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Biochemical and Molecular Aspects of Mammalian Susceptibility to Aflatoxin B_1 Carcinogenicity (43852A)

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Abstract. **Aflatoxin B_1 (AFB_1) is a fungal toxin that has been implicated as a causative agent in human hepatic and extrahepatic carcinogenesis. In this review, the mechanisms involved in AFB_1 toxicity are delineated, in order to describe the features that make a specific cell, tissue, or species susceptible to the mycotoxin. Important considerations include: (i) different mechanisms for bioactivation of AFB_1 to its ultimate carcinogenic epoxide metabolite; (ii) the balance between bioactivation to and detoxification of the epoxide; (iii) the interaction of AFB_1 epoxide with DNA and the mutational events leading to neoplastic transformation; (iv) the role of cytotoxicity in AFB_1 carcinogenesis; (v) the significance of nonepoxide metabolites in toxicity; and (vi) the contribution of mycotoxin-unrelated disease processes. Although considerable controversy remains about the importance of specific events, a great deal has been learned about biochemical and molecular actions of AFB_1.** [P.S.E.B.M. 1995, Vol 208]

Aflatoxins are a group of extremely toxic metabolites produced by some strains of the fungi *Aspergillus flavis, Aspergillus parasiticus,* and *Aspergillus nomius*. The fungi are ubiquitous and, under appropriate conditions of heat and moisture, grow on a variety of agricultural products. Aflatoxins were first identified as etiological agents for animal disease in the early 1960s following an outbreak in England of poultry deaths associated with hepatic necrosis. The disease, termed turkey X-disease, was traced to peanut meal from Brazil (1). Similar outbreaks of liver toxicity were reported in other animals consuming contaminated feeds, and hepatomas were observed in fish from hatcheries using aflatoxin-contaminated cottonseed meal food pellets (2).

Numerous outbreaks of human acute aflatoxicosis involving liver failure and gastrointestinal bleeding have occurred in Southeast Asia and Africa. The role of aflatoxins as human carcinogens has been under investigation since the realization that liver cancer incidence is high in regions with high endemic aflatoxin concentrations (reviewed in Ref. 3) and since the extreme potency of these mycotoxins as carcinogens was established in the laboratory (4). In fact, it is estimated that 250,000 deaths occur annually in certain parts of China and sub-Saharan Africa due to hepatocellular carcinoma (5). Major contributors to this high rate of hepatocellular carcinoma include both aflatoxin ingestion and hepatitis B virus infection.

For a comprehensive review of the toxicology of aflatoxins, the reader is referred to a 1994 volume ed-

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ited by Eaton and Groopman (3). The purpose of the present review is to consider the biochemical and molecular characteristics of mammalian cells and tissues which appear to be responsible for susceptibility to aflatoxin B_1 (AFB_1), the most prevalent and most potently cytotoxic and carcinogenic of the aflatoxins. Although the liver is clearly the principal target organ for AFB_1, other tissues can be affected, so we have considered extrahepatic AFB_1 effects as well as hepatic. Due primarily to the potential for occupation-related exposure to aflatoxin-containing grain dusts, the respiratory system is one such alternate target.

AFB_1 Biotransformation

Of the toxic actions associated with AFB_1 exposure, the most serious are mutagenicity and carcinogenicity, which have been linked to metabolic activation of the molecule. It has been demonstrated that AFB_1 itself is not mutagenic nor does it bind covalently to macromolecules such as DNA in the absence of a bioactivation system (6). Since bioactivation of AFB_1 is required for carcinogenicity, much attention has focused on the biotransformation of this naturally occurring mycotoxin. The initial metabolism of AFB_1 can involve four types of reactions: O-dealkylation, hydroxylation, epoxidation, or ketoreduction (Fig. 1). While the reduction of AFB_1 to aflatoxicol is believed to be catalyzed by a cytosolic NADPH-dependent reductase, the other reactions are primarily carried out by the cytochrome P450-dependent polysubstrate monooxygenase enzyme superfamily (P450, CYP). However, as discussed elsewhere in this review, other activation systems can play a role.

P450-Mediated AFB_1 Metabolism. P450 is the principal enzyme system involved in the oxidative biotransformation of AFB_1. This conclusion is based on the following observations: (i) molecular oxygen and NADPH are required for metabolism *in vitro;* (ii) the extent of microsomal activation is altered following treatment with P450 inducers (e.g., phenobarbital, 3-methylcholanthrene); (iii) inhibitors of P450 (e.g., piperonyl butoxide, cobalt chloride, carbon monoxide) inhibit microsomal activation; (iv) susceptibility of animals to AFB_1-induced hepatocarcinogenesis is affected by treatment with P450 inducers and inhibitors; (v) AFB_1 is activated in systems containing purified P450, NADPH cytochrome P450 reductase, NADPH, and phospholipid; (vi) antibodies against P450 isoforms inhibit AFB_1 activation catalyzed by microsomes or purified P450; and (vii) systems which express the cDNA of P450 isoforms are capable of AFB_1 activation (6–11).

Activation of AFB_1. The mutagenicity and carcinogenicity of AFB_1 are believed to result from the activation of AFB_1 to AFB_1-8,9-epoxide (using IUPAC nomenclature; this reactive intermediate has also been referred to as 2,3-epoxide in some publications), the ultimate mutagen/carcinogen (Fig. 1) (8, 12, 13). The 8,9-epoxide has not been isolated from biological systems due to its reactivity, but the major DNA adduct formed *in vivo* and *in vitro* in the presence of an activation system is 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy AFB_1 (AFB_1-guanine) (8, 14, 15). Thus, independent of the species or the tissue under investigation, activation of AFB_1 to the 8,9-epoxide is an absolute requirement for AFB_1 to manifest its mutagenic, carcinogenic, and DNA-binding actions. The extent of *in vivo* covalent binding of radiolabeled AFB_1 to DNA in hepatic systems has been correlated with the incidence of cancer (8, 15, 16), further supporting the importance of AFB_1-8,9-epoxide generation in carcinogenesis. Interestingly, it has been reported that AFB_1 can be activated to either an *exo-* or an *endo*-epoxide, with only the *exo*-epoxide subsequently reacting with DNA (17) (Fig. 2).

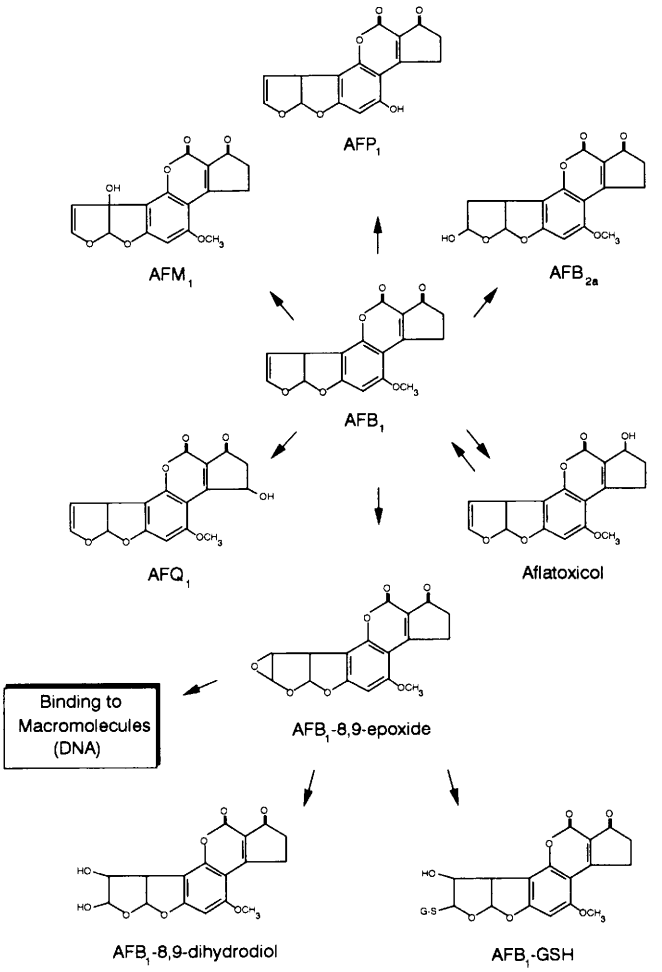

Figure 1. The biotransformation of aflatoxin B_1. G-S refers to the glutathione moiety of the glutathione conjugate.

Early studies with isozyme-selective P450 inducers and inhibitors, as well as purified and reconstituted isoforms, indicated that several forms of the enzyme are involved in AFB_1 bioactivation to a mutagenic and/

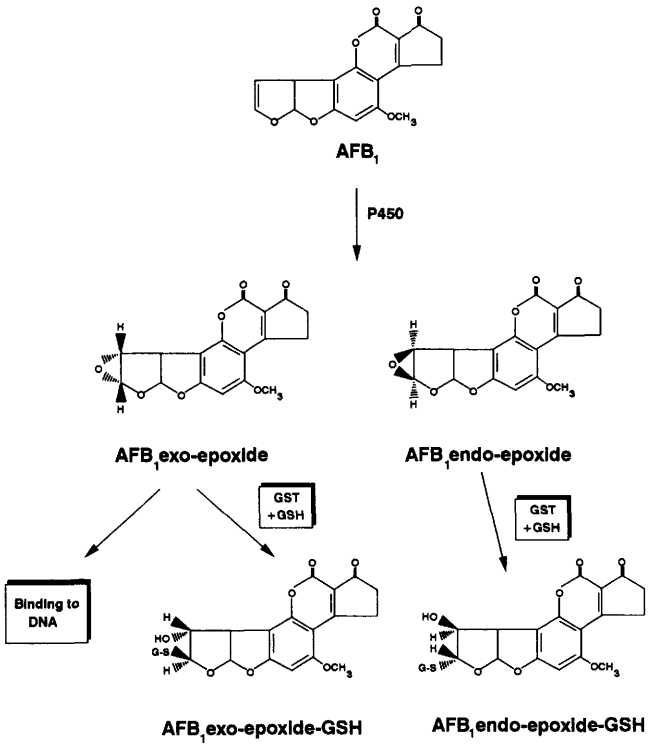

Figure 2. Formation of *exo*- and *endo*-epoxides and the corresponding glutathione conjugates from aflatoxin B_1.

or DNA-binding metabolite (Table I reviews some of the major P450 classes and their catalytic characteristics). P450s capable of activating AFB_1 in animal systems include members of the 1A, 2B, 2C, and 3A subfamilies (18–21).

Treatment of rodents with the classic P450 inducer phenobarbital resulted in a greatly increased ability of liver microsomes to generate AFB_1-nucleic acid adducts when compared with untreated animals (8, 12, 13). Similarly, based on *in vitro* studies using microsomes prepared from the livers of mice and rats (22), it has been proposed that AFB_1 activation is catalyzed by phenobarbital-inducible forms of P450. While this may be true for rats and mice, it does not appear to be the case for all species. For example, polycyclic aromatic hydrocarbon (PAH)-inducible P450 forms have been found to be more active in the hamster (23, 24), and cultured cells expressing monkey CYP1A1 cDNA also activate AFB_1 (25). We have found hepatic activation of AFB_1 in rabbits and guinea pigs to be similar to that in rats, as treatment with the PAH P450 inducer β-naphthoflavone did not significantly affect the ability of liver microsomes to generate DNA-binding metabolites of AFB_1 when compared with microsomes prepared from the livers of untreated animals (26, 27).

Table I. Cytochrome P450 Forms Involved in Aflatoxin B_1 Biotransformation

P450 subfamily	Inducer	Specific isoforms	AFB_1 Metabolite
CYP1A	PAHs	1A1, 1A2	AFB_1-8,9-epoxide, AFM_1
CYP2B	Constitutive, Phenobarbital	2B1, 2B7	AFB_1-8,9-epoxide
CYP2C	Phenobarbital	2C1, 2C2	AFB_1-8,9-epoxide
CYP3A	Glucocorticoids	3A3, 3A4	AFB_1-8,9-epoxide, AFQ_1

AFB_1 Activation by Human P450. The picture that has emerged through the use of cDNA expression systems (10, 11, 28), isoform-selective antibody inhibition (20, 29), and reconstituted P450 systems (9, 29) is that activation of AFB_1 is not limited to merely one isoform, but rather that multiple forms of human P450 are capable of this activation. In particular, these studies have implicated CYP1A2 (PAH-inducible), CYP2A3, CYP2B7, and both CYP3A3, and CYP3A4, the latter two of which are glucocorticoid-inducible. However, there is some ambiguity regarding which P450 is most important. Based on human liver P450 isoform-selective antibody and chemical inhibition, CYP3A4 appeared to play a prominent role (9, 30). Also, cDNA-expressed CYP3A4 had highest activity for AFB_1 activation and mutagenicity, and antibodies raised against CYP3A isoforms were most inhibitory to bioactivation in human liver microsomes (10, 20). Despite these findings, other research has questioned the relevance of CYP3A4 at low substrate concentrations (11, 28, 31). On the basis of bacterial mutagenicity assays and activation by cell lines expressing activities for human CYP1A2, CYP2A3, or CYP3A4, it has been suggested that CYP1A2 is the high-affinity human P450 capable of activating AFB_1 at low substrate concentrations, as would normally be encountered with human dietary exposure to the mycotoxin (11). Similarly, in studies utilizing human cell lines expressing transfected CYP1A2 or CYP3A4 cDNA, as well as microsomes prepared from human liver donors, human CYP1A2 was found to be the high-affinity P450 responsible for activating AFB_1 at low substrate concentrations, while CYP3A4 appeared to have relatively low affinity for the substrate (28). Most recently, a study using human liver samples from Thailand showed microsomal activation of AFB_1 at an intermediate concentration to be correlated strongest with CYP3A3/4 expression compared with six other P450 isoforms including CYP1A1/2 (32). Furthermore, CYP3A4 may be expressed in human livers at a constitutively higher level than CYP1A2 such that in certain individuals, CYP3A4 may be the predominant activating enzyme (28, 32). In human fetal liver, the closely related CYP3A7 is mainly involved in metabolizing AFB_1 to a genotoxic product (30).

Hydroxylation and Dealkylation of AFB_1. In addition to epoxidation, monooxygenases biotransform AFB_1 to hydroxylated products including aflatoxins M_1, (AFM_1), Q_1 (AFQ_1), P_1 (AFP_1), and B_{2a} (AFB_{2a})

(Fig. 1). The carcinogenic potency of AFB_1 is 15 times that of AFM_1 in the rat (13) and over 100 times that of AFQ_1 in trout (33). In terms of mutagenicity, these metabolites are less than 4% as potent as AFB_1, based on the Salmonella assay (8, 33). As such, they are considered to be detoxification products. Hepatic microsomal AFQ_1 formation can be increased by treating rats with glucocorticoid-type inducers such as dexamethasone (34), thus implicating CYP3A isoforms in its formation. Studies with human liver microsomes and inhibitory antibodies strongly suggested that CYP3A4 was the principal enzyme catalyzing 3α-hydroxylation to form AFQ_1 (29). Guengerich and coworkers have therefore speculated that AFB_1 can be bonded to the catalytic site of CYP3A4 in two different orientations, with either the 8,9-olefinic bond or the 3α-hydrogen bound to the allosteric site, resulting in either epoxidation or AFQ_1 formation respectively (35).

In the rat, mouse, and hamster, it appears that the formation of AFM_1 is catalyzed by P450 isoforms of the CYP1A subfamily (22, 23, 36–39). An interesting apparent human correlate to this observation has been made. In the aflatoxin-rich Fujian Province of China, a significantly higher number of cases of primary hepatoma was found among nonsmokers than smokers over 50 years old (40). This was apparently caused by greater CYP1A-mediated AFM_1 formation in smokers than in nonsmokers, due to PAHs in cigarette smoke inducing the AFB_1 detoxification pathway in the former.

The dealkylation of AFB_1 to AFP_1 has not yet been attributed to any specific P450 isoform. However, it is interesting that in contrast to other AFB_1 metabolites, AFP_1 formation was elevated in human liver tumor tissue relative to normal tissue (32). Similar to AFM_1 and AFQ_1, AFP_1 has low mutagenic potency (41).

Hydroxylation of AFB_1 at the 8 position, forming the hemiacetal AFB_{2a} produced a total loss of mutagenicity in the Salmonella assay, confirming that formation of AFB_{2a} is also a detoxification process (42).

Pulmonary Biotransformation of AFB_1 The respiratory system may be a target for the carcinogenic effects of AFB_1 following inhalation, particularly of AFB_1-laden grain dusts (43–48). In addition, there is evidence in humans that the lung is potentially at risk from aflatoxin exposure through the diet. Following acute exposure to aflatoxins during a poisoning incident in Southeast Asia in which several individuals died, AFB_1-DNA adducts were detected and measured immunochemically and by high-performance liquid chromatography in lung tissues (14). The adducts were determined to be the same as those found after exposing rats to AFB_1. Additionally, exposure of a human epithelioid lung cell line to AFB_1 resulted in formation of DNA adducts, most of which were derived from AFB_1-guanine (49). A similar profile of DNA adducts was found when cultured human tracheobronchus was exposed to AFB_1 *in vitro* (50–52).

In addition to observations with the human tissue preparations, there is a growing body of animal data implicating AFB_1 as a potential lung carcinogen. Intratracheal instillation of AFB_1 to rats resulted in tracheal carcinomas (53), and large numbers of lung tumors were induced in strain A mice following intraperitoneal or oral treatment with the toxin (54, 55).

With these few exceptions, the bulk of the data regarding AFB_1 interaction with the respiratory system have come from *in vitro* experimentation with various preparations. Using reconstituted purified rabbit lung P450 isoforms $P450_I$ and $P450_{II}$ (which correspond to CYP2B4 and CYP4B1, respectively), it was demonstrated that $P450_I$, but not $P450_{II}$, could activate AFB_1 to a mutagenic species using the Salmonella mutagenicity assay (56). However, antibodies raised against each of these isoforms were able to inhibit AFB_1 activation in rabbit lung microsomes, putting the specific role of $P450_{II}$ in AFB_1 activation in question. Since that time, tracheal explants from the hamster, rabbit, rat, and rhesus monkey have been shown to activate AFB_1 to cellular DNA adducts, as well as biotransforming AFB_1 to stable metabolites such as AFM_1 and AFQ_1 (15, 57–60).

We have demonstrated that whole rabbit lung microsomes, intact enriched isolated cells, and isolated lung cell microsomes from this species generate a DNA-binding and mutagenic metabolite of AFB_1 (26, 61, 62). Whole rabbit lung microsomes were as active as liver microsomes in catalyzing the formation of a DNA-binding metabolite of AFB_1 when the results were expressed in terms of the amount of protein present (61). When these same data were expressed in terms of microsomal P450 content, lung microsomes were found to be more active than liver microsomes in the activation of AFB_1 (61). These results demonstrate that rabbit lung contains P450 isoforms with a high capability to activate this carcinogenic mycotoxin. In addition, treatment of animals with β-naphthoflavone did not affect the activation of AFB_1, although AFM_1 formation was significantly enhanced. Thus, CYP2B4 and/or CYP4B1, which make up the vast majority of the P450 in rabbit lung, are involved in the bioactivation of AFB_1, and CYP1A1 catalyzes the detoxification of AFB_1 through hydroxylation to form AFM_1. Furthermore, based on apparent K_m and V_{max} values for these reactions, in rabbit lung, microsomal bioactivation is the preferred metabolic pathway at the low concentrations of AFB_1 expected *in vivo*. It is interesting to note that orthologs of these isoforms are present in the human (63, 64).

In an effort to identify which lung cells activate AFB_1, we used isolated rabbit lung cells that had been enriched for certain cell types by the method of centrifugal elutriation (62). These experiments indicated that AFB_1 activation in rabbit lung is not homogeneous, with activity predominating in fractions enriched in nonciliated bronchiolar epithelial (Clara) cells (62). These results provided a biochemical explanation for the previously observed susceptibility of rabbit tracheal nonciliated epithelial cells (the Clara cell equivalent in the upper airways) to AFB_1 toxicity (57, 59). In the earlier studies, tracheal explants from the rabbit incubated with AFB_1 *in vitro* showed selective necrosis of this cell type, with extensive autoradiographic localization of labeled AFB_1. Although it cannot be concluded that the Clara cell is a target for AFB_1-induced carcinogenicity, the fact that the Clara cell has a high capacity to activate AFB_1 to its ultimate carcinogenic form supports this notion. This conclusion was also supported by experiments we carried out using intact isolated rabbit lung cells and a modification of the Ames Salmonella mutagenicity assay (62). Different lung cell types were tested with their intact complement of activation and detoxification pathways, conditions not attained using only microsomes. Based on an earlier approach described by Aune *et al.* (65), lung cells were separated from direct contact with the *S. typhimurium* by a sterile Millipore Millicell-HA culture plate filter insert (Fig. 3). This separation was essential, since freshly isolated lung cell preparations are typically contaminated with bacteria that can interfere with the mutagenicity assay. Our experiments further implicated the Clara cell as the predominant cell type within the rabbit lung capable of activating AFB_1 (62). They also demonstrated that AFB_1-8,9-epoxide is stable enough to leave its site of formation in Clara cells, cross the Millicell membrane, and enter bacteria in order to produce the mutagenic response. It is therefore possible that, *in vivo*, AFB_1-8,9-epoxide might leave cells where it is generated and interact with other cell types with limited detoxification capacities. This could render cells susceptible to AFB_1 even if they lack the ability to bioactivate it to the toxic epoxide.

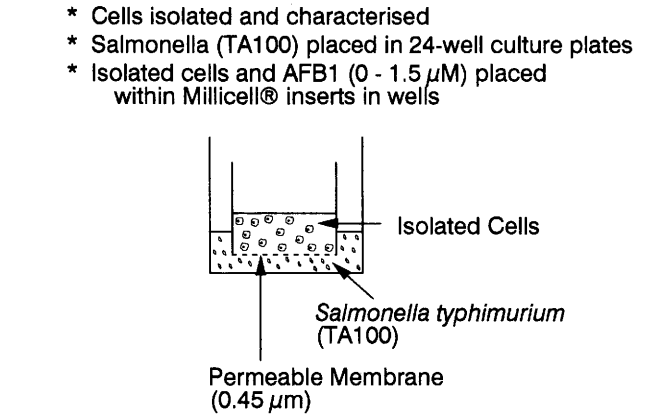

Figure 3. The incubation system used for mutagenicity testing, employing isolated lung cells for bioactivation.

Non-P450-Dependent Mechanisms of AFB_1 Bioactivation

Lipid Hydroperoxide-Dependent AFB_1 Epoxidation. Relatively recent studies have revealed activation of AFB_1 to its electrophilic DNA binding form via lipid hydroperoxide-dependent mechanisms. These reactions can be catalyzed by microsomal prostaglandin H synthase (PHS) (66, 67) and cytosolic lipoxygenases (68).

There is increasing evidence indicating the importance of PHS and lipoxygenases in xenobiotic metabolism. As shown in Figure 4, PHS and lipoxygenases catalyze the oxidation of arachidonic acid to lipid peroxy radicals, which are known epoxidizing agents for xenobiotics (69) including benzo(a)pyrene-7,8-dihydrodiol (70, 71) and cyclopenteno[c,d]pyrene (72). This process is called co-oxidation, because the epoxidation of xenobiotics occurs concomitantly with the oxidation of arachidonic acid. Since AFB_1 activation occurs via epoxidation, it is conceivable that AFB_1 could also be activated by PHS and lipoxygenases by the lipid peroxy radical-dependent mechanisms. Co-oxidative xenobiotic bioactivation may be biologically significant in chemical cytotoxicity, carcinogenicity, and teratogenicity, especially in extrahepatic tissues. Relatively high PHS and lipoxygenase activities occur in kidney, lung, and embryonic tissues (73–76), and the overall P450 activity in these organs is lower than that in liver.

Initial investigations into co-oxidative activation of AFB_1 during metabolism of arachidonic acid were conducted with C3H/10T½ mouse embryo fibroblasts (77). Blocking the release of arachidonic acid from cell membranes with a phospholipase A_2 inhibitor or inhibiting the enzymatic oxygenation of arachidonic acid resulted in a 30%–50% decrease in covalent binding of AFB_1 to cellular DNA. This suggested that the bioactivation of AFB_1 was dependent on arachidonic acid and that PHS, and likely lipoxygenases, were involved in the process. More detailed analysis of the AFB_1-DNA adduct formed by PHS in ram seminal vesicle microsomes demonstrated that the major DNA adduct was AFB_1-guanine, the same adduct produced by P450-dependent activation (66).

Using microsomal PHS and cytosolic lipoxygenases from guinea pig tissues, we found that AFB_1 can

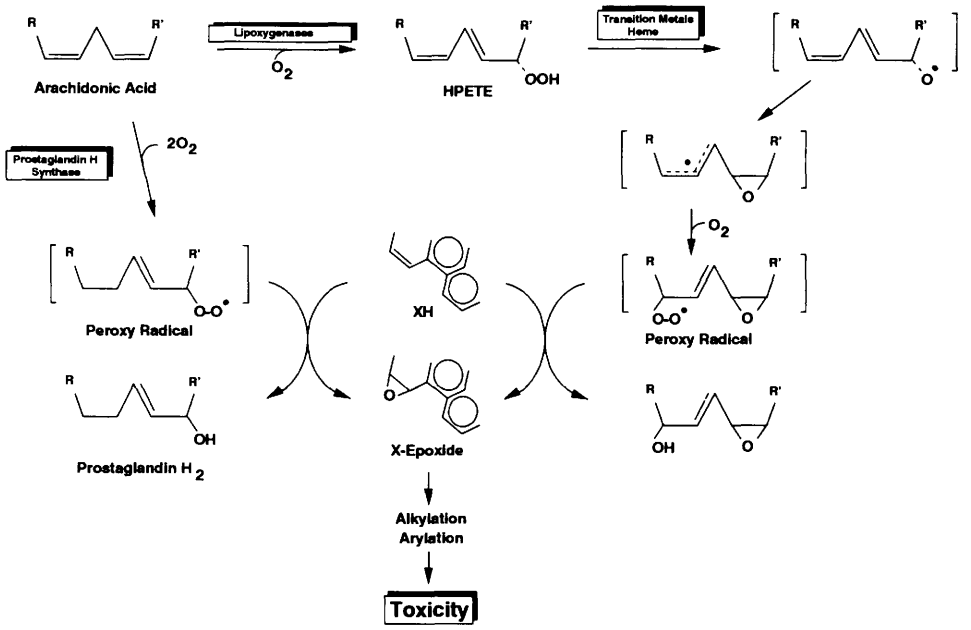

Figure 4. Co-oxidation of xenobiotics during arachidonic acid metabolism.

be bioactivated to form DNA adduct(s) by co-oxidation in liver, lung, and kidney (67, 68). AFB_1-DNA binding in the presence of arachidonic acid was inhibited by the PHS inhibitor indomethacin in microsomal incubates and by the lipoxygenase inhibitor nordihydroguaiaretic acid in cytosolic incubates, but not by the P450 inhibitor SKF-525A, indicating that the bioactivation of AFB_1 was catalyzed by PHS and lipoxygenases (67, 68). Kinetic analysis of AFB_1 activation suggested that lipoxygenase-catalyzed AFB_1 activation occurs at low substrate concentrations (68). This may be important in view of human exposure to low AFB_1 concentrations and appreciable lipoxygenase activity in some human pulmonary cells (78, 79). In fact, we have recently found evidence of co-oxidation of AFB_1 to a DNA-binding metabolite in human lung preparations, suggesting the potential importance of this mechanism of activation in human pulmonary AFB_1 effects (80).

AFB_1 has been shown to stimulate the release of endogenous arachidonic acid from guinea pig tissue membranes (68), mouse embryo fibroblast membranes (77), and human lymphocytes (81), possibly by activating phospholipase A_2. AFB_1 was also reported to stimulate the production of prostaglandins E_2 and $F_{2\alpha}$ in canine kidney endothelial cells (82). This action may be of significance for *in vivo* AFB_1 activation, because availability of free arachidonic acid is rate-limiting in prostaglandin biosynthesis. Thus, exposure to AFB_1 could initiate the arachidonic acid cascade, thereby accelerating its own activation through PHS- and lipoxygenase-mediated co-oxidation. Furthermore, the pulmonary inflammation and edema found in aflatoxicosis (83–85) might also be due in part to eicosanoid production stimulated by AFB_1.

Detoxification of AFB_1-8,9-Epoxide

Glutathione S-Transferases. Although the necessity for AFB_1 activation to AFB_1-8,9-epoxide prior to DNA binding and carcinogenesis is well accepted, levels of activating enzymes are not the sole determinants of sensitivity to AFB_1 toxicity. Activities of detoxifying biotransformation pathways are also critical, and glutathione S-transferase (GST)-catalyzed conjugation of activated AFB_1 is thought to be the most important detoxification system. The GSTs, which comprise dimeric products of a gene superfamily (composed of alpha, mu, pi, theta, and microsomal classes), are a group of cytosolic and microsomal enzymes that catalyze the conjugation of reduced glutathione (GSH; γ-L-glutamyl-L-cysteinylglycine) with a wide spectrum of compounds possessing an electrophilic center. This reaction is the first step in the mercapturic acid pathway, which leads to the excretion of many xenobiotics. Since conjugation of the electrophilic AFB_1-8,9-epoxide with GSH is an alternative fate to binding to nucleophilic sites in cellular macromolecules (Fig. 1), GST isoenzymes play a key role in the protection of tissues from AFB_1 toxicity (86).

Recent advances have been made in identification of the GSTs responsible for catalyzing the conjugation of GSH with AFB_1-8,9-epoxide. The AFB_1-GSH conjugate was identified as 8,9-dihydro-8-(S-glutathionyl)-9-hydroxy AFB_1 by proton nuclear magnetic resonance spectroscopy and mass spectroscopy (87). Recently, the two isomers of AFB_1-8,9-epoxide, AFB_1

exo-epoxide and *endo*-epoxide, were synthesized (88, 89) (Fig. 2). Although only the *exo*-epoxide reacted with DNA (17), it was demonstrated that rat and human liver microsomal activation of AFB_1 yielded a mixture of the *exo*- and *endo*- epoxides, which produced two corresponding GSH conjugates (90) (Fig. 2).

Coles and co-workers discovered that the alpha-class GSTs were the most active forms in catalyzing GSH conjugation with AFB_1-8,9-epoxide in the rat liver (91). Other researchers have characterized two ethoxyquin-inducible alpha-class GSTs from rat liver, GST 1-10 and GST 2-10, which have high activity toward AFB_1-8,9-epoxide (92). Meyer and co-workers reported that a rat alpha-class GST with high activity for AFB_1 *exo*-epoxide was also induced by dietary 1,2-dithiole-3-thione and oltipraz, and was composed of GST subunit 10 (93). The GST enzyme was thus referred to as rat GST 10-10, and was shown to exhibit activity towards AFB_1 *exo*-epoxide two orders of magnitude greater than the other rat GSTs (94). Rat GST 10-10 is not found in the uninduced adult rat liver, but is prominent in pre- and postnatal rats (95).

Experiments performed with purified rat liver GSTs and a synthetic mixture of AFB_1 *exo*- and *endo*-epoxides demonstrated that constitutive alpha- and mu-class GSTs were efficient in catalyzing the formation of AFB_1 *exo*- and *endo*-epoxide-GSH conjugates, though the AFB_1 *endo*-epoxide was by far the preferred substrate (90). Earlier research had discounted the relevance of rat hepatic mu-class GSTs in catalyzing the detoxification of AFB_1-8,9-epoxide (91). Rat pi-class GSTs are unable to catalyze this reaction (96).

Other studies using purified rat liver GSTs showed the mu-class GSTs to preferentially conjugate synthetic AFB_1-8,9-epoxide, relative to the alpha-class GSTs. When rat liver microsomes were used to generate AFB_1-8,9-epoxide, the mu-class GSTs again showed highest conjugating activity. These results suggested that the mu-class GSTs play an important role in detoxification of AFB_1 bioactivated *in vivo* in rat liver (97).

It is interesting to note that evidence in the literature implicating rat liver alpha-class GSTs as most important in AFB_1-8,9-epoxide conjugation, were based on non-rat microsomal activation systems. Studies using a rat liver microsomal activation system, such as that used by Lotlikar and co-workers (97), indicated the μ-class GSTs to have the highest conjugating activity. The conflicting evidence with respect to the importance of rat liver alpha-class GSTs versus mu-class GSTs in AFB_1-8,9-epoxide detoxification may be due to species differences in microsomal activation of AFB_1, resulting in different ratios of *exo*- and *endo*-epoxides (98). Thus, because microsomal activation generates a species-specific ratio of epoxides, and GST catalyzed detoxification also produces a species-specific profile of GSH conjugates, the selection of animal species for aflatoxin biotransformation research is critical, particularly if intended as a model for human activation and detoxification capabilities.

In contrast to the rat, the mouse is relatively resistant to the toxic and carcinogenic effects of AFB_1 and exhibits high levels of GST activity towards bioactivated aflatoxin (99). In fact, the specific activity of rat liver cytosol for the conjugation of AFB_1-8,9-epoxide was found to be approximately 1/50th that of mouse hepatic cytosol even though activity toward 1-chloro-2,4-dinitrobenzene (CDNB, a general substrate for overall GST activity) was comparable (100). The relationship between the intrinsic resistance in mice and high conjugating activity is interesting, since murine liver microsomes possess approximately 4-fold greater capacity to form AFB_1-8,9-epoxide relative to rat liver microsomes. These observations suggest that GST-catalyzed detoxification, rather than microsomal activation, is the critical determinant of susceptibility of different species to the harmful effects of AFB_1 (99).

Studies have shown mouse liver cytosol and purified GST preparations from murine liver to have a strong protective activity against AFB_1-DNA binding (101). A single homodimeric alpha-class GST (GST 4-4) was found to be responsible for the DNA protective activity against AFB_1-8,9-epoxide. Immunoprecipitation experiments confirmed the specificity of this constitutively expressed hepatic GST for AFB_1-8,9-epoxide as substrate and concluded that neither murine mu-class, pi-class nor theta-class GSTs could detoxify AFB_1-8,9-epoxide to any appreciable extent (96). The negligible contribution of the murine mu-class GST is in sharp contrast to the rat and human, where mu-class GSTs show relatively high activity for bioactivated AFB_1. Interestingly, murine alpha-class GST 4-4 was found to contribute only 2.1% to the overall cytosolic CDNB conjugation activity (102). Two research laboratories independently isolated a cDNA clone for mouse GST 4-4, and the two clones showed only four nucleotide differences with identical amino acid sequence (103, 104). Buetler and Eaton also developed rat-mouse chimeric GST constructs in order to determine the critical amino acids which confer the high catalytic activity towards AFB_1-8,9-epoxide found in murine GST 4-4. Results suggested that a region of 15 nonconserved amino acids may be important in conferring the high AFB_1-8,9-epoxide conjugating ability (105).

Experiments incubating mouse liver cytosol with a synthetic mixture of AFB_1 *exo*- and *endo*-epoxides revealed that the GSTs present in mouse liver cytosol almost exclusively conjugate the *exo*-epoxide to form AFB_1 *exo*-epoxide-GSH (90).

With the New Zealand White rabbit, we found a 5.9-fold higher rate of formation of AFB_1-GSH in liver cytosol relative to lung cytosol. In overall AFB_1-8,9-epoxide conjugating ability, rabbit liver cytosolic GSTs appear to be intermediate in activity between mouse liver and rat liver. Based on immunoprecipitation experiments, we have found rabbit lung alpha- and mu-class GSTs to be of similar importance in catalyzing conjugate formation, with pi-class possessing little or no activity.[1]

Despite the fact that AFB_1 is of great concern for human health in many parts of the world, much of the literature to date has focused upon cytochrome P450-mediated biotransformation of AFB_1 in human liver, and not detoxification reactions catalyzed by GSTs. Though humans possess GST isoenzymes which belong to all five recognized GST classes, the mu-class GSTM1 gene has attracted particular interest with respect to defense against chemical carcinogens. Substrates for GSTM1 include mutagenic and carcinogenic epoxides such as benzo(a)pyrene-4,5-oxide and styrene-7,8-oxide (106–108). GSTM1 is polymorphic in humans, with individuals possessing genetically determined combinations of the GSTM1-0, GSTM1a and GSTM1b alleles (the 1a and 1b proteins differ only on one amino acid [109]), and the deficiency in enzyme activity is caused by the inherited homozygous deletion of the GSTM1 gene (GSTM1 0/0 genotype). The frequency of the GSTM1 0/0 genotype is approximately 50% in most human populations and varies with ethnic group, ranging from 35% (Indian) to 100% (Micronesian) (110).

The importance of GSTM1 in protection against chemical carcinogens and the high frequency of the deleted genotype led Board to propose that GSTM1 0/0 individuals are more susceptible to carcinogens (111). The first studies to test this hypothesis were carried out by Seidegard and co-workers (112, 113) in patients with lung cancer. These researchers measured *trans*-stilbene oxide conjugation activity (a GSTM1 selective substrate) in peripheral leukocytes as an indicator of GSTM1 expression, and found that failure to express GSTM1 was associated with an increased susceptibility to lung cancer in cigarette smokers. Since then, others have found positive correlations between the null genotype and squamous carcinoma of the lung (114), adenocarcinoma of the stomach or colon (115), colorectal cancer (116), and bladder cancer (117).

Few studies have been carried out to investigate the potential relationship between AFB_1 carcinogenicity and the GSTM1 polymorphism. In this regard, Liu *et al.* (118) demonstrated that incubation of liver preparations from patients with high GSTM1 activity (determined using *trans*-stilbene oxide as substrate) inhibited the formation of AFB_1-DNA adducts more than those from individuals with low GSTM1 activity.

Experiments using several human liver cytosol samples and a mixture of AFB_1 *exo-* and *endo*-epoxides showed that human liver cytosol was able to catalyze the conjugation of both epoxide isomers. However, the human liver sample that showed highest activity had a conjugation efficiency with AFB_1 *exo*-epoxide approximately 25- and 400-fold lower than rat and mouse liver, respectively. These researchers also performed experiments with purified human hepatic GSTs and showed M1a-1a to have the highest activity for both AFB_1 *exo-* and *endo*-epoxides, while two alpha-class isoenzymes (A1-1, A2-2) catalyzed AFB_1 *exo*-epoxide-GSH conjugation only, and at much lower rates (90). The pi-class P1-1 and the mu-class M3-3 did not produce significant amounts of AFB_1-GSH. These results indicate that human liver GSTs conjugate AFB_1-8,9-epoxide to a much lower extent than do hepatic GSTs from rats, mice or rabbits. This diminished ability to detoxify AFB_1-8,9-epoxide is likely central to human susceptibility to the toxic effects of AFB_1.

GST-catalyzed detoxification of AFB_1-8,9-epoxide in human extra-hepatic tissues has yet to be characterized. However, our preliminary evidence suggests that human lung cytosolic GSTs possess measurable but very low conjugating activity for AFB_1-8,9-epoxide generated by rabbit liver microsomes.[2] These results are consistent with the apparent susceptibility of human lung to the toxic and carcinogenic effects of bioactivated AFB_1.

Role of Epoxide Hydrolase. AFB_1-8,9-epoxide may also be inactivated via hydrolysis to 8,9-dihydro-8,9-dihydroxy AFB_1 (AFB_1-8,9-dihydrodiol) (Fig. 1), which occurs spontaneously or is catalyzed by epoxide hydrolase. Relative to the well-established role of GST-catalyzed inactivation, the importance of epoxide hydrolase in protection against the toxic effects of bioactivated AFB_1 is apparently minimal, at least in hepatic systems. Inhibitors of epoxide hydrolase have been shown to have little effect on either AFB_1-DNA binding or AFB_1-induced mutagenesis in rat liver (119, 120). Other studies showed no relationship between the rate of formation of AFB_1-8,9-epoxide and epoxide hydrolase activity in rat and mouse liver (100).

In experiments to compare species differences in AFB_1 metabolism using tracheal explants, Coulombe and co-workers found that rabbit and hamster airway epithelia produced significant amounts of AFB_1-8,9-dihydrodiol, with diol formation higher than AFB_1-GSH in the hamster and GSH conjugate production higher than diol in the rabbit. Rat explants generated

[1] R. K. Stewart *et al.*, unpublished observation.

[2] R. K. Stewart *et al.*, unpublished observation.

lower but similar quantities of AFB_1-8,9-dihydrodiol and AFB_1-GSH (59, 60).

Further research is necessary to elucidate the importance of epoxide hydrolase in aflatoxin detoxification, both in experimental animals and humans, particularly in light of the fact that AFB_1-8,9-dihydrodiol may itself contribute to cytotoxicity (see below).

Glucuronidation and Sulfation. The hydroxylated metabolites of AFB_1, such as AFM_1, AFQ_1, and AFP_1 can readily form glucuronide or sulfate conjugates. These Phase II reactions aid in the excretion of biotransformed AFB_1. Glucuronide and sulfate conjugates of hydroxylated AFB_1 metabolites have been described in several species including trout, rat, mouse, hamster, rabbit, and monkey (60, 121–123). However, the toxicological significance of glucuronidation and sulfation is likely minimal, since hydroxylated AFB_1 metabolites have low toxicity.

The Roles of Metabolites Other Than the Epoxide in AFB_1 Toxicity

Aflatoxicol. A cytosolic NADPH-linked reductase is capable of reducing the carbonyl group in the cyclopentenone ring of AFB_1 to a hydroxyl group, resulting in formation of aflatoxicol (Fig. 1) (13, 124). Since this reaction is reversible *in vivo* (125) and *in vitro* (124), aflatoxicol formation is considered a "reservoir" for AFB_1 and its metabolites (13), and not an AFB_1 detoxification process.

AFB_1 can bind competitively to sex-determined sites on the endoplasmic reticulum of hepatocytes because of the general structural similarity between AFB_1 and C_{17}-ketosteroid molecules, and the reduction of AFB_1 to aflatoxicol is inhibited by androstenedione, androstenetrione, and estrone (126). It is therefore believed that the reaction is catalyzed by the cytosolic 17-hydroxysteroid dehydrogenase (E.C.1.1.1.51),an oxidoreductase (126).

In the past, the formation of aflatoxicol was believed to occur exclusively in the cytosol (13, 124). However, we recently reported aflatoxicol production to occur by a microsomal NADPH-dependent reductase in guinea pig kidney (27). The inhibitory effect of androstenedione on the formation of aflatoxicol in this study indicates that this reaction was catalyzed by a 17-hydroxysteroid dehydrogenase. In other animal species such as rat, mouse, hamster, rabbit, and dog, NADP-dependent 17-hydroxysteroid dehydrogenase in renal microsomes was not detected (127). Thus, the production of aflatoxicol in renal microsomes may be unique to guinea pigs. Whether this NADPH-dependent AFB_1 reductase activity is present in human renal microsomes has not been documented.

AFB_1-8,9-Dihydrodiol. Although AFB_1-8,9-epoxide is believed to be the major metabolite initiating AFB_1 carcinogenicity, several recent publications demonstrated that AFB_1-8,9-dihydrodiol may undergo opening of both the furan rings to yield a dialdehydic phenolate ion which subsequently forms Schiff bases with primary amino groups in proteins, particularly lysine (21). Acute AFB_1 toxicity in various animal species is correlated with the capacity of microsomal AFB_1-8,9-dihydrodiol production (128), and a major AFB_1 adduct in proteins such as serum albumin is apparently attributable to the reaction between AFB_1 dialdehydic phenolate and protein primary amino groups (129, 130). Accordingly, it is possible that AFB_1 dialdehydic phenolate may contribute to acute AFB_1 cytotoxicity. A novel aldehyde reductase which catalyzes the reduction of AFB_1 dialdehydic phenolate to a dialcohol has recently been characterized in rat liver (21, 131). This enzyme is induced by the antioxidant agent ethoxyquin (21, 131), and its expression is considered to play a chemoprotective role against AFB_1. It will be interesting to assess aldehyde reductase activity in different animal species and in humans. Heterogeneity of the enzyme may contribute to the resistant phenotype.

Genetic Alterations in AFB_1 Carcinogenesis

The molecular consequences of AFB_1-DNA binding have been studied in detail in hepatic systems, but very little in extra-hepatic tissues.

DNA Target Sites. As is the case with other "ultimate carcinogenic metabolites," AFB_1-8,9-epoxide preferentially binds to specific target sites in DNA. Using AFB_1-dichloride as a chemically active form of AFB_1, Yu *et al.* (132) found inhibition of polydC directed RNA synthesis and covalent binding, suggesting cytosine as a possible target. However, the N^7 position of guanine appears to be the only site of hepatic AFB_1-DNA adduct formation in humans as well as in experimental animals (133), and guanine is the preferential site of mutations by CYP1A2-metabolized AFB_1 in a shuttle vector (134). Formation of AFB_1-guanine adducts in double-stranded DNA is also sequence-specific, in that the relative formation of adducts is influenced by the flanking nucleotide sequences (135). For example, single guanine residues flanked by A-T rich sequences are poor targets, while the second guanine from the 5′ end in guanine di- and trinucleotides is favored. It must be kept in mind, however, that similar guanine-containing sequences in different parts of a DNA molecule also have different susceptibilities to alkylation by AFB_1-8,9-epoxide (136). More recent analysis of DNA conformation selectivity indicates that AFB_1 *exo*-epoxide preferentially binds with the right-handed B-form DNA helix, which is most common. There is little binding to the rare, short, wide A-form helix and no binding to the left-handed Z-form, which occurs in regions with alternating purines and pyrimidines (137). In these ex-

periments, adducts were only found to guanine. These investigators have also found that intercalation of the *exo*-epoxide in DNA orients the molecule properly for back-side S_N2 attack by N^7 of guanine on C^8 of the epoxide (17). On the other hand, intercalation of the *endo*-epoxide does not. Consequently, they found no detectable DNA adduct with AFB_1 *endo*-epoxide.

Since the AFB_1-guanine adduct is unstable, it either is lost from DNA, resulting in an apurinic site, or else the imidazole ring opens to form 8,9-dihydro-8-(N^5-formyl-2′,5′,6′-triamino-4′-oxo-N^5-pyrimidyl)-9-hydroxylaflatoxin B_1, the stable form of the principal AFB_1-DNA adduct (138).

Activation of Protooncogenes. The cellular *ras* protooncogenes code for GTP-binding p21 proteins which are important in signal transduction. These proteins are active while associated with GTP, but rapid hydrolysis of GTP in the presence of GTPase-activating protein (GAP) turns them "off." The *ras* genes are frequently found to be mutated in tumors, with point mutations occurring at specific "hot spots." The resulting oncogenes (i.e., activated protooncogenes) produce proteins with diminished GTPase activity that remain active due to decreased ability of GAP to induce GTP hydrolysis in the active p21:GTP complex. Presumably, continual transmission of signal by mutant forms causes or contributes to malignant transformation (reviewed in Ref. 139).

Initial investigations of the role of *ras* genes in AFB_1 hepatocarcinogenesis involved purifying DNA from Fischer rat liver carcinomas or from AFB_1-transformed cell lines, and transfecting it into cultured NIH3T3 mouse fibroblasts (140–143). The development of transformed foci and tumorigenicity of the transfected cells when injected into athymic nude mice, were indicative of the presence of oncogenes in a large percentage of the liver tumor DNA samples, although activated K-*ras* was identified in few of the tumors. Further sequence analysis of enzymatically amplified tumor DNA revealed that tumors with activated K-*ras* contained G-C to T-A or G-C to A-T substitutions in codon 12 (140, 142, 143), mutations which are consistent with the apparent importance of guanine as a target in DNA. Another interesting finding of the latter study was that all tumors and control liver DNA samples contained a potentially oncogenic N-*ras* allele, leading McMahon *et al.* to suggest that the presence of this allele may contribute to the sensitivity of the Fischer rat to chemically induced hepatocarcinogenesis (143).

More recently, using sensitive high-fidelity polymerase chain reaction and denaturing gel gradient electrophoresis, Soman and Wogan (144) found all of 10 less advanced rat liver tumors (adenomas and carcinomas) to contain activating point mutations at codon 12 of K-*ras*. This confirmed that K-*ras* activation is not a late event in AFB_1 hepatocarcinogenesis in the rat.

The *p53* Tumor Suppressor Gene. Considering the greater than 100 recognized cancer-related genes, *p53* tumor suppressor gene mutation is the genetic alteration most frequently associated with neoplasia. p53 protein functions include control of cell cycle, DNA repair and synthesis, cell differentiation, genomic plasticity, and programed cell death, or apoptosis (145 [and references therein]). These effects are associated with p53-mediated modulation of transcription of numerous other genes involved in control of cell growth, including other suppressor genes and protooncogenes. Consequently, if p53 expression is changed or if the protein itself is altered, for example by a point mutation in a *p53* exon, then the cell cycle becomes deregulated.

Following two simultaneous reports of high frequencies of specific G to T mutations at codon 249 of the *p53* tumor suppressor gene in human hepatocellular carcinomas from areas of China and Africa with high endemic aflatoxin (146, 147), there has been a great deal of interest in the interactions of bioactivated AFB_1 with *p53*. The specificity of the mutations for codon 249 was particularly interesting because in other human cancers, a wide spectrum of *p53* mutations is observed (148). In cancers not associated with high endemic AFB_1, sequence changes that eliminate p53 function are found throughout the gene, but are clustered in the highly conserved middle exons. The same is true for tumors from regions with low liver tumor frequency.

The highly repetitious mutation pattern (codon 249) found in AFB_1-associated human liver tumors suggests that additional factors influence the pattern. For example, the DNA sequence surrounding the mutational "hot spot" may be particularly refractory to enzymatic repair. Alternatively, the 249^{ser} p53 mutant protein may have unique biologic properties, or it may result in particularly aggressive growth of mutant hepatocytes in liver tissue chronically infected by hepatitis B virus. This would increase the likelihood of finding this particular mutation in advanced tumors (145). Furthermore, the recently published observation of increased specific codon 249 mutation frequency in normal human liver from individuals living in areas with high AFB_1 exposure, is consistent with *p53* mutation being an early mutagenic event in hepatocarcinogenesis (157).

Not all evidence has supported the importance of *p53* codon 249 mutations in AFB_1-induced carcinogenesis, however. Rat *p53* codon 243 corresponds to human codon 249. In six AFB_1-induced preneoplastic rat liver nodules, no mutations were found in codon 243. These results have been interpreted as suggesting that

mutations at this aflatoxin "hot spot" may not be involved in the early stages of rat liver tumorigenesis. Alternatively, codon 243 mutation may be relatively rare but could be an important determinant of progression to hepatocellular carcinoma. On the other hand, the rat nodule results are also consistent with the results of Fujimoto *et al.* (149). DNA analysis of nine hepatocellular carcinomas and other tumors from AFB_1-treated monkeys failed to reveal codon 249 mutations; in fact, only one of the tumors had a mutation in the conserved regions of *p53,* leading Fujimoto *et al.* to suggest that the earlier observations in human tumor DNAs were a result of species differences in gene structure, metabolism, the presence of hepatitis B virus-induced chronic active hepatitis, or that carcinogens other than AFB_1 were responsible for the codon 249 mutations (149). Also, in comparing tumors from patients in high-aflatoxin-risk and low-risk areas, frequency of codon 249 *p53* mutations did not correlate with extent of AFB_1-DNA binding (150). Thus, the relevance of specific *p53* mutations to AFB_1 toxicity remains controversial.

The Importance of Cytotoxicity in AFB_1 Carcinogenicity

Based on the Solt-Farber model of chemical carcinogenesis in the rat liver (151), carcinogen-initiated cells develop into preneoplastic foci upon addition of a strong growth stimulus. Since cytotoxicity following initiation can stimulate cell division and hence promotion of preneoplastic foci, and since AFB_1 is a potent cytotoxin in addition to being an initiator, AFB_1 may act as a "complete carcinogen." This concept is supported by the fact that, in almost all experimental systems, multiple dosing with AFB_1 is required for carcinogenicity (152). In addition to its function as a tumor initiator, AFB_1 also has been found to stimulate the promotion and progression of carcinogenesis (153). However, the process is complex. Whereas partial hepatectomy following initiator dosing is normally a potent stimulus for preneoplastic focus and tumor development, it is essentially ineffective in the case of AFB_1 (154). This is apparently due to AFB_1-mediated DNA synthesis inhibition which nullifies the potential stimulation of carcinogenicity due to partial hepatectomy (155). In contrast, repeated low-dose AFB_1 dosing causes extremely high incidence and multiplicity of liver tumors in rats (152). This is presumably because hepatocellular foci are selected which are resistant to the cytotoxicity of AFB_1, thereby potentiating its carcinogenicity (152, 155).

The significance of cytotoxicity in AFB_1 hepatocarcinogenesis is supported by the fact that chemoprotective agents such as oltipraz and ethoxyquin, which reduce the cytotoxicity of AFB_1, are reported to diminish the autopromoting capacity of the mycotoxin (153).

Conclusions

From the investigations of AFB_1 metabolism by animals and humans, it has been demonstrated that many isoforms of P450 are able to biotransform AFB_1 to DNA-binding/mutagenic species, and that the affinities of these isoforms for AFB_1 are an important consideration when determining the possible toxicological significance of exposure to the mycotoxin. Differences in P450 isoform activities, either due to genetic polymorphisms or to environmental alterations in expression, may be important contributors to human susceptibility to AFB_1. Furthermore, increasing evidence demonstrates that AFB_1 can be bioactivated by lipid hydroperoxide-dependent mechanisms, involving PHS and lipoxygenases. Although the maximum activity of this co-oxidation is low relative to P450, these processes may contribute significantly to bioactivation of AFB_1 *in vivo* in humans, who are generally exposed to low levels of AFB_1. Co-oxidation may be particularly important for AFB_1 carcinogenicity in extrahepatic tissues and for AFB_1-induced birth defects, in view of relatively low P450 activity in these organs and in embryos. Similarly, a thorough analysis of the cellular distribution of potential AFB_1 metabolizing systems will be required in the search for target cell types in the human lung.

While activation is an important aspect in understanding potential susceptibility to AFB_1, detoxification (P450-mediated as well as conjugation of the epoxide with glutathione) must also be considered. Animal studies suggest that GST-catalyzed detoxification is the crucial factor in susceptibility to AFB_1, and humans appear to lack significant GST-mediated protection against AFB_1. Further complicating the determination of the importance of AFB_1 in cancers in developing countries is the multitude of other contributing risk factors, including infection with hepatitis B and C and other parasites such as liver flukes, alcohol consumption, cigarette smoking, and familial tendency. It is also confounded by variability in the methods used to determine exposure to aflatoxins. These have included measurement of concentrations in food, as well as biomarker methods such as urinary excretion of AFB_1 and metabolites, and DNA or protein adducts.

Although the importance of the AFB_1-guanine DNA adduct is established, the roles of specific target protooncogenes and tumor suppressor genes are still a matter of controversy and intense scientific interest.

Of particular relevance to human AFB_1 poisoning is the possibility that chemopreventive agents such as oltipraz (5, 156) and possibly ellagic acid (52) will prove useful in reducing the risk of carcinogenicity.

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