MECHANISMS OF AFLATOXIN B\(_1\) LUNG TUMORIGENESIS

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Although aflatoxin B\(_1\) (AFB\(_1\)) is best known as a hepatocarcinogen, the respiratory system can also be a target of this mycotoxin. In isolated lung cells from rabbits and mice, AFB\(_1\) is bioactivated by cytochromes P450, primarily in nonciliated bronchiolar epithelial (Clara) cells. However, mutagenesis experiments suggest that the DNA-binding AFB\(_1\) epoxide metabolite can leave the cells of origin, and potentially interact with other cell types. Consistent with DNA adduct studies, AFB\(_1\)-induced AC3F1 mouse lung tumors contain point mutations at guanine residues in K-ras, with the anticipated bias for the A/J allele. Furthermore, following AFB\(_1\) treatment but prior to tumor development, K-ras mutations occur preferentially in mouse Clara cells. However, in contrast to findings with other carcinogens, AFB\(_1\)-induced mouse lung tumors demonstrate frequent, but heterogeneously distributed, overexpression of p53 protein as well as p53 point mutations, suggesting a carcinogen-specific response. Unlike lung tissue from mice and rabbits, human peripheral lung bioactivates AFB\(_1\) primarily by prostaglandin H synthase–and/or lipoxygenase-catalyzed cooxidation, with activity concentrated in macrophages. In addition, although glutathione S-transferase M1-1 has high specific activity for AFB\(_1\) epoxide conjugation, lung tissues from GSTM1-null individuals do not demonstrate diminished rates of conjugation, compared to tissues from GSTM1-positive individuals. In summary, AFB\(_1\) tumorigenesis in mice demonstrates unique properties, and processes of bioactivation show significant species differences.

Keywords aflatoxin B\(_1\), biotransformation, chemical carcinogenesis, lung cancer, oncogenes, tumor suppressor genes

Although a body of evidence supports the hypothesis that exposure of humans to the mycotoxin aflatoxin B\(_1\) (AFB\(_1\)) is an important etiological agent in hepatocellular carcinoma, both epidemiological [1] and laboratory [2–6] evidence also point towards the respiratory system as a potential target for AFB\(_1\) carcinogenesis. AFB\(_1\) also possesses a number of characteristics that make it a useful experimental tool for investigating mechanisms of chemical carcinogenesis. The mycotoxin’s biotransformation to both
nontoxic and mutagenic metabolites has been investigated extensively, and is fairly well understood. It clearly is an agent whose toxicity is determined by the balance between bioactivation and detoxification, and the fact that multiple mechanisms exist for bioactivation allows one to compare the importance of the different processes in different potential targets. Additionally, the identification of a specific DNA adduct for the bioactivated form of AFB$_1$ [7] facilitates prediction and interpretation of mutagenic responses.

**PULMONARY AFB$_1$ BIOTRANSFORMATION**

Although AFB$_1$ is converted to a number of metabolites including hydroxylated products with diminished toxicity, the major bioactivation pathway involves epoxidation, resulting in AFB$_1$ *endo*-epoxide and AFB$_1$ *exo*-epoxide (Figure 1). The latter is the major DNA-alkylating species, binding highly specifically to the N$_7$ of guanine [7]. It should be kept in mind, however, that it has been suggested that the DNA-damaging actions of AFB$_1$ may be a consequence of oxidative stress, with resultant formation of 8-hydroxydeoxyguanosine contributing to carcinogenesis [8]. Both mechanisms of AFB$_1$-induced DNA damage could potentially result in point mutations at guanine residues.

![FIGURE 1](image-url) Bioactivation of AFB$_1$ to epoxide metabolites and their detoxification by glutathione S-transferase (GST)-catalyzed conjugation with glutathione (GSH).
We found microsomes from rabbit lung to have relatively high activity for cytochrome P450 (CYP, P450) -mediated metabolism of AFB₁ to a DNA-binding metabolite, with activity similar to that in hepatic microsomes [9, 10]. Furthermore, the activity was concentrated in nonciliated bronchiolar epithelial (Clara) cells that had been isolated by centrifugal elutriation [11], and it was possible to decrease DNA binding of AFB₁ metabolite in intact Clara cells by prior treatment of animals with a cytochrome P450 1A–inducing agent [12]. This coincided with enhanced formation of a relatively nontoxic AFB₁ metabolite, indicating that shunting of AFB₁ away from bioactivation pathways in Clara cells was responsible.

Another noteworthy finding in isolated rabbit lung cells involved a modified Ames Salmonella mutagenicity assay. Using isolated intact lung cells as the bioactivating system [11], Clara cells produced a positive mutagenic result with AFB₁. For an AFB₁ metabolite produced in the lung cells to cause a positive mutagenic response, it would have to leave the lung cells, cross a 0.45-μm semipermeable membrane, and interact with the Salmonella outside the Millicell containing the lung cells. This would suggest that AFB₁ exo-epoxide, which is reported to be extremely unstable in an aqueous environment [13], is able to leave the cells that produce it, and exert a biological effect outside. Alternatively, the positive Salmonella mutagenicity could have been due to the less mutagenically active endo-epoxide. In either case, the results suggest the possibility of cells lacking substantial bioactivating activity, being targets of carcinogens due to bioactivation in neighboring cells.

**AFB₁ Bioactivation in Human Lung**

Significant differences in pulmonary cellular morphology and function between experimental animals and humans [14–16] provide a rationale for characterization of the metabolic fate of AFB₁ in human lung. Of particular note are Clara cells, which in humans are rare, and due to the lack of large amounts of smooth endoplasmic reticulum [15, 16], are not as likely to be the principal pulmonary site of cytochrome P450 activities, as they are in rodent and rabbit lungs. However, human alveolar type II cells do contain appreciable endoplasmic reticulum, and are likely to play a role in P450-catalyzed reactions [17, 18] as well as other xenobiotic-metabolizing reactions [18]. Furthermore, human alveolar macrophages possess P450 mRNAs [19, 20] as well as prostaglandin H synthase (PHS) [21] and lipoxygenase activities [22], which can be involved in xenobiotic oxidations (termed cooxidations).

Initial experiments involving whole human peripheral lung microsomes and cytosols revealed that human lung could bioactivate AFB₁ to a DNA-binding metabolite, and that there was a strong positive correlation between AFB₁ activation and cytosolic lipoxygenase activity [23]. Moreover, activation was inhibited by nordihydroguaiaretic acid, suggesting a major role
for lipoxygenase in cytosolic AFB<sub>1</sub> bioactivation. Consistent with findings in guinea pig lung [24], low levels of microsomal PHS–mediated bioactivation were detected in whole lung microsomes. NADPH-dependent microsomal activation of AFB<sub>1</sub> revealed observable but minor contributions of P450s in bioactivation in human lung. Using intact freshly isolated human lung cells, we found PHS- and lipoxygenase-mediated AFB<sub>1</sub> bioactivation to a cellular DNA-binding metabolite to be concentrated in alveolar macrophages. Similar to the finding in whole lung, cytochrome P450s appeared to play a negligible role in the isolated cells [23].

### Detoxification of AFB<sub>1</sub> Epoxides

Glutathione S-transferase (GST)-catalyzed conjugation of AFB<sub>1</sub> 8,9-epoxides with reduced glutathione (GSH) is a critical determinant of susceptibility to AFB<sub>1</sub> toxicity, and represents a significant mode of detoxification. Our rabbit lung experiments indicated a particularly important role for mu-class GSTs in AFB<sub>1</sub> epoxide conjugation [25]. Furthermore, in humans, the polymorphic mu-class enzyme hGSTM1-1, absent in approximately 50% of individuals [26], has the highest activity towards the highly mutagenic AFB<sub>1</sub> exo-epoxide [27, 28], suggesting the possibility of genetically determined interindividual differences in pulmonary susceptibility to AFB<sub>1</sub>, based on detoxification activity. In fact, whole human lung cytosol hGSTM1-1 phenotype, using the mu-class selective substrate trans-stilbene oxide, correlated with GSTM1 genotype as determined by polymerase chain reaction (PCR), asserting the functional significance of this polymorphism in human lung phase II biotransformation [29]. In contrast, conjugation of microsome-generated AFB<sub>1</sub> epoxides (both endo and exo stereoisomers) by whole lung cytosols was low and variable among lung cytosols from different specimens, and did not correlate with GSTM1 genotype. However, the activity was enriched in alveolar type II cell preparations. These results suggest that the limited conjugation activity present is contributed by GST isoforms other than GSTM1-1, but that type II cells are relatively well protected against potential toxicity of AFB<sub>1</sub> epoxides. Collectively, the human lung results demonstrate considerable activity for lipoxygenase- and PHS-catalyzed AFB<sub>1</sub> bioactivation, and very low GST-catalyzed detoxification, a likely basis for susceptibility of this tissue to AFB<sub>1</sub>.

### POST DNA-BINDING EVENTS

#### K-ras Activation

The K-ras proto-oncogene, which codes for an integral component of cell signalling pathways, is often implicated in human and mouse lung
tumors [30, 31]. The gene is activated most commonly through point mutation at specific ‘hot spots’ located at codons 12, 13, and 61. Acknowledged to be a very early event in mouse lung tumorigenesis [32], the pattern of activating point mutations in K-ras can provide valuable information about the nature of mutagenic metabolites. In our analysis of K-ras in AFB1-induced AC3F1 mouse lung tumors [33], we found that all of the 76 tumors analyzed possessed activating point mutations at guanine residues in the first exon, with almost all occurring in codon 12 (Table 1). This characteristic mutation profile was consistent with guanine being the target of AFB1 exo-epoxide.

Also of interest concerning the activation of K-ras is the parental bias for allele mutation. The AC3F1 hybrid mouse is the progeny of the strain A/J lung tumor susceptible parent and the C3H/HeJ lung tumor resistant parent. Seventy-three of 76 tumor DNA samples contained a K-ras mutation on the A/J allele, supporting the hypothesis that K-ras is a pulmonary adenoma susceptibility gene [34, 35].

With the anticipation that tumor progenitor cells should have K-ras mutations, it was of interest to determine which lung cell types had K-ras mutations after AFB1 exposure, but prior to tumor development. The fact that virtually all of the K-ras mutations in AFB1-induced tumors occurred in codon 12 allowed us to make use of a PCR-based technique, which allows for selective amplification of mutant K-ras alleles and hence detection of low-frequency mutants [36, 37]. Seven weeks following treatment of AC3F1 mice with AFB1, mutant alleles were essentially nondetectable in macrophages and polymorphonuclear leukocytes, but detectable levels of mutant K-ras alleles were found in the cell digest (isolated nonenriched lung cells), highly enriched type II cell fractions, and Clara cell fractions. In the case of the cell digest, levels were quite variable, presumably because of the presence of considerable debris, with cell fragments and DNA from Clara cells and type II cells. The most striking and consistent observation, however,
was that the highest levels of mutant alleles were found in the Clara cell fraction. Considering the evidence for the Clara cell as a key site for AFB₁ bioactivation in rabbit [11] and mouse (P. J. Donnelly and T. E. Massey, unpublished observation), it appears that the AFB₁ epoxide is exerting a direct genotoxic effect within the cell type that has the highest activity for its formation. However, the presence of detectable K-ras mutations in type II cells suggests that this cell type cannot be discounted as a target for AFB₁ epoxide as well.

**p53 Perturbation**

The p53 tumor suppressor gene is the most frequently targeted gene in human carcinogenesis, with a mutation frequency of approximately 50% in most major cancers [38–40]. The gene encodes a nuclear phosphoprotein that surveys for DNA and cellular damage. In the event of an insult to the cell, p53 arrests cell growth and allows the cell to repair itself, or p53 can initiate mechanisms leading to apoptosis [41].

A role has been established for p53 in AFB₁-induced tumorigenesis in geographical regions associated with high dietary exposure to AFB₁. p53 mutations are found at high frequency in AFB₁-associated human hepatocellular carcinomas, with a majority of the mutations occurring at the third base of codon 249 (AGG → AGT), resulting in a arginine → serine amino acid substitution [42–47]. However, in chemically induced mouse lung tumors, p53 gene perturbations are rarely observed [48–54].

In our analysis of AFB₁-induced AC3F1 mouse lung tumors, p53 alterations were examined using immunohistochemical staining, and DNA was microdissected from positive- and negative-staining regions and subsequently analyzed using single-strand conformation polymorphism (SSCP) analysis and direct sequencing [55]. Immunohistochemical staining of 71 AFB₁-induced tumor tissue sections identified heterogeneous distribution of positive nuclear p53 staining in sections from 56 tumors, with similar frequencies of positive staining in adenomas and adenocarcinomas. This result contrasted with that of Hegi and coworkers, who found evidence of positive p53 staining in only 4 of 23 in methylene chloride–induced adenocarcinomas in B6C3F1 mice [53]. In most of the AFB₁-induced tumors, staining was localized primarily to the periphery, but others displayed more widespread staining, suggesting a role of p53 in tumor progression. If p53 mutation had been a very early event, the staining pattern should have been homogenous following clonal expansion. However, mutation of p53 later during tumor development would be consistent with the heterogeneous staining pattern we observed. These results are consistent with the view of Miller, who proposed that p53 mutations are not early events in mouse lung tumorigenesis [56].
The molecular analysis of microdissected DNA [55] also points to \( p53 \) mutation occurring post adenoma development. The fact that over half of the \( p53 \) mutations were at A-T basepairs throughout exons 5 to 8 also suggests that they were not caused directly by the AFB\(_1\) epoxide but rather by subsequent genomic instability during tumor progression. Furthermore, some mutations were found in negatively staining tumor regions, albeit at a lower frequency than in positively staining regions. The lack of 100% concordance between immunohistochemical staining and presence of mutations is also consistent with previous evidence that other cellular mechanisms may be responsible for increasing the half-life of p53 protein and hence positive staining [57–59].

This unusually high frequency of \( p53 \) positive staining and mutations suggests the possibility of a carcinogen-specific response to AFB\(_1\). However, based on whole tumor DNA analysis, 1 study of urethane-induced mouse lung tumors [54] reported a \( p53 \) mutation frequency similar to what we found when we analyzed DNA from whole AFB\(_1\)-induced tumors [55]. In fact, when we performed SSCP analysis and direct sequencing on DNA isolated from whole AFB\(_1\)-induced lung tumors, it was difficult to detect mutations by direct sequencing. This emphasizes the value of using laser capture microdissection for isolation of DNA from microdissected tumor regions, and suggests that tumors induced by agents other than AFB\(_1\) may contain undetected \( p53 \) mutations.

**SOME UNANSWERED QUESTIONS**

The current state of knowledge regarding processes involved in AFB\(_1\)-induced lung tumorigenesis is summarized in Figure 2. At present, a number of questions remain to be answered, including the following.

1. **What is the overall importance of non–P450-mediated oxidation of carcinogens in human lung?** It appears that cooxidation of AFB\(_1\) by PHS and lipoxygenase is quantitatively more important than P450-catalyzed oxidation. The relevance of this process to other carcinogens deserves further attention.

2. **What is the importance of transfer of proximate or ultimate mutagenic metabolites between lung cell types?** The positive results with the modified Ames test using intact rabbit lung cells suggest that bioactivation might occur in cells other than the ultimate target cells for DNA binding and mutagenesis. For example, bioactivation in human lung macrophages, as we observed with AFB\(_1\), might result in mutagenic responses in nearby epithelial cells following transfer of AFB\(_1\) epoxide or metabolites of other carcinogens. In fact, transfer of mutagenic metabolites from cells of
origin to other targets has been proposed for benzo[a]pyrene [60] and dimethylnitrosamine [61].

3. What is the importance of DNA repair in susceptibility of Clara cells to AFB1-induced K-ras mutations? Although AFB1 bioactivation clearly is concentrated in Clara cells, the ability of a cell to repair DNA damage can also be a critical determinant of susceptibility.

4. Why does p53 perturbation appear to play a greater role in AFB1-induced mouse lung tumors, compared to those induced by other agents? The strongest evidence that AFB1 initiates a unique sequence of events resulting in p53 disruption is the contrastingly low frequency of p53 involvement (based on immunohistochemical staining of protein as well as presence of mutations) in methylene chloride–induced adenocarcinomas [53]. Other factors that may have contributed to reports of a lesser role for p53 in other studies include: analysis of a limited number of tumors; inability to examine tumors at multiple stages of progression; not conducting immunohistochemical staining for p53 protein; examining only whole tumor DNA rather than microdissected tumor regions; and use of different mouse strains and hybrids with different susceptibilities to tumorigenesis and potentially different mechanisms.

5. What is the involvement of the INK4a/ARF gene locus in AFB1-induced mouse lung tumors? This gene locus, the products of which interact with p53 and retinoblastoma (RB) pathways of cell cycle control, is a major target in carcinogenesis, and has been implicated in mouse lung tumors induced by other agents [62–65].
REFERENCES


