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MECHANISMS OF AFLATOXIN CARCINOGENESIS

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INTRODUCTION: BACKGROUND AND HISTORY OF AFLATOXINS

Discovery and Dietary Sources of Aflatoxins

Aflatoxins represent a group of closely related difuranocoumarin compounds produced by the common fungal molds *Aspergillus flavus* and *Aspergillus parasiticus* (Figure 1). A number of adverse human health effects have been associated with dietary contamination with aflatoxins, including hepatotoxicity, liver cancer, kwashiorkor, and Reye's syndrome. The link between aflatoxin exposure and both hepatoxicity (aflatoxicosis) and liver cancer are well established, whereas the association of exposure with kwashiorkor and Reye's syndrome remains tenuous, but interesting (1, 2). Aflatoxicosis, characterized by jaundice, ascites, portal hypertension, and other signs of hepatic failure, has been described in humans exposed to 2–6 mg of aflatoxin daily for approximately one month after consumption of mold-damaged corn (2, 3). Immunosuppression caused by aflatoxins has been demonstrated in laboratory animals (4, 5), although virtually no data are available on the immunosuppresive effects of aflatoxins in human populations (1).

Worldwide, aflatoxins are considered a major public health problem, especially in developing countries where long-term food storage is often inadequate for high heat and humidity, which encourage the growth of the mold. Concern for this problem focuses almost universally on the carcinogenic effects of aflatoxins; a substantial amount of experimental animal

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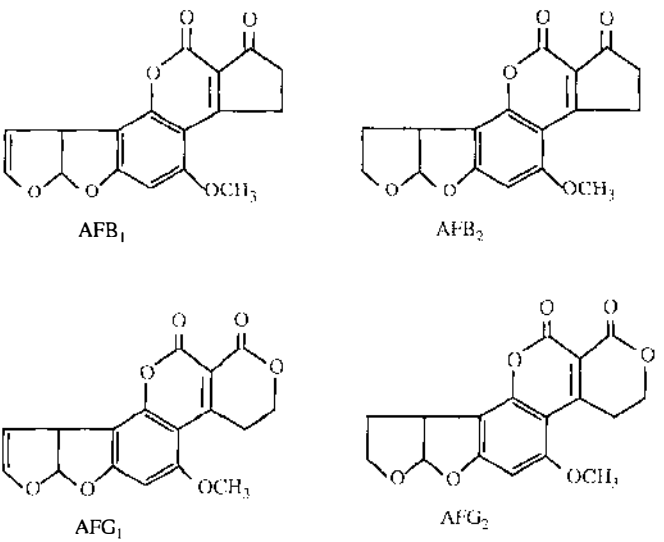


Figure 1 Aflatoxin structures.

(6–8) and human epidemiological data (1, 9–11) now supports a causative role for aflatoxins in the unusually high incidence of liver cancer in some areas of the world. Previously, the concept of a causative role for aflatoxins in human liver cancer had not been universally accepted because of the presence of endemic hepatitis B virus in high risk populations (12). Recently, however, the International Agency for Research on Cancer (IARC) reported that there is sufficient evidence to classify aflatoxin B_1 and mixtures of aflatoxins as Group 1 carcinogens in humans (13).

Experimental Animal Evidence That Aflatoxin Is Carcinogenic

Unquestionably, aflatoxins, especially aflatoxin B_1 (AFB_1), are carcinogenic in several animal species. Indeed, soon after the outbreak of hepatotoxicity in turkeys and poultry in the early 1960s, workers discovered that aflatoxin contamination was responsible for an outbreak of hepatocellular carcinomas in hatchery-reared rainbow trout, a species for which background tumor rates were very low (14). Over 20 different chronic studies in rats alone have demonstrated the potent carcinogenic effects of aflatoxins (see 7 for

a detailed review). These studies collectively utilized a variety of experimental protocols, including (*a*) different sources of aflatoxin (e.g. use of peanut meal contaminated with a mixture of aflatoxins B_1 and G_1, or use of purified AFB_1), (*b*) different routes of administration, (*c*) different periods of administration and observation, (*d*) different basal diets, and (*e*) different strains of rats. Despite such differences, all of these studies found that aflatoxin was a very potent hepatic carcinogen in rats when fed for periods of 20 weeks or longer, and a few studies found that even single, relatively high dos

dramatic demonstration of the potency of aflatoxin as a hepatocarcinogen was provided by Wogan & Newberne (15), who reported a 100% incidence of hepatic tumors in rats fed a diet containing 15 ppb (μg/kg) continuously for 68–80 weeks. Although a later dose-response study found only a 20% incidence of tumors at 15 ppb, a 100% incidence of tumors was obtained at 100 ppb for 54–88 weeks (16). From a comparative experimental point of view, these results place aflatoxin among the most potent carcinogens of all chemicals ever tested. Using the TD_{50} values for rats developed by Gold et al (30), only 2,3,7,8-TCDD ($TD_{50} = 6.7 \times 10^{-6}$ mg/kg per day) significantly exceeds AFB_1 ($TD_{50} = 9.3 \times 10^{-4}$ mg/kg per day) in potency. Using the TD_{50} parameter, AFB_1 is 1,000 times more potent a carcinogen than benzo(a)pyrene.

Remarkably, mice appear highly resistant to the hepatocarcinogenic effects of aflatoxin. Wogan (7) reported that Swiss-Webster mice fed aflatoxin-contaminated peanut meal containing 100,000 or 150,000 ppb of a mixed aflatoxin preparation ($AFB_1 + AFG_1$) for 80 weeks (postweaning) developed no hepatic tumors. However, when 1.25 μg AFB_1/g body weight was administered by i.p. injection during the first 10 days after birth (preweaning) to first generation inbred (C57BL X C3H) mice, a 100% incidence of liver tumors was obtained (18).

Although the carcinogenic potency of aflatoxins in rats is extremely high, rainbow trout exceed rats in sensitivity to the hepatocarcinogenic effects of aflatoxins and are generally considered to be the most sensitive species to aflatoxin carcinogenicity (19). For example, a dietary exposure of 20 ppb in the Shasta strain of rainbow trout for only 4 weeks resulted in a tumor incidence of 62%. Remarkably, static exposure of fertilized rainbow trout eggs (embryos) to 500 ppb (in the bathing medium) AFB_1 for 15 min resulted in a 62% incidence of hepatic tumors 12 months after hatching (19).

Human Epidemiological Evidence That Aflatoxin is Carcinogenic

Numerous epidemiological studies of human populations exposed naturally to aflatoxin-contaminated diets have been completed and have been reviewed

extensively (see 1, 9–11). Most epidemiological studies have generally supported an association between dietary aflatoxin intake and the incidence of hepatocellular carcinoma, although substantial variability in apparent response has been noted. Two limiting factors frequently confound such studies: (*a*) lack of accurate assessment of chronic aflatoxin intake, and (*b*) presence of endemic hepatitis B virus in regions where the incidence of both hepatocellular carcinoma and aflatoxin contamination are high. Accurate and applicable "biomarkers" of exposure to aflatoxin with which to assess chronic aflatoxin exposure have been developed by Groopman and co-workers (9, 11, 20–24). These studies have revealed that some, but not all, urinary metabolites of aflatoxin provide reliable assessment of dietary aflatoxin intake. Most recent studies have utilized mono- or polyclonal antibodies to specific aflatoxin metabolites. Aflatoxin M_1 has been used as a biomarker of exposure in several epidemiological studies, with generally good results. Zhu et al (25) found a correlation of 0.65 between total dietary AFB_1 intake and urinary AFM_1 excretion in 32 households in the Guangxi region of the People's Republic of China. Wild and co-workers (26) found a similar correlation between dietary intake and urinary excretion of aflatoxin metabolites, whereas Groopman et al (22) found a poor correlation between dietary exposure and urinary AFM_1 excretion.

Recently, Groopman and co-workers (11, 22–24) have demonstrated that the AFB-N^7-guanine adduct in urine represents the most reliable urinary biomarker of aflatoxin exposure. However, even with this marker, the amount of AFB-N^7-guanine in urine reflects only relatively recent exposure. There has been interest in evaluating the utility of more peristent biomarkers, such as the albumin-(ε-amino-lysine)-AFB adduct. Hall & Wild (1) found a 10-fold fluctuation in urinary aflatoxin metabolites over a 4-day period, yet the fluctuation in albumin-AFB adducts was less than 2-fold in that same period. The concern with this and other biomarkers is whether the disease process itself may affect the relationship between dietary intake and biomarker levels. For example, in a case-control study of the relationship between aflatoxin and liver cancer in the People's Republic of China, Hall & Wild (1) found that the albumin-AFB adduct level in peripheral blood was correlated with individual dietary aflatoxin intake (measured directly in food) in the controls ($r = 0.317$; $p = 0.021$) but not in the cases ($r = 0.0086$; $p = 0.959$).

As there is general agreement that hepatitis B virus infection is an important risk factor for primary liver cancer, its presence in regions where aflatoxin contamination is high provides further complexity to the interpretation of epidemiological studies. In an attempt to control this important confounder, Peers et al (27) examined the incidence of hepatocellular carcinoma in different regions of Swaziland, where the incidence of hepatitis

B was comparable, but where aflatoxin contamination of the diet varied substantially because of large differences in geographic and climatic conditions (i.e. low- versus high-elevation human populations). The results of this study suggested an important role of aflatoxin in liver cancer risk, at least in the presence of endemic hepatitis B virus.

To directly address the important question of whether aflatoxins act independently or synergistically with hepatitis B virus, Ross et al (28) conducted a prospective nested case-control study that involved the analysis of over 18,000 urine samples (collected over a period of 3.5 years) for the presence of the N^7-guanine adduct of aflatoxin. In that study, 22 subjects developed liver cancer. Urine samples from those subjects who developed liver cancer were age matched with controls and analyzed for hepatitis B virus surface antigen status and the aflatoxin exposure biomarker. Aflatoxin exposure alone (hepatitis B antigen-negative) yielded a relative risk of about two; hepatitis B virus antigen positive status alone (aflatoxin exposure negative) yielded a relative risk of about five. Combined exposure (aflatoxin plus hepatitis B virus positive antigen), however, yielded a relative risk of over 60 (28). These findings provide a basis for understanding, at least in part, the strong geographical and socioeconomic distribution of liver cancer incidence in regions of the world where both dietary aflatoxin contamination and hepatitis B virus infections are common.

BIOCHEMICAL MECHANISMS of AFLATOXIN CARCINOGENESIS

Biotransformation of Aflatoxins

The biotransformation of aflatoxins is intimately linked with their toxic and carcinogenic effects. Accordingly, differences among aflatoxin biotransformation pathways are a critical determinant underlying variations in species sensitivities to aflatoxin B_1 (AFB_1)-induced carcinogenesis. AFB_1 requires microsomal oxidation to the reactive AFB_1-8,9-epoxide (AFBO, also referred to as AFB-2,3-epoxide in older literature) to exert its hepatocarcinogenic effects (Figure 2), and the extent of covalent binding of AFBO to cellular DNA when measured in vivo is highly correlated to the carcinogenic potency of AFB_1. Microsomal biotransformation of AFB_1 also results in the production of more polar metabolites (AFM_1, AFQ_1, and AFP_1; Figure 3) that do not share the carcinogenic characteristics of AFBO. AFBO may be conjugated enzymatically with GSH, which serves as a critical pathway for AFB_1 detoxification (Figure 2). In rodents, the amount of GST activity towards AFBO is inversely related to species susceptibility to AFB_1-induced hepatocarcinogenesis. Ultimately, the amount of AFB_1 that will bind to

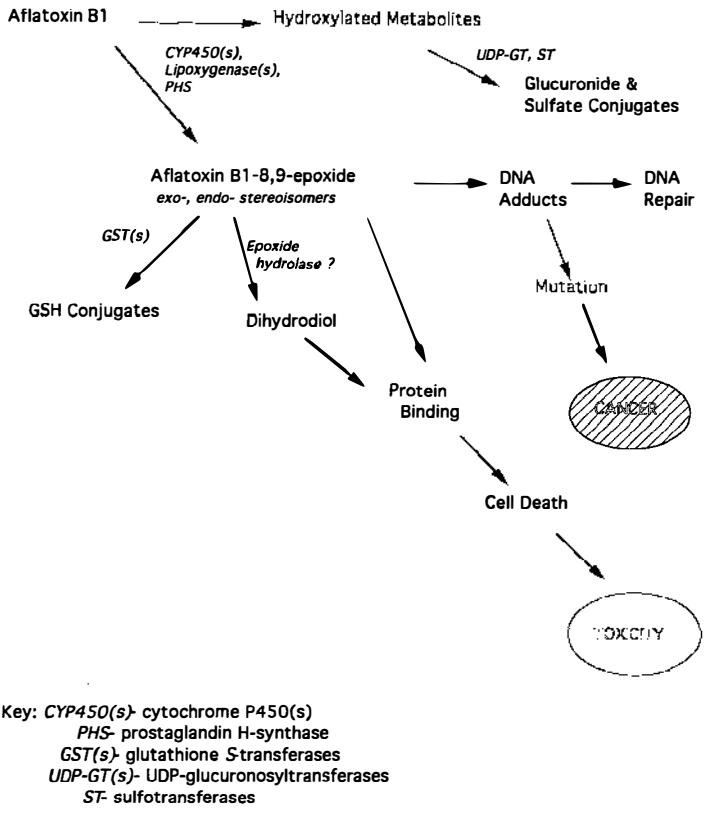
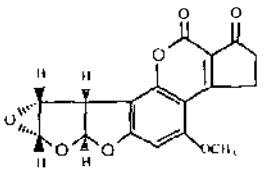


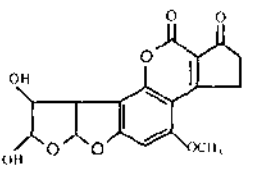
Figure 2 Overview of biotransformational pathways for aflatoxin B_1.

DNA is determined by the proportion activated to the epoxide and the fraction of the epoxide that is enzymatically conjugated with GSH. In this regard, exposure to dietary compounds that affect the rates of AFB_1 activation or AFBO elimination can ultimately affect AFB_1 carcinogenicity.

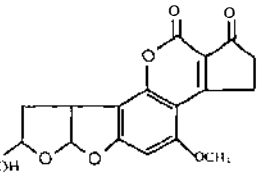
MICROSOMAL OXIDATION OF AFB_1 *Epoxidation* Microsomal cytochrome P450 (CYP450)-dependent epoxidation of the terminal furan ring of AFB_1 generates the highly reactive epoxide that is responsible for nucleic acid alkylation (29). Although AFBO has not been isolated from biological systems, its formation can be inferred by interception with trapping agents such as DNA, and also with GSH and glutathione S-transferases (GSTs). It has now been successfully synthesized and chemically characterized (30).



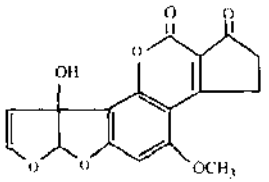
AFB_1-8,9-*exo*-epoxide (AFBO)



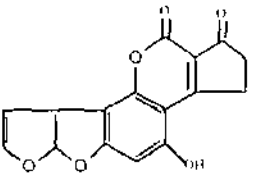
AFB_1-8,9-dihydrodiol



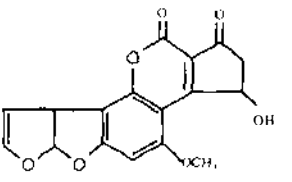
$AFB_{2\alpha}$



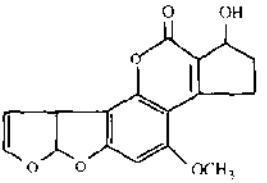
AFM_1



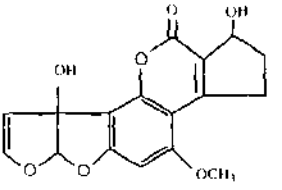
AFP_1



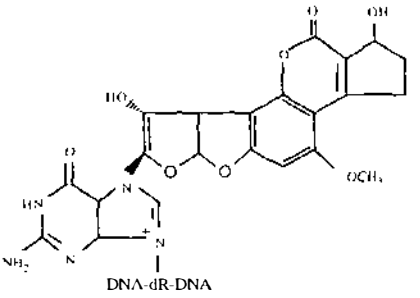
AFQ_1



Aflatoxicol



Aflatoxicol M_1



DNA-dR-DNA

trans-8,9-Dihydro-8-[N^7-guanyl]-9-hydroxyaflatoxin B_1 Adduct

Figure 3 Structures of aflatoxin B_1 primary metabolites.

When added to aqueous solutions, synthetic AFBO undergoes rapid nonenzymatic hydrolysis to AFB_1-8,9-dihydrodiol (31). AFB_1-8,9-dihydrodiol may exist in a phenolate resonance form that is capable of forming Schiff bases which react with amino acids. Sabbioni et al (32) demonstrated that AFB_1-lysine adduct is the predominant adduct found in rats after in vivo AFB_1 exposure. The rapid reaction of AFB_1 with Tris to form an AFB_1-8,9-epoxide-Tris adduct can also be exploited to monitor AFBO formation in microsomal incubations containing this buffer (33).

Striking differences exist with respect to the capacity for AFB_1 oxidation among microsomes prepared from different species. Human liver microsomes are approximately one-fourth as efficient at activating AFB_1 as are rat microsomes at high substrate concentrations (34). Mouse microsomes have higher specific activity for AFBO production than rat microsomes (34), but are resistant to the hepatocarcinogenic effects of AFB_1 because of the efficient conjugation of AFBO with GSH (35).

Recently, it has become apparent that chemical and enzymatic epoxidations of AFB_1 yield *exo*- and *endo*-AFB_1 epoxide stereoisomers (31). The epoxide ring is positioned above the plane and *trans* to the 5a and 9a protons in the *endo*-stereoisomer, whereas the epoxide ring points below the plane and *cis* to the 5a and 9a protons in the *exo*-epoxide (31). The metabolic activation of AFB_1 by human or rat microsomes produces a mixture of *endo*- and *exo*-epoxides, which can be trapped with GSH and identified by comparison with standards prepared by the reaction of the *endo*- or *exo*-steriosomers with GSH (31). Both isomers are produced by human liver microsomes, although the *exo*-epoxide predominates (31). Rat microsomes also efficiently form the *exo*-epoxide, but are far less efficient at forming *endo*-epoxide when compared to human microsomes. Although the *endo*-epoxide is less susceptible to hydrolysis than is the *exo*-conformation (43), the *exo*-epoxide is much more efficient at forming DNA adducts and is much more mutagenic than the *endo*-epoxide (T Harris, personal communication).

Although CYP450-mediated oxidation of AFB_1 is considered to be the dominant route for AFB_1 epoxidation, CYP450-independent pathways for AFB_1 activation have also been demonstrated. Battista & Marnett (36) reported that prostaglandin H synthase (PHS)-dependent epoxidation of AFB_1 can co-occur with CYP450-mediated AFB_1 epoxidation. The relative contribution of PHS in AFB_1 activation in some animals is tissue dependent. For example, PHS-dependent activation of AFB_1 accounted for $<2\%$ of the contribution of CYP450 towards AFB_1 epoxidation in guinea pig liver microsomes, whereas both PHS and CYP450(s) contributed equally to AFB_1 epoxidation in guinea pig kidney microsomes (37). Lipoxygenases from guinea pig liver and kidney are also capable of activating AFB_1 to DNA-

bound derivatives (38). Furthermore, the kinetics of lipoxygenase-dependent hepatic DNA binding suggest that this pathway could be particularly active at dietary levels of AFB_1 exposure (38). It is believed that the lipoxygenase-dependent activation of AFB_1 is catalyzed by arachidonic acid-derived peroxy radicals, which are generated through the interactions of prostaglandin G2 lipid hydroperoxide with the heme moiety of PHS (38). Due to the presence of relatively high lipoxygenase and PHS activities in human lung (39, 40), these two enzyme systems may serve as important alternative pathways for pulmonary and renal AFB_1 bioactivation.

Oxidation of AFB_1 at the 8,9 unsaturated carbon may also result in the formation of aflatoxin $B_{2\alpha}$ (8-hydroxy-8,9-dihydroaflatoxin, $AFB_{2\alpha}$) (41). While evidence based upon absorption spectra indicates that $AFB_{2\alpha}$ is formed in microsomal incubations in vitro (41), the formation of $AFB_{2\alpha}$ has not been verified by chemical analysis. Although the importance of this pathway to AFB_1 disposition is unclear, synthetic $AFB_{2\alpha}$ has been shown to form Schiff bases with amines (41).

The significance of epoxide hydrolase (EH) in AFBO hydrolysis has not been thoroughly established. Studies using isolated hepatocytes from rats and mice suggest that microsomal EH actively converts AFBO to AFB_1-dihydrodiol (42, 43). Shayiq & Avadhani (44) reported a reduction in AFB_1-DNA binding when EH was added to a reconstituted system containing purified P450s. However, other work using specific EH inhibitors in rat and mouse hepatic subcellular fractions indicates that EH does not play a significant role in the inactivation of AFBO (45, 46). Furthermore, a study from our laboratory showed that EH does not facilitate AFBO elimination in the presence of endogenous cytosolic GST in mouse subcellular fractions in vitro (35). Apparently, either AFBO is not a substrate or the K_m of EH for AFBO is relatively high as compared to that of GST. However, AFBO hydrolysis does occur, since the dihydrodiol is rapidly formed under in vitro conditions in the absence of GST.

Hydroxylation and O*-demethylation* The microsomal CYP450-dependent monooxygenases also oxidize AFB_1 to its hydroxylated metabolites AFM_1, AFP_1, and AFQ_1 (Figures 2 and 3). AFQ_1 is formed via 3a hydroxylation of AFB_1, whereas AFM_1 is produced by 9a hydroxylation of AFB_1. *O*-demethylation results in the formation of AFP_1. The acute toxicities of the hydroxylated metabolites are generally lower than the parent compound (47, 48), as are the mutagenic potencies (49–51). AFM_1 was originally isolated and identified as an AFB_1 metabolite in milk (52) (see Ref. 53 for review). Dietary AFM_1 is approximately 30% as carcinogenic as AFB_1 in trout (54) and approximately 10% as carcinogenic in rats (51). However, the acute toxicity of AFM_1 approaches that of AFB_1 in rats (55). Significant

amounts of AFM_1 are formed in a number of species including human, monkey, rat, mouse, and rainbow trout (34, 54, 56).

Although there is little information regarding AFQ_1 carcinogenicity, AFQ_1 exhibits much lower acute toxicity (48) and mutagenicity (49, 57) than AFB_1. In rainbow trout, the carcinogenic potency of AFQ_1 is approximately 1% of AFB_1 (58); this despite the extreme sensitivity of rainbow trout to aflatoxins. The oxidation of AFB_1 to AFQ_1 appears to constitute a particularly important pathway for AFB_1 detoxification in human and nonhuman primate liver (34). Raney et al (57) showed that human liver microsomes do not appreciably oxidize AFQ_1 and that synthetic AFQ_1-epoxide exhibits little mutational activity towards *Salmonella typhimurium* strain TA98. In light of this evidence it is likely that AFQ_1 has little potential for carcinogenic activity in humans.

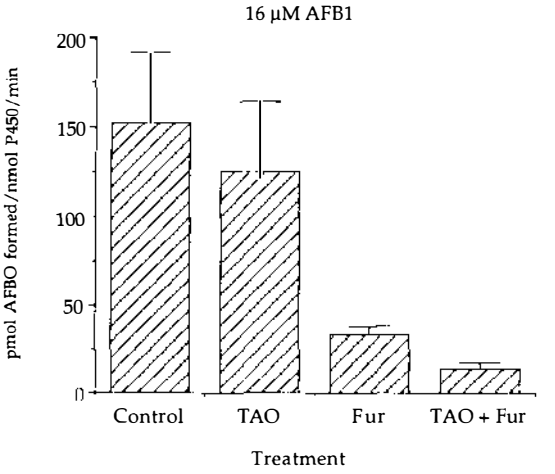
In mice, rats and monkeys, the *O*-demethylation of AFB_1 produces AFP_1 (59). Although it is not a prominent AFB_1 metabolite in human liver microsomes (56, 60), Kirby et al (61) reported that microsomal fractions from primary human liver tumors from Thai patients produced higher levels of AFP_1 than observed in microsomes prepared from normal liver tissue. Interestingly, AFP_1 is produced by human liver slices incubated with AFB_1 (DL Eaton and J Heinonen, unpublished observations) and is also a common urinary metabolite in humans exposed to dietary AFB_1 in vivo (22). In this regard, AFP_1 was the most highly correlated of all urinary AFB metabolites in humans with liver cancer (28). Other common urinary metabolites of individuals exposed to dietary AFB_1 in Shanghai, China, include AFB_1, AFM_1, and AFB_1-DNA-adducts (AFB_1-N^7-guanine) (22).

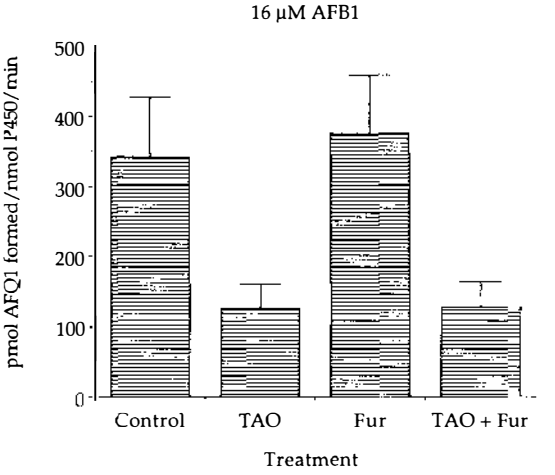
A dihydroxyaflatoxin B_1, AFM_1-P_1, can be formed by the 9a-hydroxylation of AFP_1 or the 4-*O*-demethylation of AFM_1 (41). This metabolite is formed in vivo in rats and is excreted directly in bile or as the glucuronide conjugate (41). Glucuronidation of AFP_1 must effectively compete in vivo with microsomal oxygenases responsible for the secondary oxidation of AFP_1, as the AFP_1-glucuronide conjugate is excreted in bile in greater amounts than the glucuronide conjugate of 4,9a-dihydroxy-AFB_1.

CYP450 isoenzymes involved in AFB_1 biotransformation Evidence suggests that multiple CYP450 isoenzymes contribute to AFB_1 epoxidation in the rat. CYP 2C11, a male specific CYP450 isoform in the rat, may activate AFB_1 to mutagenic metabolites, as can a polychlorinated biphenyl-inducible CYP450 in the 1A family (62). Additional, albeit indirect, evidence of a role for CYP 2C11 in AFB_1 activation in the rat was observed in our laboratory when male Sprague-Dawley rats pretreated with ciprofibrate showed a concomitant down-regulation of hepatic CYP 2C11 mRNA and decreased hepatic microsomal capacity for AFB_1-epoxidation (unpublished

observations). Metcalfe et al (63) found that AFB_1 activation by rat liver is potentiated by phenobarbital pretreatment, thus indicating that certain CYP450 forms involved in AFB_1 activation are phenobarbital-inducible. Dietary ethoxyquin increases the rate of AFM_1 formation (64) and the expression of hepatic CYP 1A2 mRNA (unpublished observations), which suggests that CYP 1A2 is capable of catalyzing the oxidation of AFB_1 to AFM_1 in rat liver. AFM_1 formation is also increased tenfold in rats pretreated with 3-methylcholanthrene, thus suggesting that the CYP450 responsible for AFM_1 formation is regulated through the Ah-receptor (63). In this regard, Koser et al (65) reported that the CYP450, which forms AFM_1 in the mouse, is CYP 1A2, which is also under regulation by the Ah receptor.

The oxidation of AFB_1 in human liver microsomes appears to be a complex process controlled by multiple P450 enzymes exhibiting different kinetic characteristics. At least five different human liver P450s, including 1A2, 2A6, 2B7, 3A3, and 3A4 are capable of activating AFB_1 to mutagenic metabolites and DNA-bound derivatives (66). Shimada & Guengerich (67) have reported that CYP 3A4 is the dominant CYP450 enzyme responsible for the activation of AFB_1 to AFBO in human liver microsomes. However, Crespi et al (66) showed that human cell lines selectively expressing CYP 1A2 were 3- to 6-fold more effective than those expressing CYP 3A4, and 40- to 50-fold more effective than CYP 2A3 at activating AFB_1 to mutagenic metabolites at low substrate concentrations. A recent study from our laboratory suggests that CYP 3A enzymes(s) are capable of AFB_1 oxidation at relatively high substrate concentrations; however, CYP 1A2 is the high affinity CYP450 active at lower substrate concentrations in human liver microsomes (60). Our conclusions were based upon experiments using specific CYP 1A2 and 3A inhibitors and human liver microsomes, and microsomes from a lymphoblastoid cell line that expresses human CYP 1A2 and 3A4 cDNAs. As observed in Figure 4, furafylline, an extremely potent and selective irreversible inhibitor of CYP 1A2 in human liver (68, 69), effectively inhibited AFB_1 epoxidation in human liver microsomes at non-saturating (16 μM AFB_1) concentrations. In contrast, troleandomycin (TAO), a specific CYP 3A inhibitor in human liver microsomes, was more effective than furafylline at inhibiting AFBO formation at saturating (128 μM AFB_1) concentrations (Figure 4). Consideration of the kinetics of aflatoxin biotransformation is important because AFB_1 concentrations that are likely to be present in the liver after dietary exposure are much lower than those concentrations commonly used in in vitro assays. Extrapolations of relative enzyme activities to low doses are not possible unless all the enzymes involved exhibit similar kinetic characteristics with regard to their substrate affinities. In human microsomes, epoxidation constitutes an increasing proportion of AFB_1 biotransformation as substrate concentrations decrease,





most likely because of the high affinity of CYP 1A2 in forming AFBO (56, 60).

The primary detoxification products of AFB_1 metabolism in human microsomes, AFQ_1 and AFM_1, appear to be catalyzed principally by CYP 3A family and CYP 1A2 isoenzymes, respectively (57, 60). As seen in Figure 3, TAO effectively inhibits AFQ_1 formation in human liver microsomes at AFB_1 concentrations of 16 μM and 128 μM. Evidence for the simultaneous production of AFM_1 and AFBO by CYP 1A2 was provided

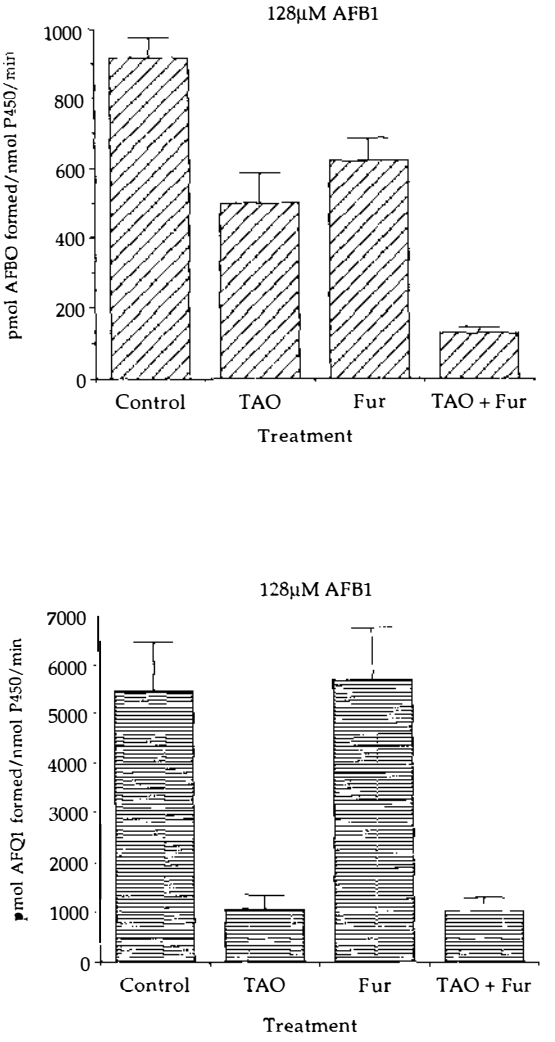


Figure 4 Effect of troleandomycin (TAO) and furafylline (FUR) on AFBO and AFQ1 formation in human liver microsomes.

Microsomes (5 mg protein/ml) prepared from liver donors (n=4) were pre-incubated for 20 min in the presence of NADPH-regenerating system and inhibitor(s) (200 μM furafylline, 40 μM troleandomycin) or DMSO carrier (control 1.3% v/v). Aliquots of inhibited microsomes were withdrawn from the preincubation mixtures and further incubated for 10 min with either 16 μM or 128 μM AFB_1 in the presence of an NADPH-regenerating system. Reactions were stopped by the addition of ice-cold methanol, centrifuged to remove precipitate, and the supernatants were analyzed for AFB_1 metabolites by HPLC (69).

by a previous study from our laboratory that showed a high correlation ($r = 0.976$) between AFBO and AFM_1 formation at 15.6 μM AFB_1 in a panel of thirteen human liver microsomes (70). CYP 1A2 produces a higher ratio of activation (AFBO) to detoxification (AFM_1) products, relative to CYP 3A4, with an activation:inactivation ratio of about 3–4:1 (60, 70). However, CYP 3A4 may be expressed in human liver at a much higher level than CYP 1A2, such that in some individuals CYP 3A4 may be the predominant source of AFBO, even though CYP 3A4 preferentially forms AFQ_1, a detoxification product. Ultimately, the relative contribution of these two principal catalysts to AFBO formation should be directly proportional to their relative amounts. However, differences in apparent kinetics of these two P450s toward AFB_1 indicate that the most important determinant of individual susceptibility to AFB_1 may well be the level of expression of CYP 1A2. Given the apparent lack of effectiveness of constitutively expressed human glutathione S-transferases (GST) in the detoxification of AFBO (71, 72), individuals with relatively high CYP 1A2 expression may be at particular risk for AFB_1-induced DNA damage.

REDUCTION Several species including rabbit, chicken, and trout may rapidly convert AFB_1 to aflatoxicol (AFL) by reduction of the 1-keto-group through a cytosolic NADPH-dependent reductase (73). AFL can be further metabolized by undergoing a 9a-hydroxylation to form AFL-M_1 (74). Although AFL is not an important reductive metabolite of AFB_1 in mammalian liver, it has been identified as the major in vivo AFB metabolite in the plasma of rats administered AFB_1 either orally or intravenously (75). AFL has been reported to be a potent frameshift mutagen and also elicits unscheduled DNA synthesis in fibroblasts incubated with a rat liver postmitochondrial fraction (76). AFL is approximately 50% as carcinogenic as AFB_1 in trout (77) and has about 70% the mutagenicity, using a trout liver activating system (49).

The formation of AFL does not appear to be an important detoxification pathway for AFB_1, as AFL may be rapidly converted back to AFB_1 by a microsomal dehydrogenase (73), thereby increasing the physiological half-life of AFB_1. Liver preparations from species that are sensitive to the acute toxic effects of AFB_1 (rabbit, rainbow trout) typically exhibit high ratios of AFB_1 reductase:AFL dehydrogenase activities, while preparations from less sensitive species (monkey, guinea pig, hamster, mouse) show the opposite pattern (73). The pattern of AFB_1-AFL interconversion in human liver preparations places humans among those species that are not extremely sensitive to the acute toxic effects of AFB_1. However, the pattern of AFB_1-AFL interconversion does not account for differences among species

in AFB_1-metabolite conjugation pathways and therefore may not be predictive of species sensitivity.

Recently, a new aldo-keto reductase involved in the reduction of AFB_1-8,9-aldehyde (AFBA) has been described (78, 79). AFBA is formed by spontaneous rearrangment of the dihydrodiol, which in turn is formed from hydrolysis of AFBO. As noted above, the dialdhyde form is capable of forming Schiff bases with amines in protein and may be responsible for some of the acute toxic effects of AFB_1. This enzyme, termed aflatoxin B_1-aldehyde reductase (AFB-AR), is dissimilar from any previously described reductase. It is inducible by ethoxyquin and is expressed in greater quantities in preneoplastic foci (78). Although the significance of this enzyme in the detoxification of AFB-aldehyde has yet to be demonstrated definitively in vivo, it may be of importance in protecting against acute and chronic hepatotoxicity of AFB_1. As cytotoxic effects of AFB may play a significant promotional role in tumor development (80), it is also possible that this enzyme pathway may offer some protection against AFB carcinogenicity, but it is unlikely to influence genotoxicity.

CONJUGATION *GST-mediated conjugation of AFBO with GSH* Several of the products of the oxidative metabolism of AFB_1 serve as substrates for phase II detoxification enzymes. Extensive interspecies variation exists in the functional importance of the different phase II enzymes in AFB_1 detoxification. In many mammalian species, the primary pathway for AFB_1 detoxification is through GST-mediated conjugation of AFBO with reduced glutathione (GSH). In contrast, AFB_1-GSH conjugation is not a significant route of AFB_1 detoxification in rainbow trout (81), coho salmon (81), or channel catfish (EP Gallagher and DL Eaton, unpublished observations). The selectivity of GST isoenzymes towards AFBO serves as a critical determinant of differences among mammalian species in susceptibility to AFB_1 hepatocarcinogenesis (46, 70, 72, 82–85). Liver cytosolic fractions from mouse have 50- to 100-fold greater AFBO conjugating activity than do those from rat, even though both species have comparable amounts of GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) (86). Accordingly, mice are resistant to the hepatocarcinogenic effects of AFB_1 when compared to rats (15), a difference reflected by 50- to 100-fold less AFB_1-DNA adduct formation by mice after in vivo AFB_1 exposure (35). The efficient detoxification of AFBO by mouse GSTs appears to be a characteristic of different mouse strains, as nine different strains exhibited similar high specific GST activities towards AFBO (87).

The GSTs comprise a multigene family of enzymes that have been divided into five classes, designated alpha, mu, pi, theta, and microsomal (88). The high activity of GST epoxide towards AFBO is largely attributable to the

expression of certain alpha class GSTs (89, 90). Unfortunately, problems exist regarding the nomenclature used to describe rodent GSTs. These inconsistencies make it difficult to identify specific alpha GSTs with high AFBO activity when comparing studies from different laboratories. In addition, functional GSTs are dimeric proteins such that each of the subunits may hybridize to form homodimers and heterodimers. In rat liver, three constitutively expressed alpha class GST isozymes, YaYa, YaYc, and YcYc have significant activity towards AFBO (99). Hayes et al (91) reported the presence of an ethoxyquin-inducible alpha class GST subunit in rat cytosol (Yc_2) that has high specific activity towards AFBO. Although not constitutively expressed in normal liver, Yc_2 is overexpressed in AFB_1-induced nodule-bearing livers, possibly underlying the acquired resistance of nodules to AFB_1 (92).

Mice contain at least three alpha class GST subunits, one of which is constitutively expressed while the other two are inducible by chemoprotective agents (93). Two alpha class GSTs from mouse liver have been cloned and sequenced (94–96). One of the GST clones, pGT41 (96), encodes a cDNA sequence that corresponds to the constitutively expressed alpha class GST-Ya_1 in rat liver. A second clone, isolated in our laboratory and termed mYc, encodes a novel sequence that exhibits 86% homology to rat Yc cDNA sequence (pGTB42) (97). Interestingly, the homology between the rat and mouse Yc isoforms (86%) is significantly lower than for mouse Ya and rat Ya isoforms (95%). When the rat Yc_1 and mouse Yc cDNAs are expressed in a bacterial expression system, the mouse Yc has nearly 100-fold higher activity for AFBO than does the rat Yc_1 isoform (98). Apparently, however, it appears now that the constitutively expressed mouse Yc form with high AFBO activity is orthologous to the ethoxyquin-inducible Yc_2 GST described by Hayes and co-workers (91, 95), and not to the rat Yc_1. Partial amino acid sequence (98), and more recently the complete cDNA sequence (JD Hayes, personal communication), indicate that the rat Yc_2 form shares 91% homology with the mouse Yc, which has high ABO activity, and that the rat form itself has high AFBO-conjugating activity (95).

As previously noted, metabolic activation of AFB_1 produces a mixture of *exo*- and *endo*-epoxide stereoisomers that can be trapped as GSH-adducts if oxidations are carried out in the presence of GSTs. Substantial differences exist regarding the ability of mouse and rat GSTs in the conjugation of the two AFB_1 stereoisomers. The *exo*-epoxide is efficiently trapped by mouse cytosolic GSTs, whereas rat cytosolic fractions are relatively inefficient at conjugating the *exo*-epoxide (99). Human liver cytosolic fractions are very poor at conjugating either stereoisomer of AFBO, although the human GST mu form, GST *M1a-M1a,* had significant conjugating activity toward the

endo-epoxide. Human alpha class GSTs had only marginally detectable activity toward either epoxide (119). GST *M1a-M1a* is inherited in an autosomal dominant fashion in human liver and is therefore absent in approximately 50% of Caucasians. Thus, it is possible that AFB_1-exposed individuals who lack this enzyme may be at increased risk for AFB_1 hepatocarcinogenesis. As noted above, however, it is questionable whether the *endo*-epoxide is of biological relevance, as it binds poorly to DNA and may not be mutagenic (T Harris, personal communication), which would make the absence of the GST *M1a* gene largely irrelevant to aflatoxin carcinogenesis.

Glucuronidation and sulfation of hydroxylated metabolites The glucuronides of AFP_1 and 4,9a-dihydroxyaflatoxin B_1 have been identified as biliary metabolites in rats treated with AFB_1 (41). Glucuronide-conjugates of AFP_1 have also been identified in chickens (100). Studies with isolated hepatocytes suggest that AFP_1 is a better substrate for glucuronidation than are AFM_1 and AFQ_1 (101). Furthermore, Ch'ih et al (102) showed that the AFP_1-glucuronide conjugate is the only significant glucuronide conjugate of hydroxylated AFB_1 metabolites formed in isolated hepatocytes prepared from rat liver. However, as the enzymatic hydrolysis of nonaromatic glucuronide conjugates with bacterial β-glucuronidase preparations can be inefficient and may underestimate the amount of conjugate present (101), the extent and significance of glucuronidation of AFM_1 and AFQ_1 remains uncertain. AFL-glucuronide and AFL-M_1-glucuronide are the major AFB_1 conjugates in rainbow trout fed control diets (103). Sulfate conjugates were not detected in rainbow trout exposed to AFB_1 (103), which is consistent with other studies indicating that rainbow trout excrete xenobiotics as glucuronides, rather than as sulfates (104). Wong et al (105) found that treatment of aqueous urinary AFB metabolites from rhesus monkeys with arylsulfatase released AFM_1, thus indicating the in vivo formation of the sulfate conjugate of AFM_1 in this species.

MODULATION OF AFB_1 BIOTRANSFORMATION A variety of dietary factors have been shown to influence the carcinogenicity of aflatoxin (106, 107). Cruciferous vegetables such as broccoli, cabbage, and brussel sprouts exhibit anticarcinogenic effects when incorporated into the diet of animals (108–111). Rats fed dietary broccoli (111), cabbage (112), or brussel sprouts (113) were protected against AFB_1 hepatocarcinogenesis, either via inhibition of enzyme-altered hepatic foci or by reduction in the number of primary liver tumors. Studies in rats and humans have shown that the antitumor effects of cruciferous vegetables are mediated through their abilities to alter the activities of drug-metabolizing enzymes (114, 115). For example, dietary

cabbage (116), brussel sprouts (117), and broccoli (111) induce hepatic GST activities in rats. Hepatic cytosolic fractions prepared from rats fed dietary broccoli had a higher rate of AFBO-GSH conjugation than those fed a control diet while in vivo AFB_1-DNA binding was lower in the rats receiving the broccoli diet (111). The rate of AFB_1 epoxidation was not significantly affected by dietary broccoli, indicating that increased AFBO detoxification, as opposed to decreased AFB_1 activation, was the critical effect of the broccoli in the preceding study (111). The breakdown products of glucosinolates appear to be some of the active ingredients in cruciferous vegetables responsible for the modulation of AFB_1 biotransformation (112, 118). The chemistry of *Cruciferae* is complex, however, and it is possible that other chemicals also contribute to the protective effects of these vegetables.

Certain dithiolthione compounds increase hepatic GST activities and GSH levels and protect against AFB_1 hepatotoxicity (107). The substituted 1,2-dithiole-3-thione, oltipraz, protects against acute and chronic AFB_1 hepatotoxicity in rodents (119). Oltipraz also reduces the size and number of enzyme-altered foci in the livers of rats treated with AFB_1 (120, 121). The in vivo covalent binding of AFB_1 to DNA in rat liver is reduced by dietary pretreatment with oltipraz, apparently through enhanced AFBO detoxification (120, 122, 123). Rats fed oltipraz prior to AFB_1 administration show a 3- to 5-fold increase in the rate of 8,9-dihydro-8-(S-glutathionyl)-9-hydroxy-aflatoxin B_1 excreted in the bile (124), increased hepatic GST activity towards CDNB, and an increase in GST Ya protein and mRNA levels (150).

Dietary treatment with synthetic antioxidants such as butylated hydroxy-anisole (BHA) and butylated hydroxytoluene (BHT) has been shown to protect against the carcinogenic effects of a variety of chemicals. These antioxidants have been employed in a number of studies to modulate the carcinogenic effects of AFB_1. Pretreatment of rats with BHA, BHT (126), and ethoxyquin (127) attenuates the carcinogenicity of AFB_1. The mechanism underlying modulation of AFB_1-induced hepatocarcinogenesis in rats by synthetic antioxidants involves, at least in part, a reduction in AFB_1-DNA binding (35, 128, 129). The protective effects of antioxidants against AFB_1 hepatocarcinogenesis have been directly linked to modulation of AFB_1 biotransformation. Pretreatment of rats with synthetic antioxidant BHA increased the level of AFB_1-GSH conjugation in vitro and also the amount of AFBO-GSH conjugate excreted in the bile (130). Similar results have been reported for ethoxyquin (128).

The enzymatic activities of CYP 1A2 and 3A4, two of the primary human liver CYP450s involved in AFB_1 activation in human liver microsomes, may be modulated by dietary drugs or chemicals. CYP 3A4 activities are

modulated by a number of steroids, antibiotics, and barbiturates (131), whereas members of the human CYP 1A family (including CYP 1A2) are inducible by polycyclic aromatic hydrocarbons such as those found in cigarette smoke or charcoal-broiled beef (132, 133). Thus, it is possible that increased enzyme expression of CYP 1A2 and/or 3A4 may potentiate AFB_1 activation and ultimately increase the risk AFB_1-hepatocarcinogenesis in exposed individuals.

Genotoxic Effects of Aflatoxins

TYPES OF DNA ADDUCTS FORMED WITH AFLATOXIN B_1 That aflatoxin B_1 is metabolized to a reactive intermediate capable of covalent modification of DNA was first reported in 1977 by three independent laboratories (29, 134–136). The identification of *trans*-8,9-dihydro-(N^7-guanyl)-9-hydroxy-aflatoxin B_1 as the putative molecular target of aflatoxin carcinogenicity thus represented a milestone in aflatoxin research obtained after 15 years of intensive research. It is now recognized that the binding of aflatoxin B_1-8,9-*exo*-epoxide to the N^7-guanine of DNA proceeds via an intercalated transition-state complex that has high preference for the B conformation of DNA (137). Although other aflatoxin metabolites, such as the epoxides of AFM, AFP, and AFQ, may contribute to DNA binding, the evidence to date strongly indicates that such secondary oxidation products are of minor importance (57, 138). Although other DNA bases such as adenosine (139) and cytosine (140) are covalently modified by aflatoxin in vitro, there is no evidence to indicate that these minor base modifications have any functional importance in aflatoxin carcinogenesis. One reason the N^7-guanine adduct may be so important is the relatively rapid rate at which it undergoes rearrangement to the ring-opened, formamidopyrimadine (FAPY) form. The FAPY-aflatoxin adduct appears to be the most stable of all AFB-DNA adducts and is relatively resistant to DNA repair processes (136). However, as Bailey points out (138), in chronic, in vivo studies the extent of initial DNA adduct formation often correlates better with ultimate tumor formation than does the level of persistent adducts (e.g. FAPY). This may be because persistent adducts accumulate over time in noninformative DNA, which is repaired at a lower rate than is transcriptionally active DNA (165). Thus, the relative importance of the FAPY adduct versus other more labile DNA adducts in the ultimate development of tumors in animals exposed to aflatoxin remains uncertain.

The specific nature of the aflatoxin DNA adduct not withstanding, numerous studies have demonstrated that carcinogenic potency is highly correlated with the extent of total DNA adducts formed in vivo. When the administered dose is normalized to target dose (e.g. DNA adducts per 10^8

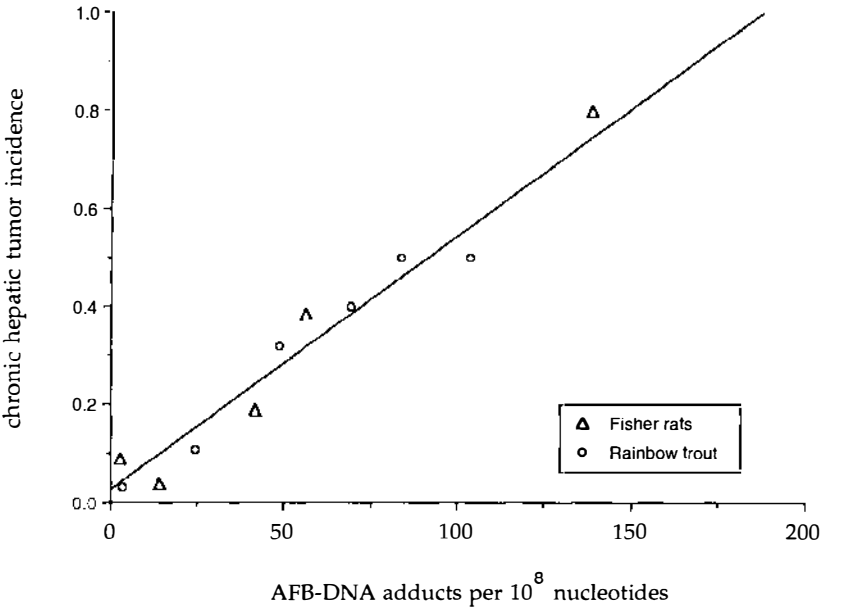


Figure 5 Chronic hepatic tumor incidence as a function of hepatic aflatoxin B_1-DNA adduct for male fisher rats (*triangles*) and rainbow trout (*open circles*). Modified from Bechtel (141). Data were fitted by least-squares regression analysis. Dose and tumor incidence data were modified from experimental data of Wogan et al (16) and Nixon et al (170) for the rat, and Lee et al (171) and Dashwood et al (143) for trout, as described by Bechtel (141).

nucleotides), a highly linear relationship between DNA adduct formation and tumor response is obtained, even when using combined data from both rats and rainbow trout (Figure 5; from Ref. 141). In both rats and trout there is also a highly linear relationship between administered dose and DNA adducts levels. Buss et al (142) demonstrated a nearly perfect linear relationship between AFB_1 dose (ng/kg/day) and AFB-DNA adducts (adducts/10^9 bases) over a dose range of 5 orders of magnitude that extended from a high dose that was expected to yield a 50% tumor incidence in rats to a low dose that encompasses the range of expected human exposure. Dashwood et al (170) also found a large range of linearity between total administered dose and AFB-DNA adduct levels in rainbow trout given aflatoxin for 2–4 weeks. These studies thus fail to provide evidence in support of a threshold hypothesis for aflatoxin genotoxicity at low doses, at least in two highly sensitive yet diverse species, rats and rainbow trout.

ACTIVATION OF ONCOGENES AND INACTIVATION OF TUMOR SUPPRESSOR GENES

Activation of ras *oncogenes* Given the strong mutagenic effects of aflatoxin, there has been considerable interest in identifying possible oncogenes and tumor suppressor genes that may serve as critical molecular targets in aflatoxin carcinogenesis. McMahon et al (144) first demonstrated that AFB_1 was capable of activating *ras* oncogenes in F344 rats. When the DNA from 12 aflatoxin-induced tumors was transfected into NIH 3T3 mouse fibroblasts, transformation occurred in 10 samples. *Hind*III restriction mapping identified the c-Ki-*ras* oncogene in 2 of the 10 transforming DNA samples. Elevated expression of p21 protein was also noted in these samples. Subsequent studies by these (145, 146) and other investigators (147), using DNA from both AFB_1-induced transformed cell lines and primary liver tumors, demonstrated that AFB_1 produces mutations in codon 12 of all three types of c-*ras* oncogenes (Ha-*ras*, Ki-*ras*, and N-*ras*). Using more sensitive techniques (PCR coupled with G+C clamp denaturing gel electrophoresis), Soman & Wogan (148) were able to demonstrate mutations at codon 12 of Ki-*ras* in all of the 10 aflatoxin-induced hepatocellular carcinomas ($n = 3$) and adenomas ($n = 7$) examined. These results suggest that the lower frequency of activated *ras* oncogenes in aflatoxin-induced tumors and/or transformed cell lines observed in earlier studies (144–147) may, at least in part, have been due to lack of sensitivity to detect such mutations, rather than to the absence of a *ras*-activating mutation. It is interesting to note that mouse liver tumors induced by neonatal exposure to aflatoxin have also been shown to have activated c-*ras* oncogenes, although the activating mutation was reported at codon 61, not codon 12 (149, 150).

Few studies have examined aflatoxin-induced tumors or transformed cell lines for other activated oncogenes. Tashiro et al (151) found an increased expression of c-*myc*, as well as c-*ras*, oncogenes in all aflatoxin-induced hepatomas. Dunn et al (152) demonstrated that aflatoxin-transformed C3H/$10T_{1/2}$ cells (7SA cell line) overexpressed protein kinase Ca (PKCa) and exhibited an abnormal responsivness to phorbol-esters, compared to the nontransformed cell line. The authors suggested that this effect of aflatoxin on PKC expression may complement or enhance the transforming abilities of Ha-*ras* oncogenes, as has been suggested by other investigators (153).

Few, if any, studies have identified activated c-*ras* oncogenes in human hepatocellular carcinomas obtained from aflatoxin-endemic areas. However, both Ha-*ras* and Ki-*ras* oncogenes are frequently activated in experimental liver tumorigenesis models, and activated *ras* oncogenes have been implicated in the early stages of carcinogenesis in a variety of human cancers (154). Whether activation of human *ras* proto-oncogenes is an important factor in the etiology of aflatoxin-induced liver cancer in humans remains

to be demonstrated conclusively, although there is supportive evidence of such a role.

Aflatoxin-induced mutations in the p53 *tumor suppressor gene* Mutation of the *p53* tumor suppressor gene has been implicated in the development of many types of tumors. Among the most intriguing recent findings associated with *p53* is the apparent carcinogen-selective mutation at codon 249 in *p53*. Since *p53* was first implicated in hepatocellular carcinoma in 1991, numerous studies have examined the presence and type of mutations in the *p53* gene in hepatocellular carcinomas in geographic regions with high (Table 1) and low (Table 2) aflatoxin exposure. Of particular interest is whether the prevalence of the G → T transversion mutation at codon 249 of *p53*, seen frequently in tumors from areas with high potential for aflatoxin exposure, is due exclusively to aflatoxins or to the presence of hepatitis B virus infection, which is also endemic in these areas. To date, at least 883 individual liver tumors (largely if not exclusively, hepatocellular carcinomas) have been evaluated for the presence of mutated *p53* (Tables 1–3). Of these, about 28% have come from regions of the world with characteristically high dietary aflatoxin contamination (Table 1). The distribution of total and codon 249-specific *p53* mutations between high and low aflatoxin regions, and/or the presence of markers of hepatitis B virus exposure, is shown in Table 3. Disregarding the status of hepatitis B virus exposure, the frequency of mutated *p53* (any site) is about twice as high in aflatoxin-endemic areas relative to its prevalence in developed countries with low aflatoxin exposure. However, the prevalence of a specific mutation at codon 249 in *p53* (usually, but not always, a G → T transversion in the third base) is more than 10 times that in low aflatoxin exposure regions. In fact, of 558 tumors examined from low aflatoxin regions, only 13 (2.3%) contained a mutation at codon 249 (Table 3). Of these 13, 7 were from Japan, 2 were from China (regions reported by the authors to be "low" aflatoxin areas), and 1 each were from South Africa, Egypt, Thailand, and the United Kingdom. Note that the assignment of the study results to either the "high" or "low" aflatoxin groups is sometimes highly speculative, and the actual history of aflatoxin exposure for the individuals has not been ascertained specifically. Given the proximity of many of these countries to aflatoxin-endemic regions, it is not difficult to imagine that some, or perhaps most, of these 13 individuals could have had substantial dietary exposure to aflatoxin at some point in their lives. Likewise, it is likely that some of the individuals classified in the high aflatoxin group may have had minimal exposure to aflatoxin. For example, of 61 samples obtained from Taiwan and classfied by the author as coming from a potentially high exposure area (155), only 4 (6.6%) had mutations in codon 249. Hosono and co-workers

Table 1 Summary of studies examining *p53* mutations in regions of the world with high aflatoxin exposure

Country/ethnicity	HB virus status	Total *p53*	Codon 249	Comments	Reference
Southern Africa	8 positive	5/8	3/8		172
	1 negative	0/1	0/1		
	1 NA[a]	0/1	0/1		
Qidong, China	16 NA	8/16	8/16		172, 186
Mozambique	15 positive	NA	8/15		172
Vietnam (3) India (1)	4 NA	NA	1/4		172
China	19 NA	NA	2/19	19 of 29 samples identified from high AFB regions	172
Bangladesh (3), Africa (3), India (1), Caribbean (1)	8 NA	NA	2/8	HBV status was measured, (3/8 +) but could not determine status of the 2 *P53* +'s; AFB exposure from authors	181
Chinese	7 positive	2/7	0/7	Assignment to high AFB region is	173
	5 negative	1/5	1/5	tenuous; inadequate detail	
African Black	9 positive	NA	0/9	Assignment to high AFB region is tenuous; inadequate detail	173
Qidong, China	34 positive	NA	21/34		174
	2 negative	NA	0/2		
Qidong, China	20 positive	14/20	NA		175
	3 negative	0/3	NA		
Taiwan	41 positive	15/41	4/41		155
	20 negative	5*/20	0.20	*1 no effect mutation	
Qidong, China	16 positive	8/16*	8/16	*exons 7–8 only analyzed	176
	4 negative	1/4*	1/4		
Senegal	13 positive	NA	8/13		177
	2 negative	NA	2/2		
	248 TOTAL	59/142	69/225		
	163 HBV+	44/92	52/143		
	37 HBV−	7/33	4/34		
	55 NA	8/17	13/48		

[a] NA = information not available.

(156, 157) inferred that their Taiwanese patients had relatively low aflatoxin exposure. Thus, because the assignment to "high" and "low" aflatoxin exposure areas of the studies summarized in Tables 1–3 is somewhat tenuous, these summary statistics should be interpreted with caution. Nevertheless, barring gross misassignment, the results do strongly support the hypothesis that mutations at codon 249 of *p53* are indicative of aflatoxin-induced hepatocellular carcinoma.

Further subclassification of these data by hepatitis B virus status is also of interest. Caution should be used in interpreting these summary statatics

Table 2 Summary of studies examining *p53* mutations in regions of the world with low aflatoxin exposure

Country/ethnicity	HB virus status1	Total *p531*	Codon 249	Comments	Reference
Japan	22 NA[a]	6/22	0/22	Referenced as "personal commun., Y Murakami and T Sekiya"	178
Taiwan	17 positive	3/17	0/17		156, 157
	12 negative	0/12	0/12		
South Africa	11 positive	NA	1/11		172
	13 NA	NA	0/13		
Various	104 NA	NA	0/104	US (27), Germany (20), Japan (12), Spain (12), China (low AFB, 10), Turkey (8) S. Korea (5), Saudia Arabia (4), Israel (3), Italy (3)	172
Japan	43 NA	8/43	0/43		179
Australia	9 positive	NA	NA		180
	10 negative	NA	NA		
Various	47 NA	NA	2/47	UK (17), Egypt (7), Italy (7), Greece (5), Turkey (4), others (7); HBV status determined (15/47 +), but status of 2 *P53* +'s not available	181
France	24 positive	7/24	NA	Both non-viral cases with *p53* mutations had alcoholic cirrhosis	182
	33 negative	2/33			
Britain	6 positive	1/6	0/6		183
	13 negative	1/13	0/13		
Chinese	24 positive	NA	0/24		173
	15 negative	NA	1/15		
	6 NA	NA	0/6		
Japanese	2 positive	NA	0/2		173
	9 negative	NA	0/9		
	2 NA	0/2	0/2		
Alaskan native	5 positive	0/5	0/5		173
	2 negative	0/2	0/2		
Caucasian	2 positive	NA	0/2		173
	7 NA	4/6	0/7		
African American	1 positive	0/1	0/1		173
Unknown*	2 positive	0/2	0/2	Assignment to LOW AFB exposure is tenuous	173
	2 negative	0/2	0/2		
	7 NA	0/7	0/7		
Germany	4 positive	1*/4	0/4		
	7 negative	3*/7	0/7		
United States	7 positive	5/7	NA		175
	10 negative	2/10	NA		
	3 NA	2/3	NA		
Japan	30 positive	10/30	3/30	Hep. C status also determined. Hep C+, B− included in HBV negative category	184
	98 negative	34/98	3/98		
	12 NA	4/12	1/12		

Shanghai, China	15 positive	3/15*	1/15**	*Exons 7–8 only analyzed	176
	3 negative	0/3*	0/3	**Case reported to have lived in high AFB region previously	
Thailand	7 positive	2/7	1/7	Biomarker data indicated low level exposure	185
	6 negative	0/6	0/6		
	2 NA	0/2	0/2		
	635 TOTAL	98/401	13/558		
	157 HBV +	32/118	6/126		
	210 HBV −	42/186*	4/167*	*includes 22/67 (total *p53*) and 3/67 (c249) Hep C+, B− samples	
	*	24/97	3/265		
	268 NA				

[a] NA = information not available.

(Table 3), however, as the presence of hepatitis B virus surface antigen, while indicative of exposure to the virus, is not necessarily indicative of a current or previously active viral infection. With this qualification, it is interesting to note that the prevalence of codon 249 mutations from the high aflatoxin exposure group is about 3 times greater in those individuals that were hepatitis B-positive. Because the number of codon 249 mutations among the low aflatoxin exposure group is very small when subdivided by hepatitis B virus status, conclusions drawn from this group would be tenuous. These summary data suggest, however, that hepatitis B virus infection may act synergistically with aflatoxin in inducing mutations at codon 249. This perhaps provides a conceptual, mechanistic basis for the apparent synergistic

Table 3 Summary of *p53* mutations in hepatocellular carcinomas worldwide reported as of July, 1993

	High AFB exposure cases		Low AFB exposure cases	
	Total *P53* mutations	Codon 249 mutation	Total *p53* mutations	Codon 249 mutation
TOTAL	59/142	69/225	98/401	13/558
	41.5%	30.7%	24.4%	2.3%
HBV +	44/92	52/143	32/118	6/126
	47.8%	36.3%	27.1%	4.8%
HBV −	7/33	4/34	42/186	4/167
	21.2%	11.7%	22.6%	2.4%
HBV NA[a]	8/17	13/48	24/97	3/265
	47.1%	27.1%	24.7%	1.1%

[a] NA = information not available.

interaction between aflatoxin and hepatitis B virus that was reported epidemiologically by Ross and co-workers (28).

Hosono et al (157) found no direct evidence that aflatoxin-DNA adducts occur at codon 249 in human liver tumor samples. Puisieux et al (158) demonstrated that a plasmid containing a full length *p53* cDNA could be mutated at codon 249 (as well as other guanine "hot spots") in vitro by incubation with AFB_1-8,9-epoxide. However, Fujimoto et al (159) failed to identify any codon 249 mutations in *p53* from aflatoxin-induced hepatocellular carcinomas obtained experimentally from nonhuman primates, and they found only one *p53* mutation (codon 175, G → T) in one out of nine tumors examined. These results demonstrate that, at least in nonhuman primates, mutation of *p53* is not required for aflatoxin-induced hepatocellular carcinoma. Aflatoxin has been shown to modify *p53* gene structure and expression in rat tumors induced with aflatoxin B_1 (106), although no rat *p53* mutations in the region corresponding to human *p53* codon 249 were detected in aflatoxin-induced preneoplastic foci (161).

There is substantial indirect evidence that inactivation of the *p53* tumor suppressor gene may play a role in the development of human liver cancer following dietary aflatoxin exposure. The high prevalence of a specific mutation in codon 249 of the human *p53* gene in human liver cancers from aflatoxin-endemic areas of the world is intriguing and may represent the first example of a "carcinogen-specific" biomarker that remains permanently fixed in the tumor tissue. Although the evidence to date does not allow the unequivocal conclusion that all tumors with codon 249 mutations are aflatoxin-derived, future prospective studies that combine biomarker assays for aflatoxin exposure with assessment of codon 249 sequence in liver tumors may ultimately allow such a remarkable conclusion to be inferred with some confidence.

RISK ASSESSMENT OF AFLATOXIN EXPOSURE AND HUMAN CANCER

Numerous studies have attempted to extrapolate laboratory animal data and/or human epidemiological data on aflatoxin exposure to human liver cancer risk (10, 162–167); this subject has been reviewed recently (168). Using the standard regulatory approach of extrapolating tumor dose response data from the most sensitive species with the Linearized Multi-stage model, a "Virtually Safe Dose" (VSD) of 0.016 ng/kg/day aflatoxin B_1 was obtained for a risk level of 1×10^{-5}, based on the rat tumor data of Wogan et al (16). When expressed as a potency value with units of $[ng/kg/day]^{-1}$, the value is 6.25×10^{-4}. Using this estimate of potency, and an estimated average daily intake of aflatoxin in the southeast United States of 110 ng/kg

per day (169), Bruce (10) estimated an excess lifetime cancer risk of 6.9 $\times 10^{-2}$, which yields an annual incidence of liver cancer of 98/100,000. This is about 20 times greatcr than the actual incidence of liver cancer in the United States from all causes. Clearly, either the potency estimate is wrong or the dietary exposure estimates in the US were substantially overestimated. Bruce, Hoseyni, and Gorelick (10, 164, 165, 168) have concluded that the rat is an inappropriate model with which to project human cancer risk for aflatoxin, primarily because of the large metabolic differences in cytochromes P450 and glutathione S-transferases discussed previously.

Using human epidemiologic data, these same authors (10, 164, 165, 168) have estimated a potency factor for aflatoxin of 4.8×10^{-5}, based on data from aflatoxin-endemic areas in Africa and Thailand, and a value of 8.2×10^{-5} using data from China (see Ref. 24 for details). These values are 13 and 7.6 times greater than the rat potency estimate of 6.25×10^{-4}, respectively. As noted by these authors, these values are likely overestimates of the true potency for aflatoxin alone, as the tumor response data for both epidemiological estimates were derived from areas with endemic hepatitis B virus. Hoseyni (165) attempted to correct for the influence of hepatitis B virus on the potency estimate for aflatoxin by using an exponential-multiplicative relative risk function applied to Chinese liver cancer incidence data. This model projected potency values of 1.6×10^{-6} and 2.8×10^{-6} for the best estimate and upper 95% confidence limit, respectively, for human cancer risk from lifetime exposure to aflatoxin in the absence of hepatitis B virus. If one uses the upper confidence limit value of the corrected risk (2.8×10^{-6}), this estimate is about 30 times less than the estimate for aflatoxin-related liver cancer risk in the presence of hepatitis B virus, and 223 times less than the estimate based on linearized multistage model extrapolation of rat data. This difference is remarkably similar to the estimated magnitude of effect of hepatitis B virus on aflatoxin carcinogenicity from the recent prospective epidemiological data of Ross et al (28). As discussed previously, these authors found a relative risk for liver cancer in aflatoxin-exposed population (Guangxi, China) of about 2 in the absence of HBV, but slightly over 60 when both hepatitis B virus and aflatoxin were considered.

SUMMARY AND CONCLUSIONS

Much progress has been made in elucidating the biochemical and molecular mechanisms that underlie aflatoxin carcinogenesis. In humans, biotransformation of AFB_1 to the putative carcinogenic intermediate, AFB-8,9-*exo*-epoxide, occurs predominantly by cytochromes P450 1A2 and 3A4, with the relative importance of each dependent upon the relative magnitude of

expression of the respective enzymes in liver. Genetic variability in the expression of these and other cytochromes P450 may result in substantial interindividual differences in susceptibility to the carcinogenic effects of aflatoxins.

Detoxification of AFB-8,9-epoxide by a specific alpha class glutathione S-transferase is an important protective mechanism in mice, and it accounts for the resistance of this species to the carcinogenic effects of AFB. This particular form of GST is expressed constitutively only at low levels in rats, but it is inducible by antioxidants such as ethoxyquin, and it accounts for much of the chemoprotective effects of a variety of substances, including natural dietary components that putatively act via an "antioxidant response element" (ARE). In humans, the constitutively expressed GSTs have very little activity toward AFB_1-8,9-*exo*-epoxide, suggesting that—on a biochemical basis—humans should be quite sensitive to the genotoxic effects of aflatoxins. If a gene encoding a high aflatoxin-active form of GST is present in the human genome, but is not constitutively expressed, and is inducible by dietary antioxidants (as occurs in rats), then chemo- and/or dietary intervention measures aimed at inducing this enzyme could be highly effective. However, as it is possible that human CYP 1A2 may also be inducible by these same chemicals (because of the possible presence of an ARE in this gene), the ultimate consequence of dietary treatment with chemicals that induce biotransformation enzymes via an ARE is uncertain. The balance of the rate of activation (*exo*-epoxide production) to inactivation (GST conjugation plus other P450-mediated non-epoxide oxidations) may be a strong indicator of individual and species susceptibility to aflatoxin carcinogenesis, if the experimental conditions are reflective of true dietary exposures.

There is strong evidence that AFB-8,9-*exo*-epoxide binds to G:C rich regions of DNA, forming an adduct at the N^7-position of guanine. Substantial evidence demonstrates that AFB_1-8,9-epoxide can induce activating mutations in the *ras* oncogene in experimental animals, primarily at codon 12. Information on the activation of other oncogenes by aflatoxin is limited, and, to date, few (if any) studies have demonstrated an activated c-*ras* oncogene in human liver tumors from aflatoxin endemic areas. In contrast, substantial evidence implicates aflatoxin-induced G:C mutations (both G → T transversions and G → A transistions) in the inactivation of the human *p53* tumor suppressor gene. These mutations occur with an extraordinary frequency at codon 249 of *p53* and may ultimately serve as a "carcinogen-specific" marker of aflatoxin-induced liver cancers in humans. The presence of hepatitis B virus appears to act synergistically with aflatoxin in the development of liver cancer. Analysis of epidemiological data of *p53* mutations in the presence and/or absence of hepatitis B virus surface antigens

suggests that the production of codon 249 mutations in *p53* by aflatoxin may be enhanced by hepatitis virus infection, providing a rational, mechanistic basis for the observed synergy between these two human cancer risk factors.

Additional studies that combine current techniques for biomonitoring of aflatoxin exposure with the putative specific gene marker (codon 249 mutations in *p53*) for aflatoxin-induced hepatocellular carcinoma are needed. Although loss of the *p53* tumor suppressor gene may be an important etiologic factor in aflatoxin-induced liver cancer in humans, animals studies suggest that it is not requisite for liver cancers to occur, and other effects of aflatoxin are likely required. The cytotoxic effects of aflatoxin, as well as other enhancers of cell proliferation—such as hepatitis B virus infection—may be important in the promotion and progression of aflatoxin-initiated cells. Thus, although the formation of AFB-DNA adducts has been demonstrated to occur linearly through a large dose-range in rats and trout, the tumorigenic response does not necessarily follow linearity at all doses because of the cytotoxic effects of aflatoxin, which are likely to occur only at higher doses.

Using both risk assessment modelling of human epidemiological data and a prospective nested case-control study of human liver cancer in aflatoxin and hepatitis B-virus endemic regions of China, one can estimate (albeit crudely) that hepatitis B virus infection enhances the carcinogenic response of aflatoxin by about 30-fold. Risk assessment using rat data and the traditional Linearized Multi-Stage model appears to overestimate human liver cancer risk from aflatoxin (in the absence of hepatitis B-virus) by perhaps 200-fold. A substantial body of in vitro biochemical data on aflatoxin metabolism suggests that humans should be equally or more sensitive to the carcinogenic effects of aflatoxin. There may be other, as yet unidentified species differences between humans and rats that act either to enhance the relative sensitivity of rats or, conversely, to provide relative protection to humans exposed to aflatoxins in the diet. Additional studies to establish a quantitative link between aflatoxin exposure and DNA damage in human liver, and to further explore the relationship between initial formation of DNA adducts, activation of specific oncogenes (and/or inactivation of tumor suppresor genes), and the ultimate development and progression to cancer are needed to fully understand how aflatoxin produces liver cancer. From such studies rational means of dietary and/or chemointervention in high-risk populations may be developed.

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