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FUNGAL TOXINS IN FOODS: Recent Concerns[1]

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INTRODUCTION

Fusarium moniliforme, the fumonisins (toxins produced by *F. moniliforme*), and the health implications of their occurrence in corn and corn products are the focus of this review. The economic and health risks of other mycotoxins

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were recently reviewed in great detail (26). In order to better understand what the future may hold for regulatory agencies and researchers, it is informative to consider how another fungus and its toxins, *Aspergillus flavus* and the aflatoxins, are perceived and acted upon by these groups. The interest of the scientific and regulatory community in both aflatoxins and fumonisins has grown in response to large increases in the incidence of unusual diseases in farm animals. For the aflatoxins this occurred in 1960 (15) and for the fumonisins, in 1989 (100). Aflatoxins (B_1, B_2, G_1, G_2) are currently the mycotoxins of greatest concern in the United States. However, the fumonisins have become the fastest growing area of mycotoxin research (93).

Perceived human health risks have spurred regulation of the aflatoxin content of foods and feeds. According to a recent General Accounting Office report to Congress concerning risks to humans, ". . . no cases of illness and/or death from eating aflatoxin have been documented in the United States" (33). In the United States the known effects on animals (26) and the perceived chronic risks to humans provide the pressure to regulate (93). The most obvious chronic risk is increased cancer incidence.

The United States is the world's major corn exporter. Three mycotoxins (or groups) commonly found on US corn are considered by the International Agency for Research on Cancer (35) to be either carcinogenic (class 1) or possibly carcinogenic (class 2B) to humans: aflatoxins (B_1 and G_1), zearalenone, and *Fusarium moniliforme* toxins (culture material and grains contaminated by *F. moniliforme* and containing fumonisins and fusarins). Currently, aflatoxin B_1 is the only mycotoxin for which the Food and Drug Administration (FDA) has set action levels in corn. An action level is the level of contamination at which the FDA may regard the food as adulterated. Action levels do not have the force of law and are not legally binding on the courts. States may set more stringent levels. In the United States, corn for use in human foods or for immature animals and dairy cows cannot exceed 20 ppb aflatoxin B_1. The action levels for aflatoxin in other animal feeds are higher; thus, corn that exceeds the 20-ppb action level is not destroyed but can often be used for various animal feeds. Recently, replacement of action levels with "regulatory limits," which are legally binding, has been discussed (34). Whether or not action levels or regulatory limits will be set for the other two carcinogenic mycotoxins on corn (zearalenone and *F. moniliforme* toxins) is uncertain. Possibly, domestic users or importing countries may set contractual specifications that could preempt any regulatory action by the FDA. Most importing countries have set aflatoxin limits that are lower than the action levels or regulatory limits set by exporting countries (31).

Human risk assessment plays a potentially important role in the economics of corn, an 8 billion bushel crop valued at $18 billion (124). Interestingly,

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less than 17% of the US corn supply is consumed as food by humans. Most corn in the US is consumed as animal feed or is exported (124) to other countries where it is used for animal feed. Nonetheless, the risk assessment of greatest significance pertains to humans. While the relationship between aflatoxin and liver cancer in humans in third world countries continues to be debated, there is much less debate about aflatoxin as a cause of liver cancer in the United States. According to a report by the General Accounting Office (33) the FDA Commissioner has stated ". . . the occasional consumption of the very few corn products that contain a measurable amount of aflatoxin are of little lifetime health consequence. . . ." However, the fact that the amount of aflatoxin consumed is small does not mean that the total amount of carcinogenic mycotoxins consumed is small. While the debate about the risks associated with consumption of aflatoxins continues, the fumonisins, a group of mycotoxins that induce cancer in laboratory rats, have been discovered. There is little doubt that the fumonisins will cause problems for producers and processors and that these problems may result in regulatory actions similar to those that govern the aflatoxins. The remainder of this review summarizes the current literature and research.

BIOLOGY OF *FUSARIUM MONILIFORME*

Fungus Description and Taxonomy

Fusarium moniliforme J. Sheldon [perithecial state *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura] belongs to the *Liseola* section of the genus *Fusarium*. The perithecial state of this fungus consists of a complex of biological species or mating populations (50) and is currently defined as having six different mating populations (63, 65) among which exist bisexual, self-sterile groups. Isolates of *G. fujikuroi* with members of the A and F mating population are the only fungi within the complex that are synonymous with *F. moniliforme* (65).

Occurrence and Pathogenicity in Plants

The fungi of mating popultions A and F, i.e. *F. moniliforme,* are distributed worldwide and are found primarily on cereal grasses in the warmer regions of the world (69). However, this distribution appears to be host species specific. Thus, fungi of mating population A and F are primarily found on corn and sorghum, respectively (50). The fungus on corn can exist as an endophyte; it may colonize portions of the developing plant but usually remains symptomless (7, 49). This symptomless expression apparently is related to the genetic nature of the fungus, the cultivar of corn (49, 85, 104), and environmental

factors. Aspects of the corn disease complex caused by *F. moniliforme* are stalk rot, seedling blight, root rot, and ear rot.

The points of entry into corn by the fungus vary, and the extent of infection as well as the nature of the expression depend on the route of entry. For example, a diseased plant might develop from a symptomless infection produced by the fungus located in the seedling root, the young shoot, or the nodal and other vegetative areas, but the corn kernels may also become infected by means other than vegetative parts. In the latter case, insects and wind may carry the fungus into the developing corn kernels, usually via the silk tracks (27). The results of ear infection vary: Some kernels are symptomless while others are obviously infected. The important point is that *F. moniliforme* is seed-borne. The fungus is located in the pedicel of kernels as a very small number of hyphae, but in kernels associated with animal toxicity the fungus is usually found as an extensive mass of sporulating hyphae that colonizes most of the internal section of the kernel, including the embryo (10). The fungus usually infects a seedling from the kernel within a two-week period. Death of the seedling may occur within three weeks (Bacon et al, manuscript submitted) or the infected seedling may show no disease symptoms as described above. In addition to being seed-borne, *F. moniliforme* is also soil-borne and survives in plant residues (83, 84), particularly roots (123a). This fungus does not produce sclerotia as survival structures; it can survive in the soil, however, as thickened hyphae within corn fragments buried 30-cm deep within a soil moisture range of 5–35%, at 5–10°C for 12 months.

Plant Toxicity

Both the A and the F mating populations of *F. moniliforme* are capable of producing mycotoxins such as the fumonisins (64), fusaric acid (C. W. Bacon, unpublished data), and fusarin C (9). The A mating population, however, is a much better producer of fumonisin B_1 than is the F population (65). The D mating population of *G. fujikuroi (F. proliferatum)* is also a good producer of FB_1 (65). *F. moniliforme* does not commonly produce moniliformin, whereas *F. proliferatum* can produce it (78). Another compound of interest that is produced by *F. moniliforme* is gibberellic acid, a plant growth regulator. Some of these compounds are phytotoxins and as such they may play important roles in the final pathology of the corn. At present, however, we have no specific information on any role these toxins play in disease expression on corn. The fumonisins are structurally similar to the tomato host-specific toxin, the AAL toxins, produced by *Alternaria alternata* f. sp. *lycopersici*. The AAL toxins produce stem canker disease in "Earlypak-7" and other susceptible cultivars of tomatoes (113). Similar disease symptoms are also produced on susceptible tomatoes by fumonisin B_1. However, *F. moniliforme* is not a natural

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pathogen of tomatoes, and all attempts at infecting tomatoes with it have failed (10, 42).

A growth-regulating response has also been observed by the addition of fumonisin B_1 to tomato plants (8). When fumonisin B_1 is applied to detached stems of resistant tomato cultivars, it induces de novo adventitious rooting. Plants dosed with a single application of fumonisin B_1 initiated callus tissue within a 24–48 hr period, and roots were produced as early as 72 hr after a dose of 10 μg per shoot. The induction of rooting was observed only in tomato cultivars that were resistant to the mycotoxin at amounts <50 μg per seedling. In one resistant cultivar, no rooting response was observed, but this cultivar showed no toxicity signs at the high doses, >50 μg per shoot, which suggests a permeability difference. The mechanism of induction may be complex and may be associated with an interaction of the rooting hormone indole acetic acid with calcium and related enzymes (14, 18). The effects of fumonisin B_1 on rooting of corn and other plants are unknown. However, it is toxic to jimsonweed (*Datura stramonium* L.) (3) and duckweed (2) (*Lemna minor* L.). This latter plant and susceptible genotypes of tomatoes (42) may be used as rapid assays for biological activity of the fumonisins and related compounds.

Control Potentials

F. moniliforme is a common cereal grain contaminant and control measures may prove difficult. Control of this pathogen on food stuff should focus on its complex infection cycle and/or the potential mechanisms for developing resistance to *F. moniliforme* in corn. Expression of the disease is highly variable, probably because of heterozygosity within a corn cultivar and genetic variation within the fungus species. Several aspects of infection appear to be under genetic control (28), and the genetics of the pericarp is considered a major determinant of resistance to air and insect transmission of the fungus into grain (104). Therefore, plant breeding is expected to be very helpful in preventing the fungus from entering the food chain. Possibly, control of soil-borne and kernel infection may be achieved with the use of microorganisms, particularly soil and endophytic bacteria (10). Of course, the successful utilization of biocontrol measures must be based on detailed knowledge of *F. moniliforme* and its association with corn—information that is not presently available.

Postharvest control of fumonisin production is possible. Two important factors are aeration and moisture content (61). Fumonisin production at low oxygen tension is minimal; thus, storage under modified atmospheres (e.g. N_2 or CO_2), or as silage, and low kernel moisture content $<22\%$ should reduce or prevent toxin production in storage (61).

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DISEASES ASSOCIATED WITH *FUSARIUM MONILIFORME* AND FUMONISINS

Equine Leucoencephalomalacia

Equine leucoencephalomalacia (ELEM) syndrome is characterized by the presence of liquefactive necrotic lesions in the white matter of the cerebrum. The name is somewhat misleading, since the gray matter may also be involved (67). This fatal disease apparently occurs only in equids. The syndrome has been recognized since the nineteenth century as a sporadically occurring condition. In 1902 (21) ELEM was experimentally produced by feeding moldy corn obtained from a field case in Kansas. The disease was known as "moldy corn poisoning," but attempts to identify the responsible fungus failed.

Wilson & Maronpot (131) succeeded in establishing the causative agent when they isolated *F. moniliforme* as the predominant contaminant of moldy corn that had caused many cases of ELEM and when they reproduced ELEM by feeding *F. moniliforme* corn culture material. Many other investigators have since demonstrated the ability of *F. moniliforme* to duplicate the symptoms of ELEM.

Shortly after the isolation of fumonisins and the identification of their structures (13, 36), Marasas et al (67) successfully produced ELEM in a horse by the intravenous administration of fumonisin B_1. This was done by avoiding hepatotoxicity as much as possible. ELEM has also been produced in horses given pure fumonisin B_1 by stomach tube, again monitoring for liver toxicity (56). Researchers have suggested (67) that high dosage levels of fumonisins induce fatal hepatotoxicity with mild brain lesions, whereas low dosage levels cause mild hepatotoxicity and severe brain lesions. However, central nervous system signs and liver lesions in the absence of elevated serum parameters, and ELEM concurrent with significant liver disease have been observed in horses fed feeds naturally contaminated with fumonisins at low levels (133). The development of brain lesions in the absence of major liver lesions does not preclude biochemical dysfunction in nonbrain tissue from contributing to the brain lesions. Current evidence suggests that horses that consume feed containing levels as low as 8 ppm of fumonisin B_1 may be at risk for developing ELEM (133).

Porcine Pulmonary Edema Syndrome

In trials with cultures of *F. moniliforme,* Kriek et al (58) fed horses, pigs, sheep, rats, and baboons. Lung edema occurred only in pigs. In 1989–1990 outbreaks of porcine pulmonary edema (PPE) were reported in different parts of the United States (25, 86, 101). Corn screenings obtained from farms (46, 86) where PPE killed pigs were predominantly contaminated with *F.*

moniliforme. Feeding *F. moniliforme* culture material produced PPE in at least one study (86) since the report by Kriek et al (58), but not all such experiments have been successful (Billy M. Colvin, personal communication). Nevertheless, the association between *F. moniliforme* and PPE appears to be strong, especially since purified fumonisin B_1 has been shown to produce the disease when administered intravenously (46, 48, 86). Also, PPE (175 ppm, 14 days) and hepatotoxicity ($\geq$23 ppm, 14 days) have been produced by feeding diets made with corn screenings naturally contaminated with fumonisins (11; G. Motelin et al, manuscript submitted).

As with ELEM, a strong correlation exists between the fumonisin content of feed obtained from different farms and outbreaks of PPE (100, 101). PPE has not yet been produced by oral administration of pure fumonisins; liver lesions have been observed, however, in swine orally dosed with pure fumonisin B_1 (47). The evidence gathered thus far suggests that the mycotoxin is responsible for PPE as well as for ELEM.

Poultry Toxicity

Several reports have been published implicating *F. moniliforme* contamination of feed in diseases of poultry (20, 30, 55). Immunosuppression in chickens was also produced in birds fed corn cultured with several different isolates of the fungus (71). Several recent studies have confirmed that *F. moniliforme, F. proliferatum,* fumonisin B_1, and moniliformin are toxic to broiler chicks (19, 29a, 52, 53, 62) and chicken embryos (54). The levels of fumonisins used in these studies were relatively high (75–644 ppm), and the co-occurrence of moniliformin in some studies posed an additional complication for interpretation. Studies at fumonisin levels closer to those found in naturally contaminated rations (1–20 ppm) should be conducted, and surveys to determine the prevalence of moniliformin in poultry diets are needed.

Human Esophageal Cancer

In some regions of the world the incidence of esophageal cancer (EC) far exceeds the "normal" occurrence of 5 or fewer cases per 100,000 population. The Transkei of South Africa has been the most extensively studied region (125) of high EC rate (50–200 per 100,000). The afflicted people consume corn as a staple, including a type of beer brewed with corn and a nonalcoholic fermented drink made with corn (98, 107). The corn is locally grown and harvested, is stored in open cribs, and is frequently visibly moldy (66). The moldiest ears of corn are hand selected for use in beer brewing (70), and *F. moniliforme* is the predominant fungus. Contamination of the corn with *F. moniliforme* (66, 94) and fumonisins (120) in the areas of high EC was statistically higher than that found in areas of the Transkei with low cancer

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rates (66, 94). The carcinogenic potential of fumonisin B_1 has been shown in laboratory rats (37). Whether fumonisins are responsible for the high rate of EC in regions of the Transkei cannot be determined conclusively, but the available evidence suggests that their presence may be at least partly responsible. Other areas of the world have high EC rates, but an association with *F. moniliforme* and/or fumonisins is not well documented (for a recent review see 79).

Studies With Laboratory Animals

In addition to studies designed to elucidate the relationship between *F. moniliforme*-induced diseases in farm animals and the fumonisins, numerous studies have been conducted with laboratory animals. Rats fed corn either contaminated with *F. moniliforme* naturally or with *F. moniliforme* culture materials develop liver tumors (51, 68, 132). Subsequent feeding studies showed that fumonisin B_1 (not $\leq$90% pure) fed at 50 ppm in a semi-purified rat diet for 18 to 26 months caused an increased incidence (10 of 15 rats) of hepatocellular carcinoma (37). Hepatotoxicity was evident after 6 months and progressed in severity with time (37). Short-term carcinogenesis studies using focal proliferation of heptocytes as an end point revealed that fumonisins B_2 and B_3 may also contribute to the toxicological and carcinogenic effects of *F. moniliforme* (39). Fumonisins were not mutagenic in the *Salmonella* mutagenicity assay (40) and they were not genotoxic in DNA repair assays using primary rat hepatocytes (80). Long-term feeding studies using pure fumonisin B_1 will be conducted by the National Toxicology Program in the near future (K. A. Voss, personal communication).

In some studies with rats, the kidney is an equal or more sensitive target for fumonisin B_1 fed either as naturally contaminated corn (127) or as pure fumonisin B_1 (126a). Interestingly, other reports for rats have indicated that the kidney is not an important target of fumonisin-induced toxicity (37, 38). The reason for these differences has yet to be explained.

Preliminary toxicokinetic studies in rats using fumonisin B_1 or [^{14}C]fumonisin B_1 have been reported (109, 110, 79a). The results indicate that orally dosed fumonisin B_1 is poorly absorbed, rapidly eliminated, and not appreciably metabolized. In the study by Norred & Plattner (79a), the liver and kidney were the organs that contained the greatest amount of radioactivity. Even though only a small amount of radioactivity was accumulated in liver and kidney, it remained constant for at least 96 hr (79a).

A study in which Vervet monkeys were fed low (0.25–to 1.0%) levels of *F. moniliforme* culture material in a corn-based, low-fat, high carbohydrate diet for periods up to 2 years indicated that *F. moniliforme* (and fumonisins) may cause atherosclerosis (32). However, the atherogenic potential was

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secondary to chronic hepatotoxicity as evidenced by liver fibrosis and elevated serum enzymes.

CHEMISTRY OF FUMONISINS AND RELATED COMPOUNDS

Isolates of *F. moniliforme* associated with disease outbreaks often produce large quantities of fumonisins (99, 122). It is reasonable to believe that the toxicities associated with consumption of *F. moniliforme* culture materials, or naturally contaminated corn, are a result of multiple toxins, toxin interactions, and synergies. However, the animal diseases associated with consumption of *F. moniliforme* culture materials or naturally contaminated corn have all been reproduced using pure fumonisin B_1 (see previous section). At the time that the structures of the fumonisins were first reported (13), two other groups had independently concluded that the hepatotoxic principle of *F. moniliforme* culture material was water soluble (60, 128) and of low molecular weight (60). The discovery of the fumonisins was made possible by the development of a short-term cancer initiation/promotion bioassay for screening the various fractions of the extracted corn culture materials (36). Interestingly, in 1981 a group of structurally similar, water-soluble compounds (AAL toxins) were isolated from culture filtrates of *Alternaria alternata* f. sp. *lycopersici* (17) after development of an appropriate plant bioassay (41). The chemistry and in vitro biological activity of these two groups of structurally related compounds are surprisingly similar. The AAL-toxins (T_A 2a and T_A 2b) are the monoesters of propane-tricarboxylic acid and 1-amino-11,15-dimethyl-2,4,5,13,14-pentahydroxyheptadecane (molecular weight = 521). The single propanetricarboxylic acid moiety is esterified at the C-13 (T_A 2a) or C-14 (T_A 2b) position of the 17 carbon aminopentol backbone. There is also a T_B series (2a and 2b), which differs from the T_A series because it lacks the C-5 hydroxyl and has a differing stereochemistry (17). Fumonisin B_1 is the diester of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyicosane (molecular weight = 721). The propanetricarboxylic acid moieties are esterified at the C-14 and C-15 positions of the 20-carbon aminopentol backbone.

Currently, the fumonisin family comprises fumonisins B_1, B_2, B_3, B_4, A_1, and A_2 (38). In naturally contaminated corn, the ratio of fumonisin B_1/fumonisin B_2 is approximately 3 and for fumonisin B_1/fumonisin B_3 it is 12 (100). Exceptions have been noted, but the isolated fungus is usually *F. proliferatum* (100). The fumonisins and AAL toxins are clearly "sphingosine-like" in their structures, and it has been suggested that the fumonisins follow a similar biosynthetic pathway (1, 91). However, other data suggest a polyketide-like pathway (77). Members of the B series differ in the number

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and location of the hydroxyl groups. Fumonisin B_1 is the most polar of the B series (22). Members of the A series are *N*-acetylated. There has been some speculation that the amides are artifacts of the extraction method (92).

The propanetricarboxylic acid moieties are easily removed by base hydrolysis (92, 111), and the amino group reacts readily with reagents commonly used for modification of protein amino groups (e.g. acetic anhydride, trinitrobenzenesulfonic acid, etc). Presumably, the carboxyl groups are equally reactive with reagents such as carbodiimide. Monomethyl and dimethylesters of fumonisin B_1 are produced during the isolation procedures when methanol is used for extraction (22). The fumonisins and AAL toxins have no ultraviolet or visible absorption, do not fluoresce, and are not easily volatilized.

Fumonisins can be purified from corn culture material (with difficulty) to a white powder (22); there is no published report of crystalline material. Often, fumonisin B_1 purified from corn culture material, which tests 100% pure based on mass comparison against a reference standard, is hygroscopic and slightly colored (B. Chamberlain, personal communication). Recently, methods have been developed for fermentation, extraction, and purification of fumonisins using liquid culture by stirred jar fermentation (77). Purification from liquid is reportedly much easier.

Nuclear magnetic resonance studies indicate that fumonisin B_1 interacts strongly with metal cations (59). In polar solvents fumonisin B_1 exists as a zwitterion, and it has been suggested that the structure favors intra and/or intermolecular hydrogen bonding and electrostatic interactions between the carboxyl and amino groups (59). Changes in chemical shifts of carbon and hydrogen of the propane-tricarboxylate esters, observed at pH 4.0, 5.7, and 7.0, are presumed to be a result of interaction between carbonyl groups (92). At present, little is known about the stereochemistry of the fumonisins; however, the chemical structures suggest a large number of possible stereoisomers (1,024 isomers, J. ApSimon, personal communication). Like sphingoid bases, which they resemble, some isomers may be biologically active and some inactive in various systems.

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MODE OF ACTION OF THE FUMONISINS

Inhibition of Sphingolipid Biosynthesis In Vitro

The fumonisins (B_1, B_2, and hydrolyzed B_1) were shown to be the first naturally occurring specific inhibitors of de novo sphingolipid biosynthesis (129). The reader is directed to a recent paper describing this discovery and the implication for the diseases caused by fumonisins (76). In addition to inhibition of de novo biosynthesis, fumonisins also appear to inhibit the reacylation of sphingosine within the sphingolipid turnover pathway and may

inhibit acylation of dietary sphingosine. This raises the interesting possibility that the animal diseases associated with the consumption of fumonisins, may result from altered sphingolipid biosynthesis. While other compounds and conditions can alter the kinetics and/or direction of carbon flux in the biosynthetic and turnover pathways, only fumonisins (including B_3 and AAL toxins; 76) have been shown to inhibit specifically ceramide synthases (sphinganine and sphingosine *N*-acyltransferase).

In primary rat hepatocytes, a consequence of in vitro inhibition of the sphinganine *N*-acyltransferase was the decrease in ceramide biosynthesis, and thus, de novo sphingosine biosynthesis (129), the rapid accumulation of sphinganine [the immediate precursor in the biosynthetic pathway of dihydroceramide (*N*-acylsphinganine)] (129), accumulation of sphingosine and sphinganine cleavage products (76), and depletion of complex sphingolipids (76, 129). In cultured cerebellar neurons, fumonisin B_1 inhibited de novo sphingomyelin biosynthesis to a greater extent than it inhibited glycosphingolipids, which suggests that fumonisins preferentially inhibit sphingomyelin biosynthesis in these cells (75). In cultured renal epithelial cells treated with fumonisin B_1, free sphingosine levels became significantly elevated but to a much lesser extent than free sphinganine (135). Unlike primary hepatocytes, the cytostatic and cytotoxic effects of fumonisin B_1 were well correlated with the inhibition of de novo sphingolipid biosynthesis (135). The accumulation of free sphingosine indicates that either reacylation of sphingosine derived from sphingolipid turnover or acylation of sphingosine derived from the serum component of the culture medium was also inhibited. The fact that sphinganine accumulated to a much greater extent than sphingosine suggested that the de novo pathway was the primary target for inhibition. As a result of this differential inhibition, the ratio of sphinganine to sphingosine increased after exposure to fumonisins, and these increases occurred long before any indication of decreased proliferation or cytotoxicity (135). In addition to implicating altered sphingolipid biosynthesis in the cytostatic and cytotoxic effects of fumonisins, the results suggested that changes in the relative amounts of free sphinganine and free sphingosine might be useful as a biomarker for animals consuming fumonisins.

Alterations in Free Sphingoid Bases In Vivo

Based on the specificity of fumonisin B_1 in inhibiting de novo sphingosine biosynthesis in primary rat hepatocytes (129), it was hypothesized that consumption of fumonisins would result in an elevated free sphinganine to free sphingosine ratio in serum. This hypothesis was first tested in horses. An increase in the serum free sphinganine to free sphingosine ratio was demonstrated, within 1 to 5 days, in ponies given feeds containing corn screenings naturally contaminated with 44 ppm fumonisin B_1 (130). The ratio became

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elevated before increases of serum enzymes indicative of cellular injury and in some ponies within 1 day after consuming fumonisin-contaminated feeds. The ratios returned to control levels when ponies voluntarily stopped eating the contaminated feed. However, the decrease in the ratio lagged by several days the decrease in fumonisin consumption. When animals resumed consumption of contaminated feed the ratios once again became elevated. In a pony given feed containing 15 to 22 ppm fumonisin B_1, no significant change in the ratio occurred until day 182 when the ratio increased dramatically and remained elevated until the animal died of equine leucoencephalomalacia on day 241. The increase in the ratio occurred at least 42 days before any other serum biochemical indices of cellular injury increased.

Similar results were obtained for pigs, confirming a dose-response relationship between the ratio of free sphinganine to free sphingosine in serum and tissues and the amount of fumonisin-contaminated feed consumed (95). Pigs were fed fumonisin-contaminated feed formulated from naturally contaminated corn screenings at 0 (<1), 5, 23, 39, 101, and 175 ppm total fumonisins (B_1 plus B_2). The results showed that the ratio was significantly elevated in liver, lung, and kidney from pigs consuming feeds containing ≥23 ppm fumonisins. Liver injury was observed in $^1/_5$ of pigs at 23 ppm, $^3/_5$ of pigs at 39 ppm, and all pigs at higher doses. However, injury to the kidney was not observed at any dose even though it contained equal or greater amounts of free sphingoid bases. In lung tissue, free sphingoid base content was elevated at doses ≥23 ppm, but lung lesions were only observed in pigs fed the diet containing 175 ppm fumonisins.

Possibly, different tissues have different tolerances to elevated levels of free sphingoid bases. Alternatively, (*a*) elevations in free sphingoid base concentrations may be a benign early response, (*b*) such elevations may not accurately gauge the extent of complex sphingolipid depletion, or (*c*) in some tissues sphingolipids may play more critical roles in maintaining cellular integrity and/or regulating cell function. In addition, many effects may be indirectly linked to sphingolipid alterations as has been proposed for pulmonary edema in swine (48). Nonetheless, it is very clear that alteration in free sphingoid bases is a very sensitive biomarker of fumonisin exposure.

In the same study with pigs (95), elevation of the ratio in serum paralleled the increase in tissues. This finding supported the earlier hypothesis (130) that the elevated ratio in serum was due to the movement of free sphinganine (accumulating as a result of inhibition of sphinganine *N*-acyltransferase) from tissues into the blood. Statistically significant increases in the serum ratio were observed at feed concentrations as low as 5 ppm total fumonisins (after 14 days) and in pigs (at higher concentrations) in which other serum biochemistry parameters were not elevated and in which there were no observable gross or microscopic lesions in liver, lung, or kidney. Thus, the

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increase in the sphinganine to sphingosine ratio appears to be a more sensitive indicator of fumonisin exposure than the development of detectable liver or lung lesions in pigs. It has been proposed that the ratio of free sphinganine to free sphingosine and the presence of elevated levels of free sphinganine in serum, urine, and tissue be used as an indicator for consumption of fumonisins by farm animals (97).

Pigs fed either naturally contaminated feed (27 ppm) or pure fumonisin B_1 at 1.5 mg/kg/day twice a day for 5 days showed qualitiatively similar alterations in free sphingoid bases in tissues and serum (47). Interestingly, in both groups, the increase in free sphingoid bases was greatest in the kidney. Liver, lung, and serum were also affected, but other tissues were only slightly or not at all altered (47).

In Sprague Dawley rats fed either *F. moniliforme* culture materials or pure fumonisin B_1 for 4 weeks (126a, 127), the kidney was a very sensitive target organ based on observed microscopic lesions. In the rats fed pure fumonisin B_1, elevation in free sphingoid bases in the kidney was closely correlated with the ultrastructural lesions (96). Rats fed *F. moniliforme* culture materials also had elevated free sphingoid bases in liver, the only organ analyzed (82).

Chickens fed feeds supplemented with *F. moniliforme* culture materials (T. S. Wiebking et al, manuscript submitted) or pure fumonisin B_1 (M. Henry, personal communication) exhibited elevated sphinganine levels and elevated ratios in tissues and serum. Thus, ingestion of fumonisins by every target animal tested to date results in elevation in free sphingoid bases in a manner consistent with inhibition of *N*-acyltransferases.

The following facts, when taken together, make a strong case for linkage between inhibition of de novo sphingolipid biosynthesis and the animal diseases shown to be caused by *F. moniliforme* culture materials and pure fumonisins. First, alterations in free sphingoid bases can be detected before or at the same time as ultrastructural lesions. Second, there is a close dose-response relationship between fumonisin levels in naturally contaminated diets or diets containing pure fumonisin B_1 and the degree of elevation of free sphingoid bases and depletion of complex sphingolipids. Third, changes in free sphingoid bases can usually be detected prior to elevation of serum biochemical parameters and at lower doses. And lastly, all the animals tested to date have responded to fumonisins with elevated levels of free sphingoid bases. However, the mechanism by which these sphingolipid alterations cause tissue damage is unknown.

Altered Sphingolipid Biosynthesis and Animal Diseases

The implications of fumonisin inhibition of sphingosine and sphinganine *N*-acyltransferases for diseases caused by fumonisins has been addressed in

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detail previously (76). A review of the sphingolipid literature is beyond the scope of this paper. Briefly, the maintenance of a low level of free sphingoid bases in tissues is important because these compounds have considerable intrinsic biological activity and can be cytotoxic in high concentrations (for review see 44, 72, 74). Thus, elevation of intracellular free sphingoid bases will disrupt the normal regulatory mechanisms within cells. In addition, complex sphingolipids have numerous important functions in cell membranes including stabilization of the membrane, sorting of lipids and proteins, binding to cytoskeletal elements, and cell-cell recognition (for review see 43, 44, 73, 114). Thus, depletion of complex sphingolipids in membranes will disrupt the normal function of the membrane. The regulated breakdown and turnover of complex sphingolipids such as sphingomyelin have been hypothesized to result in the formation of lipid second messengers (57). These hypothesized sphingolipid second messengers and effector systems (i.e. sphingosine and ceramide) may act as intracellular signals for turning on and turning off processes inside cells. Such processes include the expression of genes, activation or inactivation of specific proteins such as protein kinase C and phosphatidic acid phosphatase, the regulation of growth factor receptors, and other intracellular signalling systems (e.g. Ca^{2+}). All of the processes listed above are intimately connected to the processes of proliferation and differention of cells. Clearly, deciphering the observed in vivo effects of fumonisins in terms of altered sphingolipid biosynthesis will be complicated by the fact that there are potentially many mechanisms of action in cells.

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Other Hypothesized Modes of Action

While the inhibition of de novo sphingolipid biosynthesis is a highly attractive hypothesis for explaining the diseases caused by fumonisins, it is quite possible that there are other mechanisms. Aside from one report that fumonisins interfered with calcium in atrial muscle, there is very little other mechanistic data (103).

Since fumonisins are neither genotoxic nor mutagenic (see above), and do not appear to be metabolized to any significant extent in vivo, the mechanism of carcinogenicity will probably be difficult to ascertain. In vitro studies with cell lines indicate that fumonisins are both cytostatic and cytotoxic (29, 112, 135). There have been no published reports that fumonisins have mitogenic activity in vitro. Yet, in vivo, proliferating foci are commonly observed in both liver and kidney of rats (36, 38, 39). Under appropriate conditions, low levels of free sphingoid bases are known to be mitogenic. Thus, possibly there are conditions that could promote increased cell proliferation, subsequent to fumonisin-induced tissue damage (76).

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DETECTION AND OCCURRENCE OF FUMONISINS

Analytical Methodology

At the moment, two extraction methods are commonly cited for analysis of fumonisins in foods and feeds. Both methods involve extraction with polar solvents (methanol/water or acetonitrile/water) followed by clean-up on either strong anion-exchange column (SAX) (108) or via a C_{18} reverse phase Sep-Pak (Waters Associates, Milford, MA) (134). Use of the C_{18} method is more rapid than the SAX method; however, the SAX method produces cleaner fractions and thus detection at lower limits (B. Chamberlain, personal communication). Recovery of added fumonisins is good (>80%); however, how well these methods extract all fumonisins from natural matrices is unclear (16). Another current problem is that verified reference standards are not available.

Detection by liquid chromatography (108, 134) or thin layer chromatography (TLC) (36, 102) requires derivatization, whereas gas chromatography-mass spectrometry (GC-MS) (90) requires base hydrolysis to remove the tricarballylic acid groups and derivatization of the amino group to enhance volatility. The advantage of the GC-MS method is that it combines structural confirmation with quantification. Unfortunately, the method requires considerable time and equipment. The most frequently used method for quantification in foods and feeds is liquid chromatography, which utilizes *o*-phthaldialdehyde as the derivatizing agent (108, 118). The liquid chromatographic method can separate fumonisins B_1, B_2, and B_3 in 16 min using an isocratic solvent system, and the detection limit is 50 ppb using the methanol/water plus strong anion exchange extraction procedure (118). One problem is that the *o*-phthaldialdehyde-fumonisins derivative is not as stable as some other fluorescent derivatives (118) such as naphthalene-2,3-dicarboxaldehyde or 4-fluoro-7-nitrobenzofurazan (12, 105).

The least expensive and least sensitive method for detection of fumonisins is TLC (36). Using a two-step TLC development system made it possible to shorten the extraction procedure and quantify the para-anisaldehyde derivative using spectrophotodensitometry (61). The detection limit by this method was 50 ppm. Recently, a TLC method using fluorescamine instead of *p*-anisaldehyde allowed visualization of fumonisins from corn samples at concentrations as low as 0.1 ppm (101).

The most recent development for surveying foods and feeds is the production of monoclonal and polyclonal antibodies against fumonisins (5, 6). These antibodies have been used to screen fumonisins and related metabolites in foods, feeds, and tissues by competitive enzyme-linked immunoabsorbent assays and immunochromatography methods (88). Affinity columns have been used to isolate fumonisins from aqueous extracts of corn. Quantification of

the eluate has been carried out by HPLC of the *o*-phthaldialdehyde-fumonisin derivative or by direct fluorescence measurement (45). The antibody methods are the basis for a commercially available affinity column (Vicam, Sumerville, MA) and a laboratory test kit (Neogen Corporation, Lansing, MI).

Results of Analyses of Feeds and Foods

All of the methods described above have been used to measure fumonisins in corn, corn-based feeds, and/or corn-based foods, or other corn-based products. The first natural occurrence of fumonisins in US corn was reported in 1989 in two corn samples associated with an outbreak of ELEM (80a). Soon thereafter it was reported that fumonisins were present in moldy home-grown corn from the Transkei in southern Africa, where the rate of esophageal cancer is high (116). Since these initial reports, numerous feeds and foods have been analyzed. Several general conclusions can be drawn. First, like *F. moniliforme*, the occurrence of fumonisins on corn is widespread. Fumonisins have been detected on corn and corn-based products from the United States and Canada, Europe, South America, and Africa (e.g. 89, 94, 101, 106, 115, 117, 119–121, 126). Second, fumonisins can co-occur with aflatoxin (23). Lastly, the concentrations of fumonisins in moldy corn or feeds associated with field cases of ELEM, PPE, and other animal diseases are usually much higher than in "clean feeds" that were not associated with field cases and in foods for human consumption (e.g. see 101, 121). Nonetheless, detection of total fumonisin concentrations greater than 0.5 ppm in foods destined for human consumption is not unusual. Trace amounts (<1 to 3 ppm) of fumonisins have been found in some commercial laboratory rat rations (24, 123). Analyses for fumonisins in diets used in long-term animal studies are clearly warranted.

Potential Decontamination and Salvage Strategies

The occurrence of fumonisins in foods and feeds indicates the need for development of methods for detoxification in order to salvage contaminated grains. Atmospheric ammoniation of corn does not appear to be an effective method for detoxification (81). Ammoniation at high pressure (60 psi) and low temperature (20°C) reduced detectable fumonisin B_1 levels in corn by 79%; however, the toxic potential of the ammoniated corn was not determined (87). The use of household bleach as a means of destroying fumonisin B_1 has been reported (134); however, no data were given. Boiling culture material for 30 min had no effect on fumonisin B_1 content of corn culture material nor did it reduce its toxic potential (4). A recent report indicated that thermal decomposition of fumonisin B_1 in corn culture material followed a first-order reaction with half-life times of 10, 38, and 175 min at 150, 125, and 100°C, respectively (61). The toxic potential of the heat-treated culture material was not tested. Baking of corn meal muffins also reduced detectable fumonisin

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content as did boiling of corn meal in lime water (106). Corn products from Peru and the United States that were treated with lime water as part of the processing were also found to contain very low amounts of fumonisins (119). Ethanol fermented from moldy corn contained no fumonisins; however, the fermented mash (beer) contained appreciable amounts (16).

CONCLUSIONS

F. moniliforme and the fumonisins are clearly an area of growing concern for producers and consumers. They are a unique problem for the following reasons: (*a*) The occurrence of the fungus and toxin in corn is widespread and can be symptomless in corn; (*b*) the toxicities associated with consumption of fumonisins by farm animals are quite varied; (*c*) fumonisins are often present in feeds in the ppm range and are sometimes found in food for human consumption at ppm levels; (*d*) the actual number of fumonisins, fumonisin-like compounds, and fumonisin precursors that occur in feeds and foods is unknown; and (*e*) the mode of action is unique and the role of sphingolipids in disease is poorly understood.

The health implication of low levels of fumonisins in human foods is unknown. The levels that are safe to feed to animals are also unknown. The Mycotoxin Committee of the American Association of Veterinary Laboratory Diagnosticians recommends that concentrations greater than 5 ppm, 10 ppm, 50 ppm, and 50 ppm should not be fed to equidae, swine, beef cattle, and poultry, respectively (P. F. Ross, personal communication). These recommendations will change as more data becomes available.

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