MECHANISMS OF AFLATOXIN CARCINOGENESIS

David L. Eaton and Evan P. Gallagher
Department of Environmental Health and Institute for Environmental Studies,
University of Washington, Seattle, Washington 98195

KEY WORDS: tumor suppressor genes, biotransformation, urinary biomarkers, hepatitis B virus

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 hepatotoxicity (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 immunosuppressive 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
(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
AFLATOXIN CARCINOGENESIS 137

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 B₁ and G₁, or use of purified AFB₁), (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 doses were capable of producing hepatocellular carcinomas. The most 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 TD₅₀ values for rats developed by Gold et al (30), only 2,3,7,8-TCDD (TD₅₀ = 6.7 × 10⁻⁶ mg/kg per day) significantly exceeds AFB₁ (TD₅₀ = 9.3 × 10⁻⁴ mg/kg per day) in potency. Using the TD₅₀ₙₚ parameter, AFB₁ is 1,000 times more potent a carcinogen than benzo(a)pyrene.

Remarkably, mice appear highly resistant to the hepatocarcinogenic effects of aflatoxin. Wogan (17) reported that Swiss-Webster mice fed aflatoxin-contaminated peanut meal containing 100,000 or 150,000 ppb of a mixed aflatoxin preparation (AFB₁ + AFG₁) for 80 weeks (postweaning) developed no hepatic tumors. However, when 1.25 µg AFB₁/g body weight was administered by i.p. injection during the first 10 days after birth (postweaning) 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 300 ppb (in the bathing medium) AFB₁ 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 M1 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 AFB1 intake and urinary AFM1 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 AFM1 excretion.

Recently, Groopman and co-workers (11, 22–24) have demonstrated that the AFB-N7-guanine adduct in urine represents the most reliable urinary biomarker of aflatoxin exposure. However, even with this marker, the amount of AFB-N7-guanine in urine reflects only relatively recent exposure. There has been interest in evaluating the utility of more persistent biomarkers, such as the albumin-(e-amine-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 N7-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 B1 (AFB1)-induced carcinogenesis. AFB1 requires microsomal oxidation to the reactive AFB1-8,9-epoxide (AFBO, also referred to as AFB2,3-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 AFB1. Microsomal biotransformation of AFB1 also results in the production of more polar metabolites (AFM1, AFQ1, and AFP1; 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 AFB1 detoxification (Figure 2). In rodents, the amount of GST activity towards AFBO is inversely related to species susceptibility to AFB1-induced hepatocarcinogenesis. Ultimately, the amount of AFB1 that will bind to
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).
Figure 3 Structures of aflatoxin B1 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 $\textit{exo}$- and $\textit{endo}$-AFB$_1$ epoxide stereoisomers (31). The epoxide ring is positioned above the plane and trans to the 5a and 9a protons in the $\textit{endo}$-stereoisomer, whereas the epoxide ring points below the plane and cis to the 5a and 9a protons in the $\textit{exo}$-epoxide (31). The metabolic activation of AFB$_1$ by human or rat microsomes produces a mixture of $\textit{endo}$- and $\textit{exo}$-epoxides, which can be trapped with GSH and identified by comparison with standards prepared by the reaction of the $\textit{endo}$- or $\textit{exo}$-stereoisomers with GSH (31). Both isomers are produced by human liver microsomes, although the $\textit{exo}$-epoxide predominates (31). Rat microsomes also efficiently form the $\textit{exo}$-epoxide, but are far less efficient at forming $\textit{endo}$-epoxide when compared to human microsomes. Although the $\textit{endo}$-epoxide is less susceptible to hydrolysis than is the $\textit{exo}$-conformation (43), the $\textit{exo}$-epoxide is much more efficient at forming DNA adducts and is much more mutagenic than the $\textit{endo}$-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 lipoygenase-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 lipoygenase-dependent activation of AFB$_1$ is catalyzed by arachidonic acid-derived peroxyl 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 lipoygenase 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 $B_{2\alpha}$ ($\alpha$-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). Shariat & 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 cellubular 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$_1$, AFM$_3$, 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 AFM₁ are formed in a number of species including human, monkey, rat, mouse, and rainbow trout (34, 54, 56).

Although there is little information regarding AFQ₁ carcinogenicity, AFQ₁ exhibits much lower acute toxicity (48) and mutagenicity (49, 57) than AFB₁. In rainbow trout, the carcinogenic potency of AFQ₁ is approximately 1% of AFB₁ (58); this despite the extreme sensitivity of rainbow trout to aflatoxins. The oxidation of AFB₁ to AFQ₁ appears to constitute a particularly important pathway for AFB₁ detoxification in human and nonhuman primate liver (34). Raney et al (57) showed that human liver microsomes do not appreciably oxidize AFQ₁ and that synthetic AFQ₁-epoxide exhibits little mutational activity towards Salmonella typhimurium strain TA98. In light of this evidence it is likely that AFQ₁ has little potential for carcinogenic activity in humans.

In mice, rats and monkeys, the O-demethylation of AFB₁ produces AFP₁ (59). Although it is not a prominent AFP₁ 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 AFP₁ than observed in microsomes prepared from normal liver tissue. Interestingly, AFP₁ is produced by human liver slices incubated with AFB₁ (DL Eaton and J Heinonen, unpublished observations) and is also a common urinary metabolite in humans exposed to dietary AFB₁ in vivo (22). In this regard, AFP₁ 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 AFB₁ in Shanghai, China, include AFB₁, AFM₁, and AFB₁-DNA-adducts (AFB₁-N²-guanine) (22).

A dihydroxyaflatoxin B₁, AFM₁-P₁, can be formed by the 9α-hydroxylation of AFP₁ or the 4-O-demethylation of AFM₁ (41). This metabolite is formed in vivo in rats and is excreted directly in bile or as the glucuronide conjugate (41). Glucuronidation of AFP₁ most effectively compete in vivo with microsomal oxygenases responsible for the secondary oxidation of AFP₁, as the AFP₁-glucuronide conjugate is excreted in bile in greater amounts than the glucuronide conjugate of 4,9α-dihydroxy-AFB₁.

**CYP450 isoenzymes involved in AFB₁ biotransformation** Evidence suggests that multiple CYP450 isoenzymes contribute to AFB₁ epoxidation in the rat. CYP 2C11, a male specific CYP450 isoform in the rat, may activate AFB₁ 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 AFB₁ 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 AFB₁-epoxidation (unpublished...
observations). Metcalfe et al. (63) found that AFB\textsubscript{1} activation by rat liver is potentiated by phenobarbital pretreatment, thus indicating that certain CYP450 forms involved in AFB\textsubscript{1} activation are phenobarbital-inducible. Dietary ethoxyquin increases the rate of AFM\textsubscript{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\textsubscript{1} to AFM\textsubscript{1} in rat liver. AFM\textsubscript{1} formation is also increased tenfold in rats pretreated with 3-methylcholanthrene, thus suggesting that the CYP450 responsible for AFM\textsubscript{1} formation is regulated through the Ah-receptor (63). In this regard, Koser et al. (65) reported that the CYP450, which forms AFM\textsubscript{1} in the mouse, is CYP 1A2, which is also under regulation by the Ah receptor.

The oxidation of AFB\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{1} to mutagenic metabolites at low substrate concentrations. A recent study from our laboratory suggests that CYP 3A enzymes (s) are capable of AFB\textsubscript{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\textsubscript{1} epoxidation in human liver microsomes at non-saturating (16 \textmu M AFB\textsubscript{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 \textmu M AFB\textsubscript{1}) concentrations (Figure 4). Consideration of the kinetics of aflatoxin biotransformation is important because AFB\textsubscript{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\textsubscript{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 \(\mu\)M and 128 \(\mu\)M. 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 AFB1 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 AFB1 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 AFB1 metabolites by HPLC (69).
by a previous study from our laboratory that showed a high correlation ($r = 0.978$) 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 9α-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 frame-shift 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 AFB1-metabolite conjugation pathways and therefore may not be predictive of species sensitivity.

Recently, a new aldo-keto reductase involved in the reduction of AFB1-8,9-aldehyde (AFBA) has been described (78, 79). AFBA is formed by spontaneous rearrangement of the dihydrodiol, which in turn is formed from hydrolysis of AFBO. As noted above, the dialdehyde form is capable of forming Schiff bases with amines in protein and may be responsible for some of the acute toxic effects of AFB1. This enzyme, termed aflatoxin B1-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 AFB1. As cytotoxic effects of AFM may play a significant promotional role in tumor development (80), it is also possible that this enzyme pathway may offer some protection against AFM 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 AFB1 serve as substrates for phase II detoxication enzymes. Extensive interspecies variation exists in the functional importance of the different phase II enzymes in AFB1 detoxication. In many mammalian species, the primary pathway for AFB1 detoxication is through GST-mediated conjugation of AFBO with reduced glutathione (GSH). In contrast, AFB1-GSH conjugation is not a significant route of AFB1 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 AFB1 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 AFB1 when compared to rats (15), a difference reflected by 50- to 100-fold less AFB1-DNA adduct formation by mice after in vivo AFB1 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, \( Y \alpha_1 \), \( Y \alpha_2 \), and \( Y \kappa \) have significant activity towards AFBO (99). Hayes et al (91) reported the presence of an ethoxyquin-inducible alpha class GST subunit in rat cytosol (\( Y \kappa \)) that has high specific activity towards AFBO. Although not constitutively expressed in normal liver, \( Y \kappa \) is overexpressed in AFBI-induced nodule-bearing livers, possibly underlying the acquired resistance of nodules to AFBI (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-\( Y \alpha_1 \) in rat liver. A second clone, isolated in our laboratory and termed m\( Y \kappa \), encodes a novel sequence that exhibits 86% homology to rat \( Y \kappa \) cDNA sequence (pGT842) (97). Interestingly, the homology between the rat and mouse \( Y \kappa \) isoforms (86%) is significantly lower than for mouse \( Y \alpha_1 \) and rat \( Y \alpha_2 \) isoforms (95%). When the rat \( Y \kappa \) and mouse \( Y \kappa \) cDNAs are expressed in a bacterial expression system, the mouse \( Y \kappa \) has nearly 100-fold higher activity for AFBO than the rat \( Y \kappa \) isoform (98). Apparently, however, it appears now that the constitutively expressed mouse \( Y \kappa \) form with high AFBO activity is orthologous to the ethoxyquin-inducible \( Y \kappa \) GST described by Hayes and co-workers (91, 95), and not to the rat \( Y \kappa \). Partial amino acid sequence (98), and more recently the complete cDNA sequence (JD Hayes, personal communication), indicate that the rat \( Y \kappa \) form shares 91% homology with the mouse \( Y \kappa \), which has high AFBO activity, and that the rat form itself has high AFBO-conjugating activity (95).

As previously noted, metabolic activation of AFBI produces a mixture of \textit{exo}- and \textit{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 AFBI stereoisomers. The \textit{exo}-epoxide is efficiently trapped by mouse cytosolic GSTs, whereas rat cytosolic fractions are relatively inefficient at conjugating the \textit{exo}-epoxide (99). Human liver cytosolic fractions are very poor at conjugating either stereoisomer of AFBO, although the human GST mu form, GST \textit{M}I\textit{a}-\textit{M}I\textit{a}, 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 AFB1-exposed individuals who lack this enzyme may be at increased risk for AFB1 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 AFP1 and 4,9a-dihydroxy aflatoxin B1 have been identified as biliary metabolites in rats treated with AFB1 (41). Glucuronide-conjugates of AFP1 have also been identified in chickens (100). Studies with isolated hepatocytes suggest that AFP1 is a better substrate for glucuronidation than are AFM1 and AFQ1 (101). Furthermore, Ch'ih et al (102) showed that the AFP1-glucuronide conjugate is the only significant glucuronide conjugate of hydroxylated AFB1 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 AFM1 and AFQ1 remains uncertain. AFL-glucuronide and AFL-M1-glucuronide are the major AFB1 conjugates in rainbow trout fed control diets (103). Sulfate conjugates were not detected in rainbow trout exposed to AFB1 (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 AFM1, thus indicating the in vivo formation of the sulfate conjugate of AFM1 in this species.

MODULATION OF AFB1 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 AFB1 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 AFB1-DNA binding was lower in the rats
receiving the broccoli diet (111). The rate of AFB1 epoxidation was not
significantly affected by dietary broccoli, indicating that increased AFBO
detoxification, as opposed to decreased AFB1 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 AFB1 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 AFB1 hepatotoxicity (107). The substituted 1,2-
dithiole-3-thione, oltipraz, protects against acute and chronic AFB1 hepato-
toxicity in rodents (119). Oltipraz also reduces the size and number of
enzyme-altered foci in the livers of rats treated with AFB1 (120, 121). The
in vivo covalent binding of AFB1 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 AFB1 administration show a 3-
to 5-fold increase in the rate of 8,9-dihydro-8-(S-glutathionyl)-9-hydroxy-
aflatoxin B1 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-
amisole (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 AFB1. Pretreatment of rats with BHA, BHT (125),
and ethoxyquin (127) attenuates the carcinogenicity of AFB1. The mechanism
underlying modulation of AFB1-induced hepatocarcinogenesis in rats by
synthetic antioxidants involves, at least in part, a reduction in AFB1-DNA
binding (35, 128, 129). The protective effects of antioxidants against AFB1
hepatocarcinogenesis have been directly linked to modulation of AFB1
biotransformation. Pretreatment of rats with synthetic antioxidant BHA
increased the level of AFB1-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 AFB1 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 AFB1 activation and ultimately increase the risk AFB1-hepatocarcinogenesis in exposed individuals.

Genotoxic Effects of Aflatoxins

**Types of DNA Adducts Formed with Aflatoxin B1.** That aflatoxin B1 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-(N7-guanyl)-9-hydroxy-aflatoxin B1 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 B1-8,9-epoxide to the N7-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 adenine (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 N7-guanine adduct may be so important is the relatively rapid rate at which it undergoes rearrangement to the ring-opened, formamidopyrimidine (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 tumorigenesis 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 108...
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, dose (ng/kg/day) and AFB-DNA adducts (adducts/10^8 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 AFB1 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. HindIII 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 AFB1-induced transformed cell lines and primary liver tumors, demonstrated that AFB1 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/10T1/2 cells (7SA cell line) overexpressed protein kinase Ca (PKCa) and exhibited an abnormal responsiveness 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, 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 classified 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

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

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

<table>
<thead>
<tr>
<th>Country/ethnicity</th>
<th>HB viral status</th>
<th>Total p53</th>
<th>Codon</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japan</td>
<td>22 NA*</td>
<td>6/22</td>
<td>0/22</td>
<td>Referenced as “personal commun., Y Marakami and T Sekiya”</td>
<td>178</td>
</tr>
<tr>
<td>Taiwan</td>
<td>17 positive</td>
<td>3/17</td>
<td>0/17</td>
<td></td>
<td>156, 157</td>
</tr>
<tr>
<td></td>
<td>12 negative</td>
<td>0/12</td>
<td>0/12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Africa</td>
<td>11 positive</td>
<td>NA</td>
<td>1/11</td>
<td></td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>13 NA</td>
<td>NA</td>
<td>0/13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Various</td>
<td>104 NA</td>
<td>NA</td>
<td>0/104</td>
<td>US (27), Germany (20), Japan (12), Spain (12), China (low AFB, 10), Turkey (8), Korea (8), Saudi Arabia (4), Israel (3), Italy (3)</td>
<td>172</td>
</tr>
<tr>
<td>Japan</td>
<td>43 NA</td>
<td>8/43</td>
<td>0/43</td>
<td></td>
<td>179</td>
</tr>
<tr>
<td>Australia</td>
<td>9 positive</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>10 negative</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Various</td>
<td>47 NA</td>
<td>NA</td>
<td>2/47</td>
<td>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</td>
<td>181</td>
</tr>
<tr>
<td>France</td>
<td>24 positive</td>
<td>7/24</td>
<td>NA</td>
<td>Both non-viral cases with p53 mutations had alcoholic cirrhosis</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td>33 negative</td>
<td>2/33</td>
<td>NA</td>
<td></td>
<td>183</td>
</tr>
<tr>
<td>Britain</td>
<td>6 positive</td>
<td>1/6</td>
<td>0/6</td>
<td></td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>13 negative</td>
<td>0/13</td>
<td>0/13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chinese</td>
<td>24 positive</td>
<td>NA</td>
<td>0/24</td>
<td></td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>15 negative</td>
<td>NA</td>
<td>1/15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 NA</td>
<td>NA</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Japanese</td>
<td>2 positive</td>
<td>NA</td>
<td>0/2</td>
<td></td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>9 negative</td>
<td>NA</td>
<td>0/9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 NA</td>
<td>0/2</td>
<td>0/2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alaskan native</td>
<td>5 positive</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>2 negative</td>
<td>0/2</td>
<td>0/2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>2 positive</td>
<td>NA</td>
<td>0/2</td>
<td></td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>7 NA</td>
<td>NA</td>
<td>0/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>African American</td>
<td>1 positive</td>
<td>0/1</td>
<td>0/1</td>
<td></td>
<td>173</td>
</tr>
<tr>
<td>Unknown*</td>
<td>2 positive</td>
<td>0/2</td>
<td>0/2</td>
<td>Assignment to LOW AFB exposure is tenacious</td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>2 negative</td>
<td>0/2</td>
<td>0/2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7 NA</td>
<td>0/7</td>
<td>0/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td>4 positive</td>
<td>1*/4</td>
<td>0/4</td>
<td></td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>7 negative</td>
<td>3*/7</td>
<td>0/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>United States</td>
<td>7 positive</td>
<td>5/7</td>
<td>NA</td>
<td></td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>10 negative</td>
<td>2/10</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 NA</td>
<td>2/3</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Japan</td>
<td>30 positive</td>
<td>10/30</td>
<td>3/30</td>
<td></td>
<td>184</td>
</tr>
<tr>
<td></td>
<td>98 negative</td>
<td>34/98</td>
<td>3/98</td>
<td></td>
<td>C+, B— included in HBV negative category</td>
</tr>
<tr>
<td></td>
<td>12 NA</td>
<td>4/12</td>
<td>1/12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
AFLATOXIN CARCINOGENESIS

Shanghai, China
15 positive 3/15* 1/15**
2 negative 0/3* 0/3

*Exons 7–8 only analyzed
**Case reported to have lived in high AFB region previously

Thailand
7 positive 2/7 1/7
6 negative 0/6 0/6
2 NA 0/2 0/2

Biomarker data indicated low level exposure

635 TOTAL 98.401 13.558
157 HBV + 32/118 6/126
210 HBV – 42/166* 4/167*
*includes 22/67 (total p53) and 3/67 (c249) Hep C+, B– samples
268 NA 24/97 3/265

*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>
<thead>
<tr>
<th></th>
<th>High AFB exposure cases</th>
<th>Low AFB exposure cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total p53 mutations</td>
<td>Codon 249 mutation</td>
</tr>
<tr>
<td>TOTAL</td>
<td>59/142</td>
<td>69/225</td>
</tr>
<tr>
<td></td>
<td>41.5%</td>
<td>30.7%</td>
</tr>
<tr>
<td>HBV +</td>
<td>44/92</td>
<td>52/143</td>
</tr>
<tr>
<td></td>
<td>47.9%</td>
<td>36.3%</td>
</tr>
<tr>
<td>HBV –</td>
<td>7/33</td>
<td>4/34</td>
</tr>
<tr>
<td></td>
<td>21.2%</td>
<td>11.7%</td>
</tr>
<tr>
<td>HBV NA*</td>
<td>8/17</td>
<td>13/48</td>
</tr>
<tr>
<td></td>
<td>47.1%</td>
<td>27.1%</td>
</tr>
</tbody>
</table>

*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 AFB1-8,9-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 B1 (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 B1 was obtained for a risk level of $1 \times 10^{-6}$, based on the rat tumor data of Wogan et al (16). When expressed as a potency value with units of $[\text{ng/kg/day}]^{-1}$, the value is $6.25 \times 10^{-4}$. 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 greater 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 U.S. were substantially underestimated. 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 $4.8 \times 10^{-5}$, based on data from aflatoxin-endemic areas in Africa and Thailand, and a value of $8.2 \times 10^{-5}$ using data from China (see Ref. 24 for details). These values are 13 and 7.6 times greater than the rat potency estimate of $6.25 \times 10^{-4}$, 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 $1.6 \times 10^{-6}$ and $2.8 \times 10^{-6}$ 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 ($2.8 \times 10^{-6}$), 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 AFB1 to the putative carcinogenic intermediate, AFB-8,9-endo-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-8,9-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 chemotherapeutic 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 (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-epoxide binds to G:C rich regions of DNA, forming an adduct at the N7-position of guanine. Substantial evidence demonstrates that AFB-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 transversions) 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, animal 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 suppressor 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.

Acknowledgments
The authors would like to thank Ms. Azure Skye for her excellent assistance in the preparation of this manuscript and Dr. John Groopman for his
thoughtful comments and suggestions in reviewing this manuscript. This work was supported by NIH grants ES-05780, ES-03933, ES-04696, and T32-ES07032.

Any Annual Review chapter, as well as any article cited in an Annual Review chapter, may be purchased from the Annual Reviews Preprints and Reprints service. 1-800-247-8007, 415-259-5017; e-mail: arpreprints@annualreviews.org

Literature Cited


AFLATOXIN CARCINOGENESIS 165


67. Shimada T, Guengerich FP. 1989. Evidence for cytochrome P450(NF), the nitroreductase, being the principal enzyme involved in the bioactivation
79. Hayes JD, Judah DJ, Neal GE. 1993. Resistance to aflatoxin B1 is associated with the expression of a novel aldehyde reductase which has catalytic activity towards a cytotoxic aldehyde-containing metabolite of the toxin. Cancer Res. 53:3887-94
84. Degem GH, Nennmann H-G. 1981. Differences in aflatoxin B1 susceptibility of rat and mouse are correlated with the capability in vivo to inactivate aflatoxin B1-epoxide. Carcinogenesis 2:299-305
90. Ramsdell HS, Eaton DL. 1990. Mouse liver glutathione S-transferase isoenzyme activity toward aflatoxin B1-8,9-epoxide and benzo[a]pyrene-7,8-dihy-


113. Godlewski CF, Boyd IN, Sherman
AFLATOXIN CARCINOGENESIS


120. Kensler TW, Egner PA, Dolan PM, Groopman JD, Roebuck BD. 1987. Mechanism of action against aflatoxin mutagenicity in rats (ed 5,6-pyrazolyl)-4-methyl-1,2-dithiol-3-thione (olipraz) and related 1,2-dithiol-3-thiones and 1,2-dithiol-3-thiones. Cancer Res. 47: 2471-77.


123. Roebuck BD, Liu YL, Rogers AE, Groopman JD, Kensler TW. 1991. Protection against aflatoxin B1-induced hepatocarcinogenesis in F344 rats by 3,4-pyrazolyl-4-methyl-1,2-dithiol-3-thione.


125. Davidson NE, Egner PA, Kensler TW. 1990. Transcriptional control of glutathione S-transferase gene expression by the chemopreventive agent 5,6-pyrazolyl-4-methyl-1,2-dithiole-3-thione (olipraz) in rat liver. Cancer Res. 50: 2231-35.


microsomal fractions. Biochem. Pharmacol. 30:2451–56


patients with low aflatoxin exposure.


