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The toxicology of aflatoxins as a basis for public health decisions

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Aflatoxins have been extensively studied with respect to their mechanisms of toxicity. An understanding of metabolism, DNA adduct induction, mutagenicity and carcinogenicity has been paralleled by the development of biomarkers of aflatoxin exposure and biological effects (e.g. mutations) applied to human populations. The improvements in exposure assessment and their application in prospective epidemiological studies and the demonstration of a specific mutation in the *TP53* gene in hepatocellular carcinomas from areas of high aflatoxin exposure have contributed significantly to the classification of aflatoxins as human carcinogens. In addition to establishing the carcinogenicity of aflatoxins in humans, understanding molecular mechanisms of action has provided the scientific rationale for prevention strategies, including primary and chemoprevention approaches. Overall, integrated, multidisciplinary research on aflatoxins has provided the platform on which to base decisions regarding acceptable exposures and priorities for interventions to reduce human risk in a public health context.

Introduction

Aflatoxins are secondary fungal metabolites that frequently contaminate foods such as groundnuts and maize (Wild and Hall, 2000). The aflatoxins were structurally identified in the early 1960s and over the last 40 years the toxicology of these compounds has been extensively studied. The conditions favouring formation of the aflatoxins by *Aspergillus* fungi have been described, as has their metabolism, toxicity, DNA adduct formation, mutagenic and carcinogenic activity (Eaton and Groopman, 1994). This understanding of mechanisms of action led to the development and application of biomarkers in molecular epidemiological studies. These studies established that human exposures in parts of sub-Saharan Africa and South-east Asia are widespread and that exposure is associated with increased risk of hepatocellular carcinoma (HCC), particularly in combination with hepatitis B virus (HBV) (IARC, 1993, 2002). An interaction between HBV and aflatoxins with respect to liver cancer induction is also observed in animal models (Sell *et al*., 1991; Bannasch *et al*., 1995). Furthermore, mechanistic knowledge regarding aflatoxins has provided the scientific rationale for approaches to reduce HCC risk (Wild and Hall, 2000). For example, understanding aflatoxin metabolism has permitted chemoprevention trials to modulate the adverse effects of dietary exposure to aflatoxins. In addition, improved understanding of the interactions between HBV and aflatoxin will advise decisions regarding preventive measures in developing countries.

This paper summarizes more recent advances in understanding the mechanisms of action of this important class of chemical carcinogens and specifically links these advances to the opportunities this understanding has afforded to the study of aflatoxin-related disease in human populations. There is a clear link between the toxicology of aflatoxins, the epidemiology and, finally, public health decisions as to how to deal with these carcinogens to reduce disease risk.

Metabolism in experimental animals and humans

Aflatoxin metabolism has been extensively studied in animals and humans and several excellent reviews have been published (Eaton and Gallagher, 1994; McClean and Dutton, 1995; Guengerich *et al*., 1998). Our focus is mainly with specific reference to how this knowledge has provided the basis for development of biomarkers used in human epidemiological and intervention studies. Aflatoxin B1 (AFB1) is both the most frequently occurring and toxic of the aflatoxins and thus will be the focus of this review.

Activation

AFB1 is activated by cytochromes P450 to AFB1-8,9-*exo*-epoxide and AFB1-8,9-*endo*-epoxide, but it is the *exo*-epoxide which binds to DNA to form the predominant 8,9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB1 (AFB1–N7-Gua) adduct (Iyer *et al*., 1994; see Figure 1). AFB1–N7-Gua confers the mutagenic properties of the compound. The binding of the *exo*-epoxide to guanine reflects the geometry of intercalation between base pairs in the DNA helix; 5′ intercalation appears to facilitate adduct formation by positioning the epoxide for in-line nucleophilic reaction with the N7 guanine (Gopalakrishnan *et al*., 1990; Kobertz *et al*., 1997). The epoxide ring is positioned above the plane and *in trans* to the 5a and 9a protons in the *endo*-epoxide, hindering reaction, but in the *exo*-epoxide the epoxide ring is positioned below the plane and *in cis* to the 5a and 9a protons, assisting reaction (Raney *et al*., 1993).

Other metabolites are formed from AFB1, including AFQ1, AFM1 and AFP1 (see Figure 1). These metabolites and other naturally occurring aflatoxins (G1, B2 and G2) are poorer substrates for epoxidation and, consequently, are less mutagenic, carcinogenic and toxic than AFB1. In addition, AFG1-8,9-epoxide has a reduced ability to intercalate into the DNA helix because modification of the ring structure decreases planarity (Raney *et al*., 1990). AFB1 metabolites can be useful biomarkers of human exposure to aflatoxins and AFM1, AFQ1 and AFP1 have all been detected in human urine samples (Groopman *et al*., 1985).

The major CYP enzymes involved in human aflatoxin metabolism are CYP3A4 and 1A2 (Gallagher *et al*., 1996; Ueng *et al*., 1995). CYP3A4 results in formation of the

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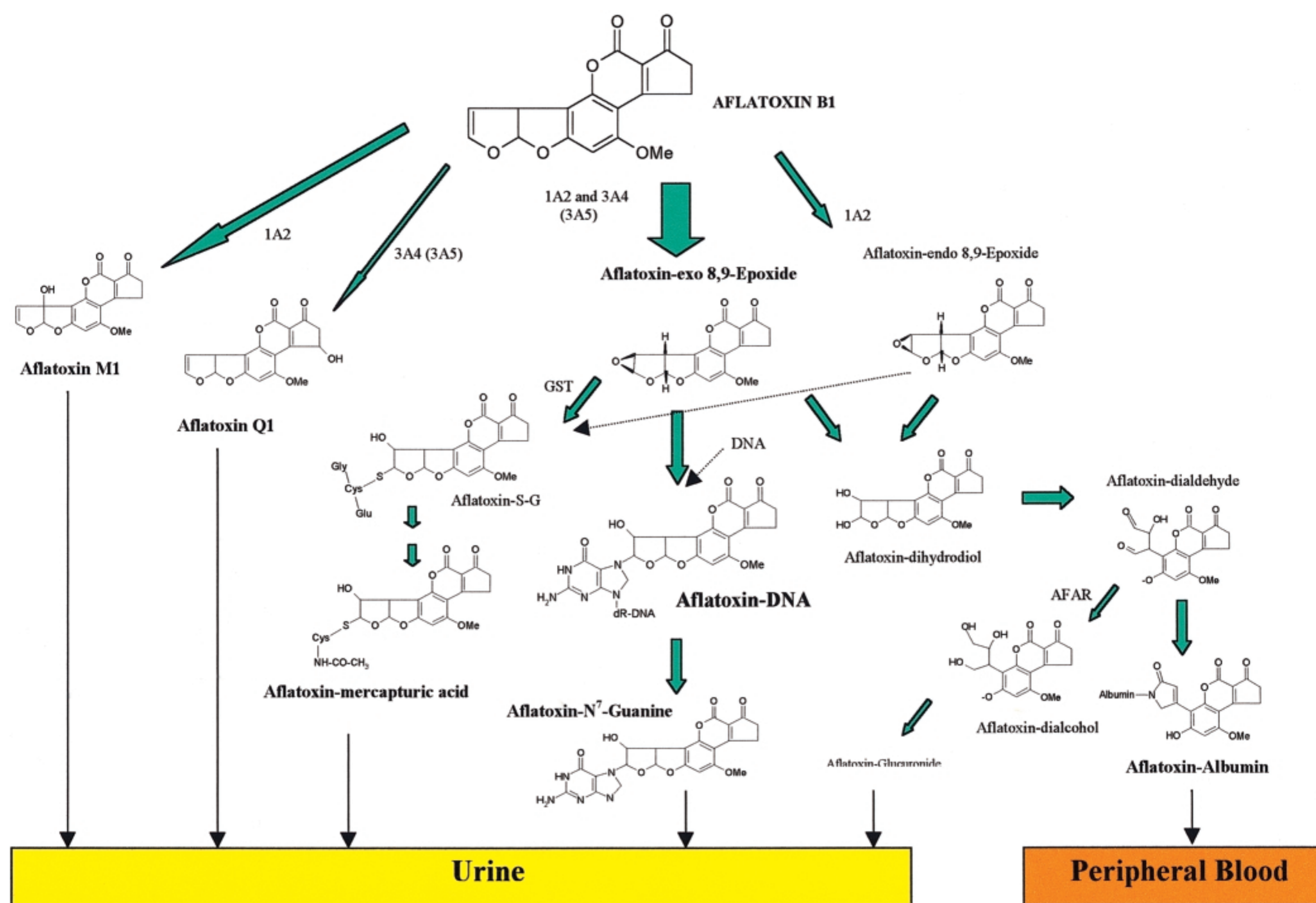

Fig. 1. Principle metabolism of aflatoxin B1 leading to reactive metabolites and biomarkers. 1A2, CYP1A2; 3A4, CYP3A4; 3A5, CYP3A5; GST, glutathione *S*-transferase; AFAR, aflatoxin aldehyde reductase; Aflatoxin-S-G, aflatoxin–glutathione conjugate.

exo-epoxide and AFQ1, whilst CYP1A2 can lead to formation of some *exo*-epoxide but also a high proportion of *endo*-epoxide and AFM1. The overall contribution of these enzymes to AFB1 metabolism *in vivo* will depend on affinity but also on expression levels in human liver, where CYP3A4 is the predominant cytochrome P450. CYP3A5 also metabolizes AFB1, mainly to the *exo*-8,9-epoxide with much less efficient formation of the detoxification product, AFQ1 (Wang *et al*., 1998). Human hepatic CYP3A5 expression is polymorphic, with a proportion of individuals showing no expression; in particular 40% of African-Americans do not express this enzyme. Recently, polymorphisms have been identified in the promoter region of CYP3A5 leading to alternative splicing and a truncated protein (Hustert *et al*., 2001; Kuehl *et al*., 2001).

Aflatoxin is known to cross the placenta and indeed aflatoxin adducts have been identified in the cord blood of newborn Gambian children (Wild *et al*., 1991). In this context it is interesting that CYP3A7, a major cytochromes P450 in human fetal liver, has the capacity to activate AFB1 to the 8,9-epoxide (Kitada *et al*., 1989). Experimental studies have further demonstrated the capacity of CYP3A7 to convert AFB1 to toxic and mutagenic metabolites (Kamataki *et al*., 1995; Yamada *et al*., 1998).

The role of extra-hepatic metabolism of aflatoxin, particularly in the small intestine, may be important in modulating the toxic and carcinogenic effects *in vivo*. Enterocytes of the small intestinal epithelium contain high levels of CYP3A enzymes and can activate aflatoxin, possibly limiting the systemic absorption of the carcinogen (Kolars *et al*., 1994). Lipoxygensase and prostaglandin H synthase may also make a significant contribution to aflatoxin metabolism in some extra-hepatic organs (Battista and Marnett, 1985; Donnelly *et al*., 1996).

Detoxification

The reactive *exo*- and *endo*-epoxides are detoxified by a number of pathways. The principal one is via glutathione *S*-transferase (GST)-mediated conjugation with reduced glutathione (GSH) to form AFB1 *exo*- and *endo*-epoxide–GSH conjugates (Johnson *et al*., 1997a; Guengerich *et al*., 1998; Raney *et al*., 1992). The *exo*- and *endo*-epoxides can also undergo rapid non-enzymatic hydrolysis to AFB1-8,9-dihydrodiol, that in turn undergoes slow, base-catalysed ring opening to a dialdehyde phenolate ion (Johnson *et al*., 1996, 1997b). The role of epoxide hydrolase in hydrolysis of the AFB1-8,9-epoxide to the dihydrodiol has not been strongly supported by experimental studies (Johnson *et al*., 1996, 1997b; Guengerich *et al*., 1998). AFB1 and AFG1 dialdehydes do not bind to DNA but form Schiff bases with primary amine groups, e.g. lysine, to form protein adducts such as aflatoxin–albumin (AF–alb) (Sabbioni *et al*., 1987; Sabbioni and Wild, 1991). A further metabolic step involves aflatoxin aldehyde reductase (AFAR), which catalyses the NADPH-dependent reduction of the dialdehydic phenolate ion to a dialcohol; this enzyme has been characterized in both rats and humans (Hayes *et al*., 1993; Ireland *et al*., 1998; Knight *et al*., 1999).

Cross-species variation in metabolism

There are marked species differences in sensitivity to aflatoxin carcinogenesis, with the rat extremely sensitive and the mouse and hamster resistant (Eaton and Groopman, 1994). The variation in carcinogenicity is paralleled by differences in DNA and protein adduct formation for a given AFB1 dose (Wild *et al*., 1996). A considerable part of the interspecies variation is a reflection of differences in expression of detoxification enzymes. The mouse but not the rat expresses high constitutive levels of a hepatic α class GST, mGSTA3-3, with a high affinity for AFB1-8,9-epoxide (Buetler and Eaton, 1992; Hayes *et al*., 1992). There is, however, an inducible α class GST (rGSTA5-5) in rats which can confer resistance to AFB1 through efficient conjugation of the 8,9-epoxide (Kensler *et al*., 1986; Hayes *et al*., 1991).

In contrast to rodents, human cytosolic fractions and liver slices demonstrate relatively low AFB1-8,9-epoxide conjugation (Moss and Neal, 1985; Kirby *et al*., 1993; Heinonen *et al*., 1996). Cross-species comparisons suggested that humans and rats formed similar levels of AF–alb for a given exposure; mice and hamsters formed far less adducts by comparison (Wild *et al*., 1996). Conclusive evidence that GST-mediated conjugation of AFB1-8,9-epoxide does occur in humans comes from the structural identification of the urinary mercapturic acid metabolite in the course of chemoprevention studies in the People's Republic of China (Wang, *et al*., 1999).

In summary, CYP3A4 and 1A2 play important roles in the metabolism of aflatoxins in humans, but CYP3A5 may also be significant and CYP3A7 could contribute *in utero*. Human GST activity towards AFB1-8,9-*exo*-epoxide is present to a limited extent, but other detoxification pathways, e.g. to the hydroxylated metabolites AFM1 and AFQ1, could play a key role in determining individual sensitivity. The above information provides the basis for chemoprevention of aflatoxin-induced hepatocarcinogenesis in humans (see below).

Sequence-specific binding to DNA and induction of mutations

Type of mutation

The positively charged imidazole ring of the AFB1–N7-Gua adduct promotes depurination and, consequently, apurinic site formation. Under slightly alkaline conditions the imidazole ring opens and forms the more stable and persistent ring opened AFB1–formamidopyrimidine (AFB1–FAPY) adduct. Investigations have been conducted as to which form of DNA damage is the most likely precursor of the mutations induced by AFB1.

The mutations induced by AFB1 in a variety of experimental systems are consistent with the above forms of DNA damage, with G→T transversions most common (Foster *et al*., 1983; Levy *et al*., 1992; Trottier *et al*., 1992; Cariello *et al*., 1994) although G→C transversions and G→A transitions are also observed (Levy *et al*., 1992).

Bailey *et al*. (1996) used site-directed mutagenesis to examine separately the effects of the AFB1–N7-Gua adduct and the apurinic site generated by adduct depurination. The predominant mutations with the AFB1–N7-Gua adduct were G→T transversions targeted to the site of the original adduct (~74%) with lower frequencies of G→A transitions (13–18%) and G→C transversions (1–3%). Using *Escherichia coli* differing in biochemical activity of UmuDC and MucAB it was shown that the mutations observed with AFB1–N7-Gua were not simply a result of depurination of the initial adduct. Unexpectedly, base substitutions were also relatively frequent at the base 5′ to the site of the original adduct, representing around 13% of the total mutations, something not observed with apurinic sites. This could reflect an interference in DNA replication following the intercalation of the AFB1-8,9-epoxide.

Sequence specificity of binding

Data on sequence-specific binding has been reviewed (Smela *et al*., 2001). Early studies suggested general sequence preferences for AFB1 binding dependent on the target guanine being in a run of guanines or with a 5′ cytosine (Benasutti *et al*., 1988). The base 3′ to the modified G appears less consistently predictive of reactivity. In a human lymphoblastoid cell line expressing recombinant human CYP1A2 enzyme a hotspot GC→TA tranversion occurred at a GG<u>G</u>GGG sequence (target base underlined) (Cariello *et al*., 1994).

Aflatoxin metabolites and DNA adducts as biomarkers in humans

Biomarkers of aflatoxin exposure include urinary metabolites such as AFB1–N7-Gua, AFM1, AFB1–mercapturic acid and serum AF–alb (Wild and Turner, 2001). Two prospective studies of HCC involved urinary aflatoxin and serum AF–alb biomarkers. In a nested control study in Shanghai, People's Republic of China, the relative risk associated with a positive test for urinary aflatoxins in HBsAg-positive subjects was 60.1 (95% CI, 6.4–561.8), whilst it was only 1.9 (95% CI, 0.5–7.5) among HBsAg-negative subjects (Ross *et al*., 1992; Qian *et al*., 1994). In the second nested case–control study in Taiwan, the relative risk for HBsAg-positive and AF–alb-positive individuals was 70.5 (95% CI, 11.8–415.4) and in HBsAg-positive subjects with a high level of urinary aflatoxins the relative risk was 111.9 (95% CI, 13.8–905.0) (Wang,L.Y. *et al*., 1996; Sun *et al*., 2001). In a separate case–control study examining 110 Taiwanese HCC patients AFB–DNA adduct levels were examined by immunohistochemistry of liver biopsies. Chronic HBsAg carrier status and AFB–DNA adducts were positively associated with HCC incidence. Where individuals had both factors the relative risk was 67.6 (95% CI, 12.2–373.2) (Lunn *et al*., 1997).

In addition to the use of exposure biomarkers in studies of the aetiology of HCC, biomarkers have also been used to assess whether polymorphisms in aflatoxin metabolizing enzymes result in altered levels of metabolites in some individuals. Wild *et al*. (1993) measured serum AF–alb in Gambian children in relation to GSTM1 genotype and in Gambian adults in relation to GSTM1, GSTT1, GSTP1 and epoxide hydrolase polymorphisms (Wild *et al*., 2000) and found no major differences in adduct levels by genotype. Only the GSTM1 null genotype was associated with a modest increase in AF–alb levels in adults and this effect was restricted to non-HBV-infected individuals. CYP3A4 phenotype, as judged by urinary cortisol metabolite ratio, was also not associated with albumin adduct level. Kensler *et al*. (1998) found no association between AF–alb and GSTM1 genotype in adults from Qidong County, People's Republic of China.

The possibility that polymorphisms in DNA repair enzymes could affect the levels of AFB1–N7-Gua adducts has been less extensively studied. Lunn *et al*. (1999) examined the levels of AFB1–DNA adducts in placental DNA from Taiwanese mothers in relation to polymorphisms in the DNA repair

Table I. *TP53* codon 249^{Ser} mutation in HCC by geographic location

Region	HCC cases	No. of *TP53* codon	Per cent $249^{Arg \rightarrow Ser}$	Reference
China				
Beijing	9	0	0	Fujimoto *et al*. (1994)
Guanxi	50	18	36	Stern *et al*. (2001)
Jiang-Su North	15	1	7	Shimuzu *et al*. (1999)
Jiang-Su South	16	9	56	Shimuzu *et al*. (1999)
Qidong	81	43	53	Scorsone *et al*. (1992); Li,D. *et al*. (1993); Fujimoto *et al*. (1994)
Shanghai	40	6	12	Buetow *et al*. (1992); Li,D. *et al*. (1993); Wong *et al*. (2000)
Tongan	21	7	33	Yang *et al*. (1997)
Xian	45	1	2	Buetow *et al*. (1992)
Hong Kong	56	5	9	Ng *et al*. (1994a,b); Wong *et al*. (2000)
Other Asian				
Taiwan	15	0	0	Oda *et al*. (1992); Hosono *et al*. (1993)
Japan	463	4	<1	Murakami *et al*. (1991); Buetow *et al*. (1992); Oda *et al*. (1992); Hayashi *et al*. (1993); Konishi *et al*. (1993); Nishida *et al*. (1993); Nose *et al*. (1993); Tanaka *et al*. (1993); Teramoto *et al*. (1994); Hsieh *et al*. (1995); Wong *et al*. (2000)
Indonesia	4	1	25	Oda *et al*. (1992)
Korea	41	0	0	Oda *et al*. (1992); Park,Y.M. *et al*. (1996)
Singapore	44	0	0	Shi *et al*. (1995)
India	21	2	10	Katiyar *et al*. (2000)
Africa				
Southern Africa	22	4	18	Bressac *et al*. (1991); Ozturk *et al*. (1991)
Senegal	15	10	67	Coursaget *et al*. (1993)
Mozambique	15	8	53	Ozturk *et al*. (1991)
Europe				
UK	189	1?	<1	Challen *et al*. (1992); Vautier *et al*. (1999)
France	100	2	2	Laurent-Puig *et al*. (2001)
Germany	33	0	0	Kress *et al*. (1992); Kubicka *et al*. (1995)
Italy	20	0	0	Bourdon *et al.* (1995)
Spain	70	0	0	Boix-Ferrero *et al*. (1999)
America				
USA	48	0	0	Buetow *et al*. (1992); DeBenedetti *et al*. (1995); Kazachkov *et al*. (1996); Wong *et al*. (2000)
Mexico	16	3	19	Soini *et al*. (1996)

enzyme, XRCC1. The presence of at least one allele of polymorphism 399^{Gln} was associated with a 2- to 3-fold higher risk of detectable AFB1–DNA adducts.

TP53 mutations in human HCC

As described above, aflatoxin has been shown to exhibit some sequence selectivity for guanine bases with a guanine or cytosine as the 5′ base and to induce predominantly G→T transversions in a number of mutational assays. On the assumption that mutational spectra in human tumours may reflect the aetiologic agent, comparisons have been made of the spectra of mutations in the tumour suppressor gene *TP53* in human HCC from regions differing in aflatoxin exposure. This molecular analysis revealed a high prevalence of an AGG→AGT (Arg→Ser) transversion at codon 249 (249^{Ser} mutation) in tumours from areas with reported high aflatoxin exposure (Montesano *et al*., 1997). A large number of studies have been published since the initial reports (Bressac *et al*., 1991; Hsu *et al*., 1991) and two recent meta-analyses examined the relationship between aflatoxin exposure, HBV infection and *TP53* mutation (Lasky and Magder 1997; Stern *et al*., 2001). Table I summarizes the published data and the key findings are described below. Many studies, particularly the earlier ones, only examined the presence or absence of the specific mutation at codon 249 and as a consequence may have overestimated the proportion of total *TP53* mutations in HCC occurring at that base pair.

Overall, the published studies show a positive correlation between population estimates of aflatoxin exposure and the proportion of HCC with a 249^{Ser} mutation. In regions of China where aflatoxin exposure is reported as high, the 249^{Ser} mutation was observed in >50% of HCC, compared with <10% in low exposure regions. In geographic regions of expected low aflatoxin exposure (including Japan, Korea, Europe and North America) the prevalence of 249^{Ser} mutations is extremely low (<1%).

One inherent limitation with the geographic correlation studies is that no account is taken of exposure and mutation occurrence at the individual level. Two studies of HCC patients in Thailand (Hollstein *et al*., 1993) and Mexico (Soini *et al*., 1996) did measure aflatoxin exposure biomarkers in addition to *TP53* mutations, but the small numbers of subjects preclude firm conclusions to date.

Chronic HBV infection is a risk factor for HCC and therefore it is important to understand whether the 249^{Ser} mutation occurs only in the case of exposure to both HBV and aflatoxin. Certainly, chronic HBV infection alone is insufficient to result in the 249^{Ser} mutation, as evidenced from studies in North America, Europe and Japan. However, the high prevalence of HBV infection in aflatoxin endemic areas has made it more difficult to define whether both risk factors are required for the mutation to occur. In a meta-analysis (Lasky and Madger, 1997) data were available on 449 patients, 201 positive for HBV markers and 248 negative. The association between level

Table II. *p53* mutations in animal tumors at codon 249 or equivalent

Species	Nucleotide sequence comparison[a]			Prevalence of codon 249 mutation	Reference
	248	249	250		
Human	CGG	AGG	CCC	See Table I	
Monkey	CGC	AGG	CCC	0/4	Fujimoto *et al*. (1992)
Rat hyperplastic nodules	CGC	CGG	CCC	0/6	Hulla *et al*. (1993)
Rat focal lesions and tumours				1/17	Lee *et al*. (1998)
Woodchuck	CGA	CGG	CCC	0/8	Rivkina *et al*. (1996)
Ground squirrel	CGC	CGG	CCC	0/7	Rivkina *et al*. (1994)
Duck	CGT	CGC	CCA	0/15	Duflot *et al*. (1994)
Tree shrew	CGG	CGC	CCC	0/5	Park,U.S. *et al*. (2000)
Mouse	CGC	CGA	CCC		

[a]The *p53* gene codon numbering refers to the human *p53* gene equivalent.

of aflatoxin exposure and 249^{Ser} mutation was still observed when restricting the analysis to HBV-positive patients in high and low aflatoxin exposure groups. However, the number of HBV-negative patients with high aflatoxin exposure was too small to make a similar comparison in HBV-negative cases.

The 249^{Ser} mutation has also been detected in blood samples from HCC patients, patients with cirrhosis and individuals without clinically diagnosed liver disease. In The Gambia, the 249^{Ser} mutation was detected in plasma DNA samples from 19 (36%) HCC patients, two (15%) cirrhosis patients and three (6%) control subjects (Kirk *et al*., 2000). Jackson *et al.* (2001) examined paired plasma and HCC samples from patients from Qidong County, People's Republic of China. Eleven tumours were positive for the 249^{Ser} mutation and the same mutation was detected in six of the paired plasma samples. Detection of the 249^{Ser} mutation in the plasma of non-cancer patients could reflect an early neoplastic event or, alternatively, chronic exposure to aflatoxin.

p53 mutations in animal tumours

In order to further test the plausibility of an association between aflatoxin exposure and *p53* mutations, HCC or preneoplastic lesions from several species have been examined for mutations at the codon corresponding to codon 249 in humans (Table II; Wild and Kleihues, 1996).

In contrast to human HCC from high aflatoxin exposure areas, no G→T transversions in codon 249 were found in tumours from rats, tree shrews and ducks. Two major limitations to this comparison are the different DNA sequences across species (a number of species are shown for comparison) and the relatively few animal tumours analysed. In ducks, for example, no mutations were observed at codon 249 (Duflot *et al*., 1994), however, the sequence difference compared with human codon 249 means that mutations at the third nucleotide would result in a silent mutation; this is also true for the other species listed except monkeys. In monkeys the codon 249 sequence is the same as humans but, to date, analyses are only available for four HCC (two from one animal) (Fujimoto *et al*., 1992). It is noteworthy that the studies in ducks, woodchucks and tree shrews did include some animals also infected with hepatitis virus in addition to aflatoxin treatment.

It should also be noted that in all animal studies the dosing regime used is several orders of magnitude higher than in humans exposed through consumption of naturally contaminated food. It is therefore possible that regulation of alternative pathways for activation, detoxification and DNA repair may be operating in these model systems.

Additional genetic alterations in human HCC

It would be unexpected that aflatoxin carcinogenesis is exclusively associated with a specific *TP53* mutation given the multiple genetic alterations observed in human HCC. For example, HCC are frequently found to exhibit loss of heterozygosity (LOH) at numerous loci. Aflatoxin can induce mitotic recombination (Stettler and Sengstag, 2001) as well as minisatellite rearrangements (Kaplanski *et al*., 1997). Mitotic recombination and genetic instability may therefore be alternative mechanisms by which aflatoxin contributes to genetic alterations in HCC. Consequently, several studies have tested the hypothesis that aflatoxin exposure is associated with specific genetic alterations other than the *TP53* 249^{Ser} mutation.

In comparisons of HCC from Qidong County and Beijing, People's Republic of China, the higher prevalence in the former region was paralleled by increased LOH at 4p11–q21, 16q22.1 and 16q22–24 loci (Fujimoto *et al*., 1992). Wong *et al*. (2000) detected double the number of genetic alterations in HCC from Shanghai (high prevalence of 249^{Ser} mutation) compared with Hong Kong (low prevalence of 249^{Ser} mutation); the most frequent changes explaining this difference were deletions on chromosomes 4q, 8p and 16q and gain of 5p. Laurent-Puig *et al*. (2001) found an association in two HCC cases between the 249^{Ser} mutation and homozygous 16q deletion.

The above data reveal some additional geographic variations in genetic alterations in HCC but are inconclusive in ascribing any of these specifically to aflatoxin exposure at this stage.

The contribution of mechanistic understanding to assessing the carcinogenicity of aflatoxin

The application of aflatoxin exposure biomarkers has clearly made a significant contribution to understanding the carcinogenicity of these toxins. In addition, understanding the metabolism of aflatoxins and the demonstration of DNA adduct formation in humans provides supporting evidence to the epidemiological data. A more recent area of research where mechanistic understanding has added to the evidence of aflatoxin carcinogenicity in humans comes from the studies of *p53* mutations described above. Whilst the geographic correlation studies provide an association between exposure and 249^{Ser} mutation frequency, a number of experimental

approaches have been taken to examine the biological plausibility of a causal link between aflatoxin-induced DNA damage and this specific mutational change.

Specificity of the 249^{Ser} mutation in the TP53 gene

An *in vitro* polymerase stop assay using a *TP53*-containing plasmid revealed the guanine at the third position of codon 249 to be a site of modification by AFB1, but neighbouring guanines were also modified (Pusieux *et al*., 1991). For human hepatocytes in culture AFB1 induced a higher frequency of mutation at the third base of codon 249 (~3-fold), but adjacent codons (247, 248 and 250) were also sites of mutation induction.

Denissenko *et al*. (1998, 1999) examined the formation of AFB1–DNA adducts in specific gene fragments in both isolated genomic DNA and HepG2 hepatocytes. AFB1 adducts were observed exclusively at guanine residues in a dose-dependent manner with 5′-GG dinucleotides a preferential site of attack. However, codon 249 was not an exceptional target and no preferential persistence of the AFB–N7-Gua adduct was observed at codon 249.

The above studies demonstrate that AFB1 does induce DNA damage at the third nucleotide of codon 249. However, there is insufficient preferential modification or mutation at this site in comparison with the adjacent guanine (second nucleotide of codon 249) or guanines in surrounding codons to explain the mutational specificity observed *in vivo* in human HCC. AFB1–DNA adducts at other sites within the *TP53* gene would also induce G→T transversions with alterations of amino acids and associated p53 protein function, but these are far more rare than the 249^{Ser} mutation in HCC from areas of high aflatoxin exposure. Consideration of the functional effects of the codon 249^{Ser} mutation in hepatocytes, particularly in the presence of co-infection by HBV, is therefore of importance. This is considered briefly below.

Functional effects of the 249^{Ser} mutation

There has been considerable interest as to whether the functional properties of the p53 protein, modified as a result of the codon 249^{Ser} mutation, has particular properties that confer a selective growth advantage on hepatocytes.

Comparisons have been made of the growth and tumourigenic properties of hepatocyte cell lines transfected with different forms of the *TP53* gene. In a human hepatoma cell line deficient in p53 expression of the 249^{Ser} p53 mutant led to an increase in mitotic activity and colony-forming ability but did not affect the tumourigenicity of the cell line when injected into nude mice (Ponchel *et al*., 1994). In a mouse hepatocyte cell line, the mouse equivalent 249^{Ser} mutant led to increased growth and colony-forming ability but no difference in tumour formation in nude mice compared with the wild-type p53 sequence (Dumenco *et al*., 1995).

The 249^{Ser} mutant is more effective than other p53 mutants, including 143^{Ala}, 175^{His}, 248^{Trp} and 282^{His}, in inhibiting wild-type p53 transcriptional transactivating activity in human liver cells (Forrester *et al*., 1995). Schleger *et al*. (1999) transfected a non-tumourigenic human liver epithelial cell line, HACL-1, which does not express detectable p53 protein, with the mutated 249^{Ser} sequence but this had no effect on the lifespan of the cells or their ability to grow in soft agar.

Overall, the above data studying the functional effects of the 249^{Ser} mutant gene product suggest that this mutation is insufficient to immortalize human liver cells, but the mutant does appear to confer a growth advantage to previously immortalized cells. This effect may differ with other cell types and HBV status.

The contribution of mechanistic understanding to assessing HBV and aflatoxin interactions

In countries with a high incidence of HCC, endemic infection with HBV is often associated with exposure to aflatoxins and there is evidence of a multiplicative increase in risk in individuals exposed to both factors (Wild and Hall, 1999). Experimental studies in HBV transgenic mice and woodchucks also suggest a synergism between the two risk factors (Sell *et al.*, 1991; Bannasch *et al.*, 1995). An understanding of the molecular mechanisms behind the interaction is of relevance to public health measures to reduce HCC incidence.

One possible mechanism of interaction is that HBV infection alters the expression of aflatoxin metabolizing enzymes and consequently the extent to which aflatoxins bind to DNA. In woodchucks, the results have been somewhat contradictory (De Flora *et al.*, 1989; Gemechu-Hatewu *et al.*, 1997), probably because of the small numbers of animals and the interindividual variation in outbred animals. Studies in HBV transgenic mouse lineages have revealed an induction of specific cytochromes P450, namely CYP1A and 2A5, in association with liver injury resulting from expression of the HBV transgenes (Kirby *et al.*, 1994a; Chemin *et al.*, 1996, 1999). The absence of cytochrome P450 induction in HBV X gene transgenic mice argue against a transactivation of the CYP genes by HBx protein (Chomarat *et al*., 1998). Furthermore, induction of cytochrome P450 enzymes has been observed in mice and hamsters where liver injury was induced by infection with bacteria and parasites (Kirby *et al.*, 1994b; Chomarat *et al.*, 1997), suggesting an effect of liver injury *per se* rather than a specific effect of HBV. One attempt has been made to assess the impact of HBV infection on cytochrome P450 activities in people exposed to aflatoxin (Wild *et al*., 2000). Cortisol metabolism was used as a marker of CYP3A4 phenotype in relation to AF–alb adducts in Gambian subjects, but no association was observed between putative CYP3A4 activity and HBV infection status. Other cytochrome P450 phenotypes were not assessed.

The effects of liver injury are not limited to cytochromes P450, for example, an increase in GST π was observed in the HBV transgenic mice (Chemin *et al*., 1999). In addition, in HepG2 cells that were HBV transfected, expression of GST α class enzymes was significantly decreased; transfection of the HBx gene into these cells also decreased the amount of GST α class protein (Jaitovitch-Groisman *et al*., 2000). A study of non-tumourous liver showed that GST activity is significantly decreased in the presence of HBV DNA (Zhou *et al.*, 1997), again suggesting that viral infection may compromise the ability of hepatocytes to detoxify chemical carcinogens. Thus, overall, effects of HBV infection on aflatoxin metabolism are likely to be complex, but there is potential for an altered balance of activation and detoxification of carcinogens during the natural history of an infection.

A more indirect approach to assessing the impact of HBV infection on aflatoxin metabolism has been to examine the level of binding of aflatoxin to DNA or proteins with respect to viral status, assuming that this will reflect interindividual differences in metabolism as well as exposure. In West Africa higher AF–alb levels have been observed in young children who were HBsAg-positive compared with those who were not (Allen *et al.*, 1992; Wild *et al.*, 1993; Turner *et al.*, 2000).

Similar observations have been reported in a study of 200 adolescents from Taiwan (Chen *et al*., 2001). In contrast, this effect of viral infection was not seen in Chinese adults (Wang, *et al*., 1996). One possibility is that viral infection has more marked effects on aflatoxin metabolism early in life, and this merits further investigation.

An alternative hypothesis regarding the mechanism of interaction between HBV and aflatoxin is that carcinogen exposure may alter viral infection and replication. In ducklings, AFB1 treatment resulted in a significant increase in serum and liver duck HBV DNA level and in liver viral RNA and duck HBV large envelope protein (Barraud *et al*., 1999). This study demonstrates that AFB1 can lead to enhanced hepadnaviral gene expression. Consistent with this, HepG2 cells transfected with recircularized HBV and treated with AFB1 (10–40 μmol/l) also showed a 2- to 3-fold increase in HBsAg level 96 h post-treatment (Banerjee *et al*., 2000).

Aflatoxin forms DNA adducts and is mutagenic, whilst chronic HBV infection can increase cell proliferation resulting in fixation of DNA adducts as mutations, perhaps at codon 249 of the *p53* gene, and selective clonal expansion of these mutant cells due to a functional growth advantage of these cells (see above). However, an alternative plausible mechanism is that the 249^{Ser} mutation could predispose hepatocytes to the carcinogenic action of aflatoxins. Human liver epithelial cells, expressing wild-type p53 and transfected with the HBx gene, were more sensitive to the cytotoxic action of AFB1-8,9-epoxide than were the parent cells (Sohn *et al*., 2000). The HBx-expressing cells were also more prone to apoptosis and to induction of mutations at codon 249 of the *TP53* gene. One possible explanation is that HBx could inhibit excision repair, thus leading to increased AFB1 adduct persistence and mutation induction.

The contribution of mechanistic understanding to aflatoxin prevention strategies

HBV vaccination is a priority for reducing the global burden of HCC. Currently, however, only ~1% of African children receive the vaccine. In addition, there are 360 000 000 HBV carriers worldwide and with the continuing restricted access to the vaccine the number of carriers will remain high for several decades. Given this and the synergistic interaction between aflatoxins and HBV (see above), intervention to reduce aflatoxin exposure is also merited (for a review see Wild and Hall, 2000).

Much of the intervention strategy is based on knowledge gained from mechanistic studies on aflatoxin. For example, the study of aflatoxin metabolism has provided biomarkers that can be used as outcome measures in intervention studies. In addition, understanding metabolism has provided the basis for chemoprevention in human populations. These are reviewed briefly here.

Primary prevention

One means of intervening to reduce aflatoxin exposure would be to alter agricultural practices such that crops like rice, with a lower incidence of *Aspergillus flavus* infestation and aflatoxin level, are consumed. However, for many communities in developing countries a change in diet is simply not feasible and thus primary intervention (pre- and post-harvest) or chemoprevention methods may need to be employed. Pre-harvest would be the most effective point of control because this is the point at which the crop is first infected by the toxin-producing fungus. Interventions at this level involve measures to reduce crop stress (e.g. improved irrigation, use of fungicides, pesticides and insecticides, use of cereal strains resistant to fungal colonization, biocontrol by introduction of competitive non-aflatoxigenic strains of *A.flavus* and genetically modified crops that inhibit fungal colonization) (Wild and Hall, 2000). However, these processes may not be economically feasible in many high risk regions.

Aflatoxins often accumulate during food storage and therefore post-harvest control at the subsistence farm aims to minimize fungal growth and aflatoxin production. The growth of *Aspergillus* is influenced most critically by temperature, moisture content and storage time.

Preliminary studies in Conakry, Guinea, revealed a high HCC incidence (Koulibaly *et al*., 1997) and aflatoxin exposure mainly attributable to contamination of groundnuts following storage (Sylla *et al*., 1999). A primary prevention study is underway where the intervention incorporates a package of post-harvest procedures, including improved sun drying prior to storage, drying on cloth rather than directly on the earth, removal of visibly mouldy nuts by hand sorting, storage in jute sacks rather than plastic, use of wooden pallets for storage to avoid contact with the earth and to improve ventilation and, finally, use of insecticides to control insect damage and spread of fungal spores. The outcomes of the study are being determined by measuring both food levels of the toxin and, more importantly, blood AF–alb biomarker levels at three time points post-harvest.

Primary intervention strategies to reduce mycotoxin exposures at the post-harvest level may have a significant impact in high exposure populations, but are unlikely to eliminate exposure. In addition, these approaches cannot be targeted specifically to high risk individuals, e.g. people with chronic HBV infection. Therefore, intervention strategies also encompass chemoprevention, using compounds that interfere with the absorption or metabolism of aflatoxins once ingested.

Chemoprevention

In experimental studies, induction of GST and AFAR decreases aflatoxin–DNA and aflatoxin–protein adduct formation and blocks aflatoxin carcinogenicity in rats (Judah *et al*., 1993; Groopman and Kensler 1999). Therefore, a similar modulation of the balance between aflatoxin activation and detoxification in humans has been sought. Oltipraz is an effective agent in blocking aflatoxin adduct formation and hepatocarcinogenesis in rodents (Roebuck *et al*., 1991; Groopman *et al*., 1992; Kensler *et al*., 1997) and, more recently, adduct formation in tree shrews (Li *et al*., 2000). The protective actions of oltipraz are believed to predominantly reflect an induction of detoxifying enzymes such as GSTs (Morel *et al*., 1993), with increased excretion of the AFB1–GSH conjugate as a mercapturic acid (AFB–NAC) and a reduced activation to the 8,9-epoxide by inhibition of CYP1A2 (Langouët *et al*., 1995).

In China, Kensler and colleagues (Jacobson *et al*., 1997; Kensler *et al*., 1998; Wang *et al*., 1999) have demonstrated that oltipraz can indeed modulate aflatoxin metabolism, as suggested by the above studies. The effects on aflatoxin metabolism *in vivo* were demonstrated by the assay of urinary AFM1, peripheral blood AF–alb adducts and the urinary AFB–NAC metabolite, with a decrease in AFM1 and an increase in AFB–NAC urinary excretion depending on treatment regimen (Wang *et al*., 1999).

In addition to oltipraz, a number of other chemoprevention agents are being developed with respect to aflatoxin. One of the most promising is chlorophyllin, a potent anti-mutagen (Dashwood *et al*., 1998) capable of forming tight molecular complexes with AFB1 and possibly of impeding aflatoxin absorption as a consequence (Kensler *et al*., 1998; Breinholt *et al*., 1999). Chlorophyllin has been evaluated in a randomized, double blind, placebo controlled chemoprevention trial in Qidong, People's Republic of China. Its consumption at each meal led to an overall 55% reduction ($P = 0.036$) in median urinary levels of AFB1–N7-Gua compared with those taking placebo (Egner *et al*., 2001).

The above clinical trials demonstrate the feasibility of chemoprevention studies with aflatoxin. However, given the multistep nature of HCC it is unlikely that these exposure biomarkers will be predictive at the individual level for HCC risk or be of value as surrogate end-points in longer term intervention trials aimed at reducing disease incidence; this is suggested indirectly in rats where a correlation between adducts and liver cancer occurred at the group but not individual level (Kensler *et al*., 1997). The specific *TP53* 249^{Ser} mutation related to aflatoxin exposure may be more predictive of individual risk and therefore be of more value in this regard. However, this requires further understanding of the temporal appearance of the biomarker in relation to the natural history of the disease.

Conclusion

Aflatoxins have been extensively studied with respect to their mechanisms of action, including their mutagenic and carcinogenic activity. This work has been paralleled by developments in biomarkers of aflatoxin metabolism, DNA adducts and mutations applied to exposed human populations. The improvements in exposure assessment in epidemiological studies and the demonstration of a specific mutation in the *TP53* gene have contributed significantly to the identification of aflatoxins as human carcinogens. In addition, the studies of animal and human aflatoxin metabolism have provided opportunities to develop chemoprevention approaches in human populations. Biomarkers can be used as outcome measures in these and primary prevention studies. Overall, the integrated, multidisciplinary research on aflatoxins has provided the scientific platform on which to base decisions regarding acceptable exposures and priorities for interventions to reduce human risk in a public health context.

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