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Fundamental and Molecular Mechanisms of Mutagenesis

DNA damage by mycotoxins

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Abstract

Mycotoxins are toxic fungal metabolites which are structurally diverse, common contaminants of the ingredients of animal feed and human food. To date, mycotoxins with carcinogenic potency in experimental animal models include aflatoxins, sterigmatocystin, ochratoxin, fumonisins, zearalenone, and some *Penicillium* toxins. Most of these carcinogenic mycotoxins are genotoxic agents with the exception of fumonisins, which is currently believed to act by disrupting the signal transduction pathways of the target cells. Aflatoxin B_1 (AFB_1), a category I known human carcinogen and the most potent genotoxic agent, is mutagenic in many model systems and produces chromosomal aberrations, micronuclei, sister chromatid exchange, unscheduled DNA synthesis, and chromosomal strand breaks, as well as forms adducts in rodent and human cells. The predominant AFB_1–DNA adduct was identified as 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy–AFB_1 (AFB_1–N^7-Gua), which derives from covalent bond formation between C8 of AFB_1–8,9-epoxides and N^7 of guanine bases in DNA. Initial AFB_1–N^7-guanine adduct can convert to a ring-opened formamidopyrimidine derivative, AFB_1–FAPY. The formation of AFB_1–N^7-guanine adduct was linear over the low-dose range in all species examined, and liver, the primary target organ, had the highest level of the adduct. Formation of initial AFB_1–N^7-guanine adduct was correlated with the incidence of hepatic tumor in trout and rats. The AFB_1–N^7-guanine adduct was removed from DNA rapidly and was excreted exclusively in urine of exposed rats. Several human studies have validated the similar correlation between dietary exposure to AFB_1 and excretion of AFB_1–N^7-guanine in urine. Replication of DNA containing AFB_1–N^7-guanine adduct-induced G → T mutations in an experimental model. Activation of *ras* protooncogene has been found in AFB_1-induced tumors in mouse, rat, and fish. More strikingly, the relationship between aflatoxin exposure and development of human hepatocellular carcinoma (HHC) was demonstrated by the studies on the *p53* tumor suppressor gene. High frequency of *p53* mutations (G → T transversion at codon 249) was found to occur in HHC collected from populations exposed to high levels of dietary aflatoxin in China and Southern Africa. Furthermore, AFB_1-induced DNA damage and hepatocarcinogenesis in experimental models can be modulated by a variety of factors including nutrients, chemopreventive agents, and other factors such as food restriction and viral infection, as well as genetic polymorphisms.

Keywords: Mycotoxin; Aflatoxin; DNA damage; Aflatoxin B_1–N^7-guanine adduct; Human hepatocellular carcinoma

Abbreviations: AFB_1, aflatoxin B_1; OA, ochratoxin A; AFB_1–N^7-Gua, 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy–aflatoxin B_1; AFB_1–FAPY, 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxy–aflatoxin B_1; 8-oxodG, 8-oxodeoxyguanosine; HBsAg, hepatitis B virus surface antigen; EQ, ethoxyquin; HCC, human hepatocellular carcinoma

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1. Introduction

Mycotoxins are structurally diverse fungal metabolites that can contaminate the ingredients of animal feed and human food. Spurred by the discovery of the aflatoxins in the early 1960s, the search for mycotoxins in food has led to the identification of over 100 toxigenic fungi and more than 300 mycotoxins [1,2]. Most mycotoxins have not been implicated in any health problems in animals or people, but their occurrence in the food supply cause concern because many are potent toxic agents under experimental conditions. The aflatoxins, certain trichothecenes, fumonisins, and ochratoxins have been associated with highly lethal episodic outbreaks of poisoning in animals and/or human populations [3,4]. To date, mycotoxins with carcinogenic potency in experimental animal models include aflatoxins, sterigmatocystin, ochratoxin, fumonisin, zearalenone, and some *Penicillium* toxins, such as citrinin, luteoskyrin, patulin, and penicillic acid [5,6]. All of these carcinogenic mycotoxins are DNA damaging agents, with the exception of fumonisins [7]. The fumonisins may induce cancer through disturbing the signal transduction pathways. Recently, aflatoxin B_1 (AFB_1) has been classified as a known human carcinogen by the International Agency for Research on Cancer [8].

This review attempts to briefly summarize currently available data on DNA damage caused by the major mycotoxins, with the emphasis on aflatoxin-induced DNA damage, its modulations, DNA base alterations in the critical oncogenes and tumor suppressor genes, and their role in human cancer development. Similar topics have also been reviewed previously [9–11].

2. DNA damage by aflatoxins

2.1. General toxicity

Aflatoxins are potent liver toxins, and the severity of effects in animals vary with dose, length of exposure, species, breed, and diet or nutritional status [9]. These compounds are lethal when consumed in large doses; however, sublethal exposures can induce chronic toxicities, and low levels of chronic exposure can result in neoplasia, primarily liver cancer, in many animal species [4,12]. In addition, AFB_1 inhibits DNA synthesis, DNA-dependent RNA polymerase activity, messenger RNA synthesis, and protein synthesis [4,13]. Mutagenicity of AFB_1 has been demonstrated using many model systems including HeLa cells, *Bacillus subtilis*, *Neurospora crossa*, *Salmonella typhimurium* (reverse and forward mutation), and CHO cells. AFB_1 is a potent carcinogen in many species of animals, including rodents, non-human primates, and fish [4]. While the liver is the major target organ, under certain circumstances, significant numbers of tumors have been found in lung, kidney and colon.

2.2. Formation of DNA adducts

The aflatoxins are among the most potent genotoxic agents known [4,14,15]. AFB_1 induces chromosomal aberrations, micronuclei, sister chromatid exchange, unscheduled DNA synthesis, chromosomal strand breaks, and forms adducts in rodent and human cells [8]. Covalent binding to DNA is generally a property of those specific aflatoxins containing an unsaturated terminal furan ring forming an epoxide. Despite the uncertainties about the specific role of individual human cytochrome *P*450s in the metabolism of aflatoxin to its two 8,9-epoxide isomers, there is no doubt that this is the critical metabolite for genotoxic damage. The initial finding supporting this pathway was the identification and confirmation in vivo of the major AFB_1–DNA adduct by Essigmann et al. [16] and Croy et al. [17] as 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy–AFB_1 (AFB_1–N^7-Gua). Approximately 1% of administered AFB_1 is bound covalently to DNA; this binding peaks at 2 h postdosing [18]. A single dose of 25 μg AFB_1 to Fischer 344 (F344) rats can cause a measurable increase in AFB_1–DNA adducts that is more pronounced following a second daily dose.

Studies on the reaction of synthetic AFB_1–8,9-epoxide with DNA in vitro strongly indicates adduct formation proceeds by a precovalent intercalation complex between double-stranded DNA and the highly electrophilic, unstable AFB_1–exo-8,9-epoxide isomer [19]. A recent study found that the *exo* isomer of AFB_1–8,9-epoxide appears to be the only product of AFB_1 involved in reaction with DNA and

reacts with the N^7 atom of guanine via an SN_2 reaction from an intercalated state [20]. Another recently published study designed an intercalation inhibitor to probe the effects of intercalation by AFB_1 epoxide on its reaction with DNA. DNA duplexes were prepared with a target strand containing multiple guanines and a non-target strand containing a *cis–syn* thymidine–benzofuran photoproduct, rendering this strand inaccessible to AFB_1 epoxide [21].

The induction of a positive charge on the imidazole portion of the formed AFB_1–N^7-Gua adduct gives rise to a ring-opened formamidopyrimidine (AFB_1–FAPY) derivative with distinct chemical properties [18]. Accumulation of AFB_1–FAPY adduct is time-dependent, non-enzymatic, and may be of biological importance because of its apparent persistence in DNA. Other minor N^7-guanyl adducts can arise through enzymatic oxidation of AFP_1, AFM_1, and others with unsaturated in the 8,9-position, implying that the hydroxylation of AFB_1 can be followed by epoxidation from electrophilic metabolites. The exception is AFQ_1, which is a relatively poor substrate for epoxidation and thus showing very low potency for DNA binding. Covalent binding of AFB_1 to adenosine [22] and cytosine [23] in DNA in vitro has also been reported.

Common oxidative DNA damage, including formation of 8-oxodeoxyguanosine (8-oxodG), was observed in rat hepatic DNA following exposure to AFB_1. A time- and dose-dependent increase in hepatic levels of 8-oxodG residues in liver DNA treated with AFB_1 has been recently reported [24,25]. Thus, toxic effects of AFB_1 can increase common endogenous DNA adducts.

2.3. Tissue specificity and dose–response of AFB_1–DNA adducts

The amount of AFB_1–DNA adducts is greater in liver than in other organs and the level of liver AFB_1–DNA adducts generally correlates with AFB_1 dosage and species susceptibility [4]. F344 rats exposed to a single dose of AFB_1 (1 mg/kg) showed 10-fold greater level of DNA adducts in liver than in kidney, a minor target organ in rats [26]. In the same study, CD-1 Swiss mice, which are relatively resistant to AFB_1, only produced 2% of the rat AFB_1–DNA adducts in liver although a 12-fold greater dosage was used. Similar results have been reported in fish. The highly sensitive rainbow trout had a 25-fold greater liver AFB_1–DNA adduct level than the resistant coho salmon, and both species showed comparable low-level adduction in the kidney, a non-target organ [27]. Single-dose exposure of 10–1000 ng/kg in male F344 rats produced liver AFB_1–DNA binding curves that were linear at low dose and less than linear at high dose [28]. Chronic exposure over the same dose range in the same model produced dose-linear steady-state levels of adducts [29]. Rainbow trout treated with carcinogenic doses of AFB_1 for 2–4 weeks also showed dose-linear accumulation of liver AFB_1–DNA adducts [30].

The lung is also a target organ for aflatoxins. Tissue derived from various portions of the lung has been used as in vitro models to study AFB_1 metabolism and activation [31]. AFB_1 can be metabolically activated by cultured intact human tracheal epithelium from the bronchus, in human epithelioid lung cells, and by rabbit lung microsomes. The two major AFB_1–DNA adducts are also formed in the pulmonary tract in ratios similar to those seen in hepatic tissues of AFB_1-treated animals [31]. Inhalation of AFB_1 on rats for 20–120 min results in an increase in the amount of AFB_1–N^7-Gua adduct formed in DNA in a dose-dependent manner [31].

2.4. Correlation with tumor formation

Numerous animal studies in many different species have been conducted over the years to examine the relationship between levels of AFB_1–DNA adducts and tumor formation, especially between chronic exposure to AFB_1 and DNA adduct formation. Correlations have been demonstrated in trout for levels of DNA adduct and formation of tumors in response to AFB_1 given with or without non-nutrient dietary components that block carcinogenesis [30]. Bechtel [32] calculated from published studies the steady-state AFB_1–DNA adducts in F344 rats fed AFB_1 in the diet for 2 years and in trout exposed in tank water for 1–20 days, and showed that tumor risk correlated with adduct content. In the rat, incidence of tumor formation shows an apparent linear increase with liver AFB_1–DNA adducts following chronic expo-

sure: 50% tumor incidence is reached at a steady-state level of approximately 1 adduct/10^6 nucleotides [32]. Similar analysis found that tumor formation in rainbow trout after 2 weeks of AFB_1 exposure parallels the rat data. This similarity has been interpreted as equal efficiency of AFB_1–DNA adducts in induction of tumors in trout and rats [30].

2.5. Urinary excretion of AFB_1–DNA adducts

In addition to monitoring AFB_1–DNA adducts in DNA of target organs, the eliminated products of these adducts can be measured in biofluids. The AFB_1–N^7-Gua adduct is removed from DNA rapidly with a half-life in rats of 8–10 h, and is excreted exclusively in urine of exposed rats [33]. A dose-dependent correlation between AFB_1 and AFB_1–N^7-Gua excreted in the urine was found in male F344 rats [33]. Following administration of AFB_1 (1 mg/kg, i.p.), 80% of the total excretion of the AFB_1–N^7-Gua adduct occurred during the 48-h period after dosing. The excretion kinetics of AFB_1–N^7-Gua were studied in rats after a single exposure to AFB_1. Urine was collected over 24 h from male F344 rats dosed orally at levels ranging from 0.03 to 1.0 mg AFB_1/kg body weight [34]. Aliquots of urine were purified by immunoaffinity chromatography and individual metabolites were quantified by HPLC analysis. AFB_1–N^7-Gua in urine accounted for 7.5% of the total aflatoxins injected on the HPLC. The relationship between AFB_1 dose and the excretion of the AFB_1–N^7-Gua over the 24-h after exposure had a correlation coefficient of 0.99. This analysis demonstrated an excellent linear correspondence between oral dose and excretion of a biologically relevant metabolite in urine. Finally, at 24 h postdosing, the residual level of AFB_1 liver DNA adducts was determined and compared with AFB_1–N^7-Gua excretion in urine; the correlation coefficient was 0.98, which supports the concept that measurement of the AFB_1–N^7-Gua in urine reflects DNA damage in the primary target organ.

In addition to its development and validation in the susceptible rat model, a systematic evaluation of the AFB_1–N^7-Gua adduct in several human populations has been reported. The first report by Autrup et al. [35,36] used synchronous fluorescence spectroscopy for the analysis of AFB_1–DNA adducts in urine samples from Murang'a district, Kenya. Over 1000 urine samples were analyzed, 12.6% of which were positive for excretion of AFB_1–N^7-Gua. Further, a regional variation was found in the excretion levels of AFB_1–N^7-Gua, the highest rate was found in the Western Highland and Central Province.

Investigations in Fusui County, Guangxi Autonomous Region, People's Republic of China (PRC), had the advantage of having dietary intake data and the measure of urinary aflatoxin metabolites. The dietary intake of aflatoxins was monitored for 1 week and the average exposure of AFB_1 was 48.4 μg/day for male and 92.4 μg/day for female [37]. Total 24 h urine samples were collected starting on the fourth day as consecutive 12-h fractions. AFB_1–N^7-Gua, AFM_1, AFP_1, and AFB_1 were the aflatoxins most commonly detected and quantified in the urine samples. The linear regression analyses for the urine levels of each of these individual aflatoxins was compared with aflatoxin intake to yield correlation coefficients for AFB_1–N^7-Gua, AFM_1, AFP_1, and AFB_1 of 0.65, 0.55, 0.02, and -0.10, respectively. Total AFB_1–N^7-Gua excretion in the urine of the male and female subjects over the complete urine collection period was plotted against the total AFB_1 exposure in the diet for each subject and revealed a statistically significant correlation coefficient of 0.80 ($p < 0.001$). This demonstrates that summation of excretion and exposure status provides a stronger association than was seen in prior statistical analyses, and supports that quantitation of the AFB_1–N^7-Gua adduct in urine is a reliable biomarker for AFB_1 exposure. Similar studies of AFB_1–N^7-Gua excretion have also been reported in The Gambia. Again, similar relationship between dietary exposure to AFB_1 and the adduct formation were revealed with the correlation coefficient 0.82 [38].

Several human studies [39,40] recently conducted in Taiwan used the measurement of levels of AFB_1–N^7-Gua in urine samples to examine effects of multiple risk factors for hepatocellular carcinoma on formation of this excreted adduct. One study included 43 asymptomatic hepatitis B virus surface antigen (HBsAg) carriers and 43 non-carriers randomly selected from an ongoing cohort study. AFB_1–N^7-Gua was detectable in 60% of individuals who smoked cigarettes but abstained from alcohol, in 64% of individuals who had a habit of drinking alcohol but

did not smoke cigarettes, and only 29% of those who neither smoked nor drank alcohol. There was a significant increase with age for the detection rate of urinary AFB_1–N^7-Gua. Age and habits of cigarette smoking and alcohol drinking were also found to be associated with a higher percentage of AFB_1–N^7-Gua in total AFB_1 metabolites excretion, indicating an increased activation of AFB_1. No significant association with the AFB_1–N^7-Gua level was observed for HBsAg carrier status, educational level, and ethnicity [39].

Another cross-sectional study in Taiwan was performed on 42 male asymptomatic HBsAg carriers and 43 male non-carriers. Urinary AFB_1–N^7-Gua was detectable in 40% of these subjects. HBsAg carriers had a higher detection rate of urinary adduct than non-carriers and the difference was statistically significant after multivariate adjustment. After taking into account the total AFB_1 urinary metabolite levels, chronic HBsAg carrier status, and other potential confounders, association of urinary excretion of AFB_1–N^7-Gua with the plasma level of cholesterol, alpha-tocopherol, lycopene, and alpha- and beta-carotene was observed at both low and high exposure levels of AFB_1 [40].

2.6. Modulation of AFB_1-induced DNA damage

Modulation of AFB_1-induced DNA damage by a variety of chemical agents including nutrients, antioxidants, herbs, pharmaceutical agents, and other factors such as food restriction and viral infection have been tested in various experimental models [9]. The risk for AFB_1 hepatocarcinogenesis can be modified in animals by using a number of chemoprotective agents including phenolic antioxidants, ethoxyquin (EQ), and dithiolethiones. Using a multiple dosing protocols, patterns of covalent modification of DNA by AFB_1 were determined [41]. EQ produced a dramatic reduction in the binding of AFB_1 to hepatic DNA: 18-fold initially and 3-fold at the end of the dosing period. Although binding was detectable at 3 and 4 months postdosing, no effect of EQ was observed, suggesting that these persistent adducts may not be involved in the initiation stage of AFB_1 carcinogenesis. The inhibitory effect of EQ on AFB_1 binding to DNA and tumorigenesis appeared to be related to induction of detoxification enzymes, since rats fed 0.4% EQ for 7 days showed a 5-fold increase in hepatic cytosolic glutathione *S*-transferase specific activities. Correspondingly, biliary elimination of AFB_1–glutathione conjugates was increased 4.5-fold in animals on the EQ diet during the first 2 h after AFB_1 dosing [41].

Studies have been further extended to the chemoprotective agent oltipraz, a substituted dithiolethione [42]. Male F344 rats were fed with a purified diet supplemented with 0.075% w/w oltipraz for 4 weeks. In this study, rats received 10 intragastric doses of AFB_1 (25 μg/rat/day at days 8–12 and 15–19). This 10-dose exposure to AFB_1 produced an 11% of hepatocellular carcinoma at 23 months with additional 9% of the rats exhibited hyperplastic nodules in their livers. In contrast, feeding rats with a diet supplemented with 0.075% oltipraz for a 4-week period surrounding the time of AFB_1 exposure afforded complete protection against AFB_1-induced hepatocellular carcinoma and hyperplastic nodules. None of these lesions was observed in the oltipraz-fed AFB_1-treated animals. Further, animals fed with oltipraz exhibited a dramatic (75–80%) reduction in liver DNA damage, which was mirrored by the reduction in the amount of AFB_1–N^7-Gua excreted in the urine. These data strongly support a conclusion that the AFB_1–N^7-Gua adduct is a relevant measure for risk of tumor development.

The effects of oltipraz on the kinetics of hepatic AFB_1–DNA adduct formation and removal in rats receiving multiple doses of 250 μg AFB_1/kg by gavage have also been studied [43]. Maximal level of AFB_1–DNA adducts were achieved following the third dose in the AFB_1-treated alone group, and declined thereafter despite continued exposure to AFB_1. This diminution of binding, particularly during the second dosing cycle, has been observed previously [26] and may be a consequence of the induction of GSTs or other enzymes involved in AFB_1 detoxification after chronic exposure to AFB_1 [44]. Inclusion of 0.03% oltipraz in the diet, beginning 1 week prior to dosing with AFB_1, resulted in substantially lower levels of hepatic AFB_1–DNA adducts throughout the exposure period. Binding was reduced by 76% over the initial 18-day period.

The analysis of AFB_1–N^7-Gua in serial 24-h urine samples collected from rats receiving 0.03% oltipraz in the diet showed the highest level of

AFB_1–N^7-Gua excretion occurred on day 2 in both groups after the third dosage of AFB_1. This outcome was identical to that observed with hepatic levels of AFB_1–DNA adducts and with the serum albumin adduct formation. Over the 15-day collection period in which AFB_1–N^7-Gua were detectable in the urine, feeding of oltipraz produced an overall reduction of 62% in the elimination of this AFB_1–DNA adduct excision product, mirroring the data on the overall levels of hepatic AFB_1–DNA adducts. The amount of AFB_1–N^7-Gua in urine represents only 1% of the total aflatoxin metabolites in urine and explains why the dramatic differences seen between treatment groups in AFB_1–N^7-Gua levels are not reflected in the levels of total urinary aflatoxin metabolites. Thus, these data indicate that excreted DNA adduct in urine and the formation of the serum albumin adducts accurately reflect the amount of genotoxic damage at the target organ site in the liver. In addition, these data indicate that the measurement of these adducts may reflect risk for disease development.

2.7. Possible role of persistent AFB_1–DNA adducts

In rat liver, the pseudo-half-life for loss of initial AFB_1 adduct from DNA has been reported at 7.5 h, with approximately 20% conversion of initial AFB_1–N^7-Gua adduct to AFB_1–FAPY in the first 24 h after exposure in the rat [18]. By comparison, AFB_1–DNA adducts show far greater persistence in the trout liver, with an overall approximate half-life of 21 days in fish held at 12°C [27]. Theoretically, the initial AFB_1–N^7-Gua adduct may decrease in concentration through several processes: DNA repair, conversion to the AFB_1–FAPY, apoptosis of heavily damaged cells, and growth dilution. Conversion of the initial AFB_1–N^7-Gua adduct to the AFB_1–FAPY has been found to be more extensive in xeroderma pigmentosum A (XPA) cells than in normal human fibroblasts, suggesting that the excision repair pathway is a major mechanism for enzymatic repair of AFB_1 adducts [45]. However, repair of these two AFB_1–DNA adducts by the UvrABC endonuclease, a nucleotide excision repair enzyme, of *Escherichia coli* was found to be in the similar rate [46]. In general, AFB_1–FAPY is more resistant to loss by spontaneous or enzymatic processes [45], which may have other mechanistic explanations.

Since the persistent AFB_1–FAPY derivative may be present during more than one round of DNA replication, it has been suggested to be important in tumor initiation, since a transversion mutation could be induced as a consequence of misparing at the time of replication [47]. However, several experiments found that initial differences in AFB_1–DNA adduct levels, rather than differences in adducts that persist over time correlate with differential tumor response. Rainbow trout exposed to AFB_1 have significantly greater initial liver AFB_1–DNA adducts than coho salmon [27], as do EQ-treated rats compared with controls [41]. In the fish, the low capacity for removal of AFB_1–DNA adducts has been interpreted as the high sensitivity of rapidly growing trout to AFB_1 and other bulky carcinogens [30]. Nevertheless, the reality that DNA adducts for aflatoxins (and other genotoxins) persist in total genomic DNA may play certain roles in the multistage carcinogenic process, such as in tumor promotion or progression. The correlation between persistence of DNA adducts and eventual tumor formation has been well demonstrated in several experimental models [48]. Recently, detection of the persistent AFB_1–FAPY adduct in liver tissues has been examined in a molecular epidemiologic study correlating exposure to AFB_1 and the risk of human hepatocellular carcinomas (HHC) [49].

2.8. Mutagenesis of AFB_1–DNA adducts

AFB_1–DNA adducts are capable of forming subsequent repair-resistant adducts, depurination, or lead to error-prone DNA repair resulting in single-strand breaks, base pair substitution, or frame shift mutations [50]. Mispairing of the adduct could induce both transversion and transition mutations [47]. Early work in the *lacI* non-sense assay developed by Foster et al. [15] for *E. coli*, showed that GC → TA transversion mutations were induced almost exclusively (> 90%) from microsomally activated AFB_1 [15]. In a plasmid-based system with a *lacZ*, mutational target, AFB_1, either when activated to its epoxide by microsomes or as the 8,9-dichloride, equally induced GC → TA and GC → AT mutations [45]. Hot spots for AFB_1 mutagenesis in the plasmid-based *lacZ* assay were found predominantly in relatively GC-riched regions of DNA, which also

are known to be hot spots for AFB_1–N^7-Gua formation [45].

Mutation spectra induced by AFB_1–8,9-epoxide in human fibroblast cells transfected with a shuttle vector plasmid (pS189) containing a *sup*F mutational target [51] showed that most mutations (> 90%) were base substitutions at G:C pairs, only about one-half of which were GC → TA transversion, one-third of the mutations at GG sites. AFB_1–8,9-epoxide also induced tandem double mutations at a high frequency in repair deficient XP human fibroblast cells.

The mutagenic activity of the major DNA adduct formed by the AFB_1 was recently investigated [52]. An oligonucleotide containing a single AFB_1–N^7-Gua adduct was inserted into the single-stranded genome of bacteriophage M13. Replication in SOS-induced *E. coli* yielded a mutation frequency for AFB_1–N^7-Gua of 4%. The predominant mutation was G → T, identical to the principal mutation in human liver tumors believed to be induced by aflatoxin. The G → T mutations of AFB_1–N^7-Gua were much more strongly dependent on MucAB than UmuDC, a pattern matching that in intact cells treated with the toxin. While most mutations were targeted to the site of the lesion, a significant fraction (13%) occurred at the base 5′ to the modified guanine. In contrast, the apurinic site-containing genome control gave rise only to targeted mutations. The mutational asymmetry observed for AFB_1–N^7-Gua is consistent with structural models indicating that aflatoxin moiety of the AFB_1–N^7-Gua adduct is covalently intercalated on the 5′ face of the guanine residue. These results suggest that the AFB_1–N^7-Gua adduct, and not the apurinic site, induces the genetic requirements for mutagenesis that best explains mutations in aflatoxin-treated cells.

2.9. Activation of protooncogenes

Studies in rats, mice, and fish have examined the activation of protooncogenes by aflatoxin exposure. Tashiro et al. [53] found an increased expression of c-*myc* and c-Ha-*ras* in all the hepatomas in rats resulting from administration of totaling 1.5 mg AFB_1 through i.p. injections. In one tumor, amplification and rearrangement of c-Ha-*ras* was observed. In a different study, McMahon et al. [54] examined 12 tumors in rats resulting from exposure to AFB_1 and found that the genomic DNA of 10 tumors was transforming in an NIH 3T3 cell assay. Of these transformation-inducing tumor DNAs, two of eight had an activated Ki-*ras*. Sinha et al. [55] examined four hepatic neoplasms induced in male Fischer rats by exposure to AFB_1 and AFG_1 and found three with N-*ras* activation and one with a G → A transversion at codon 12 in Ki-*ras*. High incidences of activated Ki-*ras* have also been recorded in AFB_1-induced liver carcinomas and adenomas in male F344 rats [56], with significant incidences of activated N-*ras* [55,57]. The mutations predominantly involved a G → A transition in codon 12, with a much lower incidence of G → T transversion in that codon. No mutations were observed in codon 61. In contrast to the rats, Bauer-Hofmann et al. [58] found that 40% of CF1 mice exposed to 6 μg AFB_1 at 7 days of age had neoplasms containing a *ras* mutation in codon 61, whereas Wiseman et al. [59] found a 70% incidence of such mutations in B6C3F1 mice.

Studies in trout have indicated that many of the AFB_1-initiated hepatic tumors occur with activating point mutations in the Ki-*ras*. In a well designed molecular dosimetry tumor study in trout treated with AFB_1, aflatoxicol, AFM_1 and aflatoxicol M_1, a similar proportion of tumors induced was found to contain activated Ki-*ras* alleles; the dominant mutation found in 58% of tumors was a codon 12 GGA → GTA transversion. Only one G → A transition mutation has been detected [30].

Riley et al. [60] recently studied in vitro activation of the human Ha-*ras* protooncogene by AFB_1 and found that the presence of G → T transversion either at the first or middle base of codon 12 in tumors resulting from transfection with the AFB_1-modified plasmid. They suggested that metabolic activated AFB_1 is capable of mutating this protooncogene to its oncogenic form, although such a mutation in the Ha-*ras* gene has not been reported for HHC occurring in AFB_1-exposed populations.

2.10. Relationship with human hepatocellular carcinoma

HCC is one of the leading causes of cancer mortality in Asia and Africa. In the PRC, this disease

accounts for at least 300,000 deaths/year with an incidence rate in some areas of the country approaching 100 cases per 100,000 per year. This malignancy is a major cause of cancer mortality in males behind cancer of the esophagus and stomach in the PRC [61]. This cancer varies worldwide by at least 100-fold. Over the past 30 years there have been extensive efforts to investigate the association between aflatoxin exposure and human HCC. Many epidemiological studies have found that increased aflatoxin ingestion corresponded to increased HCC incidence [8,62–65]. Several other investigations have not [66,67].

Studies by Yeh and Shen [68] on the epidemiology of HCC in the Guangxi Autonomous Region of China investigated the interaction between hepatitis B virus (HBV) infection and dietary aflatoxin exposure. The staple food of people living in this region during the 1960s and 1970s was corn, which was often contaminated with high levels of AFB_1. In some heavily contaminated areas, AFB_1 content ranged from 53.8 to 303 ppb, while the lightly contaminated regions showed AFB_1 levels in grains of less than 5 ppb. After 5 to 8 years of follow-up, HCC incidence was determined for these two regions of heavy and light aflatoxin contamination. Those individuals who were HBsAg positive and found to have heavy aflatoxin exposure had a HCC incidence of 649 cases per 100,000 compared with 66 cases per 100,000 in aflatoxin lightly contaminated areas. Those people who were HBsAg negative and eating heavily contaminated aflatoxin diets had a HCC rate of 99 per 100,000 compared with no cases detected in the lightly contaminated area [69].

Several recent studies have used newly developed molecular biomarkers for aflatoxins to confirm these putative interactions between aflatoxin exposure and HBV infection in the causation of HCC. A nested case-control study initiated in 1986 in Shanghai collected 18,244 urine samples from healthy males between the ages of 45 and 64. In the subsequent 7 years, 50 of these individuals developed HCC. The urine samples for these cases were age- and residence-matched with 267 controls and analyzed for both aflatoxin biomarkers and HBsAg status. The data revealed a statistically significant increase in the relative risk (RR) = 3.4 for those HCC cases where urinary aflatoxin metabolites were detected. For HBsAg positive people only, the RR = 7, but for individuals with both urinary aflatoxins and positive HBsAg status, RR = 59 [70,71]. These results show a causal relationship between the presence of two specific biomarkers (aflatoxin and HBsAg) and HCC risk.

In Qidong County, Jiangsu Province, PRC, liver cancer accounts for 10% of all adult deaths and both HBV and aflatoxin exposures are common. A nested case-control study within a cohort has followed [72], 804 healthy HBsAg positive individuals (728 male, 76 female) aged 30–65 years. Between 1993–1995, 38 of these individuals developed HCC and age, gender, residence and time of sampling matched serum samples from 34 of these cases to 170 controls. AFB_1–albumin adduct levels were determined by radioimmunoassay. The RR for HCC cases among AFB_1–albumin positive individuals was 2.4 (95% confidence interval, CI = 1.2, 4.7). Two other studies have been carried out in Taiwan. In a cohort of 8068 men followed-up for 3 years, 27 cases of HCC were identified and matched with 120 healthy controls. Serum samples were analyzed for AFB_1–albumin adducts by ELISA and AFB_1–DNA adducts were identified by immunohistochemical detection [73,74]. The proportion of subjects with a detectable serum AFB_1–albumin adducts was higher for HCC cases (74%) than matched in controls (66%) giving an odds ratio (OR) of 1.5. There was also a statistically significant association between detectable level of AFB_1–albumin adduct and HCC risk among men younger than 52 years old, showing a multivariant-adjusted OR of 5.3.

Wang et al. [64] examined 56 cases of HCC in Taiwan diagnosed between 1991 and 1995. Age, sex, residence and date of recruitment to 200 healthy controls from a large cohort study individually matched these cases. Blood samples were analyzed for hepatitis B and C viral markers and for aflatoxin–albumin adduct and urine was tested for aflatoxin metabolites. HBsAg carriers had a significantly increased risk for HCC and after adjustment for HBsAg serostatus, the matched OR was significantly elevated for subjects with high levels of urinary aflatoxin metabolites. When stratified into tertiles, a dose–response relationship with HCC was observed. HBsAg-seropositive subjects with high aflatoxin exposure had a higher risk than subjects with high

aflatoxin exposure only or HBsAg seropositivity only. In male HBsAg-seropositive subjects, adjusted OR was 2.8 (95% CI = 0.9–9.1) for detectable compared with non-detectable aflatoxin–albumin adducts and 5.5 (CI = 1.3–23.4) for high compared with low urinary aflatoxin metabolite levels.

In a recent study, urinary AFB_1 metabolites were measured in 43 HCC cases and 86 matched controls nested in a cohort of 7342 men in Taiwan. A total of 30 cases and 63 controls were also tested for AFB_1–albumin adducts. There was a dose–response relationship between urinary AFM_1 levels and risk of HCC in chronic HBV carriers. Comparing the highest with the lowest tertile of urinary AFM_1 levels, the multivariate-adjusted OR was 6.0 (95% CI = 1.2–29.0). The HCC risk associated with AFB_1 exposure was more striking among the HBV carriers with detectable AFB_1–N^7-Gua in urine. Compared with chronic HBV carriers who were negative for AFB_1–albumin adducts and urinary AFB_1–N^7-Gua, no elevated risk was observed for those who were positive for either marker. But an extremely high risk of HCC among those having both markers was found (OR = 10.0, 95% CI = 1.6–60.9) [75].

The relationship between aflatoxin exposure and development of HCC is further highlighted by the recent molecular studies of the *p53* tumor suppressor gene, the most common mutated gene detected in human cancers. Initial results came from three independent studies of *p53* mutations in HCCs occurring in populations exposed to high levels of dietary aflatoxin and found high frequencies of G → T transversion, with clustering at codon 249 [76–78]. In contrast, studies of *p53* mutations in HCCs from Japan and Western countries where there is little exposure to aflatoxins found no mutations at codon 249 [79]. These studies provide a circumstantial linkage between this signature mutation of *p53* and aflatoxin exposure in HCC from China and Southern Africa.

Fujimoto et al. [80] further examined HCC tissues obtained from two different areas in China: Qidong, where exposure to HBV and AFB_1 is high; and Beijing, where exposure to HBV is high but that of AFB_1 is low. They analyzed these tumor tissues for mutations in the *p53* gene and loss of heterozygosity for the *p53*, *Rb*, and *APC* genes. The frequencies of mutation, loss, and aberration of the *p53* gene in 25 HCC specimens from Qidong were 60, 58, and 80%, respectively. The frequencies in nine HCC specimens from Beijing were 56, 57, and 78%; however, the frequency of a G → T transversion at codon 249 in HCCs from Qidong and Beijing were 52 and 0%, respectively. These data show distinct differences in the pattern of *p53* mutations at codon 249 between HCCs in Qidong and Beijing and suggest that AFB_1 and/or other environmental carcinogens may contribute to this difference.

The observation of the codon 249 mutation in *p53* with aflatoxin exposure is not limited to only China and Southern Africa. Senegal is a country where HCC incidence is one of the highest in the world and where people are exposed to high levels of aflatoxins. A total of 15 HCC tissues from this country were examined for mutation at codon 249 of the *p53* gene [81]. Mutations at codon 249 of the *p53* gene was detected in 10 of the 15 tumor tissues tested (67%). This frequency of mutation in codon 249 of the *p53* gene is the highest described to date in the literature. Aguilar et al. [82] examined the role of AFB_1 and *p53* mutations in HCCs and in normal liver samples from the US, Thailand, and Qidong, China where AFB_1 exposures are negligible, low and high, respectively. The frequency of the AGG to AGT mutation at codon 249 parallel the level of AFB_1 exposure, which further supports the hypothesis that aflatoxin has a causative and probably early role in human hepatocarcinogenesis.

Results came from experimental studies have also linked aflatoxin as a causative agent in the described *p53* mutations. As previous mentioned, AFB_1 exposure causes G → T transversion in bacteria and human cells. Puisieux et al. [83] found that AFB_1–epoxide can bind to the particular codon 249 of *p53* in a plasmid in vitro. Further study [84] examined the mutagenesis of codons 247–250 of *p53* gene by rat liver microsome-activated AFB_1 in human HepG2 cells and found that AFB_1 preferentially induced the transversion of G → T in the third position of codon 249; however, AFB_1 also induced G → T and C → A transversion into adjacent codons, albeit at lower frequencies. Cerutti et al. [85] studied the mutability of codons 247–250 of *p53* with AFB_1 in human hepatocytes using the same strategy and found that AFB_1 preferentially induced the transversion of G → T in the third position of codon 249, generating

the same mutation which is found in a large fraction of HCCs from regions of the world with AFB_1-contaminated food. These experimental results support a role for AFB_1 as an etiological factor for HCCs in heavily AFB_1-contaminated areas. Using the human *p53* gene in an in vitro assay, codon 249 has been shown to be a preferential site for formation of AFB_1–N^7-Gua adducts [83], evidence consistent with a role for aflatoxin in the mutations seen in human tumors.

3. DNA damage by other mycotoxins

3.1. Ochratoxins

Ochratoxins are a group of structurally related metabolites that are produced by *A. ochraceus* and related species, as well as *P. viridicatum* and certain other *Penicillium* species [86]. The major mycotoxin in this group is ochratoxin A (OA) which appears to be the only one of major carcinogenic significance. Chemically, OA contains an isocoumarin moiety linked by a peptide bond to phenylalanine. OA is genotoxic in *E. coli* by means of induction of the SOS DNA repair activity and is mutagenic in NIH 3T3 cells expressing selected cytochrome *P*450s and carrying a shuttle vector containing the bacterial *lacZ* gene as a reporter gene [8]. Increases in sister chromatid exchange rates has been observed in CHO cells in the presence of S9 mixture and in peripheral human lymphocytes cultured in a conditioned medium. OA was also mutagenic in several *Salmonella* tester strains when this conditioned medium from OA-treated hepatocytes was used. By contrast, OA is not mutagenic in standard Ames assays, with or without S9 [8,86]. OA induced DNA single-strand breaks in cultured mouse and CHO cells. OA induced weakly positive response for induction of unscheduled DNA synthesis in primary hepatocytes from ACI C3H strain mice and ACI strain rats. Dietary feeding of OA induced renal adenomas and HCCs in mice and in rats. Carcinogenic effects on humans is suspected because of the high incidence of kidney, pelvis, ureter, and urinary bladder carcinomas among patients suffering from Balkan endemic nephropathy. In regions with Balkan endemic nephropathy, high levels of OA were found in human blood [8].

Formation of DNA adducts was studied by Pfohl-Leszkowicz et al. [87,88] using the nuclease P_1 enrichment version of the ^{32}P-postlabeling method. Several adducts were found in kidney, liver, and spleen of mice treated with three dose of OA ranging from 0.6 to 1.2 mg/kg body weight. The DNA adduct level was dose-dependent and time related. Most of the adducts disappeared within 3 days [87]. DNA adduct levels were also measured in the tissues of mice treated with 2.5 mg/kg of OA over a 16-day period. Adducts were 2-fold lower in liver and 19-fold lower in spleen than in kidney at 24 h, indicating that the kidney may also be the main target for the genotoxicity of OA as it is for nephrotoxicity. Total DNA adducts reached a maximum at 48 h when 103, 42 and 2.2 adducts/10^9 nucleotides were found, respectively, in kidney, liver and spleen. The pattern of major adducts differed between kidney and liver. All adducts disappeared in liver and spleen 5 days after administration of OA, whereas some adducts persisted for at least 16 days in the kidney [88].

3.2. Sterigmatocystin

Several species of *Aspergillus*, *Penicillium luteum*, and a *Bipolaris* species produce Sterigmatocystin. Chemically, sterigmatocystin resembles aflatoxins and is a precursor in the biosynthesis of aflatoxin. Sterigmatocystin is a hepatotoxin, but is less potent than AFB_1. It was mutagenic in the *S. typhimurium* test, the *Rec* assay, and the *B. subtilis* assay. Sterigmatocystin is carcinogenic to rats and mice, mainly inducing liver tumors. It can covalently bind to DNA and formed DNA adducts. The DNA adduct formation of sterigmatocystin was recently reviewed by McConnell and Garner [10].

3.3. Zearalenone

Zearalenone is produced primarily by *Fusarium graminearum* and is among the most widely distributed *Fusarium* mycotoxins [89]. Zearalenone has toxic effects in domestic pigs and experimental animals and is a causative agent for F-2 toxicosis and

hyperestrogenism in pigs [90]. Its carcinogenicity was tested by administration in the diet in one experiment in mice and in two experiments in rats. A dose-related increased incidence of hepatocellular adenomas was observed in female mice and of pituitary adenomas in mice of both sexes. No increase in the incidence of tumors was observed in rats [8].

Zearalenone was negative in the Ames *S. typhimurium* assay. It was also negative in a eukaryotic cell mutation assay with *Saccharomyces cerevisae*. However, zearalenone and its estrogenic metabolites showed a positive DNA damaging effect in recombination tests with *B. subtilis* [5]. It also induced sister chromatid exchange, chromosomal aberration and polyploidy in CHO cells in vitro. Its ability to form DNA adducts has been recently studied in female mice and rats using a ^{32}P-postlabeling method [91]. Treatment of mice with zearalenone (2 mg/kg i.p. or orally) leads to the formation of several DNA adducts in the liver and kidney. No DNA adducts were detected in the genital organs. The total DNA adduct levels reached 1393 ± 324 and 114 ± 37 adducts/10^9 nucleotides, respectively, in liver and kidney after i.p. treatment, indicating a 12-fold higher level in the liver than in the kidney. Oral treatment with the same dose only caused one-half of the DNA adducts in liver (548 ± 50 adducts/10^9 nucleotides). In contrast, no DNA adducts could be detected in rats after i.p. treatment. Repeated treatment with zearalenone 1 mg/kg i.p. on days 1, 5, 7, 9 and 10 caused the total DNA adduct levels were 1008 ± 235, 61 ± 1 and 17 ± 5 adducts/10^9 nucleotides, respectively, in liver, kidney, and genital organs of the mice at the end of the last dose [91].

4. Summary

Mycotoxins are toxic fungal metabolites which are structurally diverse, common contaminants of the ingredients of animal feed and human food. To date, mycotoxins with carcinogenic potency in experimental animal models include aflatoxins, sterigmatocystin, ochratoxin, fumonisin, zearalenone, and some *Penicillium* toxins, such as citrinin, luteoskyrin, patulin, and penicillic acid. Most of these carcinogenic mycotoxins are genotoxic agents with the only exception of fumonisins, which is currently believed to act by disrupting the signal transduction pathways of the mycotoxins. The aflatoxins are the most potent genotoxic agents. AFB_1 has been demonstrated to be mutagenic in many model systems including HeLa cells, *B. subtilis*, *N. crossa*, *S. typhimurium* (reverse and forward mutation), and CHO cells. AFB_1 produces chromosomal aberrations, micronuclei, sister chromatid exchange, unscheduled DNA synthesis, and chromosomal strand breaks, as well as forms adducts in rodent and human cells. AFB_1 is a potent carcinogen in many species of animals, including rodents, non-human primates, and fish. AFB_1 was classified as a category I known human carcinogen by the International Agency for Research on Cancer in 1993.

The predominant AFB_1–DNA adduct was identified as AFB_1–N^7-Gua, which derives from covalent bond formation between C8 of AFB_1–8,9-epoxides and N^7 of guanine bases in DNA. The formation of AFB_1–N^7-Gua is linear over the low-dose range in all species examined, and liver, the primary target organ, has the highest level of the adduct. Formation of initial AFB_1–N^7-Gua adduct has been proven to be correlated with the incidence of hepatic tumor in trout and rats. Initial AFB_1–N^7-Gua adduct can convert to a ring-opened AFB_1–FAPY derivative. Accumulation of AFB_1–FAPY adduct is time-dependent, non-enzymatic, and may have potential biological importance because of its apparent persistence in DNA. The AFB_1–N^7-Gua adduct is removed from DNA rapidly and is excreted exclusively in urine of exposed rats. A dose-dependent correlation between AFB_1 dose and AFB_1–N^7-Gua excreted in the urine was observed in male F344 rats. In addition, a correlation between the residual level of AFB_1–DNA adducts in liver and AFB_1–N^7-Gua excretion in urine over 24 h postdosing was found, which supports the concept that measurement of the AFB_1–N^7-Gua in urine reflects DNA damage in the liver. Several human studies have validated the similar correlation between dietary exposure to AFB_1 and excretion of AFB_1–N^7-Gua in urine.

The role of AFB_1–DNA damage in the mechanism for AFB_1 carcinogenesis has been extensively studied. Replication of DNA containing AFB_1–N^7-Gua adduct-induced G → T mutations in an experi-

mental model. Activation of *ras* protooncogene has been found in AFB_1-induced tumors in mouse, rat, and fish. More strikingly, the relationship between aflatoxin exposure and development of HHC is further demonstrated by the studies on the *p53* tumor suppressor gene. High frequency of *p53* mutations ($G \rightarrow T$ transversion at codon 249) was found to occur in HHC collected from populations exposed to high levels of dietary aflatoxin in China and Southern Africa. In contrast, no this kind of mutation in *p53* gene was found in HHC from Japan and Western countries where there is little exposure to aflatoxins.

Furthermore, AFB_1-induced DNA damage and AFB_1 hepatocarcinogenesis in experimental models can be modulated by a variety of factors including nutrients, antioxidants, herbs, chemopreventive agents, and other factors such as food restriction and viral infection, as well as genetic polymorphisms. How these factors work in high risk population for HHC are under active investigation.

In striking contrast to the vast literature on DNA damage induced by the aflatoxins, there are far fewer studies using the other major mycotoxins. To date these investigations have included ochratoxin A, sterigmatocystin, and zearaleone and this limited data set is summarized in this report. The lack of information on these other mycotoxins should not be interpreted as a statement on the public health risk of these agents. Similar to the polycyclic aromatic hydrocarbon literature, where benzo[*a*]pyrene dominates experimental studies, the aflatoxins have been extensively characterized with respect to chemistry, biology and toxicology. Indeed the aflatoxin literature serves as a template for future investigations of other mycotoxins. Finally, the recognition that human exposure to multiple mycotoxins in the diet is the rule and not the exception warrants complex mixture studies to examine the interactive toxicologic effects of mycotoxins across species.

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