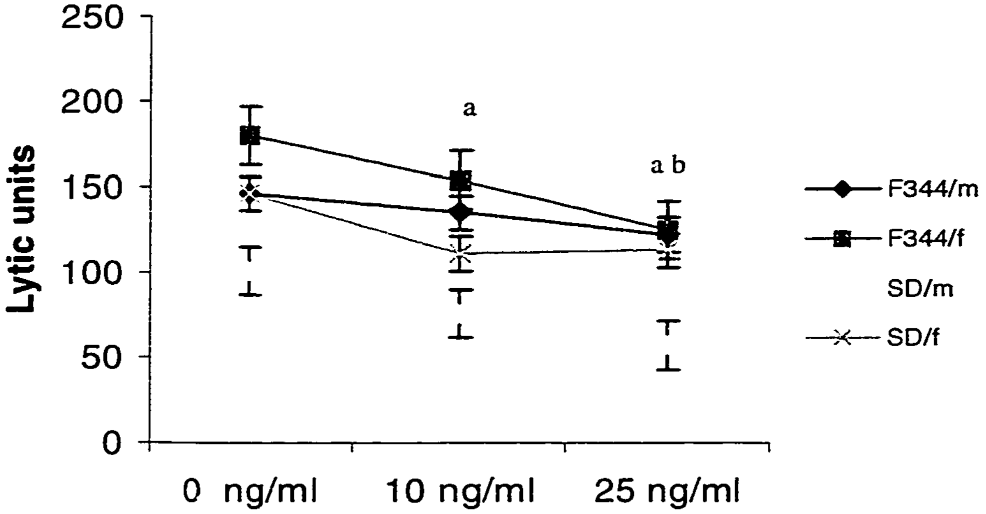

Figure 3: The effect of PGE_2 on hepatic NK acitivity of F344/N and SD rats was expressed as lytic units at different concentrations of PGE_2 (N=3/group). 0, 10 and 25ng/ml three concentration were set as the final concentration in respective wells. /m=male, /f=female. 10ng/ml PGE_2 significantly inhibited the hepatic NK activity compared with untreated hepatic cells from both rat strains($p<0.05$), and the hepatic NK activity at 25ng/ml PGE_2 was significantly inhibited compared with hepatic cells from both rat strain treated with 10ng/ml PGE_2($p<0.05$)

a: The NK activity in both strains and genders was significantly different from the controls

b: The NK activity at 25 ng/ml PGE_2 was significantly different from the control and 10ng/ml PGE_2 groups.

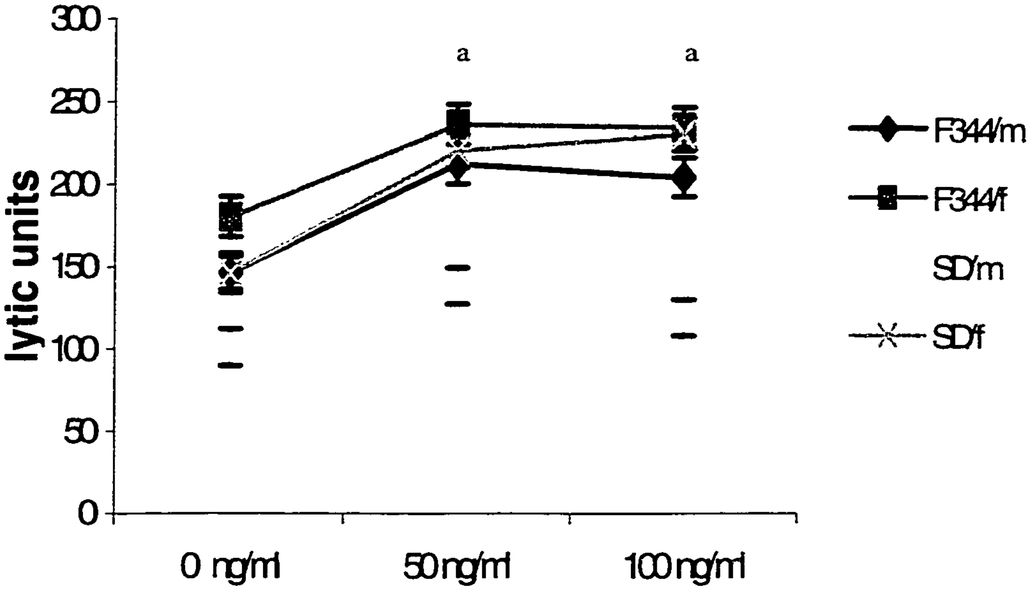

Figure 4: The effect of PGF_{2a} on NK activity of F344/N and SD rats was expressed as lytic units at different concentration (N=3/group). 0, 50 and 100ng/ml were set as the final concentration in respective wells. /m=male, /f=female. $PGF_{2\alpha}$ significantly boosted the hepatic NK activity of F344/N and SD rats at both 50 and 100 ng/ml in comparison with the control hepatic NK activity ($p<0.05$).

a: The NK activity of both strain and gender was significantly different from control groups.

REACTION OF FUMONISIN WITH GLUCOSE PREVENTS PROMOTION OF HEPATOCARCINOGENESIS IN FEMALE F344/N WHILE MAINTAINING NORMAL HEPATIC SPHINGANINE: SPHINGOSINE

A paper accepted by the *Journal of Agricultural and Food Chemistry*

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ABSTRACT

The reaction of the primary amine of fumonisin B_1 (FB_1) with glucose was hypothesized to detoxify this mycotoxin. Eighty 10-day old female F344/N rats were injected intraperitoneally with diethylnitrosamine (DEN, 15mg/kg body weight). At 4 weeks of age, the weaned rats were randomly assigned to one of the 4 treatment groups with 20 rats each. At 9 weeks of age, 4 rats from each treatment group were killed. At 12 weeks, another 5 rats from each group were killed. At 20 weeks of age, remaining rats were killed. In comparison with the rats fed basal diet or FB_1-glucose (containing 25ppm FB_1), rats fed 8 ppm (residual amount of free FB_1 in the FB_1-glucose mixture) or 25 ppm

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FB_1 had greater alanine aminotransferase activity at 9 and 20 weeks of age($P<0.001$), greater endogenous hepatic prostaglandin E_2 production at 20 weeks of age($P<0.05$), and significantly lower plasma cholesterol at 20 weeks of age($p<0.01$). Placental glutathione S-transferase-positive (PGST) and γ-glutamyl transferase (GGT)-positive altered hepatic foci occurred only in rats fed 25 ppm FB_1 at 20 weeks of age. Hepatic natural killer (NK) cell activity was similar among the four groups, but the percentage of total liver-associated mononuclear cells exhibiting the NKR-P1^{bright} marker was significantly greater in rats fed FB_1-glucose , FB_1(8ppm) and FB_1 (25ppm) than in control rats at 9 weeks of age, and FB_1-glucose treated rats had significantly lower NKR-P1^{bright} cells as a percentage of total liver-associated mononuclear cells than in rats fed 25ppm FB_1 at 20 weeks of age($P<0.05$). PGST- or GGT-positive AHF were not detected in any treatment group at 9 or 12 weeks of age. At 20 weeks of age, half of the rats fed 25ppm FB_1 had PGST and GGT- positive AHF. The sphinganine (Sa) concentration as well as the Sa/sphingosine (So) ratio were significantly greater in the rats fed 25 ppm FB_1 diet as compared with the control groups respectively at 12 or 20 week age. Therefore, modifying FB_1 with glucose seems to prevent FB_1-induced hepatotoxicity and promotion of hepatocarcinogenesis. Sa/So ratio was not the most sensitive biomarker of FB_1 toxicity.

INTRODUCTION

The carcinogenic and toxic effects of fumonisin B_1 (FB_1) , a mycotoxin produced by the commonly occurring corn fungi, *Fusarium moniliforme* and *Fusarium proliferatum*, have been studied intensively. Fumonisin B_1 (69.3μM/kg, 50ppm) was hepatocarcinogenic in rats fed the toxicant for approximately two years (*1*). The incidence of *F. moniliforme* in corn for human consumption has been correlated with the incidence of esophageal cancer in Transkei, Southern Africa (*2*) and in China (*3*).The concentration of FB_1 in corn reached approximately 11.1 µmol/kg in areas of southern Africa where human esophageal cancer rate was high(*4*). Corn products for human and animal consumption were determined to have FB_1 concentration between 0.3-4.2 µmol/kg in the U.S. (*5*, *6*, *7*).

Several biomarkers have been used to study FB_1 hepatocarcinogenicity. Fumonisin B_1-promoted rat hepatocarcinogenesis was readily quantified by measuring placental glutathione S-transferase (PGST) positive altered hepatic foci (AHF) (*8*) and γ-glutamyltransferase (GGT)-positive AHF (*9*). Plasma alanine aminotransferase (ALT) activity was increased during fumonisin hepatotoxicity (*10*), and hepatocarcinogenesis in rats (*11*). Increased plasma total cholesterol was observed in FB_1-treated vervet monkeys (*12*), and in rats (*11*) in short term studies. Greater hepatic prostaglandin $F_{2\alpha}$ production was also observed in FB_1 tumor promotion in rat liver (*13*). *In vivo* administration of 50ppm FB_1 significantly suppressed hepatic natural killer (NK) cell activity while stimulating hepatic preneoplasia (*13*). Natural killer cell activity suppression by FB_1 during tumor promotion may be mechanistically significant, but this remains to be determined. Thus numerous possible biomarker of FB_1 toxicity and tumor promotion may be used to probe mechanism of action of mycotoxin.

Recent studies regarding the biological effects of fumonisins indicated that they selectively inhibit ceramide synthase, a key enzyme in the sphingolipid biosynthetic pathway (*14*). It was suggested that the subsequent accumulation of the sphingoid bases, sphinganine (Sa) and sphingosine (So), could have an important role in the toxicological effects of fumonisin in the kidney and the liver of rats (*15, 16*). In addition, as the sphingoid bases are important regulators of cellular growth and differentiation (*17*), the continued disruption of sphingolipid biosynthesis has been implicated in the hepatocarcinogenicity of fumonisin (*18*).

Currently, there has been increased attention directed at reducing the human and animal exposure to these fungal toxins. Biological, chemical, and physical processes have been explored to salvage fumonisin-contaminated corn. Thermostability of FB_1 proved to be great. When dry corn was heated at 50, 75, 100 and 125°C for 40 minutes, only a small amount of FB_1 was lost (*19*). 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 its toxicity in rats (*15*). Nixtamalization, the traditional process to produce masa or tortilla flour, reduced the amount of FB_1 by hydrolyzing FB_1 to hydrolyzed FB_1 (HFB_1), but HFB_1 was similar in toxicity to FB_1 when the nutritional status of rats was adequate (*11*). In vitro toxicity studies of several FB_1 analogs showed that the analogs containing FB_1 amine groups and the tricarballylic side chains were more toxic than analogs containing only the tricarballylic side chains (*20*), and naturally occurring N-acetyl –FB_1 was not toxic (*21*). Therefore, the primary amine of FB_1 is likely to be critical for its toxicity. Murphy et al. (*22*) reported a method to detoxify FB_1 by derivatizing the amine group with a reducing sugar, fructose, in a nonenzymatic browning reaction. Diethylnitrosamine-initiated (15 mg/kg body weight) male F344/N rats were fed for 4 weeks either 69.3 μM FB_1 or 69.3 μM FB_1 reacted with fructose (FB_1–fructose). Rats fed FB_1 had significantly increased levels of several markers of hepatocarcinogenicity, while rats receiving FB_1 – fructose showed no signs of hepatocarcinogenicity or hepatotoxicity(*13*). A more practical and efficient method to block FB_1's amine group by reacting the amine group with glucose had been developed in Dr. Murphy's lab (*23*) .The FB_1-glucose reaction was more complete than the reaction with fructose, and the reaction products were more easily isolated than FB_1-fructose products. It was hypothesized that modifying FB_1 with glucose would prevent promotion of hepatocarcinogenicity by FB_1. Our experiment was designed to test the effectiveness of this detoxification method by examining effects of FB_1 –glucose on several markers of FB_1 promotion of hepatocarcinogenesis.

MATERIALS AND METHODS

Preparation of Fumonisin B_1-glucose adduct

FB_1-glucose was prepared by heating 1.39 mM FB_1 (total 8.1 mmol) with 0.1M D-glucose in 50 mM potassium phosphate buffer, pH 7.0 at 80°C . After 48 hours, the pH of the reaction mixture was adjusted to pH 2.7 to stop the reaction. Reversed-phase C_{18} SPE cartridges (Supelco, Bellefonte, PA) were preconditioned with 50 ml 100% methanol at apparent pH 2.7 followed by 100 ml deionized water at apparent pH 2.7. An aliquot of 50 ml of the 1.39 mM FB_1/0.1 M D-glucose reaction mixture was loaded on the cartridge. The cartridge was washed with 100 ml deionized water and 100 ml 30% methanol at an apparent pH of 2.7. The D-glucose was washed out at this step. The FB_1-glucose was eluted with 50 ml of 40% methanol and 100 ml 100% methanol at apparent pH 2.7. The eluant was evaporated to dryness with Brinkmann rotavapor R110 (Westbury, NY) at 35°C. The residue was redissolved in Milli Q water, and brought to 10.0 ml in volumetric flask. The unreacted free FB_1 was quantified with HPLC OPA derivative method (*5*). The solution was freeze-dried and the total mass was determined on an analytical balance. The amount of FB_1-glucose was determined by subtracting the free FB_1 mass from the total mass.

Diets

Four experimental diets were fed to rats. The control group was fed basal diet AIN-93G (American Institute of Nutrition, 1993). The FB_1 group was fed a diet containing highly purified FB_1 which was prepared by incorporating 25ppm FB_1 into basal diets. The FB_1 – glucose group was fed a diet in which purified FB_1 reacted with glucose was incorporated into the basal diet at a level equivalent to 25 ppm FB_1 diet. Analysis of the FB_1 –glucose products showed that approximately 8ppm unreacted FB_1 remained in the FB_1-glucose containing diet. Thus another control group was fed 8ppm FB_1.

Animals

The experimental procedures were approved by the Iowa State University Animal Care Committee. Eighty 10-day old female F344/N rats obtained from Harlan Sprague-Dawley (Madison, WI) were injected intraperitoneally with diethylnitrosamine (DEN, 15mg/kg body weight) in 0.1 ml corn oil. At 4 weeks of age, the weaned rats were randomly assigned to one of the 4 treatment groups with 20 rats each. At 9 weeks of age, 4 rats from each treatment group were killed. At 12 weeks, another 5 rats from each group were killed. At 20 weeks of age, remaining rats were killed. The carcasses of the rats were store at -20°C freezer. Before scanned by dual-energy X-ray absorptiometry (QDR 2000, Hologic Inc. Waltham, MA), the carcasses were thawed at 0°C for about 2 hours. the composition of soft tissue lean, and fat in the body were expressed as the percentage of the body weight (*24*). Rats were given free access to the experimental diets and with a 12-h light/dark cycle maintained at 22-25°C and 50% humidity. Body weight and feed intake were recorded weekly.

Plasma and liver samples preparations

Before the liver was perfused, 1ml sodium chloride solution (contain 100 unit of heparin) was injected into the abdominal vein, and about 3ml blood was drained out into the syringe. Part of the plasma obtained from heparinized blood was analyzed within 24 hours for alanine aminotransferase(ALT) activity. The remaining plasma was stored at -80°C for later plasma total cholesterol analysis.

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

Each of the left, median, and right lateral lobe of the livers was sliced into 1cm slices. Three slices, one from each lobe, were immediately frozen as a block on dry ice and store at -80°C. From each of the frozen liver blocks, 5 10-µm serial sections were cut with a Histostat Microtome (Model 855, Leica Inc., Deerfield, IL) for later staining of GGT and PGST.

For each rat, 0.5 g minced liver portion were immediately homogenized in an ice bath with 10 passes of a Potter-Elvehjem homogenizer in 5ml, PH 7.4, 50 mM potassium phosphate buffer containing 4.2 mM acetyl salicylic acid (Sigma Chemical Co., St. Louis, MO). The liver homogenates were frozen at -80°C for later analysis of endogenous hepatic PGE_2 and $PGF_{2\alpha}$.

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 for the ALT assay (Sigma Chemical Co., St. Louis, MO).

Determination of Sphingosine (So) and Sphinganine (Sa)

Thawed liver tissues were homogenized in 4 volumes 0.05 M potassium phosphate buffer. Homogenate (0.1 ml) was transferred to a cold (13 X 100mm) glass tube with a Teflon lined screw cap. The extraction method was perforemed as described by Riley et al (*25*). Sphingosine and sphinganine were quantified by HPLC as described by Riley et al (*25*) but using C17-phytosphingosine (Sigma Chemical Co., St. Louis, MO) as the internal standard. Sphinganine and So standard (Sigma Chemical Co., St. Louis, MO) mixture at different concentrations (1, 3, 5, 7 9 nmol) were prepared for standard curves.

Prostaglandin assays

PGE_2 and $PGF_{2\alpha}$ were determined in a radioimmunoassay as described by McCosh et al (*26*). Anti-PGE_2 antiserum (0.2ml /per assay tube) and anti-$PGF_{2\alpha}$ antiserum (0.2ml / per assay tube) were obtained from Sigma Co.(St Louis, MO). H^3-PGE_2 (10^{-6}µCi/ per aliquot) and H^3- $PGF_{2\alpha}$ (10^{-6}µCi/ per aliquot) was obtained from NEN Products (Boston, MA). PGE_2 and $PGF_{2\alpha}$ were quantified by using a computer program based on a logit transformation of the standard curve (*27*)

Natural killer cell assays

Natural killer cell assays were performed as previously described (*28*). Cells were plated in triplicate at the following effector to target ratios in 96 well plates: 25:1, 12.5:1, 6.25:1, 3 : 1. The target cells for the assay were YAC-1 cells(ATCC Co, Rockville, MD) (8×10^3/well) which had been labeled with 200µCi ^{51}Cr. The amount of ^{51}Cr released by dying cells was counted using a Gamma Trac 1191(TM Analytic, Inc., Elk Grove Village, IL). Lytic units were calculated using a computer program based on the equation of Pross and Maroun(1984).

Fluorescent staining of lymphoid cells

Leukocyte suspensions were diluted with an equal volume PBS/0.1%azide (cold) and incubated at 4°C (5min). Separate aliquots were stained with 0.2µg/2×10^5(mononuclear cell) of anti-rat NKR-P1A-Biotin(mAb 3.2.3), or an equivalent amount of isotype control (murine IgG1-biotin). A second step of 0.1µg/2×10^5 (mononuclear cell) Strep-AvidinCychrome was used(All from Pharmingen, San Diego,CA). All incubations were performed at 4°C in the dark for 30min, and washed with

PBS/0.1%azide. The contaminating red blood cells were lysed using 10% ammonium chloride buffer (pH=7.4). Cells were fixed with PBS/1% paraformaldehyde prior to analysis using an EPICS-XL-MCI flow cytometer (Coulter, Miami, FL). According to the density of fluorescence, three cell populations were distinguished: NKR-P1^{bright} , NKR-P1^{dim} and negative, and gates were set to help measure the percentage of different populations (*29*)(Fig 1).

Immunohistochemical Staining

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). Anti-PGST antiserum was prepared as described previously (*30*). The second frozen serial section was stained for GGT activity as described by Rutenburg et al. (*31*). The substrate for GGT was glutamyl-4-naphthylamide (GMNA) (United States Biochemical Corp., Cleveland, OH). Altered hepatic foci were quantified via computerized stereology. A Sony 3-chip color video camera DXC-3000A took the images of the liver section stained for GGT and PGST, which were digitally transferred from the camera to an Apple Power Mac G3 computer (Apple computer, Inc., Cupertino, CA), and analyzed with IP Lab image analysis software (version 3.2.3, Scanalytics, Fairfax, VA). Lung, kidney, brain, tibia and additional liver samples were processed by routine histopathological methods for hematoxylin-eosin staining (*32*).

Statistical analysis

Multi-variant regression analysis was used to compare the four growth curves. One-way ANOVA was performed to analyze plasma total cholesterol, alanine aminotransferase activity, the percentage of leukocytes that carry NKR-P1^{bright} and NKR-P1 ^{dim} population markers as well as the amounts and ratio of Sa/So. The hepatic NK activity was compared using one-way ANOVA combined with covariance for the percentage of NKR-P1^{bright} population. Two-way ANOVA was performed to analyze endogenous PGE_2 and $PGF_{2\alpha}$ at different ages. Student's t-test was performed to compare all possible group differences after ANOVA. A p-value of <0.05 was considered to be statistically significant.

RESULTS

Effect on body weight gains, relative liver weight and body mass composition

The body weights and food intake did not differ among treatments. Relative liver weights were not different among the four treatment groups at any time point, and body composition (%fat) was similar among all groups at 20 weeks of age. Body weight and food intake did not differ among treatments (Table 1).The growth curves of four treatment groups were similar over a period of 20 weeks (Data not shown).

Plasma total cholesterol levels and alanine aminotransferase activity

At 9 weeks of age, the total plasma cholesterol concentration was not different among the four treatment groups. At 12 weeks of age, in comparison with the rats fed basal diet and the rats fed FB_1-glucose, the rats fed 25ppm FB_1 diet had significantly greater plasma total cholesterol concentration ($p<0.05$). At 20 weeks of age, in comparison with the rats fed basal or FB_1-glucose diets, the rats fed 8ppm or 25ppm FB_1 diets had significantly decreased plasma total cholesterol ($p<0.05$) (Figure 1). Alanine aminotransferase activity of the rats fed 8ppm or 25ppm diet was significantly greater than the rats fed basal or FB_1-glucose diet at 9 weeks age. At 12 weeks of age, only the rats fed 25ppm FB_1 exhibited significantly greater ALT activity than did other groups. At 20 weeks of age, the rats fed 8ppm or 25ppm FB_1 exhibited greater ALT activity compared with rats fed basal or FB_1-glucose diet at 20 weeks of age (Figure 2).

Hepatic NKR-P1^{brigth} and NKR-P1^{dim} population

Flow cytometric analysis using mAb 3.2.3 (natural killer cell receptor protein 1 (NKR-P1)) revealed two distinct subsets of hepatic mononuclear cells expressing variable levels of NKR-P1. The NKR-P1^{bright} population, which expresses a high level of NKR-P1, has been identified as the population causing NK associated lytic activity (*33*). The NKR-P1^{dim} population, which expresses a low level (2-10 fold lower) of NKR-P1, has been linked to a subset of T lymphocytes that have NK-like cytolytic function under activation by interleukin-2 (*34*). The results showed that all three treatment groups had greater percentage of NKR-P1^{bright} population at 9 weeks of age than did the control group. FB_1-glucose lowered the percentage of NKR-P1^{bright} mononuclear cells as compared with the group fed 25 ppm diet at 20 weeks of age (Figure 3A). The NKR-P1^{dim} population was not different among each group at three time points, but with increasing age, each group had a greater percentage of NKR-P1^{dim} mononuclear cells as compared with the same group at 9 weeks of age (Figure 3B).

Hepatic natural killer cell activity

Before covariation with the percentage of NKR-P1^{bright} population, all groups of rats exhibited similar NK lytic activity at 9 and 12 weeks of age. The rats fed FB_1 – glucose exhibited significantly lower NK lytic activity as compared with the rats fed 25ppm FB_1 diet at 20 weeks of age (Figure 4A). After covariation with the percentage of NKR-P1^{bright} mononuclear cells, all treatment groups exhibited similar NK lytic activity at each age (Figure 4B).

Hepatic concentration of PGE_2 and $PGE_{2\alpha}$

The hepatic PGE_2 concentration was similar among each group at both 9 and 12 weeks of age, but hepatic PGE_2 concentration in rats fed both 8ppm or 25ppm diet was significantly increased by approximate 50% as compared with the control group at 20 weeks of age. With the age increase, PGE_2 concentrations in rats at 12 or 20 weeks of age were significantly greater than that of rats fed the same diets at 9 weeks of age (Figure 5) . The $PGF_{2\alpha}$ concentration were not different among treatments at any time. Hepatic $PGE_{2\alpha}$ was greater in all treatments at 12 and 20 weeks of age than at 9 weeks of age (Figure 6).

Altered hepatic foci indicated by PGST or GGT staining

There were no detectable PGST- or GGT-positive AHF in any treatment group at 9 or 12 weeks of age. At 20 weeks of age, half of the rats fed 25ppm FB_1 had PGST and GGT- positive AHF. The average PGST-positive AHF area percentage was 0.4 ± 0.7, and the average GGT-positive AHF area 0.6 ± 0.8. There were no detectable PGST- or GGT-positive AHF foci in the groups fed 25ppm FB_1 reacted with glucose, 8ppm FB_1, or basal diet at 20 weeks of age (Table 2). Histopathology studies showed that all the groups exhibited similar mild fatty change in periportal hepatocytes and mild increase of bilirubin in epithelium of proximal convoluted tubules. No lesions was found in lung, brain or tibia.

Sphingolipid analysis

The levels of Sa, So, and the ratio Sa/So were not affected in any treatment group at 9 weeks of age. The Sa concentration as well as the ratios of Sa/So were significantly greater in the rats fed 25ppm FB_1 diet as compared with the control group at 12 or 20 weeks of age. The So concentration was unaffected among all treatment groups at 12 or 20 weeks of age (Table 3).

DISCUSSION

This study demonstrated that subjecting FB_1 to a nonenzymatic browning reaction with glucose avoid FB_1 toxicity as reflected in plasma total cholesterol concentration, ALT activity, development of GGT- and PGST-positive AHF , concentration of endogenous hepatic PGE_2, accumulation of Sa , or Sa/So ratio. These results agreed with the findings of Lu et al (*13*) in which FB_1 was detoxified by reaction with fructose. Because the FB_1-fructose and FB_1 – glucose products are likely to be similar, both reactions probably had similar detoxification effects. The present study showed a lack of toxicity of FB_1 –glucose over 15 weeks treatment, compared with a lack of toxicity over 4 weeks in an earlier FB_1 – fructose detoxification study (*13*)

The effectiveness of the detoxification of FB_1 by reaction with glucose is probably not explained by diminished bioavailability. FB_1 when reacted with fructose had much greater absorption than did FB_1 (*35*). Because FB_1-fructose and FB_1-glucose products are likely to be similar, it is likely that FB_1-glucose products were also absorbed. The addition of glucose to FB_1 may have prevented the inhibitory binding of FB_1 to ceramide synthase, thought to be a pathway of FB1 toxicity (*14*). The observation that sphingolipid ratios remained at control values in the rats exposed to FB_1-glucose in this study further supports this assumption.

The rats fed 25ppm FB_1 diet had noticeably greater plasma total cholesterol concentration as compared with the control group and with rats fed FB_1-glucose at 12 weeks of age in the present study. The observation of FB_1-induced hypercholesterolemia was reported in vervet monkeys (*12*), as well as in rats (*11*, *13*). The reason that the increase of cholesterol in our experiment was not as great as the reported in earlier experiments might be that basal diets contained only 7% fat, which was much less than the fat content (20%) used by Lu et al. (*13*). The mechanism underlying the effect of FB1 on plasma cholesterol is unknown. The increase in plasma total cholesterol by FB_1 might result from stimulation of cholesterol synthesis in hepatocytes, or impaired cholesterol removal by liver. We also observed that the rats fed 8ppm FB_1 or 25 ppm FB_1 diet had significantly lower plasma total cholesterol level as compared with controls or rats fed FB_1-glucsoe at 20 weeks of age. The cholesterol levels in primary rat hepatocytes were decreased under the effect of 500 μM FB_1 (*36*), The mechanism of the decrease in the levels of cholesterol is not clear, but it could be the result of decreased level of sphingomyelin (SM) in cell membranes which influenced cholesterol synthesis and/or metabolism.

The plasma ALT activity was significantly increased in rats fed 8 and 25ppm FB_1 as compared with the control group at 9 weeks of age. At 12 weeks of age, only the rats fed

25 ppm FB_1 exhibited greater ALT activity as compared with the control group. This result is partially in agreement with the finding of Lu (*37*), in which ALT activity in rats fed 25ppm FB_1 for 4 weeks increased significantly compared with controls, but ALT did not increase in rats fed 12.5ppm FB_1 diet. This difference between our results and the results obtained by Lu may be explained by the greater time of FB_1 exposure in the present study, which might have caused more hepatocellular damage. the sphigomyelin synthesis. In contrast, FB_1 treatment increased the hepatocellular concentration of phosphatidylcholine (PC) and phosphatidylethanolamine (PEA).

Groups fed 8 or 25ppm FB_1 but not the group fed FB_1-glucose showed significantly greater endogenous hepatic PGE_2 as compared with the control group at 20 weeks of age. This result differed from the findings of Lu (*37*), in which rats fed 50ppm FB_1 showed greater amount of PGE_2 and $PGF_{2\alpha}$, but no significant increase of PGE_2 or $PGF_{2\alpha}$ was observed in rats fed 25 or 12.5ppm FB_1. As with ALT, the longer period of FB_1 exposure in the present study may have permitted a lower dose of FB_1 to increase PG levels. Also, as with ALT, we have previously proposed that increased PG production was a hallmark of promotion of rat hepatocarcinogenesis caused by FB_1 (*13*). But in our experiment, only rats fed 25 ppm FB_1 showed induction of AHF in the liver, yet rats fed 8 ppm FB_1 also exhibited greater amounts of PGE_2, but had no induction of AHF. This suggests that the increase of PG production may precede the occurrence of preneoplasia.

The NKR-P1^{bright} population in rats fed FB_1 or FB_1-glucose was significantly greater than in the control group at 9 weeks of age. This might reflect a general immune response of the host to exogenous antigen. *In vitro* experiments have shown that NK percentage significantly increased under the effect of antigen (*38*). At 12 weeks of age, all four groups had similar NKR-P1^{bright} percentage. At 20 weeks of age, both FB$_1$-fed groups had similar percentages of NKR-P1^{bright} cells compared with the control group. But the percentage of NKR-P1^{bright} mononuclear cells in FB$_1$-glucose treated rats was significantly decreased as compared with 25 ppm FB$_1$–treated group. Before covariation with the percentage of NKR-P1^{bright} population, all groups exhibited similar lytic activity at 9 or 12 weeks of age, but FB$_1$-glucose-treated rats had significantly decreased NK lytic activity as compared with 25 ppm FB$_1$-treated rats. After covariation with the percentage of NKR-P1^{bright} population, all four groups had similar NK lytic activity per NK cell. Thus, FB$_1$-glucose was not metabolically inert. It remains to be seen whether the suppression of NK cells by FB$_1$-glucose is physiologically significant, and if so, by what mechanism this occurs. The inhibition of NK lytic activity in rats fed FB$_1$-glucose at 20 weeks of age probably resulted from the decreased percentage of NKR-P1^{bright} population.

Our results do not agree with the findings of Lu et al.(*13*), which showed that hepatic NK lytic activity was significantly inhibited when rats were fed 50ppm FB$_1$ as compared with control group. Lu et al. (*13*) proposed that the inhibition of NK activity might be through the effect of increased PG production (*39, 40*). A dose-response inhibition by PGE$_2$ of NK activity had been reported by Liu et al.(*41*) in *in vitro* cocultures of hepatic NK cells with PGE$_2$. In our experiment, we observed the increased production of PGE$_2$ by FB$_1$ feeding, but NK activity was not affected . The reason that we did not observe the inhibition of NK lytic activity might be that less (25ppm) FB$_1$ as well as less fat (7%) was fed to the rats as compared with the experiment of Lu et al.(*13*)

(50ppm FB_1 and 20% fat in the diet). The possible explanation for the lack of effect of FB_1 on NK activity in our study may lie in interactions between carcinogenesis and NK activity. Lee et al. (*42*) showed that, in male F344/rats given 40ppm DEN in drinking water for 10 weeks, as PGST-positive AHF developed, splenic NK activity changed. After 5 weeks, DEN treated and control rat spleen NK activity was similar, but at 10 weeks, NK activity was significantly greater in DEN treated rats compared with control rats. At 20 weeks, DEN-treated rats had significantly lower NK activity than did control rats. Lower NK activity was accompanied by PGST-positive AHF. Lu et al.(*13*) showed 20-fold greater area of PGST-positive AHF as compared with our study, which implies that with greater extent of carcinogenesis , NK lytic activity was affected adversely.

In the present study, only half of the rats fed 25 ppm FB_1 showed PGST- and GGT-positive AHF, in comparison with Lu (*37*) in which 11 of 12 animal fed 25 ppm FB1 showed PGST and GGT-positive AHF. The area occupied by AHF was much greater than in our study. The fat content of the diet was 20% (*37*), which was much greater than the 7% fat content in our experimental diet . It has been shown that high levels of dietary fat markedly shorten the time between ultraviolet exposure and its induction of skin tumor (*43*). Many studies have shown that dietary fat is a tumor promoter (*44*, *45*). In our study, although the feeding time was longer than in the study by Lu (*37*) , the lesser dietary fat in the present study might have produced fewer and smaller GGT- and PGST-positive AHF.

The increase in free sphingosine and sphinganine in animal tissue, serum and urine have been used extensively as an experimental biomarker for fumonisin exposure (*46*). In our study, the accumulation of Sa and increase of Sa/So ratio only appeared in rats fed 25 ppm FB_1 at 12 and 20 week age. This did not agree with previous observations that animals exposed to FB_1 developed altered Sa/So ratio before other signs of intoxification were observed (*47*, *14*). In the present study, we observed increased ALT in both FB_1 treated groups at 9 weeks of age and in rats fed 25 ppm FB_1 at 12 weeks of age, but we did not observe the Sa/So ratio change at 9 weeks of age. Our study agreed with Gelderblom et al. (*48*) who indicated that no significant change in Sa/So ratio was observed at the lowest dietary level of FB_1 that induced cancer promotion and inhibition of cell proliferation. Further studies are needed to determine the role of Sa/So ratio in fumonisin toxicity and how the effect of FB_1 on this ratio interacts with other dietary components.

It was noticed that both FB_1-glucose and FB_1(8ppm) diets contained the same amount (8ppm) of free FB_1, but only FB_1(8ppm) treated rats exhibited adverse effects, which was reflected in increased ALT activity, and total cholesterol and PGE_2 concentrations. FB_1-glucose which contain 8ppm free FB_1 treated rats exhibited similar effect as control rats. It seemed that FB_1-glucose can prevent the adverse effect of FB_1 when FB_1 and FB_1-glucose were fed together. Perhaps the FB_1-glucose prevented the free FB_1 from interacting with its site of action.

In conclusion, reaction of FB_1 with glucose can detoxify FB_1 in a rat hepatocarcinogenesis model.The mechanism might be that binding ability of FB_1-glucose to ceramide synthase was inhibited, as was reflected in the lack of accumulation of Sa when FB1-glucose was fed. Sa/So did increase when the same amount of FB_1 was fed (25ppm). Further study is needed to determine the bioavailability of FB_1-glucose and to determine its effects in other animal models.

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Table 1

	Body weight (g)	LW/BW(x 10^2)	Body Composition (% fat)	Food intake (g/day)
Control	204 ± 12	4.1 ± .5	32.8 ± 3	13.5 ± 3
FB1-glucose	205 ± 12	3.9 ± .6	34.2 ± 5	14.5 ± 2
FB1(8ppm)	198 ± 15	3.9 ± .4	33.4 ± 3	12.8 ± 2
FB1(25ppm)	197 ± 16	3.7 ± .5	31.5 ± 2	11.5 ± 2

Table 1: Comparison of the final body weight, body mass composition, relative liver weight and food intake among four treatment F344/N female rats. No difference was found in these four indicators among these groups under each treatment over 20 weeks (N=10/ group). The data entries in the table were expressed as mean ± standard error of mean (SEM).

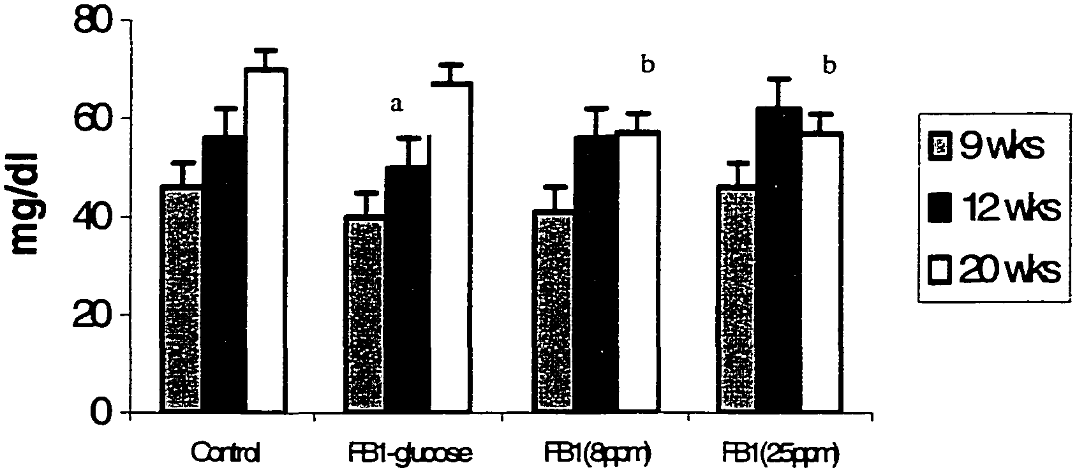

Figure 1: Comparison of the total plasma cholesterol concentration at three time points. At 9 weeks of age, no difference was found among four treatment groups. At 12 weeks, the rats fed 25ppm FB_1 exhibited significantly greater cholesterol concentration as compared with FB_1-glucose group. At 20 weeks of age, both the rats fed with 8ppm and 25ppm FB_1 exhibited significantly lower cholesterol concentration as compared with control group. The error bar represented the SEM.

a: significantly different as compared with FB_1(25ppm) group at 12 weeks of age (N= 5/group).

b: Significantly different as compared with control group at 20 weeks of age (N=10/group).

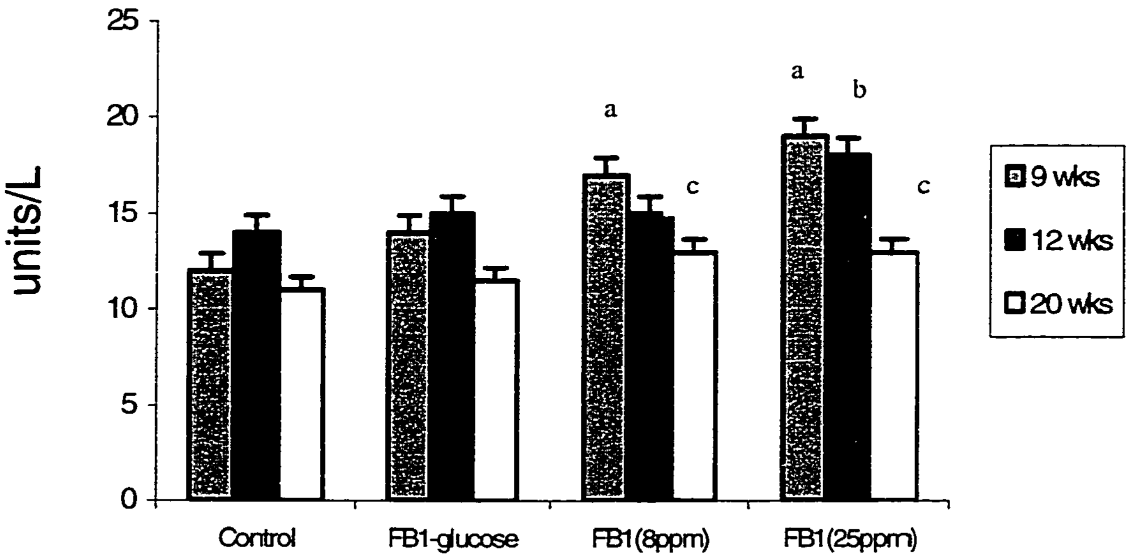

Figure 2: Comparison of the alanine aminotransferase (ALT) activity at three age points. At 9 weeks of age, both the rats fed 8ppm and 25ppm FB_1 showed significantly greater ALT activity as compared with control group. At 12 weeks of age, the rats fed 25ppm FB_1 showed significantly greater ALT activity as compared with control group.

At 20 weeks of age, both the rats fed 8ppm and 25ppm FB_1 showed significantly greater ALT activity as compared with control group. The error bar represented the SEM.

a: Significantly greater than control group at 9 weeks of age (N= 4/group)

b: Significantly greater than control group at 12 weeks of age (N= 5 /group)

c: Significantly greater than control group at 20 weeks of age (N=10/group)

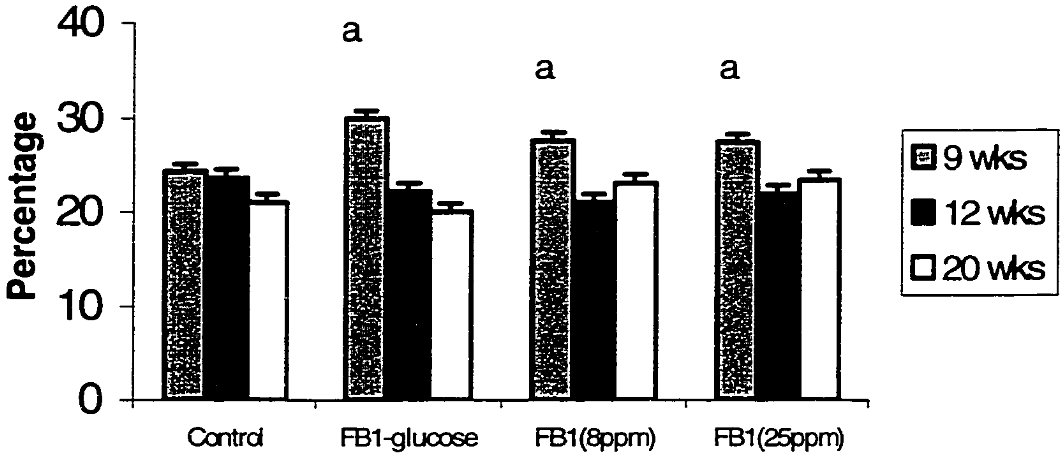

Figure 3A: Comparison of the percentage of hepatic NKR-P1^{bright} mononuclear cells at three time points. At 9 weeks of age, all the rats fed FB_1-glucose , 8ppm FB_1 or 25ppmFB_1 exhibited greater NKR-P1^{bright} percentage as compared with control group. No difference was found among treatment groups at 12 or 20 weeks of age. The error bar represented the SEM.

a: Significantly greater than control group at 9 week age (N= 4/group). The error bar represented the SEM.

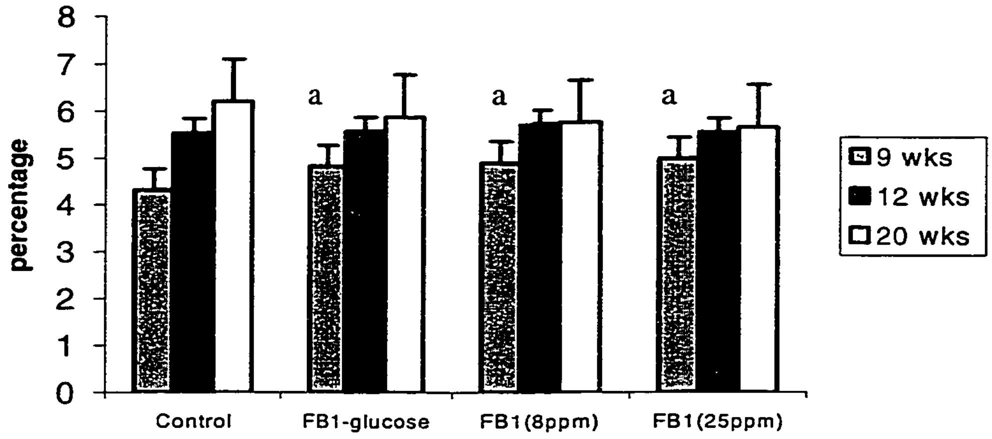

Figure 3B: Comparison of the percentage of hepatic NKR-P1^{dim} cells at three time points. All the rats showed similar percentage among treatment within the same time point. a: Significantly greater than control group at 9 week age (N= 4/group). The error bar represented the SEM.

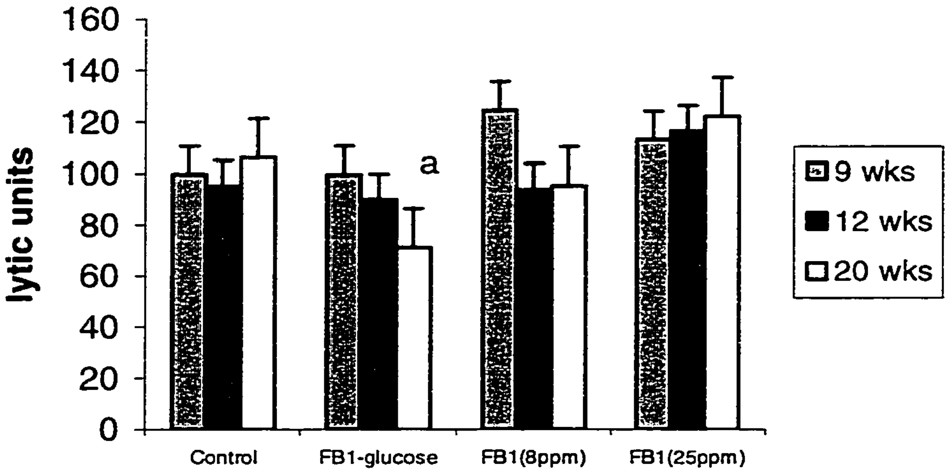

Figure 4A: Comparison of hepatic NK activity at three time points prior to covariation with the percentage of NKR-P1^{bright} mononuclear cells. Activity was expressed as lytic units, calculated from the specific lysis curve. At 9 and 12 weeks of age, all the rats exhibited similar NK lytic activity. At 20 weeks of age, the rats fed FB_1-glucose exhibited significantly lower NK activity as compared with the rats fed 25ppm FB_1. The error bar represented the SEM

a: Significantly different as compared with the group fed 25ppm FB_1 (N=10 /group)

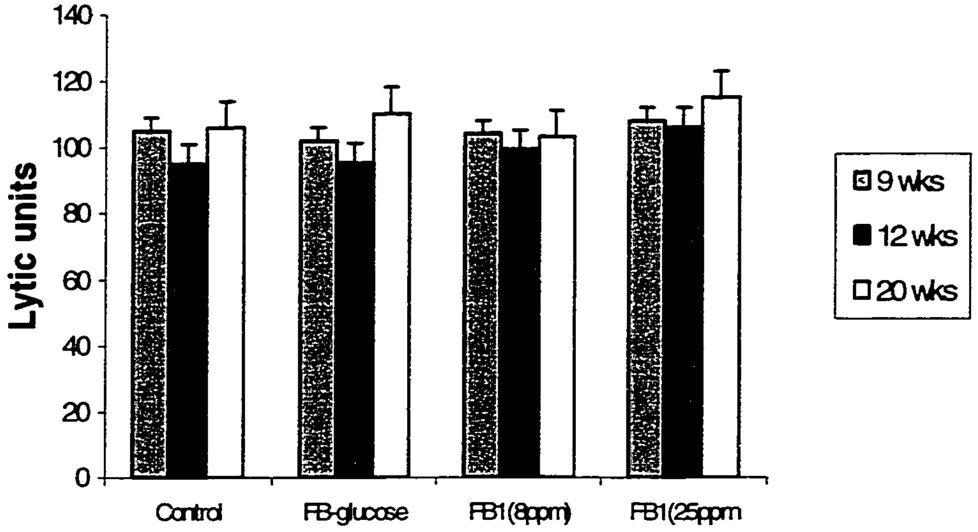

Figure 4B: Comparison of hepatic NK activity at three age stages after covariation with the percentage of NKR-P1^{bright} population. At three age stages, no difference was found among treatment within each age stage. The error bar represented the SEM.

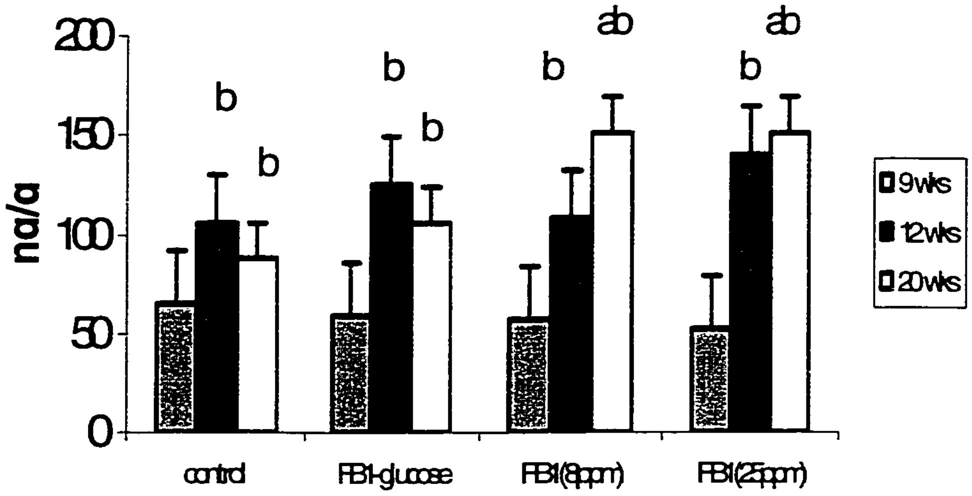

Figure 5: Comparison of the hepatic PGE_2 concentration in four treatment groups at three time points. The concentration of PGE_2 is expressed as ng per gram liver tissue. At 9 weeks of age, there is no difference among the treatment groups. At 12 weeks of age, no difference was found among the treatment groups. At 20 weeks of age, the rats fed 8ppm or 25ppm FB_1 exhibited significantly greater concentration of PGE_2 as compared with control group. Rats at both the 12 and 20 weeks of age had greater PGE_2 concentration as compared with same treatment at 9 weeks of age. The error bar represented the SEM.

a: significantly greater than control group at 20 weeks of age (N=10 /group).

b: Significantly greater than 9 weeks of age group of the same treatment

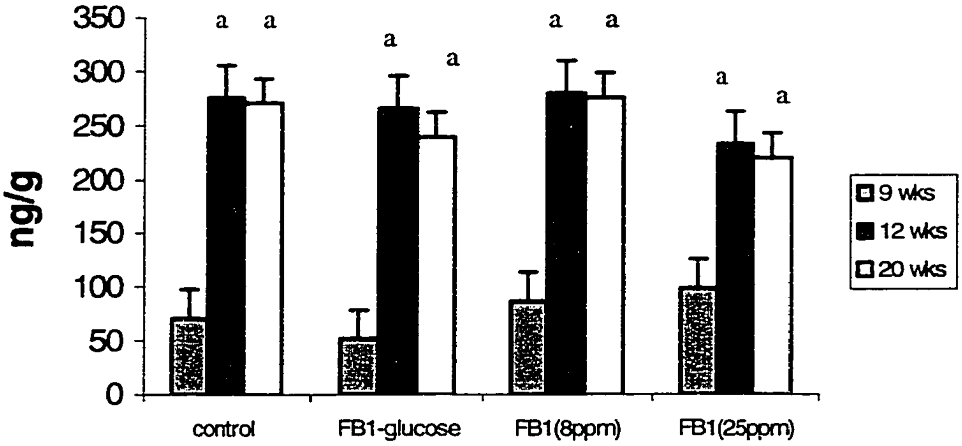

Figure 6 : Comparison of the hepatic $PGF_{2\alpha}$ concentration in four groups at three time points . The concentration of hepatic $PGF_{2\alpha}$ was expressed as ng per g liver tissure. The is no difference among treatment groups at three time points. Both the rats at 12 and 20 weeks of age had greater hepatic $PGF_{2\alpha}$ concentration as compared with same treatment at 9 weeks of age. The error bar represented the SEM.

a : Significantly different as compared with rats of 9 weeks of age within the same treatment.

Table 2: Placental glutathione S-transferase (PGST) and γ-glutamyl transferase (GGT)-positive altered hepatic foci (AHF)

	#of rats with PGST AHF	% PGST Area	# of rats with GGT AHF	% GGT Area
Control	0	0	0	0
FB1-glucose	0	0	0	0
FB1(8ppm)	0	0	0	0
FB1(25ppm)	5	0.4 ± 0.7	5	0.6± 0.8

The AHF occurred only in rats fed 25 ppm FB_1 at 20 weeks of age. The data entries in the table were expressed as mean ± standard error of mean (SEM).

Table 3: Comparison of sphingosine (nmol/ml) (So), sphinganine (nmol/ml) (Sa) as well as the ratio of Sa/So at three time points

	9 weeks of age (nmol/ml)			12 weeks of age (nmol/ml)			20 weeks of age (nmol/ml)		
	So	Sa	Sa/So	So	Sa	Sa/So	So	Sa	Sa/So
Control	5.6±1.2	0.7±0.2	0.1±0.1	6.5±2.2	0.9±0.3	0.12±0.1	5.9±2.5	1±0.5	0.15±0.1
FB1-Glucose	6.5±1.5	0.6±0.2	0.1±0.1	5.7±2.5	0.8±0.2	0.12±0.1	5.8±1.5	0.8±0.5	0.12±0.1
FB1(8ppm)	5.4±2.3	0.8±0.2	0.15±0.1	6.3±3.0	1.3±0.5	0.18±0.2	6.5±2.9	1.2±0.8	0.2±0.1
FB1(25ppm)	5.5±2.8	1.1±0.3	0.2±0.1	7.5±3.5	3.5±1.5**a**	0.5 ±0.2**a**	5.8±1.6	4.5±1.5**b**	0.8±0.3**b**

The concentration of the sphingolipids was expressed as nmol per g liver tissue. At 9 weeks of age. The Sa, So and the ratio were similar among treatment groups. At 12 weeks of age. The rats fed 8ppm or 25ppm FB_1 diet exhibited greater Sa concentration and increased ratio of Sa/So as compared with control group. At 20 weeks of age, the rats fed 8ppm and 25ppm FB_1 diet exhibited greater Sa concentration and increase ratio of Sa/So as compared with control group. The data entries in the table were expressed as mean ± standard error of mean (SEM).

a: Significantly different as compared with control group at 12 weeks of age (N= 5/group)

b: Significantly different as compared with control group at 20 weeks of age (N=10/group)

INCREASED DIETARY FAT AND ENERGY INTAKE DURING FUMONISIN PROMOTED HEPATOCARCINOGENESIS INCREASE HEPATIC PROSTAGLANDINS, SPHINGANINE, AND DEVELOPMENT OF PLACENTAL GLUTATHIONE TRANSFERASE (+) FOCI, WHILE INHIBITING NATURAL KILLER CELL NUMBER

A paper to be submitted to *Carcinogenesis*

Hongjun Liu, Joan E. Cunnick*, Patricia A. Murphy and Suzanne Hendrich**

ABSTRACT

We propose that greater dietary fat and energy intake promotes FB_1 carcinogenesis, inhibition of NK cell activity parallels the enhanced development of preneoplasia by greater dietary fat, and the endogenous production of prostaglandins and sphingolipids are involved in the modulation of NK cell activity. In our previous studies, we observed that rats fed 50ppm FB_1 and 20% dietary fat for four weeks developed preneoplasia, and the liver-associated natural killer (NK) cell activity was inhibited compared with a control

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group, but, when rats were fed 25ppm FB_1 and 7% fat for 20 weeks, only half of the rats developed preneoplasia, and NK activity did not change compared with a control group. In the present study, 24 10-day old female F344/N rats were injected intraperitoneally with diethylnitrosamine (DEN, 15mg/kg body weight). At 4 weeks of age, rats were randomly assigned to AIN93G, AIN93G modified to contain an additional 13% beef tallow (by weight) substituted for corn starch, or either diet supplemented with fumonisin B_1 (FB_1, 50mg/kg). At 9 weeks of age, in comparison with rats fed high- or low-fat control diets, the groups fed FB_1 showed greater alanine aminotransferase activity (ALT), total plasma cholesterol, endogenous hepatic prostaglandin E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$), accumulation of sphinganine (Sa), increased ratio of Sa/So (sphingosine), and significantly less natural killer (NK) cell activity and total percentage of liver-associated NKR-P1^{bright} cells. The group fed a high fat diet and FB_1 exhibited greater total plasma cholesterol, PGE_2 , and lower NK cells as a percentage of liver-associated lymphocytes than the group fed a low fat diet and FB_1. Placental glutathione S-transferase-positive (PGST) and γ-glutamyl transferase (GGT)-positive altered hepatic foci (AHF) occurred only in rats fed FB_1. The same number of animals developed preneoplasia in these two groups, but the group fed a high fat diet and FB_1 had greater average hepatic area occupied by PGST (+) AHF than did the group fed low fat and FB_1. From these findings, high fat and greater energy consumption promoted FB_1 carcinogenesis in comparison with the low fat diet. The inhibition of total NK activity paralleled the development of preneoplasia, and sphinganine did not seem to be a factor modulating the hepatic NK cell activity. Prostaglandin E_2 seemed to be a key factor of NK cell activity modulation in the present study. But in one of our previous study, we found the increase of PGE_2. we did not see the inhibition of NK activity. Our results implicated some other factors than PGE_2 produced by neoplasms as down-regulators of NK activity accompanying growth of preneoplasia.

INTRODUCTION

Fumonisin B_1 (FB_1) and related compounds occur in grain and grain products as the result of infestation and growth of fungi of the *Fusarium* species (1). FB_1 was identified as the major toxic substance produced by cultures of *Fusarium moniliforme* (2), which caused equine leukoencephalomalacia (ELEM) (3), porcine pulmonary edema (PPE) (4), and was hepatotoxic and hepatocarcinogenic in rats (5, 6).

FB_1 toxicity and carcinogenicity were evaluated in female F344/N rats (7), initiated by diethylnitrosamine (DEN, 15mg/kg body weight) at 10 days of age, and given free access to the control diet (AIN93, 7% soybean oil + 13% beef tallow) or treatment diet (AIN93, 7% corn oil + 13% beef tallow + 50ppm FB_1) for 5 weeks, FB_1 - fed rats developed altered hepatic foci (AHF), and hepatic NK activity in FB_1–fed rats was significantly inhibited as compared with the control group. When female F344/N rats were initiated by DEN (15mg/kg bw), and given free access to control diet (AIN93, 7% soybean oil) or the same diet containing 25ppm FB_1 for 16 weeks, only half of the animals of the FB_1–fed rats developed GGT- and PGST-positive foci (8), and no difference in hepatic NK cell activity was observed between control and FB_1 -fed rats. These two experiments suggested that there was an interaction between dietary fat and FB_1 carcinogenesis, as reflected in the altered hepatic foci and hepatic NK activity.

Evidence from experimental animal models strongly suggests that liver-associated NK cells and Kupffer cells are the first line of defense against blood-borne metastasizing solid tumor cells invading the liver. Thus NK cells protect the parenchyma. The primary role of NK cells in neoplasia is directed against blood-borne tumor cells during the intravascular phase of tumor metastasis(9). In male F344/N rats given 40ppm DEN in drinking water for 10 weeks, as GST-P^+ foci developed, splenic NK activity changed. After 5 weeks, DEN-treated and control rat spleen NK activity was similar, but at 10 weeks, NK activity was significantly greater in DEN treated rats compared with controls. At 20 weeks, DEN-treated rats had significantly lower NK activity than did controls (10). This suggests an interaction between chemical carcinogenesis and NK activity, at the very early stage of carcinogenesis, the NK cell activity may not change or even increase, with the progression of neoplasia, the inhibition of NK cell activity occurs, and may parallel the development of neoplasia.

Increased dietary fat increased the development of mammary tumors induced by chemical carcinogens in rats (11, 12). Hopkins and Carrol (13) reported that in rats initiated with 7,12-dimethylbenz[a]anthracene one week before dietary treatment, rats fed 3% sunflower seed oil and 17% of either tallow or coconut oil developed twice as many tumors as those fed 3% sunflower seed oil. Rats were first intubated with diethylnitrosamine (DEN, 10 mg/kg) 20 hr after partial hepatectomy; 1 week later, rats were fed one of three purified diets (a low-fat diet similar to the AIN-76 diet, a high saturated fat diet, or a high polyunsaturated fat diet) with or without 0.05%

phenobarbital in the diet for 10 months. Increasing the fat level of the diet did not increase the number of GGT-positive foci arising spontaneously or induced by DEN alone. When phenobarbital was present in the diet, both high-fat diets enhanced the induction of GGT-positive foci. Increasing the dietary fat level enhanced promotion of hepatic AHF by phenobarbital (14). We hypothesized that greater dietary fat and energy intake promotes FB_1 carcinogenesis, the inhibition of NK cell activity parallels the development of preneoplasia, and the inhibition of NK activity may be modulated by prostaglandins and/or sphingolipids which had been observed to accumulate in previous experiments of FB_1 promotion of hepatocarcinogenesis (7, 8).

MATERIAL AND METHODS

Diets

Four experimental diets were fed to rats (Table 1) : AIN93G (American Institute of Nutrition, 1993, which contain 7% soybean oil), AIN-93G supplemented with 13% beef tallow (in substitution for corn starch), and both low and high fat diet supplemented with 50mg FB_1 /kg diet. Fumonisin B_1 was purified from liquid cultures of *F. proliferatum* strain M5991, which predominantly produces FB_1 (15). Fumonisin B_1 was purified by the same procedure as described by Dantzer et al. (15). The purity of the FB_1 was >95%.

Animals

The experimental procedures were approved by the Iowa State University Animal Care Committee. Twenty-four 10-day old female F344/N rats obtained from Charles River (Wilmington, MA) were injected intraperitoneally with diethylnitrosamine (DEN, 15mg/kg body weight) in 0.1 ml corn oil. At 4 weeks of age, the weaned rats were randomly assigned to one of the 4 treatment groups with 6 rats each. At 9 weeks of age, all the rats were killed by cervical dislocation. Rats were given free access to the experimental diets and maintained at 22-25°C and 50% humidity with a 12-h light/dark cycle. Body weight and feed intake were measured weekly.

Plasma and liver samples preparations

Before the liver was perfused, 1ml sodium chloride solution (0.1%, containing 100 units of heparin) was injected into the abdominal vein, and about 3ml blood was removed by syringe. Part of the plasma obtained from heparinized blood was analyzed within 24 hours for alanine aminotransferase(ALT) activity. The remaining plasma was stored at -80°C for later plasma total cholesterol analysis.

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

Each of the left, median, and right lateral lobe of the livers was sliced into 1cm slices. Three slices, one from each lobe, were immediately frozen as a block on dry ice and store at -80°C. From each of the frozen liver blocks, 5 10-μm serial sections were cut with a Histostat Microtome (Model 855, Leica Inc., Deerfield, IL) for later staining for gamma glutamyl-transferase (GGT) and placental glutathione-transferase (PGST). Additional liver samples were processed by routine histopathological methods for hematoxylin-eosin staining (16).

For each rat, 0.5 g minced liver portion were immediately homogenized in an ice bath with 10 passes of a Potter-Elvehjem homogenizer in 5ml, PH 7.4, 50 mM potassium phosphate buffer containing 4.2 mM acetyl salicylic acid (Sigma Chemical Co., St. Louis, MO). The liver homogenates were frozen at -80°C for later analysis of endogenous hepatic PGE_2 and $PGF_{2\alpha}$.

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 for the ALT assay (Sigma Chemical Co., St. Louis, MO).

Determination of sphingosine (So) and sphinganine (Sa)

Thawed liver tissues were homogenized in 4 volumes 0.05 M potassium phosphate buffer. Homogenate (0.1 ml) was transferred to a cold (13 X 100mm) glass tube with a Teflon lined screw cap. The extraction method was performed as described by Riley et al. (17). Sphingosine and sphinganine were quantified by HPLC as described by Riley et al. (17) but using C17-phytosphingosine (Sigma Chemical Co., St. Louis, MO) as the internal standard. The HPLC system include two Beckman model 110B pumps (Beckman, San Ramon, CA), a Dynamax® - 100 A pore size, 3 μ particle size, C18, 4.6 x 50 mm column

(Varian Chromatography Associates, Walnut Creek, California), and a Waters model 470 scanning fluorescence detector (Waters, Milford, MA). Sphinganine and So standard (Sigma Chemical Co., St. Louis, MO) mixture at different concentrations (1, 3, 5, 7 9 nmol) were prepared for standard curves.

Prostaglandin assays

PGE_2 and $PGF_{2\alpha}$ were determined in a radioimmunoassay as described by McCosh et al (18). Anti-PGE_2 antiserum (0.2ml /per assay tube) and anti-$PGF_{2\alpha}$ antiserum (0.2ml / per assay tube) were obtained from Sigma Co.(St Louis, MO). H^3-PGE_2 ($10^{-6}\mu Ci$/ per aliquot) and H^3- $PGF_{2\alpha}$ ($10^{-6}\mu Ci$/ per aliquot) was obtained from NEN Products (Boston, MA). PGE_2 and $PGF_{2\alpha}$ were quantified by using a computer program based on a logit transformation of the standard curve (19)

Natural killer cell assays

Natural killer cell assays were performed as previously described (20). Cells were plated in triplicate at the following effector to target ratios in 96 well plates: 25:1, 12.5:1, 6.25:1, 3 :1. The target cells for the assay were YAC-1 cells(ATCC Co, Rockville, MD) (8×10^3/well) which had been labeled with 200μCi ^{51}Cr (NEN, Boston, MA). The amount of ^{51}Cr released by dying cells was counted using a Gamma Trac 1191(TM Analytic, Inc., Elk Grove Village, IL). Lytic units were calculated using a computer program based on the equation of Pross and Maroun(1984).

Fluorescent staining of lymphoid cells

Leukocyte suspensions were diluted with an equal volume PBS/0.1%azide (cold) and incubated at 4°C (5min). Separate aliquots were stained with

0.2μg/2×10^5(mononuclear cell) of anti-rat NKR-P1A-Biotin(mAb 3.2.3), or an equivalent amount of isotype control (murine IgG1-biotin). A second step of 0.1μg/2×10^5(mononuclear cell) Strep-AvidinCychrome was used(All from Pharmingen, San Diego,CA). All incubations were performed at 4°C in the dark for 30min, and washed with PBS/0.1%azide. The contaminating red blood cells were lysed using 10% ammonium chloride buffer (pH 7.4). Cells were fixed with PBS/1% paraformaldehyde prior to analysis using an EPICS-XL-MCI flow cytometer (Coulter, Miami, FL). According to the density of fluorescence, three cell populations were distinguished: NKR-P1$^{\text{bright}}$, NKR-P1$^{\text{dim}}$ and negative, and gates were set to help measure the percentage of different populations.

Immunohistochemical staining

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). Anti-PGST antiserum was prepared as described previously (21). The second frozen serial section was stained for GGT activity as described by Rutenburg et al. (22). The substrate for GGT was glutamyl-4-naphthylamide (GMNA) (United States Biochemical Corp., Cleveland, OH). Altered hepatic foci were quantified via computerized stereology. A Sony 3-chip color video camera DXC-3000A took the images of the liver section stained for GGT and PGST, which were digitally transferred from the camera to an Apple Power Mac G3 computer (Apple computer, Inc., Cupertino, CA), and analyzed with IP Lab image analysis software (version 3.2.3, Scanalytics, Fairfax, VA).

Statistical analysis:

One-way ANOVA was performed to analyze the final body weight, plasma total cholesterol, alanine aminotransferase activity, the percentage of leukocytes that carry NKR-P1^{bright} and NKR-P1 ^{dim} population markers as well as the amounts and ratio of Sa/So. Two-way ANOVA was performed to analyze fat and fumonisin main effect as well as their interaction on the above-mentioned biomarkers. The correlation among total cholesterol, prostaglandins, and the area of AHF was analyzed by Pearson test. The hepatic NK activity was analyzed by analysis of covariance (ANCOVA) by taking the NKR-P1^{bright} population as covariable . Student's t-test was performed to compare all possible group differences after ANOVA. A p-value of <0.05 was considered to be statistically significant.

RESULTS

Effect on body weight gains, relative liver weight and feed intake

The food intake did not differ among treatments (Table 1). But the final body weight in groups fed FB_1 was significantly lower than in the control group fed a high fat diet (Table 1). Relative liver weights were not different among the four treatment groups, and the average daily food consumption was similar among the four groups (Table 1). The average daily energy intake was much greater for rats fed a high fat diet than for rats fed a low fat diet (Table 1).

Plasma total cholesterol levels and alanine aminotransferase activity

Alanine aminotransferase (ALT) activity of the rats fed 50ppm FB_1 diet was significantly greater than that in the rats fed low or high fat control diets. Alanine aminotransferase activity was not different between the two groups fed FB_1 (Figure 2). In comparison with the rats fed a low fat diet, the control group fed a high fat diet and both FB_1 -fed groups had significantly greater total plasma cholesterol concentration. The total cholesterol concentration in rats fed a low fat diet and FB_1 was significantly greater than in rats fed high fat control diet, and rats fed a high fat diet and FB_1 had significantly greater total cholesterol concentration as compared with rats fed a low fat and FB_1 (Figure 1). There was no interaction between fat and fumonisin effects on ALT or total cholesterol (data no shown).

Hepatic natural killer cell activity and NKR-P1^{brigth} and NKR-P1^{dim} population

Flow cytometric analysis using murine antibody 3.2.3 (natural killer cell receptor protein 1 (NKR-P1)) revealed two distinct subsets of hepatic mononuclear cells expressing variable levels of NKR-P1. The NKR-P1^{bright} population, which expresses a high level of NKR-P1, has been identified as the population causing NK associated lytic activity (23). The NKR-P1^{dim} population, which expresses a low level (2-10 fold lower) of NKR-P1, has been linked to a subset of T lymphocytes that have NK-like cytolytic function under activation by interleukin-2 (24). The results showed that two FB_1 treatment groups had significantly lower per NK cell activity as compared with both basal diet and high fat diet control groups. Per NK cell activity was not different between two FB_1 treatment groups (Figure 3). The percentage of NKR-P1^{bright} population in both FB_1 fed groups was significantly lower than in either control groups, and the percentage of NKR-P1^{bright} mononuclear cells in the rats fed 50ppm FB_1 and high dietary fat was significantly lower those fed 50ppm FB_1 and low fat (Figure 4). The NKR-P1^{dim} population was not different among each group (data now shown). Based on the percentage of NKR-P1^{bright} in leukocytes and the total leukocytes in the hepatic perfusate, we calculated the total hepatic NK cells in each group. After covariance of the total hepatic NK cell number with liver weight, the rats fed FB_1 had lesser total NK cell numbers as compared with either control groups, and the rats fed high fat diet and FB_1 had lesser total NK cell numbers than those fed low fat diet and FB_1 (Figure 5)

Hepatic concentration of PGE_2 and $PGE_{2\alpha}$

The hepatic PGE_2 was greater in rats fed FB_1 and high fat diet than the rats fed FB_1 and low fat diet. The rats fed FB_1 and low fat diet had greater hepatic PGE_2 than the rats fed high fat diet. The rats fed high fat diet had greater hepatic PGE_2 concentration than the rats fed low fat diet, there is no interaction between fat and FB_1. They had additive effect on the increase of hepatic PGE_2(Figure 6). Hepatic $PGF_{2\alpha}$ was greater in rats fed FB_1 and high fat diet or low fat diet than rats fed high fat diet. The rats fed high fat diet had greater hepatic $PGF_{2\alpha}$ than rats fed low fat diet. Hepatic $PGF_{2\alpha}$ was not different between FB_1 -fed groups (Figure 7). There was no interaction between fat and fumonisin effect on the either PGE_2 or $PGF_{2\alpha}$.

Altered hepatic foci indicated by PGST or GGT staining

There were no detectable PGST- or GGT-positive AHF in either control group. Five out of six rats fed 50ppm FB_1 and basal diet had PGST-positive AHF, and four out 6 rats fed 50ppm FB_1 in basal diet had GGT- positive AHF. The average PGST-positive AHF area was 3.8 ± 1.3 percent of the total hepatic area, and the average GGT-positive AHF area was 1.4 ± 0.9 percent of the total hepatic area. Five out of 6 rats fed 50ppm FB_1

in high fat diet had PGST- or GGT- positive AHF foci, and the average PGST-positive AHF area as a percent of total hepatic area was 6.1 ± 1.7, whereas the average GGT-positive AHF area was 1.3 ± 0.8 percent of the total hepatic area (Table 2). The average PGST-positive AHF area in the rats fed 50ppm FB_1 in high fat diet is significantly greater than in the rats fed 50ppm FB_1 and low fat diet.

Sphingolipid analysis

The levels of Sa, So, and the ratio Sa/So were not affected by dietary fat content of control diets. Both FB_1 –fed groups had similar hepatic Sa and Sa/So ratio, which was greater than either control group (Table 3). There was no interaction between fat and fumonisin effect on Sa, So, or Sa/So ration (data not shown).

DISCUSSION

The present study showed that rats fed high fat diet without FB_1 had greater total plasma cholesterol concentration than did rats fed a low fat control diet (Figure 2). This is consistent with previous findings that high dietary beef tallow intake can increase the total cholesterol concentration. Six-week-old male Fischer 344 rats were placed on a high-fat [7% (wt/wt) soybean oil + 15% (wt/wt) beef tallow] or a normal-fat (7% soybean oil, AIN-93G) diet. At 10 weeks of age, the rats fed a high fat diet showed increased blood cholesterol as compared with the rats fed a low fat diet. The observation of FB_1-induced hypercholesterolemia was reported in vervet monkeys (25), as well as in rats (26, 7). In the present experiment, both FB_1 –fed groups had greater total cholesterol concentration than either control group. 50ppm FB_1 seemed to have similar ability to increase the total cholesterol as did feeding a high fat diet without FB_1, FB_1 and high fat showed a additive effect on cholesterol concentration (no interaction between fat and FB_1 based on two-way ANOVA test, data not shown). The mechanism of FB_1 increasing plasma cholesterol is not clear yet. 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 diet containing 0.25-1% of *F.moniliforme* culture material (25). It was therefore proposed that impaired removal from the plasma rather than increased cholesterol synthesis in the liver probably occurred (25).

The plasma ALT activity was significantly increased in FB_1 –fed groups compared with the two control groups (Figure 1). This result is in agreement with the finding of Lu (7), in which ALT activity in rats fed 50ppm FB_1 and 20% total dietary fat for 5 weeks increased significantly compared with controls. 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 (26). The ALT activity was not different between two FB_1 –fed groups suggesting that quantity of total dietary energy intake did not affect FB_1 hepatoxicity per se.

Groups fed 50ppm FB_1 with high or low dietary fat intake showed significantly greater endogenous hepatic PGE_2 and $PGF_{2\alpha}$ than the control groups (Figure 6, 7). This result is consistent with the findings of Lu (7), in which rats fed 50ppm FB_1 and 20% dietary fat showed greater amount of PGE_2 and $PGF_{2\alpha}$. Liu et al (8) reported that F344/N female rats fed AIN93G diet supplemented with 25ppm FB_1 showed greater hepatic PGE_2 in the rats fed control diet (AIN93G). In the present study, the group fed 50 ppm FB_1 and high dietary fat had greater hepatic PGE_2 than rats fed 50ppm FB_1 and a low fat diet. It was also observed that the total hepatic area occupied by PGST-positive AHF was greater in rats fed 50ppm FB_1 and high dietary fat than in rats fed 50ppm FB_1 and low dietary fat. The development of preneoplasia was highly correlated with the PGE_2 metabolism in this study (data not shown). Gupta et al. showed that feeding a choline deficient (CD) diet, an efficient liver tumor promoting regimen, to male Sprague-Dawley rats initiated by a single dose of diethylnitrosamine induced 2-2.5 fold increased levels of PGE_2 in the liver. The addition of indomethacin to a CD diet suppressed the diet-induced elevations of PGE_2 and a substitution of fats in a CD diet with menhaden oil had the same effect. Furthermore, both indomethacin and menhaden oil added to a CD diet suppressed the induction of GGT-positive hepatocyte foci in the liver of rats . Lin et al.(27) showed greater PGE_2 in NZB/W F1 mice fed diets containing 200 g dietary fat/kg (equal amount of lard and soybean oil) than in mice fed 50 g dietary fat/kg (equal amount of lard and soybean oil) respectively. The study suggested that greater amount of PGE_2 might be produced by macrophages under the situation of high fat intake. Parhar et al. (28) showed that PGE_2 production by tumor-host-derived macrophages was significantly greater than that produced by normal splenic macrophages. All our studies (7, 29) observed that increased production of prostaglandins accompanied the development of FB_1 carcinogenesis. This suggests that prostaglandins may play a important role during FB_1 carcinogenesis, and the inhibition of immune function by prostaglandins might be one of the factors that promote the FB_1 carcinogenesis, as observed by Lu et al. (7) and the present experiment.

The observation of the inhibition of total NK activity by FB_1 as compared with both control groups (Figure 3,4,5) is consistent with previous findings in which rats fed 50ppm FB_1 and 20% dietary fat showed significant lower total NK activity than the control group (7). In another experiment, when F344/N rats were fed 25ppm FB_1 and 7% dietary fat, the total NK activity was not changed, but the production of endogenous PGE_2 was much greater than in control groups(29). Although NK activity per cell was similar between the two FB_1-fed groups, the total mature NK population was greater in rats fed 50ppm FB_1 and low dietary fat than in rats fed 50ppm FB_1 and high dietary fat, which paralleled the production of PGE_2 and the extent of the neoplasia in these two groups. These results suggested that the PGE_2 might be a factor modulating NK activity during the development of neoplasia. Parsha et al.(30) reported NK cells are progressively inactivated during tumor development by PGE_2 secreted by host macrophages. Parhar et al.(28) also revealed that tumor-host-derived macrophages derived from CBA mice bearing 21-day intraperitoneal Ehrlich ascites tumors or C3H/HeJ mice bearing 21-day subcutaneous T58 mammary adenocarcinomas (but not normal macrophages) markedly suppressed NK activity. Indomethacin and anti- PGE_2 antibody prevented the suppression of NK activity in this model. This finding ran parallel with high levels of PGE_2 production by tumor-host-derived but not normal splenic macrophages, and pure PGE_2 (10^{-6}M) but not $PGF_{2\alpha}$ (10^{-6}M) mimicked these suppressor effects. Another in vitro experiment also showed similar results that the hepatic NK cell activity was inhibited with the increase of PGE_2 concentration from 0 to 25ng/ml, but NK activity was enhanced with the increase of $PGF_{2\alpha}$ from 0 to 50ng/ml (29). Our experiment further verified these *in vivo* and *in vitro* findings, the present study showed greater PGE_2 and decreased NK activity in rats fed FB_1 and high fat diet, but the concentration of $PGF_{2\alpha}$ was similar in both FB_1-fed groups. This implied that PGE_2 overwhelmed the effect of $PGF_{2\alpha}$ in the group fed FB_1 and high fat diet, and caused the inhibition of NK activity in this group. It has been showed that PGE_2 activates adenylate cyclase with a subsequent rise in cyclic AMP (31), which acts as a "second messenger". Cyclic AMP itself is an inhibitor of lymphocyte activation (31, 32). The presence of receptors for PGE_1 and PGE_2 on the lymphocyte surface had been demonstrated, while there were no binding sites for PGA, $PGF_{1\alpha}$ or $PGF_{2\alpha}$. The relative potency of seven prostaglandins in inhibiting cytolytic activity correlated very well with their potency in stimulating cyclic AMP accumulation in lymphocytes: $E_1=E_2>A_1=A_2>F_{1\alpha}=F_{2\alpha}\approx 0$ (33). But effects of PGs on NK cells could be mediated in other ways. In the present study, we observed the NK activity per cell was similar between two groups, but the percentage of NK population paralleled the change of PGE_2 between two FB_1-fed groups, and the total NK cell number (Figure 5), after adjusting by their liver weight, was much lower in rats fed high fat diet and FB_1 than those fed low fat diet and FB_1. This suggested that total NK activity was much lower in rats fed high fat diet and FB_1 than those fed low fat diet and FB_1. The present study seemed to suggest that PGE_2 may play a role in the NK cell activity modulation, but in our previous study (8), we did not observe the inhibition of NK cell activity while the endogenous production of hepatic PGE_2 increased under effect of FB_1. Further study is needed to clarify the effect of PGE_2 on the NK cell activity during FB_1 carcinogenesis. It was noticed that percent of PGST (+) AHF in rats fed FB_1 and high fat diet was 2 fold of the rats fed FB_1 and low fat diet, and was 12 fold of the percent of PGST (+) AHF in the previous study (8). This suggested that key difference between these two studies seemed to be that tumor developed had progressed further in the present study, which had greater preneoplasms in the present study than the previous study (8). This was probably part due to feeding less FB_1 in the previous study (25ppm) than in the present study. A similar FB_1 dose/response was observed by Lu et al.(7). The suppression of NK activity were probably mediated significantly by the products of the preneoplasms than PGE_2, because PGE_2 was increased in both the studies whereas preoneoplasms were much greater in the present study than in the previous study. This implies that some other factors than PGE_2 produced by preneoplasms seem to be more important down-regulators of NK activity during development of carcinogenesis.

In our study, the accumulation of Sa and increase of Sa/So ratio agreed with previous observations that animals exposed to FB_1 feeding increased Sa and altered Sa/So (34). Sphinganine is a known inhibitor of protein kinase C (PKC) (35), and PKC is considered to play an essential role in the lytic mechanism of NK cell-mediated cytolysis (36). We hypothesized that accumulated sphinganine might inhibit the NK activity through inhibition of the PKC. In the present study, although we observed change of total NK cell activity between the groups fed FB_1 and different dietary fat, we did not see the difference of Sa and the ratio of Sa/So between these two groups. Thus Sa might have played some role in the inhibition of NK activity, but Sa did not play a role in the difference of total NK activity between groups fed FB_1 and differing dietary fat contents.

The same number of animals (5/6) developed neoplasia in two FB_1 treatment groups, but the average area of PGST-positive AHF in rats fed a high fat diet was greater than in the rats fed a low fat diet. There was no difference in the area of GGT-positive AHF between the two groups fed FB_1. It is hard to explain why PGST (+) foci were stimulated by high fat diet, but not GGT (+) foci. It had been observed that when female F344/N rats dosed with diethylnitrosamine (DEN) 24 h after partial hepatectomy were treated with the promoting agents, phenobarbital (PB) or 3,4,7,8-tetrachlorodibenzo-p-dioxin (TCDD), or the peroxisome proliferating agent, WY 14,643, for 6 months, PGST scored more foci in all groups than GGT (37). The greater area of PGST-positive AHF indicated that the group fed a high fat diet and FB_1 had more preneoplasia than did rats fed low dietary fat and FB_1. Birt et al., (38) compared diets of different fat content and composition during pancreatic carcinogenesis. Pancreatic cancer was induced with N-nitrosobis(2-oxopropyl)amine (BOP) 20 mg /kg body wt, hamsters were assigned to one of five diet treatments: (i) 4.3% corn oil (control); (ii) 20.5% corn oil (high corn oil); (iii) 0.5% corn oil + 3.8% beef tallow (low beef tallow); (iv) 0.6% corn oil + 19.9% beef tallow (high beef tallow); and (v) 5.1% corn oil + 15.4% beef tallow (high fat mixture). These diets were fed until the study ended 84 weeks after BOP treatment. Hamsters were pair fed to consume the same calorie allotment as the control corn oil group. Pancreatic adenoma incidence and multiplicity (no./effective animal) were higher in hamsters fed beef tallow than in those fed corn oil diets. Carcinoma in situ multiplicity was elevated in hamsters fed high-fat diets irrespective of the nature of fat fed. Pancreatic adenocarcinoma multiplicity was elevated in hamsters fed the low- or high-beef tallow diets compared with the low- or high-corn oil diets. This study showed that high saturated fat (beef tallow) intake promoted the carcinogenesis when fed isocalorically compared with a lower fat diet. In our study, it is possible that increased fat intake promoted that carcinogenesis not only by increased energy intake, but that the fat per se promoted FB_1 carcinogenesis by increasing the cholesterol concentration. It has been suggested that increased cholesterol concentration correlated with the development of neoplasia (39). Gregg et al. (40) measured GGT (+) AHF and 3-hydroxy-3-methyl glutaryl CoA reductase (HMG) activity in rat liver after treatment with DEN, phenobarbital and partial hepatectomy. Increased [^{14}C] acetate incorporation into cholesterol and HMG reductase activity were associated with high levels of gamma-glutamyl transpeptidase and foci formation. Elevated cholesterol levels and enhanced cholesterogenesis were consistent observations in proliferating normal, preneoplastic and neoplastic cells (41). In addition, the cholesterol biosynthetic pathway and the hexose monophosphate pathway were found to be stimulated in proliferating cells. In deed, the NADPH generated by this pathway is presumed to be needed for cholesterol and DNA synthesis and, in addition, the pentose phosphates generated by this pathway are utilized for DNA synthesis. Based on this evidence, it has been suggested that cholesterol and DNA synthesis are linked (42), but the exact nature of this linkage is not established. Perhaps some intermediates of the cholesterol synthesis pathway are mediators of DNA synthesis (43)

Increased caloric consumption instead of the high fat intake per se has also been shown to enhance the development of tumors. In carcinogen-treated rats, reducing energy consumption by 25% or as little as 12% of that consumed ad libitum-fed control negate the significant mammary tumor stimulation of a high-fat diet (44, 45). Furthermore, carcinogen-treated rats fed low- and high-fat diets and restricted in food consumption to a level equivalent to that consumed by the animals consuming the least amount of food showed no differences in mammary tumor development (46). All these studies suggested that reducing caloric intake strongly inhibited carcinogenesis. In our present study, because animals were fed *ad libitum*, the rats fed the high dietary fat also consumed more calories. Overall, this study showed that the consumption of a high-fat/high-calorie diet increased FB_1 carcinogenesis compared to the consumption of a low-fat/low-calorie diet. Greater hepatic area occupied by PGST-positive AHF in rats fed FB_1 and high fat might be caused by the enhancement of focal lesion DNA synthesis and inhibition of apoptosis. Mice were initiated by DEN (35 mg DEN/kg body weight injected intraperitoneally, twice per week for 8 weeks), then placed into four groups: NIH-07 control diet/no PB (group 1); NIH-07 diet/500 mg PB per liter of drinking water (group 2); diet restricted NIH-07 diet/no PB (group 3); and diet restricted NIH-7 diet/ 500 mg PB per liter of drinking water (group 4) (47). The unrestricted group with PB in drinking water had enhanced hepatic focal lesion volume and number compared with the control group. Mice fed PB and an unrestricted diet had inhibited apoptosis in normal and focal hepatocytes compared with the control group. In contrast, mice fed PB and a restricted diet exhibited a significantly lower focal lesion volume and number compared with mice given PB and unrestricted diet. Restricted mice did not show inhibition of focal apoptosis, in fact, the incidence of focal apoptosis was increased in these mice compared with unrestricted mice. These results suggest that inhibition of focal lesion DNA synthesis and enhancement of apoptosis may be a mechanism for the inhibition of cancer by the diet restriction.

In summary our study suggested that high fat/high caloric intake enhanced FB_1 carcinogenesis. Inhibition of total NK activity paralleled the development of PGST-positive preneoplasia (highly correlated, data now shown). Sphinganine and $PGF_{2\alpha}$did not seem to modulating NK cell activity, because they did not differ between the group fed high fat and FB_1 and the group fed low fat and FB_1. Hepatic PGE_2 might be one of the factors modulating NK activity in the present study (highly correlated, data not shown), because the change of NK activity came along with the change of PGE_2 in the present study. In our previous study, we did not observe the NK activity change along with increased PGE_2. The key difference between the present study and previous study was that much greater preneoplasia in the present study. This implied that some other factors might be important down-regulators of NK activity.

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Table 1. The rats fed FB$_1$ had significantly lower final body weight than the rats fed high fat diet. Average daily food consumption was similar among four treatments at any time point. The daily total energy intake was greater in rats fed high fat diet.

		Food Consumption, g/day/animal			Energy intake Kcal/animal /day		
Diet group	Final BW	Week 5	Week 7	Week 9	Week 5	Week7	Week 9
Low fat	142 ± 4 b	11.5 ± 2	12.5 ± 2	13.3 ± 3	48 ± 5 c	50 ± 5 b	51 ± 5 b
High fat	147 ± 5 b	10.6 ± 2	13.2 ± 2	12.8 ± 2	54 ± 6 b	62 ± 6 a	60 ± 4 a
Low fat/FB1	137 ± 4 a	11.4 ±2	12.5 ± 3	12.2 ± 2	44 ± 4 c	47 ± 6 b	48 ± 5 b
High fat/FB1	139 ± 4 a	12.5 ±2	11.5 ±2	12.5 ± 3	60 ± 4 a	57 ± 4 a	62 ± 7 a

Comparison of the final body weight, average daily food consumption, and daily total energy intake at 5, 7, and 9 weeks of age. The data entries in the table were expressed as mean ± standard error of mean (SEM). Treatments with different letters were significantly different.

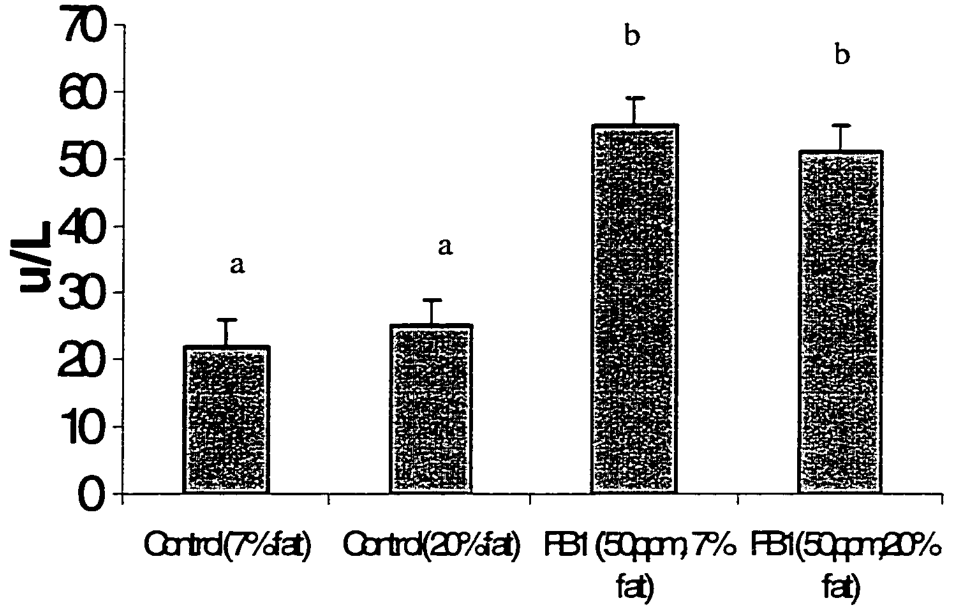

Figure 1: Comparison of the alanine aminotransferase (ALT) activity at 9 weeks of age, both the rats fed 50ppm FB_1 and low or high-fat diet showed significantly greater ALT activity as compared with two control groups. No difference was found between two control group, and no difference was found between two FB_1 treatment group. Vaules are means ± SEM. Treatments with different letters were significantly different.

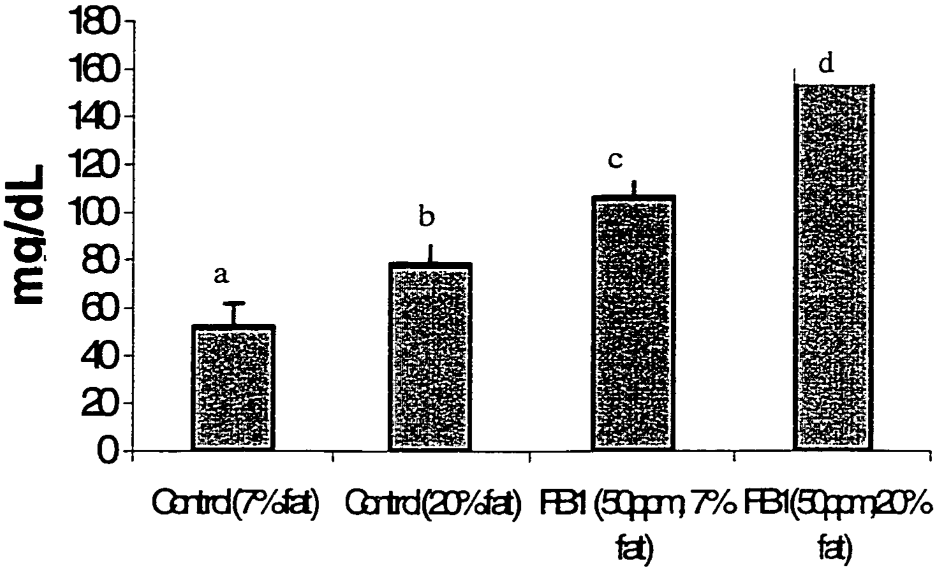

Figure 2: Comparison of the total plasma cholesterol concentration at 9 weeks of age.The two FB_1 treatment group and high diet control group exhibited significantly greater cholesterol concentration as compared with control group, and two FB_1 treatment group exhibited significantly greater cholesterol concentration as compared with high fat control group, and the group fed 50ppm FB_1 and high fat had greater amount of cholesterol than the group fed 50ppm FB_1 and low fat diet. The error bar represented the SEM. Treatments with different letters were significantly different.

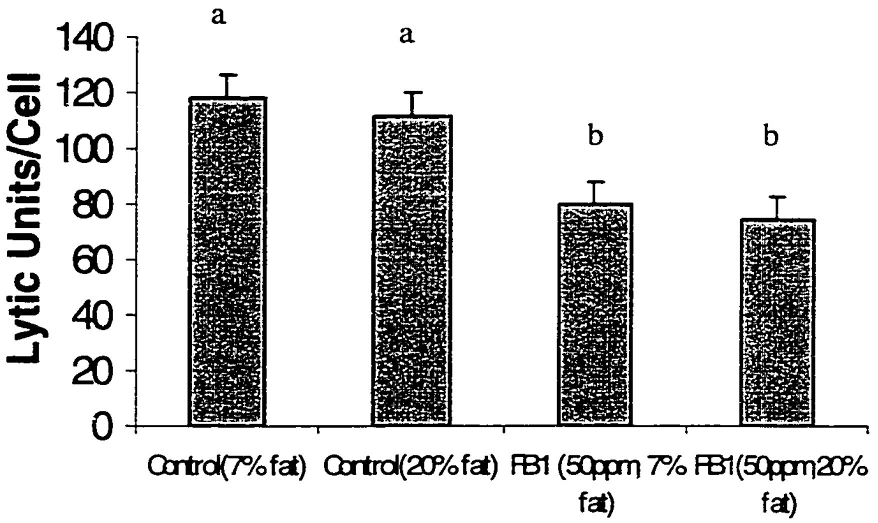

Figure 3: Comparison of hepatic NK activity at 9 weeks of age by adjusting with the percentage of NKR-P1^{bright} mononuclear cells. Activity was expressed as lytic units/per cell, calculated from the specific lysis curve. The rats fed FB$_1$ and high or low fat diet exhibited significantly lower NK activity as compared with the two control group. No difference was found between two FB$_1$ treatments, and no difference was found between two control groups. The error bar represented the SEM. Treatments with different letters were significantly different.

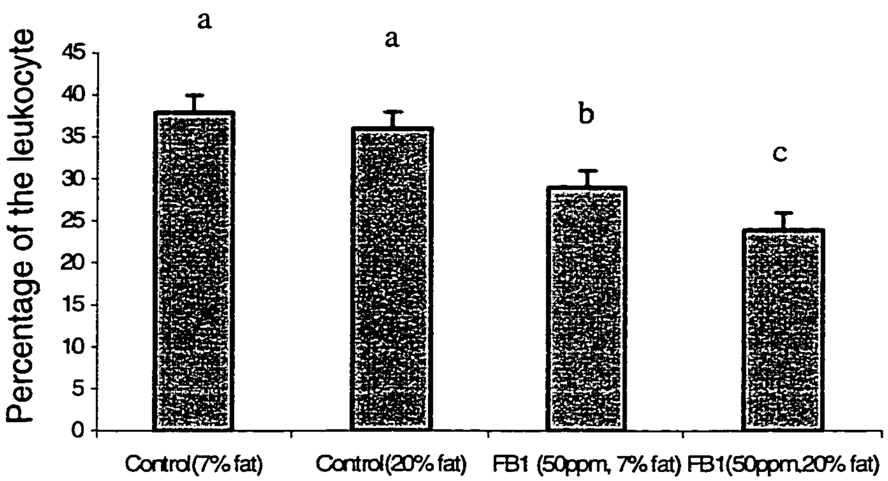

Figure 4: Comparison of the percentage of hepatic NKR-P1^{bright} mononuclear cells at 9 weeks of age. Both the rats fed 50ppm FB$_1$ and low or high fat diet exhibited significantly lower NKR-P1^{bright} percentage as compared with two control groups. No difference was found between two control groups, the group fed 50ppm and high fat diet had significantly lower NKR-P1^{bright} percentage than the group fed 50ppm FB$_1$ and low fat diet. The error bar represented the SEM. Treatments with different letters were significantly different.

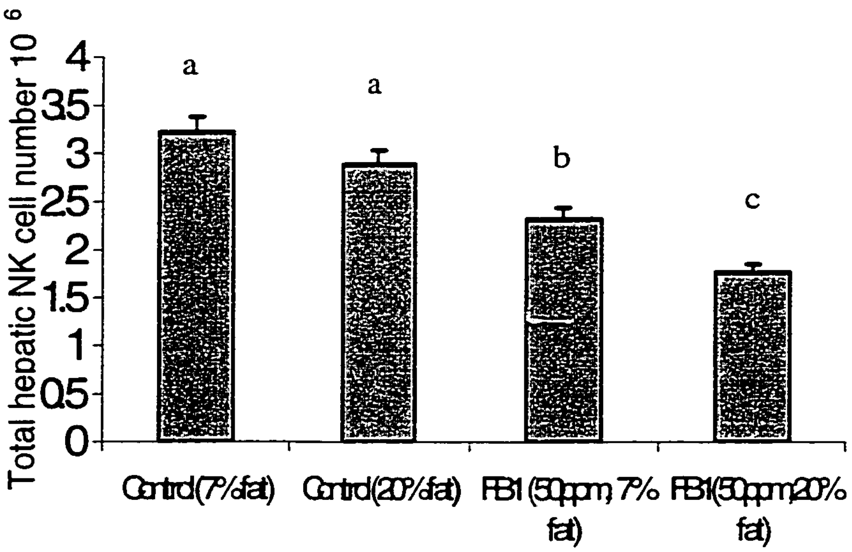

Figure 5. Comparison of the total hepatic NK cell number at 9 weeks of age. Both the rats fed low or high fat diet and 50ppm FB$_1$ and exhibited significantly lower total NK cell numbers compared with either control groups. No difference was found between two control groups, the rats fed high fat diet and 50ppm FB$_1$ had significantly lower total hepatic NK cell numbers than the rats fed 50ppm FB$_1$ and low fat diet. The error bar represented the SEM. Treatments with different letters were significantly deferent.

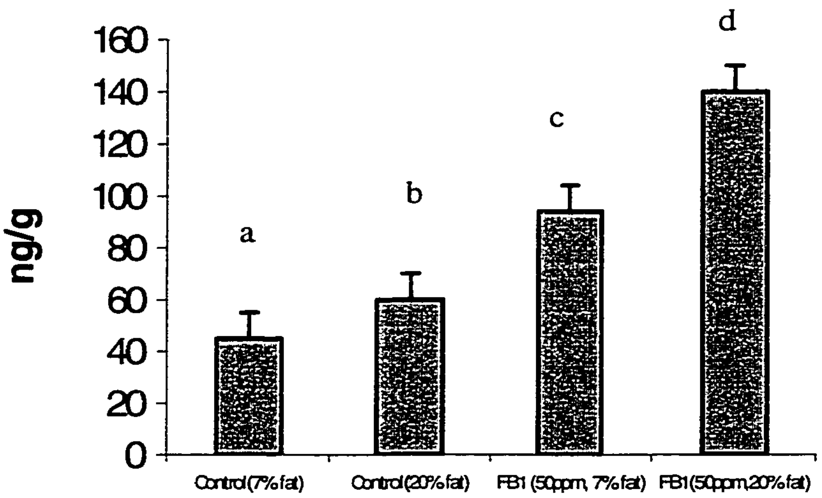

Figure 6: Comparison of the hepatic PGE_2 concentration in four treatment groups at 9 weeks of age. The concentration of PGE_2 is expressed as ng per gram liver tissue. Both FB_1 treatment group and high fat control groups had greater hepatic PGE_2 than low fat control group, two FB_1 treatment groups had greater hepatic PGE_2 than high fat control group, and the group fed $FB_1$and high fat diet had greater hepatic PGE_2 than the group fed 50ppm FB_1 and low fat diet. The error bar represents the SEM. Treatments with different letters were significantly different.

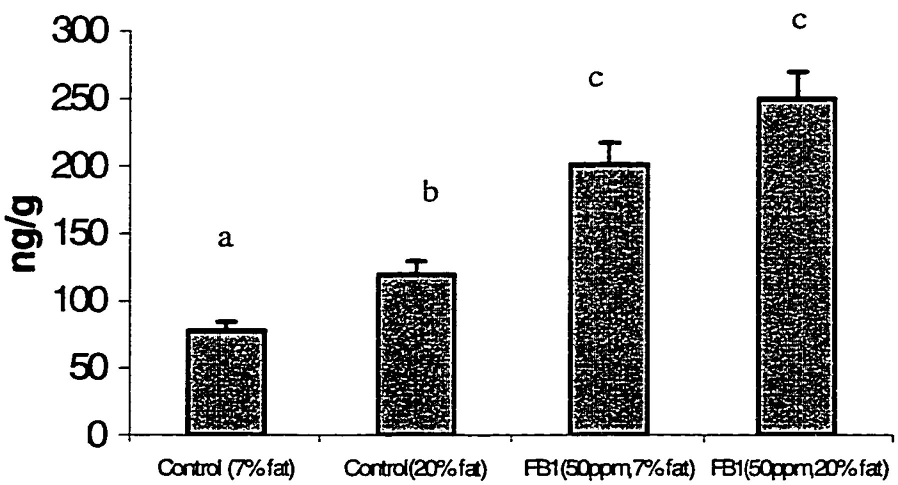

Figure 7 : Comparison of the hepatic $PGF_{2\alpha}$ concentration in four groups at 9 weeks of age. The concentration of hepatic $PGF_{2\alpha}$ was expressed as ng per g liver tissure. Both FB_1 treatment and high fat control groups had greater hepatic $PGF_{2\alpha}$ than low fat control group, and the group fed 50ppm FB_1 and high fat diet had greater hepatic $PGF_{2\alpha}$ than group fed 50ppm FB_1 and low fat diet and the high fat control group. There was no difference between the group fed 50ppm FB_1 and low fat diet and the high fat control group. The error bar represented the SEM. Treatments with different letters were significantly different.

Table2. The rats fed FB_1 developed PGST- and GGT- positive AHF.

Treatment	#of rats with PGST AHF	% PGST Area	# of rats with GGT AHF	% GGT Area
Low fat	0/6	0	0/6	0
High fat	0/6	0	0/6	0
Low fat / FB1	5/6	3.8 ± 1.3 b	4/6	1.4 ± 0.9 b
High fat / FB1	5/6	6.1 ± 1.7 a	5/6	1.3 ± 0.8 a

Placental glutathione S-transferase (PGST) and γ-glutamyl transferase (GGT)-positive altered hepatic foci (AHF). The AHF occurred only in two FB_1 treatment groups at 9 weeks of age. The average area of PGST-positive AHF was significantly greater in the group fed 50ppm FB_1 and high fat diet than that fed 50ppm FB_1 and low fat diet, no difference was found for the GGT-positive AHF between two FB_1 treatment group. The data entries in the table were expressed as mean ± standard error of mean (SEM). Treatments with different letters were significantly different.

Table 3. The rats fed FB1 had greater hepatic sphinganine (nmol/g) and Sa/S ratio Than two control groups

Treatment	Sphingosine (So)	Sphinganine (Sa)	Sa/So
Low fat	45 ± 4	9 ± 2 b	0.12±0.1 b
High fat	57± 6	8 ± 2 b	0.12±0.1 b
Low fat / FB1	63± 7	13 ± 3 a	0.21±0.2 a
High fat / FB1	75 ± 11	15 ± 5 a	0.28 ±0.2 a

Comparison of sphingosine (nmol/g) (So), sphinganine (nmol/g) (Sa) as well as the ratio of Sa/So at three time points. The concentration of the sphingolipids was expressed as nmol per g liver tissue. At 9 weeks of age, the hepatic So were similar among four groups. The rats fed 50ppm FB_1 diet exhibited greater Sa concentration and increased ratio of Sa/So as compared with two control groups, and the group fed 50ppm FB_1 had greater Sa and Sa/So ratio than the group fed 50ppm FB_1 and low fat diet. The Sa, So and Sa/So ration were not different between two control groups. The data entries in the table were expressed as mean ± standard error of mean (SEM). Treatments with different letters were significantly different.

GENERAL CONCLUSION

Natural killer cells are known to help the body fight cancer metastasis and certain viral infections. Their role at earlier stages in carcinogenesis is uncertain. Our studies showed that the development of fumonisin-promoted neoplasia in rat liver was accompanied by suppression of liver-associated NK activity and increased liver prostaglandin PGE_2 levels. Unknown factors other than PGE_2 produced by preneoplasms seemed to be more important down-regulators of NK activity as they occurred during development of carcinogenesis, because we did not observe inhibition of NK activity in a previous study, but we did see the inhibition of NK activity in the present and other studies, and the hepatic PGE_2 increased to a similar extent in all studies. 5 out 6 rats developed AHF in the study showing the inhibition of NK activity, but only half the animals developed AHF in the study that did not show inhibition of NK activity, and the area of AHF was much greater in the former study than the latter study. This suggested that the inhibition of NK activity paralleled the development of preneoplasia. The down-regulation of NK activity might be a signal or biomarker of developing preneoplasia. This could potentially be used for human cancer diagnosis, and might permit easier and earlier cancer screening.

Previous studies had suggested that Sprague-Dawley rats were much less susceptible to fumonisin promotion of carcinogenenesis than F344. The comparison of immune function between F344/N and SD rats suggested that SD rats would experience greater total liver-associated NK activity may partly explain their lesser cancer susceptibility than do F344/N rats under the condition of fumonisin promotion of carcinogenenesis. *In vitro* observation that PGE_2 and $PGF_{2\alpha}$ had opposite effect of on NK activity respectively, may suggest that these two prostaglandins can negate the effect of each other on NK cell *in viv*. Our latest study suggested that increasing PGE_2 may overwhelm effect of $PGF_{2\alpha}$, causing net suppression of NK activity.

Reacting FB_1 with glucose eliminated FB_1 toxicity as reflected in plasma total cholesterol concentration, ALT activity, development of GGT- and PGST-positive AHF , concentration of endogenous hepatic PGE_2 the accumulation of Sa, as well as the ratio of Sa/ So. This work also suggested that fumonisin-induced increased PGE_2 did not inhibit liver associated NK activity, although increased PGE_2 accompanied fumonisin promotion of carcinogenesis. The histopathology examination indicated that the tumor development stayed at the preneoplasia stage.

Greater dietary fat and energy intake can promote FB_1 carcinogenesis, and caused inhibition of NK cell activity. The findings suggested that high fat and greater energy consumption promoted FB_1 carcinogenesis in comparison with the low fat diet. The inhibition of total NK activity paralleled the production of PGE_2 and the development of preneoplasia. Increased PGE_2 , and some other factors produced during further development of preneoplasia may be crucial down-regulators of hepatic NK activity.

Future studies are needed to understand the mechanism of FB_1 hepatotoxiciy and hepatocarcinogenesis, as well as the role of NK cell during the hepatocarcinogenesis. The immunomodulation factors during the development of preneoplasia as well as the effect of PGE_2 on the NK cell activity during carcinogenesis are needed to be investigated too.

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