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by

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GENERAL INTRODUCTION

Mycotoxins are fungal metabolites that possess diverse properties which could be used in fermentation, food flavorings, and antibiotics. Some mycotoxins cause plant, human, and animal diseases. Thus, their control is of considerable economic and social importance. Mycotoxins are ubiquitous in nature, with their contamination of foodstuffs recognized for decades as a problem. "Turkey X" disease caused by aflatoxin B_1 (AFB_1), a mycotoxin produced by *A. flavus*, was first reported about 30 years ago (Sargeant et al., 1961); AFB_1 is a potent natural hepatocarcinogen. Fumonisins, mycotoxins from *Fusarium moniliforme* and *Fusarium proliferatum*, are also gaining reputation as other natural hepatocarcinogens, but their mode of action has yet to be determined.

Fumonisins have been implicated in disease outbreaks causing brain disease in horses in the U.S.A. (Marasas et al., 1988). They are present in high concentrations in maize used as a staple food in the Transkei, South Africa, an area characterized by one of the world's highest rates of human esophageal cancer (Sydenham et al., 1990). Successful isolation and characterization of fumonisins has made it possible to determine their possible health effects on human and animals. Most research has shown that fumonisins are

hepatotoxic as well as hepatocarcinogenic to male rats (Gelderblom et al., 1991). However, it has not been established that this also applies to female rats. The gender difference is important in carcinogenesis because there is often a sex-dependence in the ability of chemicals to induce cancer (Deml and Oesterle, 1982). Thus, this research will examine hepatocarcinogenesis in females due to *F. proliferatum* mycotoxins. Numerous preneoplastic markers of enzyme altered foci (EAF), sometimes referred to as altered hepatic foci (AHF), are used in carcinogenesis to quantify the effects of initiating or promoting agents. So far, only one marker viz., γ-glutamyl transferase (GGT), has been used to quantify the effects of fumonisins as hepatocarcinogens. The second objective of this research is to study the hepatocarcinogenicity of fumonisin B1 using placental glutathione S-transferase (PGST) marker.

There is a positive relationship between carcinogenic and teratogenic chemicals and, since FB_1 is a carcinogen in rat liver, it may also be a teratogen. Aflatoxin B_1 is a hepatocarcinogen as well as a teratogen in a variety of animal species. The third objective is to determine the teratogenic potential of FB_1 in rats.

Research is still lacking in various areas such as the metabolism, biosynthesis and mode of action of fumonisins. But in order to address these questions it is imperative to

develop a suitable substrate medium that will allow production of labelled fumonisins in significant quantity. Fumonisin production from a solid substrate (corn) is very easy, but the isolation and purification processes are tedious and expensive. Therefore, it is important to develop a liquid medium that would support production of efficiently labelled fumonisins. Thus, the fourth objective of this research is to develop a liquid medium that would produce fumonisins in sufficient quantities that could be used for metabolism studies.

Explanation of dissertation format

This dissertation is composed of three papers. The first two are modified manuscripts formatted for publication in *Carcinogenesis* and *Journal of Food and Chemical Toxicology*, respectively. The third paper will also be submitted to a technical journal as yet undetermined. Each paper has its own abstract, introduction, material and methods, results, discussion and bibliography. Following the three papers is a general summary of the entire dissertation. References cited for the general introduction and literature review follow the general summary.

LITERATURE REVIEW

Mycotoxins

Mycotoxins are secondary metabolites produced by fungi. They are produced by a large number of fungi contaminating diverse foods and commodities. *Aspergillus, Fusarium* and *Penicillium* genera are the major mycotoxin-producing fungi that contaminate foods. Stress factors such as water, temperature, and insect damage of the host plant determine the fungal infestation and ultimate production of the mycotoxin. Mycotoxins have chemically diverse structures, thus their biological activities differ greatly. One fungal species can produce a variety of chemically diverse mycotoxins, e.g., moniliformin, fumonisins, fusarin, etc., are produced by *F. moniliforme* and *F. proliferatum*. A particular mycotoxin might also be produced by a variety of species or genera, e.g., tricothecenes are produced by *Fusarium, Cephalosporium, Myrothecium, Stachybotrys* and *Trichoderma*. Mycotoxins can display specific organ toxicity (e.g., ochratoxin A - a nephrotoxin), and can be mutagenic, carcinogenic and/or teratogenic (CAST, 1989).

Mycotoxin-associated diseases have long been known to occur. Ergotism, caused by a toxin from *Claviceps purpurea,* a fungus that contaminated rye, was the earliest disease

reported. Disease epidemics such as alimentary toxic aleukia (ATA), caused by tricothecenes from *Fusarium*, occurred around World War II in the Soviet Union (Marasas and Nelson, 1987). Several mycotoxins considered to be of human or animal health concern are cited in the literature (CAST, 1989; Marasas and Nelson, 1987). Aflatoxins are the most studied and the only regulated mycotoxins in the U.S.A. Fumonisins, mycotoxins isolated from *F. moniliforme* and *F. proliferatum,* are newly described mycotoxins (Gelderblom et al., 1988a). Because of their predominance in several food commodities such as corn, sorghum, and millet (Nelson et al., 1991), and their toxic effects on animals as well as possible health risks to human, the Food and Drug Administration-USDA Interagency Working Group on Fumonisins is considering their regulation in the near future (Hansen, 1993). Although clear-cut toxicity of many mycotoxins to humans has not been demonstrated, the effects of these compounds on experimental animals and *in vitro* leaves little doubt about their potential toxicity to humans.

Mycotoxins as teratogens

There is sparse evidence concerning the toxicity of mycotoxins to embryonic development because most research has been done on adult animals. The importance of mycotoxins in reproduction may be indirect; i.e., they may reduce fertility

by having effects on parental gametogenesis. For example, zearalenone, a metabolite of *Fusarium roseum*, induces feminization syndrome in male pigs and hyperestrogenism in female pigs (Hayes, 1981). Mycotoxins may also affect intrauterine development by causing toxic effects on the vital maternal organs such as liver, kidneys and the immune system, thus impairing maternal health. Invariably, poor maternal health may result in inefficient nutrient transfer, and increased xenobiotic transfer to the developing fetus (Abramovici, 1977). Depending on the metabolism, mycotoxins may be transferred as electrophilic metabolites after detoxification by the mother, thus impairing fetal development (Abramovici, 1977). Depending on the dosage and gestation period, mycotoxins may also impair fetal development by inducing abortions or inducing variable malformations in the fetuses.

Teratogenic effects of other *Fusarium* mycotoxins besides zearalenone have been documented in experimental and farm animals. Fusarenon-X fed to mice at 5, 10 or 20 ppm during gestation resulted in stunting and abortions either as a single or chronic dose (Hayes, 1981). Mycotoxins from *Aspergillus* and *Penicillium* genera have also been shown to be teratogenic. Ochratoxin A (OA) was shown to cause mainly craniofacial anomalies, as well as other skeletal malformations (Arora & Frolen, 1981). Aflatoxin B_1 has also

been found to cause exencephaly, open eyes and protrusion of eyes in mice when dosed on day 7 of gestation (Arora et al., 1981). Aflatoxin B_1 has also been reported to cause fetal growth retardation when given to hamsters on day 8 of gestation by i.p. injection at 4 or 6 mg/kg (Schmidt & Panciera, 1980).

Mycotoxins as carcinogens

Carcinogenic mycotoxins, like chemical carcinogens, are biotransformed to DNA-adducts which ultimately result in genetic alterations. However, some carcinogens may not be genotoxic but are still capable of inducing neoplasia. Most mutagenic mycotoxins have also been found to be carcinogenic. Among carcinogenic mycotoxins, AFB_1 has been studied extensively (Stark, 1980). Aflatoxin B_1 causes liver cancer in rats and trout at 20 ppb in diet fed for 12 months. There is also a greater than average prevalence of liver cell cancer (LCC) in humans in some Asian and African populations where high levels of AFB (3 - 222 ng/kg body weight) are ingested in foods. There is a positive association between high intakes of aflatoxins and incidence of LCC in these populations (CAST, 1989). Carcinogenicity of other toxins from *Fusaria* species such as trichothecenes is still questionable (Wilson, 1982).

The relationship between carcinogenesis and teratogenesis has been established for a variety of chemicals. The

relationship between the two processes is that they both involve rapid cell growth, therefore somatic cell division is a prerequisite for both. The critical distinguishing feature is that cancerous growth lacks organization while embryonic growth is well organized with each primordial cell destined to a particular function (Di Paolo and Kotin, 1966). Aflatoxin B1 and ochratoxin A are both teratogenic and carcinogenic to rats (CAST, 1989).

Fusarium species

Fusarium moniliforme is a fungus placed under section *Liseola* of the genus *Fusarium*. Species included in the section *Liseola* are *F. moniliforme, F. proliferatum, F. subglutinans* and *F. anthophilum*. These species do not form chlamydospores, and are differentiated by the morphology of their macroconidia, such as shape (club or oval), and whether the conidia are borne in chains or false-heads (Nelson, 1992). *F. moniliforme* is a common corn contaminant causing corn ear rot and kernel infections. It can cause corn diseases by systemic contamination (soil borne) or contamination of corn kernels during silking (Nelson, 1992).

Fusarium moniliforme produces several metabolites among which fumonisins are the most predominant. Fusaric acid, produced by *F. moniliforme* and other *Fusaria*, affects plant cell respiration by inhibiting iron porphyrin oxidase (Nelson,

1992). Fusaric acid is also a chelator of metals such as copper, cobalt, and zinc (Bacon and Williamson, 1992). Fusarins are mutagenic metabolites of *F. moniliforme* which require activation by S-9 liver enzymes (Gelderblom et al., 1988b; Lu et al., 1988). They are also very unstable, thus they are found in minute amounts in nature (Thiel et al., 1986). Moniliformin is commonly produced by *F. proliferatum* and by very few isolates of *F. moniliforme* (Nelson, 1992). It is a highly toxic compound causing rapid death and intestinal and thymic hemorrhage when fed to rats at concentrations ranging from 3 - 8 mg/g of diet (Abbas et al., 1990).

Fumonisins are mycotoxins recently isolated from *Fusaria*. They are produced predominantly by *F. moniliforme* and to a lesser extent by *F. proliferatum* (Ross et al., 1990) and *F. nygamani* (Thiel et al., 1991a). Fumonisins have been isolated mostly in corn products including human food, and in lesser quantities in sorghum and millet (Nelson et al., 1991; Murphy et al. 1993; Thiel et al., 1992). They have been characterized as diesters of propane-1,2,3-tricarboxylic acid and either 2-acetylamino- or 2-amino-12,16-dimethyl-3,5,14,15-pentahydroxyicosane or its C-10 deoxy analogue. The first four fumonisins isolated were named fumonisin A1, A2, B1 and B2 (Bezuidenhout et al., 1988); fumonisins B3 and B4 have also been reported (Cawood et al., 1991; Plattner et al., 1992). Their structure is similar to the tomato host-specific toxin,

the AAL toxin, produced by *Alternaria alternata f.* sp. *lycopersici*. This mycotoxin lacks one of the tricarboxylic acid side chains (Shier, 1993). Fumonisin B1 is the most studied and it is produced in greater amounts than other fumonisins by most *F. moniliforme* strains.

Under laboratory conditions, isolates of *F. moniliforme* cultures producing fumonisins have been successfully grown on corn, a solid medium substrate. Levels of about 6.4 g FB_1/kg of corn were reported when the culture was grown for four weeks at room temperature (Nelson et al., 1991). Unsuccessful attempts have been made to produce high levels of FB_1 in liquid cultures. The highest levels of fumonisins (140 ppm) produced from liquid cultures were from *Alternaria alternata* isolates (Chen et al., 1992), whereas only 74 ppm and 53 ppm FB_1 have been produced from *F. moniliforme* isolates by Jackson and Bennett (1990) and Plattner and Shackelford (1992), respectively. Production of FB_1 in liquid cultures will significantly facilitate the purification of fumonisins.

Diseases resulting from fusarium mycotoxin infections, especially the trichothecenes, include chronic diseases such as eye, skin, subcutaneous and systemic infections (CAST, 1989). Animal diseases implicating fumonisins have been documented as far back as 1850 in the midwestern states of the U.S.A. Similar outbreaks occurred again in 1930 and from 1983-84 in the U.S.A. But it was only in the 1980s that

fumonisins were isolated and implicated in disease outbreaks in horses. They cause leucoencephalomalacia in horses (Keller et al., 1990, Thiel et al., 1991b), pulmonary edema in pigs (Osweiler et al., 1992; Colvin et al., 1993), hepatocarcinoma in rats (Gelderblom et al., 1991), rickets in chicks (Brown et al., 1992), and have been implicated as a possible agent causing esophageal cancer in humans in Southern Africa (Rheeder et al., 1992). The atherogenic potential of fumonisins has been reported in vervets fed 0.25 - 1.0% of *F. monilforme corn* cultures in a high protein and low fat diet for 2 years. The corn culture materail was also hepatotoxic to the vervets (Fincham et al., 1992). The liver seems to be the common organ affected in pigs, horses and rats, although development of liver cancer has been reported in rats only (Gelderblom et al., 1991). Some feeding studies have reported renal toxicity in rats fed corn naturally contaminated with *F. moniliforme* from confirmed cases of ELEM (Voss et al., 1989).

Fumonisins as hepatocarcinogens

Several models used for inducing cancer in the rat liver have been identified (Farber, 1984). Some of these models have been employed in establishing whether fumonisins are complete or incomplete carcinogens. Gelderblom and associates (1988a) were able to demonstrate that the methanol-water (CH_3OH-H_2O) fraction containing FB_1 fed for 4 weeks to male

rats, strain BD IX at 0.1% in diets for 1 week after DEN initiation induced significantly greater numbers of GGT-(+)AHF when compared to DEN initiated rats without FB_1. Cancer promoting potential of other strains of *F. moniliforme* from corn in the Transkei (South Africa) were assayed using the initiation--promotion regimen. *Fusarium moniliforme* strains MRC 826 and MRC 4319 were found to be the most hepatocarcinogenic, inducing the most GGT-(+)AHF (Gelderblom et al., 1988b).

Feeding purified FB_1 at 50 ppm incorporated into the diet for up to 26 months to BD IX male rats showed that FB_1 is a complete carcinogen. Gamma-glutamyl transferase GGT-(+)AHF were induced. Hepatocellular carcinomas as well as metastases to the heart, lungs, or kidneys were noted (Gelderblom et al., 1991). Chronic feeding of FB_1 without prior initiation showed that FB_1 is a complete carcinogen. But most complete carcinogens are also initiators. Subsequent research by Gelderblom et al. (1992) on the cancer-initiating potential of fumonisins showed that they are poor initiators. To prove this, they employed two regimens for multistage carcinogenesis. The first regimen involved continuous feeding of FB_1 at 0.1% in diets for 26 days, then exposure to a "selective agent" or promoter, namely, partial hepatectomy (PH), followed by 2-acetylaminofluorene (AAF) and/ or carbon tetrachloride (CCl_4) in the diet. The other regimen involved

administration of varying doses (50 - 200 mg/kg) of fumonisins either as single or multiple doses to partially hepactomized male Fischer rats. Two weeks later, the rats were exposed to 2AAF/CCl_4 and killed 2 weeks after the promoting treatment. Rats that were fed FB_1 for 26 days had greater number of GGT-(+)AHF than the rats fed basal diets only. Selection of these AHF with 2AAF/CCl_4 treatment after chronic exposure to FB_1, produced a two-fold increase in the number of GGT-(+)AHF and a 15 fold increase in the percentage area of foci/liver section compared to the rats fed FB for 26 days without PH nor 2AAF/CCl_4 treatment. Groups that were exposed to single or multiple doses of FB either before or after PH, had significantly lower number of GGT-(+) AHF than the diethylnitrosamine (DEN) positive control group, and the number of GGT-(+) foci were not significantly different from the negative control (group fed basal diet). Fumonisins failed to induce hyperplastic foci when fed as a single or discontinous dose indicating that fumonisins are poor cancer initiators, and are possibly promotors because neoplasia was induced with chronic feeding.

The carcinogenicity of other fumonisins, and some of their derivatives, have been evaluated using a short-term carcinogenesis assay. During 21 days of feeding 0.1% fumonisins and their derivatives in the diet, male Fischer rats fed FB_1, FB_2 and FB_3 lost weight, and had GGT-(+)AHF after

the selection treatment. The data indicated that FB_3 was more toxic than FB_2, while FB_2 was more toxic than FB_1. All other derivatives (AFs and APs) were not toxic (Gelderblom et al., 1993). Studies on the carcinogenicity of fumonisins indicate that fumonisins have both initiation and promotion capabilities when fed chronically.

The mechanism of cancer induction by fumonisins in rats still remains to be explained since fumonisins are neither mutagenic in the Ames *Salmonella* test (Gelderblom and Snyman, 1991) or genotoxic in the DNA repair assays in primary hepatocytes (Gelderblom et al., 1989). Moreover, they are poorly absorbed, and rapidly eliminated almost entirely in feces (Shephard et al., 1992). They have been found to alter sphingosine (SO)/sphinganine (SA) ratios in vitro (Wang et al., 1991). Alteration of SO/SA ratios in the liver, lungs and kidneys of pigs exposed to $\geq$23 ppm fumonisin B_1 in the diet has also been reported (Riley et al., 1993). Assay of serum levels of sphingosine and sphinganine in horses dosed with 15 - 44 μg/g FB_1 (Wang et al., 1992) and chicks dosed with 75 mg/kg FB_1 in diet (Weibking et al., 1993) indicated a change in these sphingolipids before the onset of the disease, hence the suggestion that the change in sphingosine-sphinganine ratios could be used as an early marker of fumonisins exposure in animals (Wang et al., 1992; Riley et al., 1993).

Normally, sphingosine inhibits protein kinase C (PKC), and thus modulates neoplastic transformation (Borek et al., 1991). Protein kinase C plays a role in signal transduction and cell growth (Hansen et al., 1990), but its role in multistage carcinogenesis has not been firmly established (Weinstein, 1988). PKC has receptors for tumor promotors such as phorbol esters, and sphingosine had been shown to block the tumor promoting effects of phorbol esters by inhibiting the activity of PKC (Borek et al., 1991). Fumonisins block ceramide synthetase, an enzyme involved in converting sphinganine to ceramide (Wang et al., 1991). Sphingosine is a product of ceramide breakdown, therefore, fumonisins may increase sphinganine and decrease sphingosine. Because serum SO/SA ratios were altered in pigs exposed to fumonisins before the onset of disease (Riley et al., (1993), the alteration of this ratio might be critical in cancer induction by FB. Seemingly, the alteration of SO/SA ratios interfere with the ability to inhibit tumor promotion. The interference of these modulators by fumonisins might indicate a mechanism by which fumonisins induce cancer. Fumonisins cause atherosclerosis in vervets, and their atherogenic plagues contain sphingolipids. Since FB's also inhibit PKC, they may provide templates for therapeutic agents in cancer and atherosclerosis treatments (Norred, 1993).

Another mechanism by which fumonisins induce different

diseases in different animals has been speculated to be by their less polar derivatives, namely; the hydrolyzed fumonisins or aminopolyol derivatives (HFB or AP) (Abbas et al., 1993). Fumonisins are water-soluble, therefore the lipophilic aminopolyols might be more toxic. *In vivo*, the hydrolysis of fumonisins might occur through the action of carboxyl esterases found in many tissues and subcellular fractions. The esterases will hydrolyze the diester bonds to release the two tricarboxylic side chains. The somewhat lipophilic molecule formed could then be taken up more readily by membranes than the parent compound. Structurally, the hydrolyzed fumonisins are more similar to the sphingosine or sphinganine structures than are the fumonisins. Therefore, the aminopolyols might also alter sphingolipid metabolism in the liver. The synthetic analog of FB_1 without the tricarboxylic side chains was as toxic as the parent analog in rhesus monkey renal cells, which suggests that HFB might be a toxic metabolite of fumonisins (Kraus et al., 1992). Increased toxicity of HFB has also been noticed in jimsonweed leaves and mammalian cell lines. Hydrolyzed FB_1 and FB_2 were cytotoxic to mammalian cell lines and more phytotoxic to jimsonweed leaves than the parent compounds (Abbas et al., 1993). A food survey on corn based products (Hopmans and Murphy, 1993) reported the presence of hydrolyzed FB_1 (HFB or AP) in masa, a corn based product that is treated with lime

(Sydenham et al., 1991). The presence of HFB in masa could be of human health concern since lime-treated *F. proliferatum* infected corn containing 10 ppm HFB_1 seemed to be at least as toxic as *F. proliferatum*-infected corn containing 50 ppm FB_1, with respect to cholesterol and glutamate pyruvate transaminase and incidence of neoplasia (Hendrich et al., 1993). A recent study on the comparative toxicity of fumonisins (B_1, B_2 and B_3) and N-acetyl derivatives of FB_1 and FB_1 (AF_1 and AF_2) as well as their respective aminopolyols (AP_1 and AP_2) indicate that the APs are toxic in cultured cells and not toxic *in vivo*. The AP or HFB showed greater cytotoxicity to rat hepatocytes (125 μM) than their parent compounds (FBs), while the N-acetyl derivativatives were the least toxic at 1000 μM. In contrast, weanling male Fischer rats that were fed 0.05 - 0.1% FB or the amonopolyols in diet for 21 days, followed by the 2-AAF/PH selection treatment 14 days after dietary exposure and 14 days before sacrifice lost more weight than the rats on aminopolyols (AP) diets and control diet (Gelderblom et al., 1993). The conflicting results concerning the toxicity of APs or HFB in vivo might be due to the fact that a corn culture material containing HFB was fed to rats (Hendrich et al., 1992) compared to feeding a purified toxin (Gelderblom et al., 1993), which suggest that there might be other unidentified components in the nixtamilized corn culture material responsible for the observed toxicity.

Carcinogenesis

Carcinogenesis is a multistage process involving initiation, promotion of initiated cells and progression to tumor formation. Several models have been developed in an attempt to study the different stages of tumorigenesis. The earliest models involved mouse skin tumorigenesis (Boutwell, 1974). Rat liver is also useful because it is highly perfused by chemicals and hepatocarcinogenesis is readily quantified by numerous histochemical assays of preneoplastic markers coupled with computerized stereology. Tumors induced by carcinogenic agents can either be genotoxic or epigenetic. Genotoxic compounds are mutagens and alter the DNA directly or may require metabolic activation to form electrophiles which will form DNA adducts. Therefore, genotoxic agents have initiating activity. In contrast, epigenetic carcinogens are non-mutagens and thus do not alter DNA directly. Their mechanisms as carcinogens are still incompletely understood. Carcinogens may also effect different stages of carcinogenesis, thus they maybe termed incomplete or complete carcinogens. Incomplete carcinogens are often initiators, while complete carcinogens have initiating, promoting (Miller and Miller, 1981) and progressing properties (Pitot et al., 1988a). There is also a dose-response for most carcinogens, which decreases with higher doses because of cell death (Iversen, 1988).

Although cancer development is a multistage process, it is not a continuous process involving a series of changes in a cohort of cells; rather, it is a discontinuous process involving the selection of "rare events" that occur in the cell. Normally, the initiated cells are repaired, but a "rare event" may prevent this natural repair process, resulting in altered cells. Another "rare event" occurs when a promoting agent is present, this event will selectively stimulate the growth of these altered cells. The next "rare event" stimulates the autonomous growth of this population of altered cells to form neoplasia. Since these processes depend on the occurrence of the "rare events", this might be the reason why cancer development tends to be a slow process (Farber, 1980). Sometimes the focal areas may develop within existing foci producing "nodules within nodules" or foci-within foci (Farber, 1973) however, this is an uncommon occurrence (Pitot et al., 1978).

Histopathological analysis of stages of cancer indicate that during initiation, lesions smaller than the liver lobule develop. These are altered foci which appear within one week of exposure to a carcinogen. These altered foci do not compress the adjacent liver tissue, although they are clearly demarcated and different biochemically from the normal tissue. The persistent altered foci develop into neoplastic nodules which are generally larger than the area of several nodules,

and elevated from the rest of the surrounding hepatocytes. These nodules lose some of the characteristic altered enzyme activities of the foci. The nodules progress to carcinomas that are benign (if genotypic alteration did not occur) or malignant (if genotypic alteration occurred). The benign lesions are called adenomas, while the cancerous ones are called hepatocellular carcinomas (Williams, 1980).

Initiation

Initiation, the first stage of carcinogenesis, is induced by any chemical that alters the genetic structure of the DNA and it is irreversible. The initiation stage can also occur spontaneously in dividing cells (Ward, 1983). Initiation can be induced by a necrogenic dose of a carcinogen (e.g., >30 mg/kg DEN). A single non-necrogenic dose of an initiator must be followed by a cell proliferator (e. g., CCl_4) or partial hepatectomy before the damaged cells undergo DNA repair. If a carcinogen is given after a cell proliferator or PH, then initiation induction fails (Scherer and Emmelot, 1975). The number of focal lesions or enzyme-altered hepatic foci are used to quantify this stage (Pitot et al., 1988a). Initially, these islands of altered hepatocytes grow at the same pace as the unaltered hepatocytes, during promotion they are selected to proliferate faster. Quantitation of these altered foci is by biochemical markers which either indicate the loss of or

the increased activity of enzymes or cell components in the altered cells (Farber, 1980).

The stability and heterogeneity of these markers has been reported by several scholars. For example, Pitot and associates (1978) demonstrated the phenotypic heterogeneity of enzyme-altered hepatic foci (AHF) in rats treated with diethylnitrosamine at 10 mg/kg after 24 hour partial hepatectomy, followed by a diet containing 0.5% phenobarbital (PB) eight weeks after DEN initiation. Rats which received this treatment had twice the relative proportion of foci exhibiting glucose-6-phosphatase (G-6-P), canalicular ATPase (ATPase) and gamma glutamyl transferase (GGT) compared to the controls, indicating the heterogeneity of the markers.

The stability of enzyme-AHF markers has been demonstrated in several laboratories (Goldsworthy and Pitot, 1985; Farber, 1980). Rats were treated according to the Pitot et al., (1978) regimen described above, except that some rats were given PB-free diet for 6 months after 3 or 4 months of the treatment. Removal of PB for 6 months did not have a significant effect on the presence of all three markers (G-6-P, ATPase, GGT) demonstrating the stability or persistence of the markers. GGT scored the vast majority of markers at all times (Goldsworthy and Pitot, 1985). Despite the fact that these markers are persistent, they can revert depending on the time of exposure to promotion agent after initiation. Xu et

al., (1990) using GGT, PGST, ATPase and G-6-Pase markers, demonstrated that rats exposed to a promoting agent within 1 day to 2 months after initiation did not show any variability in the volume percentage of the number of AHF, but an 11 month delay between initiation and promotion resulted in 20% fewer AHF. Therefore, initiation induction depends on the dose and time of initiation. High doses of the carcinogen cause cell death, and non-necrogenic doses must be coupled with partial hepatectomy within 24 hours of exposure to avoid the natural repair process of the initiated (mutated) cells depending on the age of the rats. By taking advantage of the proliferative nature of cells in growing animals, initiation can be induced by a non-necrogenic dose without partial hepatectomy in female and male rats at a few days of age (Peraino et al., 1981).

Placental glutathione S-transferase is also an accurate marker for different stages of carcinogenesis, because very small foci were detected 48 hr after a single dose of diethylnitrosamine (10 - 80 mg/kg body weight) in 6 weeks old male Fischer rats (Moore et al., 1987).

Promotion

Promotion, the second stage of carcinogenesis, is induced by any substance that alters the genetic expression of information within the cell to stimulate growth of initiated cells. Promotion is the stage where the initiated cells are

selected for preferential growth; i.e., the initiated cells are selected to grow under conditions where normal cell growth may actually be inhibited (Farber, 1980). This stage is partially reversible and requires continuous exposure to the promoting agent. Promoting agents are effective only if given after an initiator and not alone (Miller and Miller, 1981). Unlike initiators, promoting agents exhibit a no-effect level and a maximal dose response in that any dose of promotor has the same effect on the promotion of initiated cells (Pitot, 1990; Pitot and Sirica, 1980). The promotion stage has been demonstrated by Pitot protocol where a single dose of DEN was given to rats 24 hour after partial hepatectomy (PH), and 2 months later the rats were fed diets containing 0.5% PB for 6 months. The number of AHF was increased 5 times in PB fed rats. Some rats developed hepatocellular carcinomas (Pitot et al., 1978). Using a similar protocol except feeding PB diet 1 week after DEN, and withdrawing and re-administering PB at certain intervals, Hendrich and associates were able to demonstrate the reversible nature of the promotion stage (Hendrich et al., 1986).

The mechanisms of promotors during carcinogenesis has been hypothesized as being through "differential inhibition, differential stimulation or differential recovery" of initiated cells. The differential inhibition involves rapid growth of initiated cells due to the promotor, while

differential stimulation is the selective growth stimulation of altered cells. The third mechanism could be due to the inability of the initiated cells to recover; thus, in the presence of a promotor, these cells revert to the proliferating stage resulting in papillomas as in skin tumorigenesis (Farber, 1980).

Progression

Progression, the ultimate stage of carcinogenesis, is irreversible, and characterized by benign and/or malignant tumors. Agents that induce progression act on promoted cells to advance the initiated cells to neoplastic cells (Pitot et al., 1988a).

Models of hepatocarcinogenesis

Induction of liver tumors in the rats has been used as a model of cancer research since 1932 (Yoshida, 1932). Numerous models have been developed to investigate the mechanisms of neoplastic induction in rat liver. These models are based on the identification and characterization of preneoplastic lesions, especially altered hepatic foci (AHF), which precede hepatic adenomas and carcinomas (Goldsworthy et al., 1986). Variability in these models may be influenced in part by diet (Glauert and Pitot, 1986), age, sex and strain of the animal (Xu et al., 1990). A review by Goldsworthy and associates

(1986) places the different models in categories based on whether they prove initiation and/or promotion. The end stage of the models is the presence of AHF or obviously neoplastic cells.

Historically, the earliest demonstration of the different stages of carcinogenicity were conducted by Sasaki and Yoshida (1935). Long term exposure of rats to carcinogens resulted in death or tumors. Removal of the carcinogen at certain periods stopped the progression of tumor to cancer (Rabes et al., 1972). Other models are now available for analyzing stages of rat liver carcinogenesis. All the models involve a brief exposure to the initiating carcinogen, either in very young animals in which the liver is proliferating (Peraino et al., 1984), or in partially hepatectomized livers of adult rats (Scherer and Emmelot, 1975; Solt and Farber, 1976). In these models, promotion is stimulated by a brief or prolonged exposure to a chemical or an environment that selects the initiated cells.

Enzyme-altered hepatic foci (AHF)

Enzyme altered hepatic foci are of clonal origin and used as markers of preneoplasia. These markers represent either a decrease or increase in a cell component or enzyme activity of initiated cells which indicate metabolic dysfunctions during hepatocarcinogenesis (Bannash et al., 1989). Altered hepatic

foci have decreased xenobiotic metabolism reflecting molecular alterations in neoplastic cells (Roomi et al., 1985). Table 1 shows some of the altered enzyme or cellular components reported in AHF.

The use of histochemical and biochemical stains has made it easy for the differentiation and identification of AHF. Histochemical stains are used to identify altered foci according to their staining reaction to hematoxylin and eosin (H & E) stains, while immunohistochemical stains are used to identify the different altered enzymes. These foci stain differently from the surrounding normal tissue. Using H & E

Table 1. A selected list of altered enzyme or cellular components found in altered hepatic foci

Markers of loss of cell component or enzyme activity	Markers of increased cell component or enzyme activity
iron storage	glycogen
β-glucuronidase	γ-glutamyl transferase
serine dehydratase	placental glutathione S-transferase[a]
canalicular ATPase	
glucose-6-phosphatase	

(adapted from Emmelot and Scherer, 1980)

[a] (Sato et al., 1984)

stains, the AHF may be basophilic, eosinophilic or clear. The latter foci consists of vacuolated cells and thus, do not take up the stains. The persistent altered foci become neoplastic nodules, which are generally larger than the area of several lobules of the liver, and elevated from the rest of the liver tissue. Nodules maintain most of the altered or cellular component or enzyme activities similar to the foci (e.g., iron accumulation deficiency), but some activities such as GGT are lost (Williams, 1980). This loss of enzyme activity accounts for the difference in quantifying liver lesions using histology (H & E stain) and immunohistochemical stains.

Gamma-glutamyl transferase (GGT) has been used most frequently in hepatocarcinogenesis. GGT is normally present in trace amounts in adult and regenerating liver cells, but it is very high in fetal hepatocytes (Pitot and Sirica, 1980). GGT is a membrane bound enzyme found in the secretory and absorptive tissues. It is mainly concentrated in the kidneys, the pancreas, and seminal vesicles, with trace amounts found in other tissues. GGT hydrolyses glutathione by cleaving the gamma-glutamyl group releasing glutamate (Hendrich and Pitot, 1987). Abnormally high levels of GGT are found in liver tumors in mice and human hepatocellular carcinomas (Gerber and Thung, 1980). The leaking of GGT from hepatic cells during liver damage can be detected in serum at an early stage of

carcinogenesis (Sawaba et al., 1983). Induction of liver GGT during hepatocarcinogenesis might be a protective mechanism of the cells in an attempt to preserve their glutathione (GSH) by utilizing extracellular (GSH) (Hendrich and Pitot, 1987).

Another AHF marker that has gained recognition is the placental glutathione-S-transferase (PGST). It belongs to a family of GST enzymes involved in the hepatic detoxification process. The GSTs exit as several isoenzyme viz., GST-A, GST-B, GST-C and several subsections (Kitahara et al., 1983). Like GGT, the role of GST during hepatocarcinogenesis is to utilize the glutathione in an attempt to protect the AHF; thus, a combination of GST and GGT presence may enhance proliferation of preneoplastic cell (Hendrich and Pitot, 1987).

The scoring of AHF by PGST and GGT markers seem to decrease with age, but PGST is more persistent and scores a greater number of AHF compared to GGT, i.e., GGT scored 5 -10% less AHF, and 37% of the AHF were GGT negative (Tatematsu et al., 1988). Antioxidants such as BHA and BHT inhibit the growth of GGT -(+) AHF that have been initiated by DEN, and BHA inhibits the growth of PGST-(+) foci (Tatematsu et al., 1988). Placental glutathione S-transferase is also an early marker of hepatocarcinogenesis. Single PGST-(+) foci appeared in rats that had been partially hepatectomized within 4 hour of initiation with DEN (80 mg/kg bodyweight). The rats that

were kept for 12 weeks still demonstrated large number of single PGST foci. The uninitiated rats without PH also had small PGST-(+) foci. Their results indicate that PGST is an early markers of neoplasia, it is persistent and could be used as markers of putative "initiated" cells (Moore et al., 1987). However, the use of PGST as a marker of spontaneous foci seems to be male specific. Mitaka and Tsukada (1987) demonstrated that in 18 and 23 month old rats, PGST scored 90% of the putative AHF whereas GGT-scored 75% of AHF in males, and in females both markers scored 20% of the AHF. While these results indicate the efficiency of PGST as a marker of putative preneoplasia, PGST markers might be useful more in male rats only.

Quantitation of enzyme-altered foci (EAF)

The quantitation of EAF for the characterization of the effects of putative initiating and promoting agents has been developed in explaining the models of hepatocarcinogenesis (Pitot and Sirica, 1980). Quantitation of EAF by computerized stereology is based on the mathematical principles of particle enumeration in solid mass. Stereological quantitation is applied to quantify discrete lesions in solid tissues. The methods transform the two dimensional analysis of EAF (Foci/cm^2) to a three dimensional analysis - number of foci/cm^3; number of foci/liver, the volume percentage occupied

by foci, and the mean volume occupied by foci (Campbell et al., 1982). Transformation of the number of foci per area into three dimensional data allow direct inferences about the effect of the carcinogen on the number and size of the foci. Analysis of AHF by stereological quantitation is based on the assumptions that foci are spherical, foci centers are located randomly within the sphere, and their size and location is independent of other foci. The main limitation of this method is that the three-dimensional estimation of foci with very small diameters cannot be computed (Morris, 1989).

The use of stereological quantitation of EAF provides information on the analysis of the effect of different promotors during hepatocarcinogenesis. A comparative study on the mechanism of PB and a peroxisome proliferator (WY-14,643) showed that PB increased the number of AHF while WY-14,643 increased the size of the AHF. This difference in the number of AHF demonstrated that the mechanisms of the two promotors are different (Popp and Goldsworthy, 1989). Schulte-Hermann et al. (1989) used radiolabelled ^{3}H-thymidine to demonstrate that the increase in number of AHF during promotion is due to enhanced phenotypic expression of the foci and not due to formation of new foci with altered enzyme. This induced expression resulted in a larger volume occupied by the foci. The quantification of GGT-(+) AHF after withdrawal of PB in the Pitot protocol (1978) demonstrates the irreversibility of

initiated cells. The reversible nature of promotion is demonstrated by quantifying the volume AHF when PB was administered and then withdrawn, and readministered (Hendrich et al., 1986). Computerized stereological analysis can also be used to quantify multiple marker operations, thus enabling one to establish if a carcinogen induces multiple AHF (Hendrich and Pitot, 1987).

PAPER 1. *FUSARIUM PROLIFERATUM*-FERMENTED CORN STIMULATES DEVELOPMENT OF PLACENTAL GLUTATHIONE S-TRANSFERASE-POSITIVE ALTERED HEPATIC FOCI IN FEMALE RATS

Fusarium proliferatum-fermented corn stimulates development of placental glutathione S-transferase-positive altered hepatic foci in female rats

By

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ABSTRACT

Groups of eight six-week old female Sprague-Dawley rats were initiated with diethylnitrosamine (DEN, 30 mg/kg). Control and initiated groups were fed a semipurified diet, or diets supplemented with *Fusarium proliferatum*-contaminated corn to contain 20 or 50 mg/kg fumonisin B_1 (FB_1). Histochemical staining for gamma-glutamyltransferase (GGT) and immunochemical staining for placental glutathione S-transferase (PGST), markers of altered hepatic foci (AHF), were performed on serial frozen hepatic sections. Gamma-glutamyltransferase -(+) altered hepatic foci (AHF) were not found in any group. Treatment with DEN significantly increased the number of PGST(+) hepatocytes compared with the uninitiated groups ($P<0.0004$). Groups fed *F. proliferatum*-containing diets also had a significantly increased number of PGST-(+) AHF compared with those fed no *F. proliferatum* ($P<0.003$). The volume percentage of liver occupied by PGST-(+) foci was significantly greater in the groups treated with DEN ($P<0.01$) or *F. proliferatum* ($P<0.03$) compared with the controls. The mean focal volume occupied by AHF in the groups given DEN was also significantly greater than in the uninitiated groups ($P<0.02$). *Fusarium proliferatum* treatment also significantly increased mean focal volume ($P<0.04$). In conclusion, the feeding of *F. proliferatum* containing only 20

mg/kg FB_1 promotes the development of DEN-initiated AHF in rats. Placental glutathione S-transferase is a more useful marker than GGT in detecting AHF produced by small amounts of *F. proliferatum* mycotoxins fed after a mild initiating treatment.

INTRODUCTION

Fusarium proliferatum and *F. moniliforme* mycotoxins are natural contaminants of corn which may be found in human food and animal feed. The presence of *F. moniliforme* in corn is associated with human esophageal cancer in Southern Africa (1). Mycotoxins from this fungus, especially the fumonisins, are highly toxic to experimental animals, causing hepatocellular carcinomas (2) and cholangiocarcinomas in rats (3), equine leukoencephalomalacia (ELEM) (4, 5), and porcine pulmonary edema (PPE) (6). Fumonisin B_1 (FB_1) is present in feeds associated with known cases of ELEM and PPE at 1-126 μg/g feed (7). Fumonisin B_1 is found in corn at approximately 7.9 μg/g in regions where human esophageal cancer is more prevalent (1). Corn products for human consumption commonly contain 0.2 - 2 μg/g FB_1 (8). But, the cancer risk associated with a usual human intake of FB_1 has not been established.

Carcinogenesis is a prolonged process that produces a new population of cells with altered organizational, structural and biochemical properties. Altered expression or activity of enzymes such as gamma-glutamyl transferase (GGT), canalicular adenosine triphosphatase, glucose-6-phosphatase (9), and placental glutathione S-transferase (PGST) (10) is a biochemical marker of carcinogen-induced altered hepatic foci

(AHF). Placental glutathione S-transferase may be the most useful of these markers in quantifying hepatocarcinogenesis (10), because PGST appears quite early (11) and persists strongly throughout preneoplasia and neoplasia in human and animal tissues (12).

The purpose of this study was to assess FB_1 toxicity by exposing rats to *F. proliferatum*/corn culture material containing smaller amounts of FB_1 than have been previously studied, and to assess the utility of PGST as a marker of FB_1-promoted AHF. Studies feeding male rats either purified FB_1 (50 mg/kg) incorporated into the diet (2,13) or oven- or freeze-dried *F. moniliforme*/corn culture material containing approximately 40 mg/kg FB_1 (4% corn culture) (14) have been conducted. Gamma-glutamyltransferase has been the only marker studied of FB_1-promoted AHF (13). In this study, for six months, rats were fed 20 or 50 mg/kg FB_1 supplied from crude *F. proliferatum* culture material, and preneoplasia was measured by computerized stereology (15).

MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified. Diet ingredients were obtained from Harlan Teklad (Madison, WI).

Preparation of diets

Lyophilized cultures of *F. proliferatum* strain M5991 (obtained from Dr. Paul Nelson, Pennsylvania State University) were reconstituted in sterile phosphate-buffered saline, 1 ml of which was used as an inoculum for 300 g sterile aflatoxin-free corn. The corn culture was incubated in the dark for 6 weeks at 22-25° C. Corn culture was freeze-dried, assayed for FB_1 content by P. F. Ross, National Veterinary Services Lab, Ames,IA (7), ground, and incorporated into basal diet in amounts of 3 - 10 g corn culture /kg diet to make diets containing 20 and 50 mg/kg FB_1 (FB_{20} and FB_{50}). The basal diet was modified from the AIN-76 semipurified diet to contain 20% of kcals from fat (Table I, 16).

Animals and treatments

Forty-eight six-week-old female Sprague-Dawley (SD) rats were divided into 6 groups. Three groups were initiated with diethylnitrosamine (DEN, 30 mg/kg body weight) by oral

intubation in 0.1 ml dimethylsulfoxide (DMSO). One week later, rats were fed a diet containing 0, 20 or 50 mg/kg FB_1 from *F. proliferatum* corn culture. Three other groups were fed diets containing 0, 20 or 50 mg/kg FB_1 from corn culture, without initiation. These rats were initially dosed with 0.1 ml DMSO each. The levels of FB_1 incorporated into the diets were periodically confirmed by high pressure liquid chromatography performed by P. F. Ross, National Veterinary Services Laboratory, Ames, IA (7). Animals were caged individually and had a twelve hour light cycle. This type of housing and animal care was approved by the Iowa State University Committee on Animal Care. Semipurified diet (Table I) and water were available *ad libitum*. Body weights were recorded each month throughout the experiment. Rats were euthanized under a carbon dioxide atmosphere 6 months after DEN treatment. Slices from each of the three lobes of the liver were immediately frozen as a block on dry ice and then stored at -80° C for later immunohistochemical staining. Additional slices were fixed in phosphate-buffered formalin for histopathological evaluation.

Immunohistochemical staining

Two serial sections (10 μM thick) from each rat liver were stained for the presence of GGT- or PGST-positive AHF. Gamma-glutamyltransferase activity was determined (17). Liver

sections from DEN-initiated rats promoted with phenobarbital for 6 months (18) were used to verify the GGT staining. Immunohistochemical staining for PGST-(+) AHF was performed with a Vectastain ABC kit (Vector Laboratory, Burlingame. CA). Rabbit anti-rat PGST serum had been prepared previously in our laboratory (19). Computerized stereology was used to quantify the number of AHF, the volume percent of liver occupied by AHF, and the mean volume of AHF (15). Additional liver slices were processed by routine histopathological methods for hematoxylin-eosin staining (20). Data were analyzed by analysis of variance using the Statistical Analysis System (SAS, Cary, NC) on the mainframe computer of Iowa State University. Data for numbers and percent volume of AHF were processed by the SAS Stem and leaf program before being analyzed by the PROC GLM analysis of variance program. The Stem and leaf procedures involved taking the cube root or fourth root of the numbers or percent volume PGST-(+) AHF respectively, thus normalizing the distribution of the data. For hematoxylin and eosin-stained hepatic sections, Fisher's exact test was used to determine differences from the control group.

RESULTS

A few rats, randomly dispersed among treatments, were euthanized before the end of the experiment because of mammary tumor development and dental malocclusions which are common in this strain. Diet analyses for levels of fumonisins were consistent with the amounts that were supposedly incorporated into the diets. Whereas there was no statistical difference between groups with respect to body weight (Table II), the relative liver weight was significantly greater ($P<.06$) in the groups given DEN alone or given DEN and fed *F. proliferatum* to provide 20 mg/kg FB_1 than in the control group.

Gamma-glutamyltransferase-positive AHF were not noted in any group. The number of PGST-(+) hepatocytes was significantly greater in the rats treated with DEN than in the uninitiated groups ($P<0.0004$). Rats fed either dose of *F. proliferatum* had significantly greater numbers of PGST-(+) hepatocytes than did rats fed no *F. proliferatum* ($P<0.003$). *F. proliferatum* providing 50mg/kg FB_1 produced more PGST-(+) hepatocytes than did the lower dose of *F. proliferatum* ($P<0.09$). There was no interaction between DEN and *F. proliferatum* (Table III). Altered hepatic foci in the groups treated with DEN and FB_1 were very large with occasional single PGST-(+) hepatocytes (Figure 1).

The percentage of the volume of liver occupied by PGST-(+) AHF was significantly greater in the DEN-initiated groups than in uninitiated groups ($P<0.01$). Rats fed DEN/*F. proliferatum* (FB_{20}) showed greater hepatic volume occupied by PGST-(+) AHF compared with the higher dose. The groups fed either dose of *F. proliferatum* had significantly greater hepatic volume occupied by PGST-(+) AHF than did the groups fed no fumonisin ($P<0.03$). There was a significant interaction between DEN and FB_1 treatments ($P<0.074$), with a dose response in uninitiated rats but not in the initiated rats (Table III).

Mean focal volume of PGST-(+) hepatocytes in DEN-initiated groups was also significantly greater than in the uninitiated groups ($P<0.02$). The groups treated with DEN and fed *F. proliferatum*-corn culture material containing 20 mg FB_1 /kg had significantly greater mean focal volume than the groups treated with DEN and fed *F. proliferatum* containing 50 mg FB_1 /kg ($P<0.04$) (Table III).

Histopathology studies showed that all the groups treated with DEN had clear cell foci (CCF), with a significantly greater incidence of foci than in the control group ($P<0.04$, based on 2-tailed Fisher's exact test). The groups given DEN and *F. proliferatum* showed a dose response to *F. proliferatum* treatment, with the group fed *F. proliferatum* containing 50 mg/kg FB_1 having significantly more CCF than the control group

($P<0.002$, Fisher's exact test). The uninitiated group fed the lower dose of *F. proliferatum* had no CCF, and the uninitiated group fed the higher dose of *F. proliferatum* (50 mg/kg FB_1) had an incidence of CCF not significantly different from the control. DEN-initiated groups with or without *F. proliferatum* treatment also showed eosinophilic foci, subcapsular hemorrhage, and karyomegaly of the liver in conjunction with CCF. One rat from the group fed the lower dose of *F. proliferatum*-fermented corn had bile duct proliferation (Table IV).

DISCUSSION

Cancer development is thought to be a multistage process involving initiation, production of heritable genetic alterations; promotion, expression of growth-promoting characteristics; and progression (21). Although FB_1 has not been shown to be genotoxic, Gelderblom et al. (2) reported complete hepatocarcinogenicity of 50 mg/kg purified FB_1 fed to male rats for 18-26 months. Previous studies feeding FB_1-containing *F. proliferatum* corn cultures showed promoting effects in DEN-initiated rats (13). Our study tends to support the finding of Gelderblom et al. (2), in that AHF developed in uninitiated rats fed FB_1, and suggests that as small an amount as 20 mg/kg FB_1 may be tumor promoting, if not a complete hepatocarcinogen (Table III). Although our study was performed with FB_1 supplied in *F. proliferatum*/corn culture material, fumonisins were probably the crucial components in initiating carcinogenesis. The fat-soluble fusarins, also likely components of *F. proliferatum* corn culture, have been shown not to be initiating agents when given to rats at 50 or 100 mg/kg before phenobarbital promotion (22) although they are mutagens (23). Cultures producing large amounts of fumonisins are likely to produce only small amounts of other *F. proliferatum* mycotoxins (1).

Other components in the corn culture material which remain to be identified may be partly responsible for the observed development of AHF. But, it is likely that FB_1 is primarily responsible for the development of AHF.

The difference in relative persistence of markers of carcinogenesis might account for the absence of GGT from AHF in this study. GGT is unstable compared with PGST (12). Several studies have shown that GGT(+) AHF foci may disappear or are diminished over a period of time compared with PGST(+) foci (12,24,25,26). Greater stability of PGST-(+) hepatocytes than GGT-(+) hepatocytes during promotion is seen in data from hepatic sections of DEN-initiated, partially hepatectomized rats fed diets mixed with either 2-acetylaminofluorene, phenobarbital, or butylated hydroxyanisole which showed that 37% of PGST-(+) foci were GGT-negative at 50 weeks after initiation (12). Perhaps GGT activity of AHF is only stimulated by more stringent carcinogenic regimens than used in the present study. A single dose of DEN was administered at six weeks of age, which is a very mild initiating stimulus in comparison with protocols such as the Solt-Farber (18), Pitot (27) or Peraino protocols (28) where GGT-(+) AHF are observed. These protocols initiate with DEN at a few days of age or after partial hepatectomy, and a very large dose of DEN (200 mg/kg) may be given (18). DEN at varying doses given at 6 weeks of age in a protocol similar to the present study

produced PGST-(+) single cells and AHF, but no attempt was made to detect GGT-(+) AHF (11).

Spontaneous development of PGST-(+) AHF was noted in some of the animals fed only basal diet (Table III). This is commonly age-associated (29,30,31). Placental glutathione S-transferase, a persistent marker of hepatocarcinogenesis (12) seems to be more reliable than GGT in examining fumonisin carcinogenesis because even when small amounts of *F. proliferatum* mycotoxins (20 ppm FB_1) were fed, PGST-(+) AHF could be detected (Table III). Therefore, PGST may be useful in quantifying *F. proliferatum* hepatocarcinogenesis.

Lesions noted in the hematoxylin and eosin-stained hepatic sections do not correspond with the presence of PGST-(+) AHF, although all the DEN-initiated rats showed clear cell foci (CCF), eosinophilic foci and other hepatocellular changes (Table IV). The relationship between the presence of PGST-(+) AHF and other hepatocellular changes is unclear. However, 90% or 20 % of eosinophilic foci in rats dosed with aflatoxin B_1 and either phenobarbital (50 mg/kg diet) or nafenopin (100 mg/kg diet) respectively, expressed CCF, eosinophilic foci and GGT-(+) AHF (32). Mitaka and Tsukada (31) also reported the presence of GGT-(+) and PGST-(+) AHF adjacent to CCF. Hepatocellular alterations such as clear cell, eosinophilic and basophilic foci may progress to neoplastic nodules (33,34). The progression of PGST-(+) foci was not examined

(33). Increased PGST is a characteristic of single preneoplastic hepatocytes (11) which persists throughout the development of neoplasia, and is a marker of several forms of neoplasia (35). Therefore, the presence of PGST-(+) AHF in hepatic sections from uninitiated rats fed *F. proliferatum* corn cultures providing 20 mg FB_1 /kg diet suggest that FB_1 may be a complete carcinogen even at this low dose and exposure to corn naturally contaminated with *Fusarium spp.* containing as little as 20 ppm FB_1 probably poses a cancer risk.

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Table I: Basal Diet

Ingredient	g/kg
corn oil	33
beef tallow	56
casein	199
corn starch	409
dextrose	199
cellulose	50
AIN-76 vitamin mix	10
AIN-76 mineral mix	35
$CaCO_3$	4.5
choline bitartrate	2.0
dl-methionine	3.0
ascorbic acid*	0.1

* As an antioxidant

Table II. Body weight and liver weight as percentage of body weight in rats dosed with DEN and *Fusarium proliferatum*

Treatment	Body weight (g)	Relative liver weight (%)
Control (n=5)	315.2 ±15.4	3.0 ±0.47[b]
DEN (n=7)	317.0 ±31.1	3.4 ±0.26[a]
FB_{20} (n=7)	304.1 ±24.5	3.1 ±0.25[ab]
DEN-FB_{20} (n=8)	295.8 ±31.5	3.3 ±0.21[a]
FB_{50} (n=8)	294.5 ±26.4	3.2 ±0.28[ab]
DEN-FB_{50} (n=7)	296.4 ±25.0	3.2 ±0.25[ab]

N represents the number of rats in each treatment. All data are expressed as means ± standard deviations. Relative liver weight is the (liver weight/body weight) x 100%. Significant differences are noted by superscripts. Groups not sharing a letter are significantly different ($p<0.06$).

Table III. Quantitative results of Placental glutathione S-transferase-positive altered hepatocyte foci [PGST-(+)-AHF] in rats dosed with DEN and *Fusarium proliferatum* corn culture material

Treatment	No. of PGST-(+) AHF per liver	% Volume occupied by PGST-(+) AHF	No. of PGST-(+) AHF per cm^2
Control (n=5)	260±420	0.18±0.27	20±34
DEN (n=7)	2120±1180[a]	0.57±0.11[a]	153±96[a]
FB_{20} (n=7)	630±450[b]	0.39±0.31[b]	52±38
DEN-FB_{20} (n=8)	3670±3910	0.78±0.38[c]	282±302[c]
FB_{50} (n=8)	1570±1640[b]	0.63±0.24[b]	134±152
DEN-FB_{50} (n=7)	1460±1178	0.61±0.36	120±100

N represents the number of rats in each treatment. All data are expressed as means ± standard deviations.

[a] The DEN-treated groups had significantly greater number of foci per liver ($P<0.0004$); percentage of liver occupied by PGST-(+) AHF ($P<0.01$); and mean focal volume ($P<0.02$) than the uninitiated groups.

[b] Groups treated with *F. proliferatum* culture to provide 20 or 50 FB_1 mg/kg had significantly greater number of foci/liver ($P<0.003$), and percentage of liver volume occupied by PGST-(+) AHF ($P<0.03$) than the groups fed no *F. proliferatum* corn culture.

[c] The groups treated with DEN and *F. proliferatum* corn culture containing 20 mg FB_1/kg had significantly greater percentage of liver occupied by PGST-(+) AHF ($P<0.07$) and PGST-(+) mean focal volume ($P<0.04$) than the groups treated with DEN and 50 mg FB_1/kg.

Table IV. Results of microscopic examination of hematoxylin and eosin-stained hepatic sections from rats dosed with DEN and *Fusarium proliferatum*

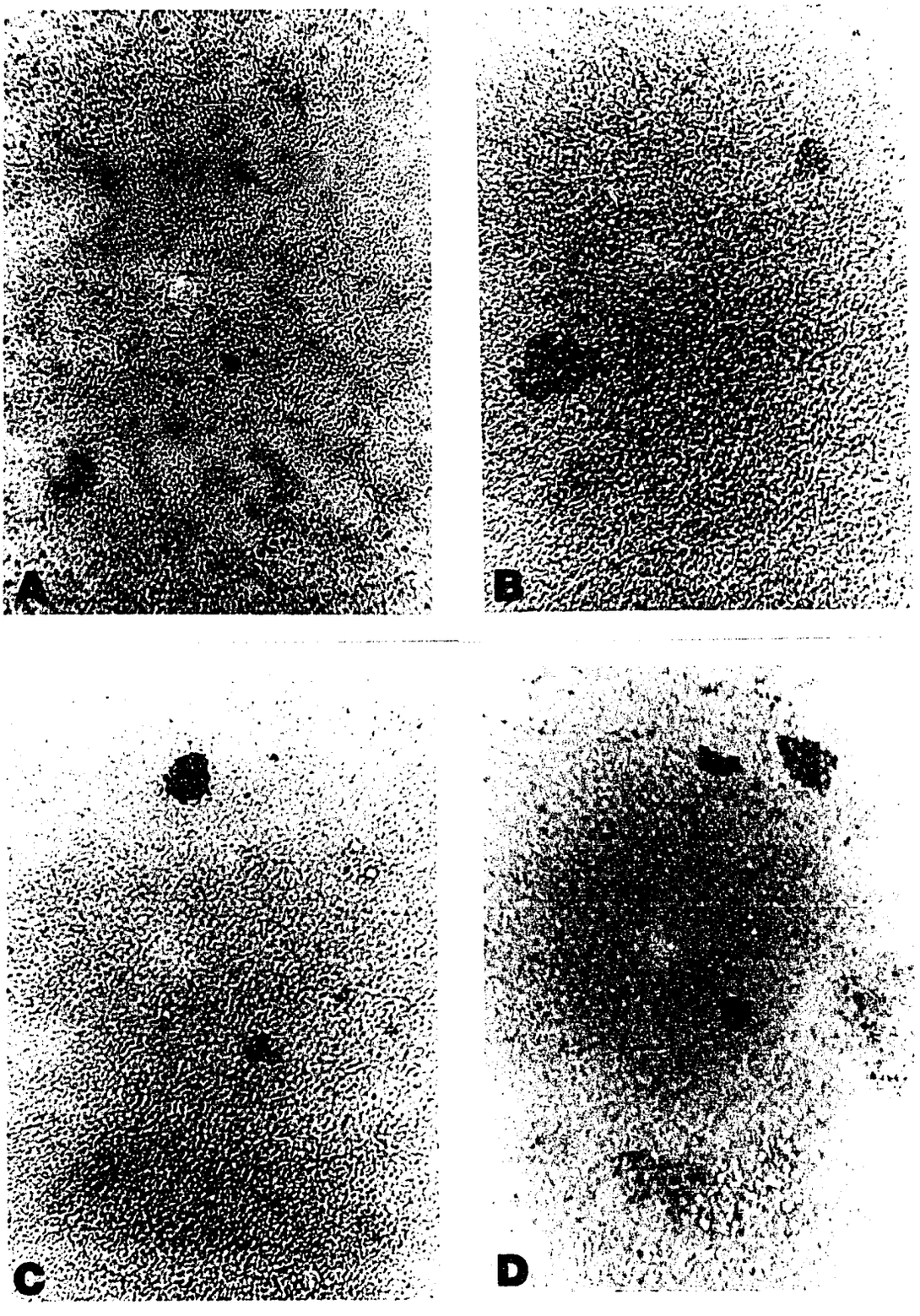
Treatment	n	NML	CCF	EF	Other
Control	5	100%	0%	0%	0%
DEN	7	14%	86%[a]	14%	0%
FB_{20}	7	100%	0%	0%	14%*
DEN-FB_{20}	8	62%	38%[ab]	0%	12%**
FB_{50}	8	88%	12%	12%	0%
DEN-FB_{50}	7	43%	57%[ab]	29%	14%***

Hepatic sections were paraffin-embedded, sectioned and stained with hematoxylin and eosin before microscopic examination. NML indicates that no microscopic lesions were observed in the hepatic sections. Some individuals had both CCF (clear cell foci) and EF (eosinophilic foci). Other hepatocellular changes noted were: * hemorrhage and bile duct proliferation; ** subcapsular hemorrhage; and *** karyomegaly of the liver.

[a] These groups were significantly different from the control ($p<0.004$, Fisher's exact test).

[b] These groups were significantly different from the control ($p<0.002$, Fisher's exact test).

Figure 1: Immunohistochemical stained liver sections showing placental glutathione S-transferase altered hepatocytes [PGST-(+) AHF] in (A) control, (B) diethylnitrosamine (30 mg/kg) initiated, (C) *Fusarium proliferatum*-fermented corn cultures containing 20 ppm FB_1 and (D) DEN-initiated and fed *Fusarium proliferatum*-fermented corn cultures containing 20 ppm FB_1.


A
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C
D

PAPER 2. FUMONISIN B_1 IS FETOTOXIC TO RATS

Fumonisin B_1 is fetotoxic to rats

By

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ABSTRACT

Groups of 5-6 pregnant F344/N rats were dosed by oral intubation from days 9-13 of gestation with 30 or 60 mg purified fumonisin B_1 (FB_1)/kg body weight, or with a fat-soluble extract of *Fusarium moniliforme*/corn culture derived from an amount of corn culture that would provide approximately 60 mg/kg FB_1. Control rats were dosed with water or corn oil. Food intake was monitored daily during dosing. Fetal bone development was examined after staining with alizarin red, whereas internal organ development was examined in hematoxylin and eosin-stained tissue sections. Although group differences in maternal body weight were not statistically significant, weight was 10% less in dams dosed with 60 mg/kg FB_1 compared with the control group ($p<0.119$). Relative litter weight was significantly suppressed by 60 mg/kg FB_1 ($p<$ANOVA, 0.003). Ossification of the sternebrae and vertebral bodies was significantly impaired by FB_1 treatment (Chi-square test, $p<0.01$). Litters from mothers treated with a fat-soluble extract of *F. moniliforme*/corn culture did not show suppression of litter weight or impairment of bone development. Therefore, fumonisin B_1 is fetotoxic to rats, suppressing growth and fetal bone development.

INTRODUCTION

Mycotoxins are natural environmental contaminants that have been implicated as teratogens in animals (Hayes, 1981). Fumonisins, mycotoxins produced by *Fusarium moniliforme* are novel water-soluble carcinogens (Gelderblom et al., 1991). *F. moniliforme* contamination of corn is associated with human esophageal cancer (Sydenham et al., 1990). Fumonisins are also hepatotoxic and hepatocarcinogenic to rats (Gelderblom et al., 1991), and they cause equine leucoencephalomalacia (Thiel et al., 1991). Other mycotoxins produced by this fungus are the fusarins, which are mutagenic in the Ames *Salmonella typhimurium* test (Bjeldanes and Thompson, 1979). The carcinogenicity and/or mutagenicity of compounds is positively correlated with their teratogenicity (Juchau, 1989). Therefore, carcinogens and/or mutagens are possible teratogens. The mechanisms of action of mycotoxins as teratogens have been categorized in three ways. Mycotoxins can interfere with hormonal biosynthesis: e.g., zearalenone which have estrogen-like activity in mammals, can damage parental gametes producing infertility or abnormal offspring if successful fertilization occurs. Mycotoxins may cause fetal malformations especially during organogenesis by interfering with nucleic acid and protein biosynthesis of the fetus. This would interrupt normal cell differentiation and

organogenesis resulting in malformations. Mycotoxins may affect the fetus indirectly by affecting vital maternal organs such as the liver, reducing nutrient transfer to the fetus or increasing toxin transfer (Hayes,1981).

Tricothecenes and zearalenone (F-2 toxin), mycotoxins produced by other *Fusarium* species, have been shown to be teratogenic in several animal species including rats (Hayes, 1981). But the teratogenic effects of *F. moniliforme* mycotoxins such as fusarins and fumonisins have not been reported. Thus, the purpose of this study was to determine possible teratogenic effects of *F. moniliforme* mycotoxins, especially the fumonisins.

METHODS

Preparation of *Fusarium moniliforme* mycotoxins

Sterile, aflatoxin-free feed corn analyzed by P.F. Ross (National Veterinary Services Lab, Ames, IA.; Ross et al. 1991) was inoculated with lyophilized cultures of *F. moniliforme* strain M5991 obtained from Dr. Paul Nelson (Pennsylvania State University), that had been reconstituted in phosphate buffered saline, pH 7.4. This corn culture was incubated in the dark for three weeks at room temperature (22° C). The corn culture was freeze-dried, ground and extracted with acetonitrile:water (1:1). The extract was partitioned with ethyl acetate after which the water-phase was loaded onto an Amberlite XAD-16 column (Sigma Chemical Co., St Louis, MO). The column was washed with water and FB_1 eluted with methanol. The eluate was dried under vacuum, redissolved in water and loaded onto a Lobar C_8-column (25x310 mm, Merck, Darmstadt, Germany). The column was washed with acetonitrile:0.1% trifluoroacetic acid (TFA)) in water (80:20), and FB_1 was eluted with acetonitrile:0.1% TFA in water (50:50). The fraction was further purified using HPLC equipped with a semi-preparative C_{18}-column (Partisil 10 ODS-3; 9.4x250 mm, Whatman, Hillsboro, OR). The FB_1 was eluted with 20% acetonitrile:0.1% TFA in water for 5 minutes, followed by a gradient from 20 to

70% acetonitrile every 5 minutes. The purity of the isolated FB_1 was determined by analytical HPLC of the o-phthalaldehyde-derivative and confirmed by GC-mass-spectrometry. The isolated FB_1 was approximately 80% pure.

Five hundred grams of *F. moniliforme*/corn culture prepared as above was extracted with 1500 ml chloroform:isopropanol (1:1) solution overnight. The extract was concentrated to 30 mls by Buchi rotavapor (Fisher Scientific, Pittsburgh, PA) (Bjeldanes and Thomson, 1979). This was the fat-soluble extract.

Dosing of rats

The animals were housed individually in plastic cages and exposed to a 12 hr light and dark cycle. This housing was approved by the Iowa State University Committee on Animal Care.

Supposedly timed pregnant F344/N rats (SASCO, Omaha NE) were divided into four groups, 9 rats in the experimental groups and 8 in the control group. The control group was subdivided into two equal groups which were dosed with water as a control for the water-soluble extract, and corn oil as a control for the fat-soluble extract respectively. One group was orally intubated with 60 mg/kg per day purified fumonisin B_1 (FB_{60}), a second group was given 30 mg/kg per day purified fumonisin B_1 (FB_{30}). The last group (FusX) was dosed with fat-

soluble extract of *F. moniliforme*/corn culture material from an amount of corn culture which would provide 60 mg/kg FB_1 per rat/day (i. e. 12 mg/rat). The *F. moniliforme*/corn culture contained 3700 ppm FB_1, therefore 3 g corn culture/day would provide 12 mg FB_1. The amount of fat-soluble extract equaled 3/500 x 30 ml, or approximately 0.2 ml per rat/day, because 500 g corn culture was extracted and condensed to a volume of 30 ml. Diets and water were available *ad libitum*.

The day when vaginal plugs were noticed after mating was designated day 1 of gestation according to the supplier. The dosing was carried out between days 9-13 of gestation which is within the critical period of organogenesis (days 6 to 15) (Wilson, 1973). During days 9-13, food intakes were recorded. Maternal body weights were recorded on arrival and on the day of necropsy. Throughout the study, rats were fed semipurified diets based on AIN 76 (Table 1) (National Research Council, 1978).

Maternal necropsy and fetal examination

On day 20 of gestation the rats were euthanized under carbon dioxide atmosphere. The uterine horns were examined for resorption sites before the pups were removed. Litter weights and maternal carcass weights were recorded. Gross fetal malformations were examined before incising their abdomens above the umbilicus, approximately two-thirds of the

litter were placed in 95% alcohol. The remaining fetuses were placed in 10% formalin. The litters in alcohol solution were saved for skeletal analysis and the formalin-fixed fetuses were saved for crown-rump measurements and for histological screening of congenital malformations of internal organs (Persuad, 1985).

Histological analysis

From the formalin-fixed pups, three sections, each 3-4 mm thick from the thoracic, hepatic and urogenital regions were cut and embedded in paraffin (Palmer, 1978). These were further cut into 10 micrometer sections using a microtome. Four serial sections were mounted on slides and stained with hematoxylin-eosin stains by methods described by Sheehan and Hrapchak (1980). Harris' hematoxylin was used as a primary stain after the tissues had been hydrated. One percent HCl in 70% ethanol was used as a differentiator and eosin as a counterstain. Histological analysis for organ development was done by examination of slides under a light microscope.

Skeletal analysis

Ethanol-fixed pups were stained by the modified Dawson technique for staining fetal skeletons (Lorke, 1977). Alizarin red S dye (0.8 g/100 ml of 0.7% potassium hydroxide) was used to stain the skeleton after the tissue and fat had

been dissolved in 0.7 % potassium hydroxide solution for 1 week. The stained fetuses were then stored in glycerol for examination under a dissection microscope. Skeletal development was assessed for hypoplasia, under- or extraossification, misshapes, supernumeracy (for ribs) and branching of the vertebral arch (Persuad, 1985; Taylor, 1986).

Analysis of data

The Statistical Analysis System computer program (SAS, Cary, NC) for one way analysis of variance was used to analyze animal data, and the least significant differences between treatment means were determined by Duncan's multiple range tests. Differences between each treatment and the control group in the incidence of skeletal variations were calculated by Chi-square tests using the two-way contingency analysis (Denenberg, 1976).

RESULTS

Data analysis was based on pregnant rats only because about three rats in each group were not pregnant, therefore data were combined for the controls dosed with oil or water.

Maternal body weights on day of arrival and at sacrifice were not significantly different among groups ($p<0.1189$), although rats dosed with 60 mg/kg FB_1 weighed 10% less compared with control dams. Diet intake during dosing was similar in all groups (Table 2). No resorption sites were noted in any group.

Growth impairment in the fumonisin-treated rats was reflected in crown-rump lengths because the FB_{30} group differed significantly from the FusX group ($p<0.001$) (Table 2). Although litter sizes were the same, litter weights per number of pups in the fumonisin-treated groups was significantly reduced compared with the control ($p<0.003$) with a dose response to fumonisin treatment. Litters from the FusX-treated dams did not show any sign of toxic effects (Table 2). Maternal and fetal weights were significantly correlated ($r= 0.56$, $p<0.005$).

Histological examinations

No microscopic sections showed congenital abnormalities. There were normal chondrocytes and osteoblasts in the vertebral and sternebrae portions of the hematoxylin and eosin-stained tissues suggesting delayed ossification, which was seen in the alizarin-stained fetuses of the FB_1-treated dams.

Skeletal examinations

Fetotoxic effects of FB_1 were noted in litters from the FB_1-treated rats (Table 3). The most outstanding anomalies were the lack of ossification and absence of the sternebrae, which were found to a significantly greater extent in FB_1-treated groups compared with the control group ($p<0.01$). All groups showed incomplete ossification of the sternebrae where either the third and/or fourth sternebrae were not ossified. Underossification of the vertebral bodies was also present to a significantly greater extent than the control in the FB_{60} litters ($p<0.01$) but not in the FB_{30} litters. Fumonisin B_1-treated groups also had misshapen ribs, skull hypoplasia or unossified phalanges (Figure 1B) although their incidence was not significantly greater than in the control group.

DISCUSSION

The reduced body weights of dams indicate possible maternal toxicity which correlates with fetal body weights and skeletal defects (Khera, 1984). This positive correlation has been noted in mice using a variety of structurally unrelated compounds that produced toxic effects in the mothers (Ryan et al., 1991). Although maternal body weights were not monitored continuously, diet intakes were similar during dosing (Table 2), suggesting that any birth defects noted were not due to food restriction (Johnson, 1965; Anderson et al., 1980).

Litter weight is the most sensitive and consistent measure of toxicity (Ryan et al., 1991) compared with other parameters such as fetal resorption and percent abnormal fetuses, which may occur spontaneously in some animals (Gaylor, 1978). Fetal growth suppression in the fumonisin-treated rats may be due to impairment of fetal nutrition, placental function or altered fetal metabolism due to the toxin (Manson and Wise, 1991). But, probably the suppressed fetal weights are due to decreased maternal weights in the fumonisin-treated rats as suggested by a review of the teratogenicity of numerous chemicals (Khera, 1984). Because FB_1 is hepatotoxic, it might have caused damage to the livers of the mothers thus interfering with nutrient transfer to the

fetuses (Hayes, 1981; Manson and Wise, 1991). *F. moniliforme*-contaminated corn extracts inhibit protein synthesis in cultured hepatocytes (Norred et al., 1990), which suggest a mechanism of growth inhibition in vivo. Another possibility could be that the fetuses developed biochemical defects early in gestation due to FB_1 and thus were unable to use nutrients efficiently, producing low birth weights. FB_1 alters sphingosine metabolism (Wang et al., 1991), and sphingosine inhibits Ca^{2+}-dependent Na^+/K^+ ATPase and protein kinase C (Oishi et al., 1990). Therefore, it is possible that FB_1 inhibition of sphingosine metabolism might have increased Na^+/K^+ pump activity resulting in less energy deposition and a decrease in body weight. Until a definite stereochemistry and mode of action of fumonisins are clarified, only speculation is possible regarding the biochemical bases of the effects of fumonisin on fetal development.

Normal ossification of the sternebrae is complete at birth except for the fifth and sixth sternebrae (Taylor, 1986), but in this study even some of the controls had one or two sternebrae not ossified. This might be due to spontaneous malformations which may occur in some strains of laboratory rats (Persuad, 1985; Szabo, 1989). Osteogenesis of the vertebral bodies occur from day 18 of gestation until after birth (Gaylor, 1978; Strong, 1925) but in this study litters from the fumonisin-treated rats showed lack of ossification at

day 20 even in the lumbar and thoracic regions which are normally ossified by day 18 or 19 of gestation. The lack of ossification of sternebrae and vertebral bodies could be caused by general growth impairment due to FB_1 or to FB_1-altered intracellular metabolism of calcium. Rat placenta contains calcium binding proteins (CaBP) immunologically similar to vitamin D-dependent CaBP found in the duodenal mucosa (Garel, 1987). The chemical structure of FB_1, by virtue of its propane-1,2,3-tricarboxylic acid plus 2-acetylamino side chain structure (Bezuidenhout et al., 1988), indicates a possible Ca-binding capacity of this mycotoxin. It is possible that FB_1 interferes with placental CaBP, thus affecting Ca absorption by the fetuses. The mechanism of FB_1-impaired bone development deserves further study.

Ossification defects induced by an unknown toxin from *Fusarium roseum* have been reported in female broiler chicks which have been exposed to either 2% *F. roseum* incorporated into the diet (Haynes et al., 1985), or 3 - 5 mg/ml aqueous solution (Haynes and Wasler, 1986) from day one to two weeks. Tibial dyschondroplasia was noted as early as day 4, and was severe at 2 weeks (Haynes and Wasler, 1986; Haynes et al., 1985). Although this defect is common in chickens, an unidentified *F. roseum* mycotoxin exacerbated the defect. Due to effects on bone development, fumonisins might be related to this unidentified mycotoxin. The absence of fetal or embryo

toxicity in the fat-soluble extract might be due to the low levels of mutagenic fusarins (80 mg/kg culture material) in fumonisin-containing *F. moniliforme* (Sydenham et al., 1990). Although they are mutagenic, fusarins have been shown not to be carcinogens (Gelderblom et al., 1986), and therefore they may not cause embryotoxicity or teratogenicity. Although other unknown factors in the 80 % pure FB_1 may have contributed to the observed suppression of fetal body weight and improved ossification, these results suggest that FB_1 is fetotoxic if not teratogenic to rats. A small dose of a fat-soluble extract of *F. moniliforme* was not fetotoxic. However, more studies need to be conducted using fumonisins of greater purity and larger numbers of rats. The U.S. and Canada have established a standard (20 rats/group) to allow for 0.02-0.85% spontaneous congenital malformations that occur in rats (Persuad, 1985). But these numbers are to be used after initial studies. In this study, 8 to 9 rats/group were used as a recommended number for testing teratogenic potential of a new chemical (Manson and Wise, 1991). Furthermore, more studies are needed because fumonisins are commonly found in corn products in the human diets in amounts of 0.2 - 2 ppm (Sydenham et al., 1991). Therefore, fumonisins pose a potential human health risk during pregnancy.

Acknowledgements

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$CaCO_3$	4.5
choline bitartrate	2.0
dl-methionine	3.0
ascorbic acid*	0.1

* As an antioxidant

Table 2. Animal data

Treatment	Dose of FB (mg/kg/rat)	Initial body weight (g)	Final body weight (g)	Diet intake during dosing (g)	Mean litter weight (g) /no. of pups	Mean length (cm)
Control	0	155.3	218.0	86.3	1.9[a]	2.7[ab]
n=6		(16.3)	(9.8)	(3.6)	(0.2)	(0.1)
FB_{60}	79.6	151.2	206.0	89.8	1.5[b]	2.6[bc]
n=5	(4.6)	(8.9)	(7.6)	(8.6)	(0.2)	(0.1)
FB_{30}	40.3	149.4	213.2	85.0	1.7[a]	2.5[c]
n=6	(2.9)	(11.5)	(13.3)	(19.7)	(0.2)	(0.1)
FusX	...	158.6	221.7	91.5	2.0[a]	2.8[a]
n=6		(4.6)	(10.2)	(5.3)	(0.1)	(0.1)

All data are expressed as means ± standard deviations (). N represents the number of rats in each treatment. Groups not sharing a letter are significantly different, $p<0.05$, one way ANOVA, with least significant differences determined by Duncan's multiple range test.

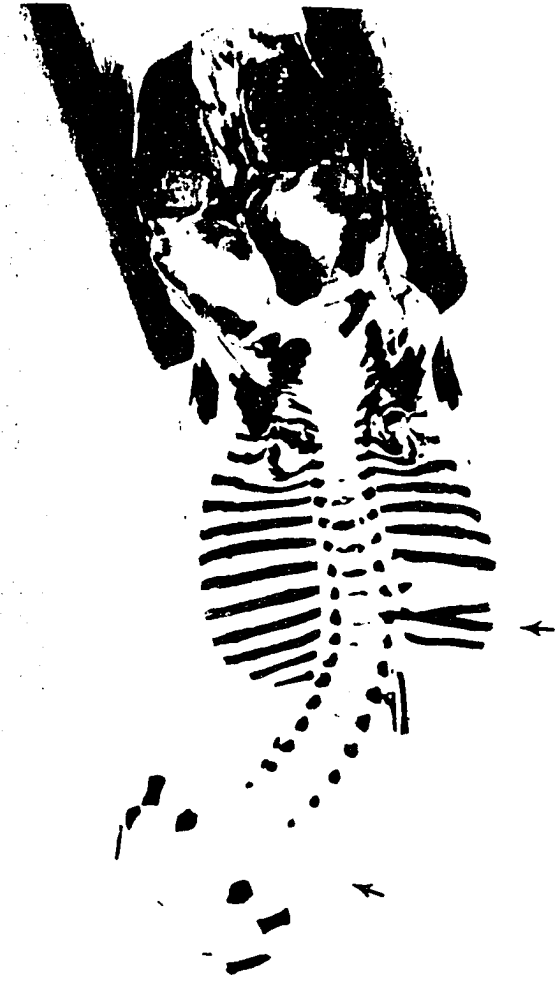
Table 3. Percent incidence of skeletal anomalies in 20-day old fetal rats dosed with FB_1 or FusX on days 9-13 of gestation

Treatments	1 or 2 sternum ossified	Sternum hypoplasia	Hypoplasia of vertebral bodies
Control n=22	41%	0%	0%
FB_{60} n=18	28%	72%[a]	33%[a]
FB_{30} n=31	19%	77%[a]	29%[a]
FusX n=23	48%	4%	0%

N represents the number of pups in each treatment.[a] By a Chi-square test, two-way contingency plan, these groups showed greater than expected frequencies of anomalies, $p<0.01$.

Figure 1: Skeletal development of (A) control and (B) fumonisin-treated pup. Panel A shows a pup from a control dam, with normally shaped ribs, and normal ossification of the phalanges and vertebral bodies. Panel B shows a pup taken from a dam treated with 60 mg/kg fumonisin B_1. It shows under-ossification of the vertebral bodies of the cervical and lumbar regions as well the phalanges.

A



B

PAPER 3. PRODUCTION OF FUMONISINS BY *FUSARIUM PROLIFERATUM* M5991, M6104 AND M6290 IN LIQUID CULTURE MEDIUM AND THE METABOLISM OF 14C-LABELLED FUMONISIN B_1 IN RATS.

Production of fumonisins by *Fusarium proliferatum* M5991, M6104 and M6290 in liquid culture medium and the metabolism of 14C-labelled fumonisin B_1 in rats.

By

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ABSTRACT

Fumonisins are readily produced by various *Fusaria* cultures on corn, a solid substrate medium. The production of fumonisins in most basal liquid medium is either very low or requires a long incubation period. Liquid media used to produce fumonisins were used either singly or in various combinations to find the most suitable medium for fumonisin production. Fumonisin B_1 was produced by *F. proliferatum* lyophilized cultures grown in basal salt medium that was supplemented with thiamin, riboflavin, pantothenate, niacin, pyridoxamine, and thiotic acid (500 μg of each/L), and folic acid, biotin and vitamin B_{12} (50 μg of each/L), and 25 mg l-methionine/100 mL medium. The cultures were incubated for 48 h at 22 ± 2° C while shaking at 50 rpm, in a pre-enrichment medium containing a malt extract as the main carbohydrate source. A 50 mL aliquot of the culture was centrifuged and 20 mL of the supernatant was inoculated into a 100 mL basal medium in Roux bottles (VMR Scientific, Philadelphia, PA). The amount of FB_1 produced was 340 μg/mL after 24 days incubation at 22±2° C and 50 rpm. Supplementing the basal salt medium with methionine increased the production of FB_1 by 10 folds compared to medium without methionine after 16 days of incubation, therefore methionine supplemetation is

important for FB_1 production. The metabolism of ^{14}C-labelled fumonisin B_1 showed that a single dose (0.1 μCi) of FB was excreted almost entirely within 24 hours. The amount of ^{14}C-labelled FB_1 recovered in feces was 96%, 2.5% in urine, 0.7% in liver and kidneys. There was no quenching in the blood. Only 4% of the counts were not accounted for. This implies that very small amounts of FB_1 are required to cause disease.

INTRODUCTION

Fusarium moniliforme, a fungus found contaminating various grains, especially corn, implicated in outbreaks of leukoencephalomalacia in horses (Marasas et al., 1988), hepatocarcinogenicity and hepatotoxicity in rats (Gelderblom et al., 1991), pulmonary edema in pigs (Osweiler et al., 1992); has been successfully grown in solid substrate medium but not liquid medium. High levels of fumonisins B1 (FB_1) (6.4 g/kg of corn) (Nelson et al., 1991) and >7 g FB_1/kg (Nelson et al., 1992) have been produced on corn medium using *F. moniliforme* and *F. nyagamai* cultures, respectively. Purification of fumonisins from the solid (corn) medium is very cumbersome and expensive due to co-extraction of numerous interfering compounds (Gelderblom et al., 1988; Vesonder et al., 1990), hence the need to develop a liquid medium that will support the production of fumonisins. Production of tricothecenes (T-2), mycotoxins produced by *Fusarium tricinctum*, has been successful in yeast extract sucrose (YES) liquid medium. Levels close to 1000 ppm T-2 were produced after 6 weeks of incubation which declined to 50 ppm at 7 weeks (Gabal et al., 1983). Fungi producing trichothecenes are commonly found in corn, cattle feed and mixed feed (CAST, 1989) similar to fumonisin producing fungi.

Successful production of FB_1 (74 mg/L) on a basal liquid

medium supplemented with glucose and ammonium sulfate and maintained at pH 5.0 has been reported by Jackson and Bennett (1990). Plattner and Schackelford (1992) were able to produce FB_1 at levels ranging from 20 - 53 μg/mL in liquid culture used for growing *Alternaria alternata*, a tomato fungus that produces a mycotoxin structurally similar to FB_1. The medium used contained potato dextrose broth and 0.1% yeast (Gilchrist and Grogan, 1976). When this medium was supplemented with 25 mg methionine/100 mL of medium, 53 μg/mL FB_1 was produced after 13 days of incubation at 25° C, and only 30 μg/mL was produced after 21 days of incubation when 100 mg methionine. So far, the highest level of FB_1 produced from a liquid culture is 140 ppm when *A. alternata* culture were used (Chen et al., 1992). This method however, requires a long incubation period (110 days) to produce this amount. Aeration of the growth medium during incubation seem not to play an important role in FB_1 production as suggested by Jackson and Bennett (1990). Plattner and Schackelford (1991), Gabal et al., (1983), and Chen et al. (1992), incubated their liquid cultures without aeration (stationary). During storage, production of fumonisins from *Fusaria*-contaminated corn require temperate moist conditions (Romer and Maune, 1993). These conditions have been simulated in the laboratory using mycotoxin-free cracked corn kernels that had been autoclaved twice before inoculation with lyophilized cultures of *F.*

moniliforme or *F. proiliferatum*. Suitable incubation temperature was found to vary from 20 -25° C, with maximum FB_1 (17.9 g/kg) produced by corn cultures incubated at 20° C for 13 weeks (Alberts et al., 1990).

The metabolism and ultimate fate of FB_1 is not yet well characterized. Fumonisins seem to be entirely excreted in feces when the exposure route is oral and 66% in feces when the route of exposure is through intraperitoneal injection (Shephard et al., 1992a).

The aim of this experiment was to develop a liquid medium that would produce sufficient levels of fumonisins within a reasonable period that could be radiolabelled, and furthermore, to determine the disposition of labeled fumonisin B1 after a single dose.

MATERIALS AND METHODS

Materials: Malt extract broth was obtained from BBL (Cockeysville, MD). Potato dextrose broth, yeast extract, and peptone were obtained from Difco Lab (Detroit, MI). ScintiVerse™ Bio-HP scintillation analyzer, $(NH_4)_2SO_4$, $CaCl_2.2H_2O$, $FeSO_4.7H_2O$, KH_2PO4, NaCl, $MnSO_4.H_2O$, $MgSO_4.7H_2O$, $(NH_4)Cl$ and $NH_4H_2PO_4$ were obtained from Fisher Scientific Chemical Co. (Springfield, NJ). Glucose, thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thiotic acid, folic acid, biotin and vitamin B12 were obtained from Sigma Chemical Co. (St. Louis, MO). l-Methionine was obtained from Gibco Lab. (Grand Island, NY). *Fusarium proliferatum* strains M5991, M6104 and M6290 were obtained from Dr. Paul Nelson (Pennsylvania State University).

Experiment 1

Fusarium proliferatum cultures were reconstituted in 5 ml sterile phosphate buffer and inoculated into a 500 ml sterile pre-enrichment medium (Table II) in an culture flask. The medium was incubated at room temperature (25 °C ± 2 °C) for 48 hours while shaking at 5 rpm. An aliquot of the pre-enrichment medium was transferred to a previously autoclaved growth medium (Table II) which was supplemented with 500 µg/L

each of thiamin, riboflavin, pantothenate, niacin, pyridoxamine, and thiotic acid, and 50 μg/L each of folic acid, biotin and vitamin B12 (Jackson and Bennett, 1990; Table I) to make a 1:10 dilution (250 mL total). The cultures were grown in 2 L culture flasks. Incubation was at room temperature (22 - 27 °C) for 24 days on a shaker (50 rpm). Approximately 5 ml of the culture medium was sampled every other day to determine FB production over time. It was assayed by thin layer chromatography, and confirmation of FB was made by high performance liquid chromatography (Ross et al., 1992). An aliquot was taken each day to determine the pH of some cultures, whereas in other batches the pH was not adjusted. The pH was adjusted to 5.0 with either HCl or NaOH. Several attempts were made to produce FB_1 in liquid culture using the methods employed by Jackson and Bennet (1990) (Table 1) in order to compare FB production of the two media.

Production of FB_2 and FB_3 by *F. proliferatum* strains M6104 and M6290 respectively were cultured similarly, except that pH was not adjusted.

Experiment 2

Table II medium was used but pH was not adjusted. On day 8 of incubation, which was established as the day when FB production began, 100 μCi L-[methyl ^{14}C]-labeled methionine (with specific activity of 2.0 GBq/mmol or 55.0 mCi/mmol)

(DuPont, Wilmington, DE) was added to the 250 mL of growth medium and incubated for 4 days. Fumonisin B_1 was extracted from the medium by methods of Cawood et al. (1991). At the end of 12 days of incubation, the liquid culture produced 3.8 μCi ± 1.4 of ^{14}C-labeled FB_1 (specific activity of 0.14GBq/mmol). The FB_1 was 85% pure, therefore, the total amount of ^{14}C-labeled fumonisin was 33.5 mg. The material was diluted in 3.8 mL distilled water to give a ^{14}C-FB_1 concentration of 1 μCi/mL. Four rats with an average weight of 114 g were placed in metabolic cages and had free access to food and water. Three rats were gavaged with 0.1 μCi ^{14}C-FB_1 (0.1 ml). The fourth rat was dosed with water to be used for calculating the amount of quenching. After 24 hours rats were killed by CO_2 anesthesia and immediately decapitated to collect blood. Liver, kidneys, and thigh muscle, and all urine and feces were collected. Approximately 1 g of liver, muscle or feces, or 0.3 g of kidney, or 1 mL blood or urine were placed in 10 mL scintillation fluid. Radioactivity was counted on Beckman LS3801 counter (Beckman Instruments Inc., Irvine,CA) within 24 hr after sacrifice.

F. proliferatum M6104 and M6290 cultures were grown using the same procedures as for producing FB_1, but were not labelled with ^{14}C-methionine. These cultures were incubated for 40 days. *Other F. proliferatum M5991* cultures were also incubated for 40 days.

Experiment 3

Glucose in the pre-enrichment medium was substituted with a malt extract because it has been reported that production of fumonisins in cultures require a simple carbohydrate medium (Nelson, 1992). The culture flasks were substituted with flat Roux bottles (VMR Scientific Co, Philadelphia, PA) to increase the surface area and allow more air circulation. On a subsequent trial, 25 mg l-methionine/100 mL medium (Table II) and vitamins were added to the medium. Production of FB in this medium was compared to the medium used by Plattner and Schakelford (Table III). The medium used by Plattner and Schakelford was supplemented with vitamins (Table I) in one flask.

Production of FB2 and FB3 from *F. proliferatum* (M6104 and M6290) was attempted using similar procedures except that Gilchrist and Grogan medium was not supplemented with vitamins.

RESULTS AND DISCUSSION

Production of FB_1

Fusarium proliferatum M5991 culture grown on Jackson and Bennetts' (1990) medium produced <100 ppb FB_1 at all times, whereas, the medium in Table II supplemented with vitamins produced more fumonisin (10 ppm) after 24 days of incubation compared to 6 ppm when pH was adjusted (Table IV). But when methionine, [L-methyl ^{14}C]-labelling was incorporated into medium (described in Table II, without pH adjustment) in the second experiment, 33.5 ppm ^{14}C-labelled FB_1 was produced after 14 days incubation. Attempts made to reproduce or improve the amounts of FB_1 production using the second medium (Table II) failed initially. Less than <100 ppb FB_1 was produced each time. The failure to reproduce our results might have been due to temperature variability (20 - 35 °C), which was uncontrollable during this time. The cultures seemed to undergo alcoholic fermentation within 3 days when temperatures were >27 °C. Another reason could be that *F. proliferatum* was used instead of *F. moniliforme*. FB_1 is the main metabolite of *F. moniliforme*, and it is produced to a lesser extent by *F. proliferatum* (Ross et al., 1990). The ^{14}C-labelled cultures used for metabolism studies produced 33.5 mg FB_1/L. Methionine is probably required in the biosynthesis of FB_1

(Plattner and Shackelford, 1992). *F. proliferatum* M5991 culture that were incubated for 40 days produced 5 ppm FB_1, and the FB_1 production increased over time (Figure 1).

Substituting a malt extract for glucose in the third experiment and using flat culture bottles produced 5 - 10 ppm FB_1 after 21 days of incubation at room temperature (Table IV). The initial pH was 6.1 and remained at 3 from day 4 of incubation. Adding 25 mg methionine to the medium increased the production of FB_1 extensively (230 μg/mL) compared to 22.2 μg/mL in medium without methionine after 16 days of incubation. The addition of vitamins to the medium used by Plattner and Schakelford stimulated FB_1 production such that within 10 days of incubation, 68.2 μg FB_1/mL was produced compared to 51.7 μg FB_1/mL of medium without vitamins (Table V). Using this medium, Plattner and Shackelford (1992) obtained 53.0 μg FB_1/mL within 13 days.

Attempts to produce FB_2 and FB_3 within 14 days of incubation were not successful ($\leq$100 ppb) was produced, thus the cultures were incubated for 40 days. Production of FB_2 or FB_3 by *F. proliferatum* (M6104 or 6290, respectively) ranged from 0.05 - 0.2 ppm FB_2 or 0.1 - 0.5 ppm FB_3, with optimum production at various time points (Figures 2 & 3). These strains also supported production of FB_1 at earlier stages of incubation, and once the FB_1 production declined, then FB_2 or FB_3 production occurred. These results show that FB_2 and FB_3

require longer incubation, and suggest that FB_2 and FB_3 may be derived from FB_1, or that FB_1 inhibits FB_2 or FB_3 production i.e., FB_1 production must be shut off before FB_2 or FB_3 are produced. Results from the third experiment however, indicate that the availability of suitable substrates for the fungi induce FB_2 and FB_3 production at an earlier stage of incubation. *F. proliferatum* M6104 produced 64.3 μg/mL FB_2 within 11 days of incubation when Table II medium was used (with malt extract), and 84.3 μg/mL FB_2 was produced after 11 days of incubation when 25 mg l-methionine was added to 100 mL of the medium. The Gilchirst and Grogan medium produced 43.8 μg/mL FB_2 after 11 days of incubation. Production of FB_3 from *F. proliferatum* M6290 was 7 μg/mL in Table II medium without methionine and only 4.6 μg/mL was produced in the methionine supplemented medium within 11 days of incubation. Production of FB_1 by these strains ranged from 0.03 - 0.84 μg/mL in all the media and did not seem to inhibit production of FB_2 or FB_3 unlike the earlier experiments (Figures 1 & 2). The use of basal salt medium supplemented with l-methionine seem to support independent production of FB_2 and FB_3 from *F.proliferatum*. There was a 430 and 23 fold increase in the production of FB_2 and FB_3 from *F. proliferatum* grown in methionine and vitamin supplemented medium, previously pre-enriched in medium containing malt extract compared to the unsupplemented medium with glucose used in the pre-enrichment.

Production of fumonisins by *F. proliferatum* in less than a month of incubation, and without the need of sophisticated equipment might facilitate the production of labeled FB_1 for metabolic studies. In this study, basal salts were used with l-methionine and vitamin B supplementation. The ability of *F. proliferatum* to increase fumonisins production in the presence of vitamins implies that the fungus requires vitamins. Most fungi require thiamin and biotin, and fewer fungi require pyridoxamine, while riboflavin, nicotinic acid, pantothenate and vitamin B_{12} are required by very few fungi isolated in nature (Robinson, 1978). *Fusarium proliferatum* seem to require all of these vitamins in order to synthesize fumonisins. *F. proliferatum* utilizes some of the carbons from methionine and the malt extract and seems to prefer the malt extract to glucose at early stages of growth. Other carbon sources for the fungi are from sucrose and glycerol during the later stages of growth. The use of basal salts in combination with the nitrogen (from NH_4Cl and $NH_4H_2PO_4$) and carbon sources serve as growth enzyme activators and increased rate of absorption of the nutrients (Robinson, 1978). The defined nature of this medium (basal salts, methionine and vitamins Table II) makes it well suited for biosynthetic and nutritional requirements of FB production by *Fusaria* spp. This medium could provide a suitable substrate for the production of fumonisins using C-14 labelled methionine.

Metabolism of fumonisins

^{14}C-labelled FB_1 was mainly disposed of in the feces (96%) with 2.5% excreted in urine. The counts in feces include biliary FB_1 excretion. Some radioactivity was found in the liver (0.7%) and to a lesser extent in the kidneys (0.07%) and sample of thigh muscle. There was no radioactivity in the blood. Based on the number of radioactive counts given to the rats and the number of cpm expected, only 4% was absorbed by the body. The amount of radioactivity in the GI tract was not determined; therefore, they might be part of the unaccounted counts. Similar results reported by Shephard et al. (1992a) showed that 101% of methionine-labelled ^{14}C FB_1 was recovered in the feces, and only trace amounts were recovered in the urine and muscles when 28 μCi/mmol FB_1 was dosed at 7.5 mg/kg bodyweight to 6 week old rats. Fumonisin B_1 given through intraperitoneal injection was found (66%) in the feces, 32% in urine, 1% in liver and only trace amounts in kidney and blood. A similar study carried out by the same group but using non-labelled FB_1 showed that after i.p. injection, FB_1 was absorbed rapidly and maximum concentrations were detected in plasma within 20 minutes of injection. Sixteen percent of the dose of FB_1 was recovered intact in feces when the route of exposure was through i.p. injection and only 0.4 % was recovered in urine when exposure by gavage (Shephard et al., 1992b). However, a recent review article points out that some

groups have recovered the greatest amount of radioactivity in the liver and kidney, with small amounts accumulating in these organs for at least 96 hours when the rats were dosed orally (Riley et al., 1993). Results from our study and Shephard's study imply that fumonisins must be highly toxic, because almost all the fumonisin seems to be excreted intact in feces within 24 hours, and only small amounts are seemingly absorbed by the body.

These conflicting results about the disposition of fumonisins indicate the need to produce an efficiently labelled pure FB for toxicokinetic studies.

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Table I: Submerged liquid culture medium used for growing *Fusarium moniliforme* cultures to produce fumonisin B_1

Ingredients[1]	Amount (g/L)
Glucose	90.0
$(NH_4)_2SO_4$	3.5
KH_2PO_4	2.0
$MgSO_4.7H_2O$	0.3
$CaCl_2.2H_2O$	0.4
$MnSO_4.H_2O$	0.2

Ingredients[1]	Amount (µg/L)
Thiamin	500
Riboflavin	500
Niacin	500
Pyridoxamine	500
Thiotic acid	500
Folic acid	50
Biotin	50
Vit B12	50

[1] Glucose, Thiamin, riboflavin, niacin, pyridoxamine, thiotic acid, folic acid, biotin and vit B12 were obtained from Sigma Chemical Co. (St. Louis, MO). $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$ and $MnSO_4.H_2O$ were obtained from Fisher Scientific Chemical Co. (Springfield, NJ).

Table II: Basal salt medium used for growing cultures of fumonisin-producing *Fusaria*

Pre-enrichment medium

Ingredients[1]	Amounts (g/L)
$(NH_4)Cl$	3.0
$FeSO_4.7H_2O$	0.2
$MgSO_4.7H_2O$	2.0
KH_2PO_4	2.0
Peptone	2.0
Yeast	2.0
Glucose*	20.0

Growth medium

Ingredients[1]	Amounts (g/L)
$(NH_4)_2HPO_4$	1.0
NaCl	5.0
$MgSO_4.7H_2O$	2.0
KH_2PO_4	3.0
Sucrose	40.0
Glycerol	10.0

* Glucose was substituted with malt extract[2] in experiments 2 and 3.

1 $(NH_4)Cl$, $FeSO_4.7H_2O$, $MgSO_4.7H_2O$, KH_2PO_4, $(NH_4)_2HPO_4$ and NaCl were obtained from Fisher Scientific Chemical Co. (Springfield, NJ). Glucose was obtained from Sigma Chemical Co. (St. Louis, MO). Peptone and yeast extract were obtained from Difco Lab (Detroit, MI).

2 Malt extract was obtained from BBL (Cockeysville, MI).

Table III: Gilchrist and Grogan medium adapted to permit fumonisins production by *F. moniliforme* and/or *F. proliferatum* cultures.

Ingredients[1]	Amounts (g/100 mL)
Potato dextrose broth	2.4
Yeast extract	0.1
l-methionine	0.25

[1] Potato dextrose broth and yeast extract were obtained from Difco Lab. (Detroit, MI). l-methionine was obtained from Gibco Lab (Grand Island, NY).

Table IV: Amounts of fumonisin B1 produced by *Fusarium proliferatum* M5991 on basal salt medium (medium II) supplemented with vitamins with and without pH adjustment and incubated at 22 ± 2 °C for 24 days

Medium[1]	Amount of FB_1 produced (µg/mL)		
	Day 14	Day 18	Day 24
Medium II (A)	2	5	ND
Medium II (B)	ND[3]	5	10
Medium II (A) pH adjusted[2]	1	ND	3
Medium II (B) pH adjusted	2	4	6

[1] Medium II (A) is a basal salt medium with glucose used in the pre-enrichment medium.
[2] Medium II (B) is a basal salt medium with malt extract used in the pre-enrichment medium. Medium II (A) or (B) pH adjusted indicate that the pH was adjusted to 5 using NaOH or HCl throughout the experiment.
[3] ND means amounts not determined.

Table V: Amounts of fumonisin B1 produced by *Fusarium proliferatum* M5991 incubated on basal salt medium (medium II) supplemented with 25 mg methionine, Gilchrist & Grogan (G & G) medium, at 22 ± 2 °C for 24 days

Medium	Amount of FB_1 produced (µg/mL)		
	Day 10	Day 16	Day 24
(1) G & G	51.7	70.4	71.0
(2) G & G +vit[1]	68.2	79.1	79.8
(3) Medium II	1.9	22.2	ND[3]
(4) Medium II+ met[2]	89.5	230.2	349.0

[1] Gilchrist and Grogan medium #2 was supplemented with 500 µg/L each of thiamin, riboflavin, pantothenate, niacin, pyridoxamine, and thiotic acid, and 50 µg/L each of folic acid, biotin, and vitamin B12.
[2] Medium II is a basal salt medium which was supplemented with 25 mg l-methionine/ 100 mL of medium, and the B vitamins.
[3] ND means amounts not determined.

Table VI: Disposition of a single dose (0.1 μCi) methionine L-[methyl^{14}C]-labelled FB_1 in 3 rats over 24 hrs

Sample	Rat #	Avg. cpm	Quenching factor[1]	Corrected cpm[2]	Total Corrected cmp[3]	% Dose recovered[4]
Feces	1	2007	8.6	17260	50918	95%
	2	2865	8.6	24639	101002	97%
	3	1990	8.6	17111	56462	96%
Urine	1	226	0.8	181	1898	3.0%
	2	240	0.8	192	2112	2.0%
	3	244	0.8	305	1525	2.6%
Liver	1	70	1.4	98	578	1.0%
	2	98	1.4	137	768	0.7%
	3	44	1.4	62	316	0.6%
Kidney	1	23	1.8	41	54	0.1%
	2	27	1.8	49	58	0.1%
	3	13	1.8	24	27	0.05%
Muscle	1	14	1.6	22	68	0.1%
	2	15	1.6	24	62	0.1%
	3	10	1.6	16	38	0.6%
Blood	1	1	1			
	2	-7	1		No quenching	
	3	-3	1			

[1] Quenching factor = (Average # of cpm in spiked sample (or control)- cpm for the blank) ÷ (Average # of cpm obtained by placing the corresponding amount of 14C-labelled FB1 in 10 mL of scintillation fluid - cpm for the blank)

[2] Corrected cpm = Average cpm x quenching factor

[3] Total corresponding cpm = corrected cpm x total amount of sample in g or mL

[4] % dose recovered = 100 x Total corresponding cpm sample ÷ total cpm recovered in all samples for each rat

Figure 1. Production of fumonisins by *Fusarium proliferatum* M5991, a predominate producer of fumonisin B1, in a basal salt medium supplemented with vitamins.

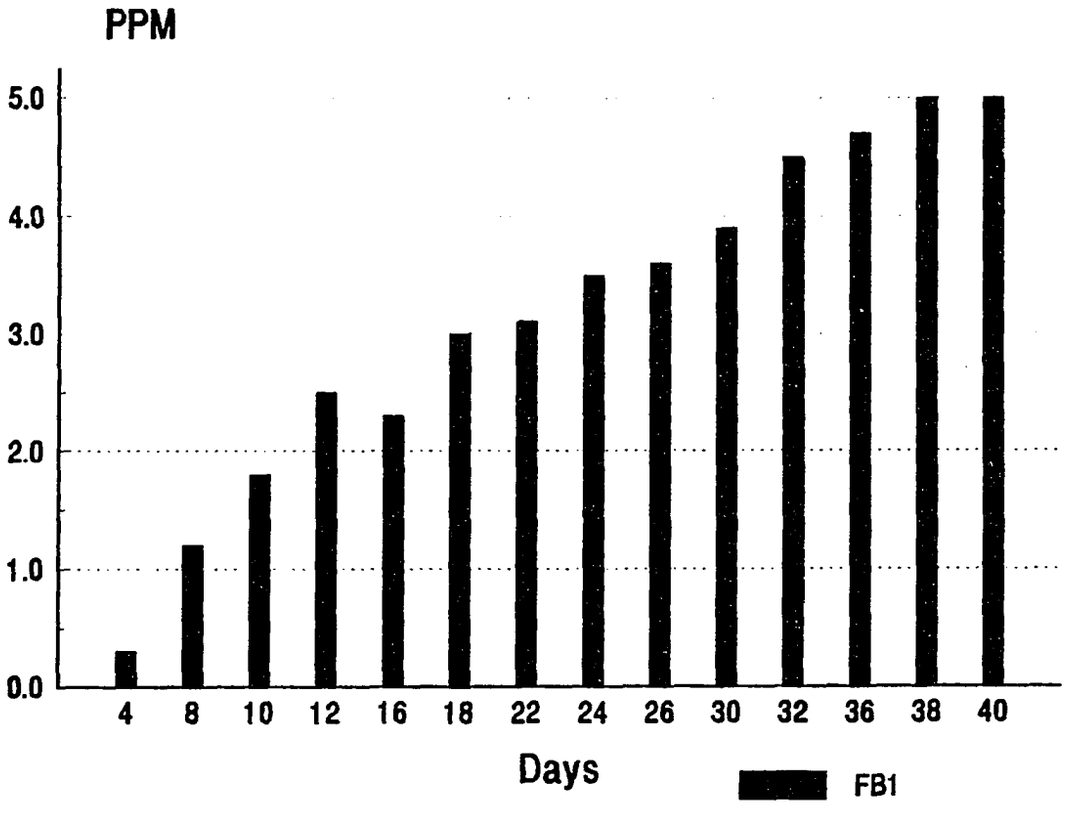

PPM
5.0
4.0
3.0
2.0
1.0
0.0
4
8
10
12
16
18
22
24
26
30
32
36
38
40
Days
FB1

Figure 2. Production of fumonisins by *Fusarium proliferatum* M6104, a predominate producer of fumonisin B_3, in a basal salt medium supplemented with vitamins.

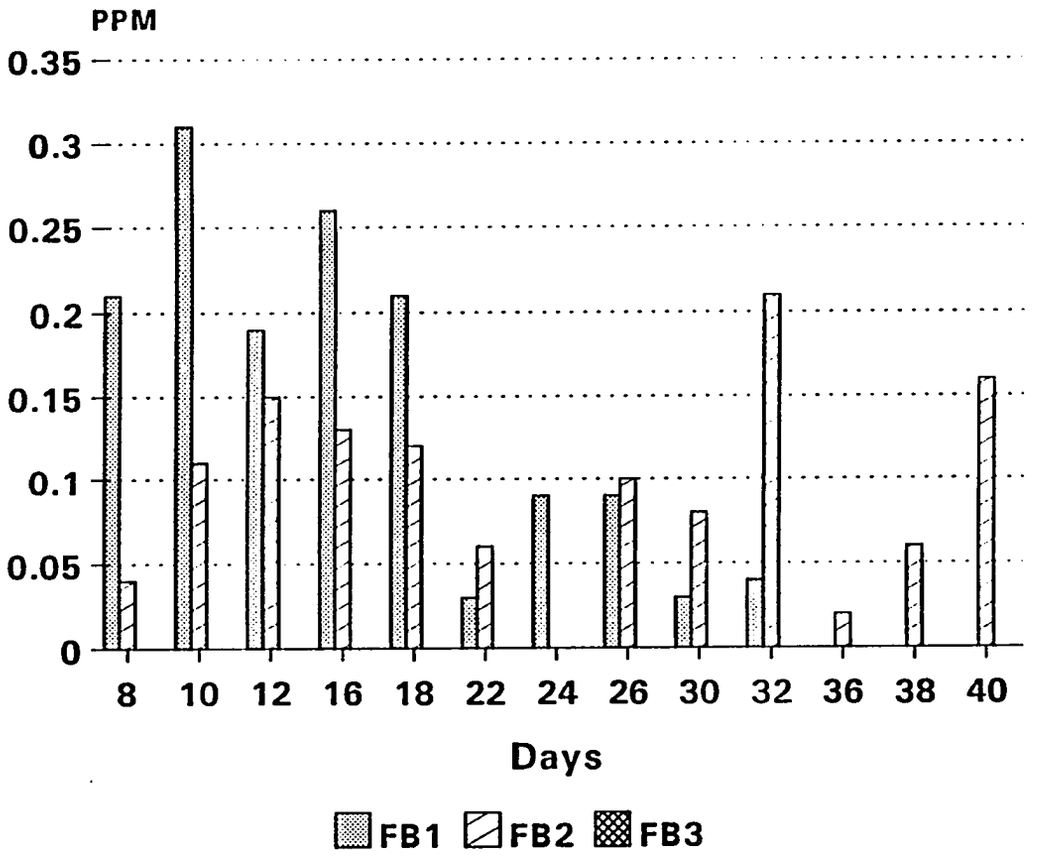
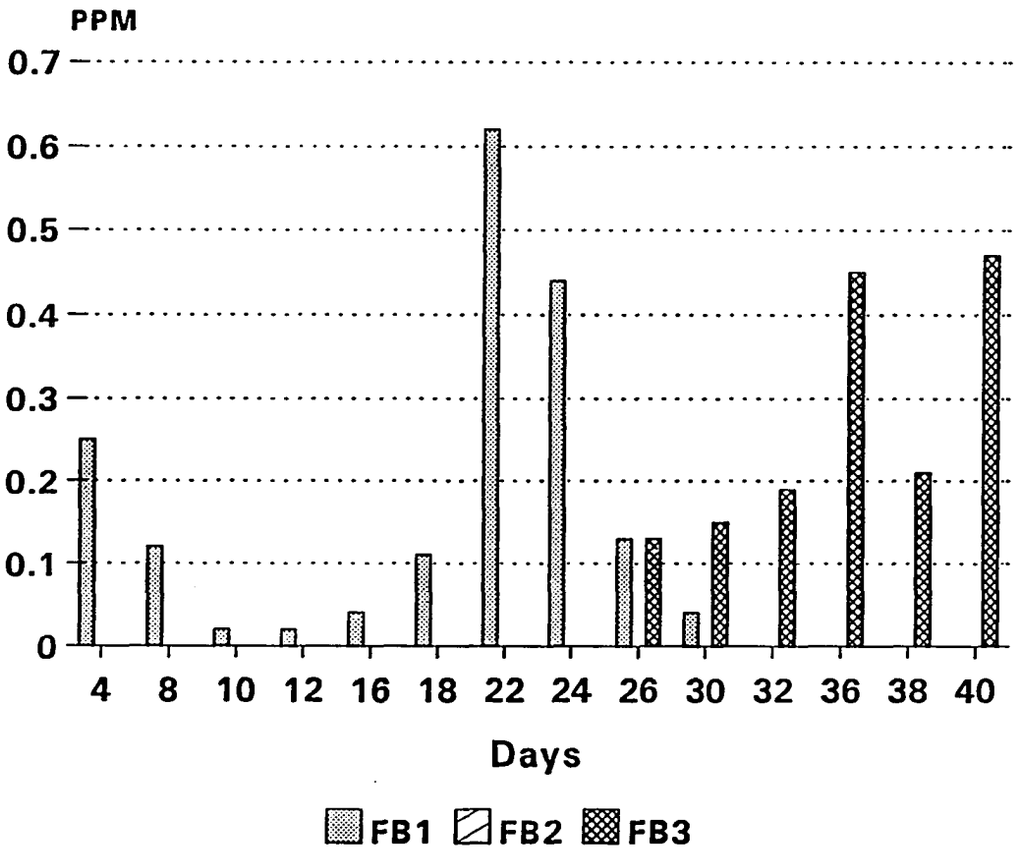

PPM
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
8
10
12
16
18
22
24
26
30
32
36
38
40
Days
FB1
FB2
FB3

Figure 3. Production of fumonisins by *Fusarium proliferatum* M6290, a predominate producer of fumonisin B_3, in a basal salt medium supplemented with vitamins.


PPM
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
4
8
10
12
16
18
22
24
26
30
32
36
38
40
Days
FB1
FB2
FB3

GENERAL CONCLUSIONS

Feeding of *Fusarium proliferatum*-fermented corn culture material containing 20 or 50 ppm FB_1 for 6 months induced liver cancer in female F344/N rats. Placental glutathione S-transferase (PGST) proved to be a better marker of altered hepatic foci (AHF) than γ-glutamyltransferase (GGT) in that only PGST-(+) AHF were noted when a single dose of diethylnitrosamine (DEN, 30 mg/kg body weight), a very mild initiating stimulus was administered to rats. The persistence of PGST-(+) AHF also imply that this marker is more reliable than GGT in examining FB_1 carcinogenesis at small amounts of exposure. Future research is necessary in determining if PGST is also a stable marker of FB_1-induced hepatocarcinogenicity in male and females using purified FB_1.

These studies also showed that FB is fetotoxic, therefore it is potentially teratogenic. The metabolism studies showed that FB_1 is rapidly excreted almost entirely in feces, and only a small amount is absorbed in the body (4%). These results raise other issues concerning the minimum exposure level (dose) of fumonisins, given that only a small amount is absorbed in the body. Specifically, is the proposed mechanism of alteration of sphigolipid metabolism by fumonisins critical in cancer induction, fetotoxicity and other toxic effects caused by fumonisins or is there another mechanism? The

proposed role of fumonisins in sphingolipid metabolism is of importance and needs to be addressed further in future research as to what dose of FB alter the sphingolipid metabolism, and the relationship between the alteration of the sphingoid base ratios and FB toxicity.

In this study, it was shown that the biosynthesis of fumonisins from *F. proliferatum* cultures requires nutrient supplementation beyond the basal salts and carbohydrate source. Supplementing the medium with 25 mg l-methionine/100 mL medium improved the production of fumonisins tremendously, showing a 10 fold increase of FB_1, and a 430 and 23 fold increase in FB_2 and FB_3, respectively. In the presence of methionine production of fumonisins B_2 and B_3 was not delayed; in contrast to when the basal salt was without methionine. The medium without methionine produced the highest concentrations (0.2 μg/mL) FB_2 or (0.45 μg/mL) FB_3 after day 32 and 36 of incubation, respectively. This study could provide a useful basis for subsequently determining whether FB_2 and FB_3 are byproducts of FB_1 or are synthesized independently.

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