INTEGRATIVE TOXICOLOGICAL EVALUATION OF AFLATOXIN B_1 EXPOSURE IN FISCHER 344 RATS AND BIOMARKER BASED EXPOSURE ASSESSMENT OF AFLATOXIN B_1 IN HUMAN POPULATIONS

by

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Under the Direction of JIA-SHENG WANG

ABSTRACT

Aflatoxin B_1 (AFB_1) is a potent toxic and carcinogenic mycotoxin, which can adversely affect the immune system in animals and humans. Traditional toxicological evaluation of AFB_1 has focused on alternations in the histopathological, serum biochemical, and immunohistological aspects, an integration of molecular biomarkers (such as AFB_1-lysine adduct, AFB-Lys) data into these changes might provide useful information for a risk assessment purpose. Biomarkers based AFB_1 exposure or risk assessment is currently favored copmared to food survey based exposure assessment. A rapid non-antibody based method for measuring serum AFB-Lys adduct in animals and humans was developed. The solid-phase-extraction based high performance liquid chromatography (HPLC) method has a limit of detection of 0.4 pg/mg albumin and recovery rates of 72.0-94.4%. The elimination kinetics of serum AFB-Lys and the toxicities of AFB_1 in F344 rats were further studied using single- (50-1000 μg/kg b.w.)

and repeated-dose (5-75 μg/kg b.w./day) designs. Several kinetic parameters of serum AFB-Lys were determined: the peak time (4 h), the half-life (2.31 days) and a conversion ratio of 1.12-1.98% at 24 h, as estimated by a physiologically based pharmacokinetic model. A linear increase in the adduct level was found only after repeated low doses (5-25 μg/kg). Liver damage, bile duct proliferation and necrosis, were most prominent at 3 days after a single dose (≥ 25 μg/kg), correlating with clinical biochemistry changes (ALT and AST) and liver glutathione S transferase placental form positive (GST-P^+) hepatocytes formation. Repeated treatment induced concurrent appearance of liver GST-P^+ foci and bile duct proliferation after 3 weeks. One-week treatment decreased the percentages of splenic $CD4^+$ and $CD8^+$ T cells as well as their production of IL-4 and IFN-γ (≥ 25 μg/kg), and TNF-α production by $CD3^-CD8a^+$NK cells (75 μg/kg). Five-week treatment increased IFN-γ production by $CD4^+$ cells (25 μg/kg), and TNF-α production by NK cells (75 μg/kg) and inhibited IL-4 production by $CD4^+$ and $CD8^+$ cells, suggesting an inflammatory response. These results provide an integrative toxicopathological evaluation of AFB_1 exposure in F344 rats. Human exposures were also assessed based on the measurement of serum AFB-Lys levels. The rank from high exposure to low is: West Africa countries (Ghana and Burkina Faso) > Southeast Countries (China and Malaysia) > Uganda > Haiti > United States. Currently, AFB_1 exposure remains prevalent in West Africa and Southeast Asia (detection rates > 90%), which warrants urgent and effective interventions. These data may facilitate human aflatoxin exposure assessment and improve our understanding of how AFB_1 can affect immune response.

INDEX WORDS: Aflatoxin, aflatoxin B_1 lysine, biomarker, toxicity, liver, spleen, exposure

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CHAPTER 1

INTRODUCTION

Problem Statement

Aflatoxins represent a group of naturally occurring mycotoxins that have long been recognized as hazardous contaminants of food, especially in peanuts and corn. Among the naturally occurring aflatoxins (B_1, B_2, G_1, G_2), aflatoxin B_1 (AFB_1) is the most potent hepatotoxic and genotoxic, and has been categorized as a known human carcinogen (Group I) by the International Agency for Research on Cancer (IARC) [1]. Acute exposure to high levels of AFB_1 via the diet causes disease (aflatoxicosis) and death in animals and humans [2]. Chronic exposure to low levels of aflatoxins is one of the major risk factors in the etiology of human hepatocellular carcinoma (HCC) in several regions of Africa and Southeast Asia [3]. In addition, AFB_1 has also been shown to be an anti-nutritional agent in animals and humans and also a potential immunotoxic agent in humans [4, 5], which may aggravate infectious diseases in the developing world, such as HIV infection [6]. Aflatoxin contamination in food remains a serious burden in the developing countries where poverty and contaminated food supplies present a major and persistent challenge. Human exposure can occur by consumption of aflatoxin contaminated commodities and the products derived from them as well as from tissues, eggs, and milk (AFM_1) of animals that have consumed contaminated feeds [7]. Occupational exposure, as noted in poultry workers though inhalation, has gained much

concern [8, 9] and deserves caution. Therefore, accurate assessment of global aflatoxin exposure is urgently needed.

Biomarkers are cellular, biochemical, or molecular alterations that can be accurately measured in biological media, such as human tissues, cells or fluids, to indicate the exposure, effect or susceptibility in individuals. The knowledge of the toxicology and metabolism of aflatoxins leads to the validation of several biomarkers that have gained wide application in human exposure assessment and efficacy evaluation of mitigation strategies for reducing aflatoxin exposure. These biomarkers include urinary AFB_1-N^7-guanine adduct, aflatoxin M_1, AFB_1-mercapturic acid, and serum AFB_1-albumin adduct. AFB_1-albumin adduct, or more specifically, AFB_1-lysine adduct, has a relative longer biological half life than other urinary biomarkers and serves as a good marker for chronic exposure for up to several months. Chemical-specific biomarkers can be used to identify stages in the development of the toxic effects/disease of environmental agents and to provide important information for critical regulatory, clinical and public health problems.

Currently available methods for measuring serum AFB_1-lysine adduct consists of enzyme linked immunosorbent assay (ELISA), immunoaffinity column based HPLC method, radioimmunoassay (RIA), and the isotope dilution mass spectrometry (IDMS) method. The results of antibody-based assays are influenced by the specificity and the sensitivity of the antibodies used. Since the major AFB-albumin adduct was identified as the AFB_1-lysine adduct, the measurement of the latter has been widely used in human studies. A non-antibody based method that doesn't require sophisticated mass spectrometry [10] seems promising towards initiation of a global network for monitoring

aflatoxins exposure or assessing efficacy of intervention strategies to reduce aflatoxin exposure.

Traditional toxicological evaluation of AFB_1 has focused on alternations in the histopathological, serum biochemical, and immunohistological aspects [1], an integration of molecular biomarkers (such as AFB_1-lysine adduct) data into these changes might provide useful information for a risk assessment purpose. Such data are relatively scarce, especially the correlation between changes in biomarker and toxicity in a time course.

The overall objective of my research is to develop a non-antibody method for measuring serum AFB_1-lysine adduct, to take an integrative toxicological evaluation of AFB_1 exposure in Fischer 344 rats, and to assess exposures to AFB_1 in different human populations based on the measurement of serum AFB_1-lysine adduct.

The hypothesis of the dissertation

We propose to establish a global network of collaborators to study aflatoxin exposure using serum AFB_1-lysine adduct as a biomarker. Our **working hypothesis** is that levels of AFB_1-lysine adduct in human serum are highly stable and correlated with aflatoxin intake, and will be a reliable biological response indicator for human aflatoxicosis in high-risk populations.

The **immediate goal** of this dissertation project is to develop a highly sensitive, non-antibody, non-radioactive, and non-mass spectrometry (MS) based analytical method for rapidly measuring serum AFB_1-lysine adduct. The method will be validated in animals treated with single or repeated doses of AFB_1 and in archived blood samples previously collected from high risk populations in developing countries. Analysis will be performed in samples from human biomonitoring and/or intervention studies conducted

in China, Ghana, and Burkina Faso. Our **long-term goal** is to establish a global network for studying aflatoxins exposure and evaluating the efficacy of chemoprevention strategies based on measuring the molecular biomarker of AFB_1-lysine adduct in serum.

The specific aims of this dissertation include:

Phase I: To develop a highly sensitive, non-antibody, non-radioactive, and non-mass spectrometry (MS) based HPLC-fluorescence method for rapidly measuring serum AFB_1-lysine adduct;

Phase II: To validate the method in animals (rats) treated with a single dose (50, 250, and 1000 μg/kg body weight) or repeated doses (5, 10, 25, and 75 μg/kg body weight for 5 weeks) of AFB_1 and in archived human blood samples previously collected from high risk populations in US, Ghana, and China.

Phase III: To evaluate the integrative toxicological approach in AFB_1 treated rats. The adverse effects of AFB_1 on the splenic immune functions, liver preneoplastic changes, and serum biochemical alternations will be compared to concurrent measurement of serum AFB-Lys adduct levels.

Phase IV: To assess human exposure in samples from human biomonitoring and intervention studies conducted in China, Malaysia, the United States, Ghana, Burkina Faso, Uganda and Haiti.

This dissertation is intended to validate an integrative toxicological evaluation of AFB_1 exposure in Fischer 344 rats and the assessment of AFB_1 exposure in different human populations using the biomarker serum AFB_1-lysine adduct. AFB_1 exposure has been linked to many human diseases including aflatoxicosis, Reye syndrome, hepatocellular carcinoma, immune and growth retardation in children. The role of AFB_1

biomarkers including serum AFB_1-lysine in the process of clinical diseases or adverse health effects related to AFB_1 remains largely unknown. The effects of AFB_1 exposure on the histological, immunohistological, clinical biochemical parameters, as well as on the cell-specific cytokine secretion in splenic lymphocytes will be explored in Fischer 344 rats through both single-dose and repeated-dose treatment protocols. Temporal correlations among these changes will be explored. The temporal changes in serum AFB_1-lysine adduct will be monitored and the kinetic parameters (e.g., peak time, half-life and elimination kinetics) of this biomarker will be validated using the non-antibody method. The results from the animal experiment will increase our understanding of the potential use of molecular biomarker into the toxicity assessment of AFB_1. Such data could be useful for delineation of functional and microstructural changes in liver and for evaluation of various intervention strategies to diminish the acute and chronic toxic effects due to AFB_1 exposure. Human exposure data from populations worldwide can serve as the first step towards a global network that reflects current human AFB_1 exposure. Such data are also important for identifying high risk populations and can be used, together with the human demographic information, to reveal potential correlations with other human disease or adverse health effects.

CHAPTER 2

LITERATURE REVIEW

General Overview

Aflatoxins were discovered in the early 1960s as the causative agent of turkey X disease in Great Britain, which caused the death of thousands of young turkeys and ducklings that were fed with a contaminated peanut meal originating in Brazil [11, 12]. The most significant changes of turkey X disease, as described by Wannop [13], were in the liver, including areas of eosinophilic parenchymatous cells with homogenous cytoplasm, swollen parenchymatous cells and their nuclei, obligation of many sinusoids, loss of the structure of the liver cord, biliary proliferation, necrosis and regeneration near the portal tract and large vacuoles. In the contaminated feed, the fungi *Apergillus flavus* and *Aspergillus parasiticus* were identified as the culprits that produce toxins [14, 15]. The toxins were characterized chemically and were designated aflatoxins [16]. From a retrospective standpoint of view, the outbreaks of turkey X disease were preceded by a number of less well-described episodes of epizootics in different animal species [17]. The outbreak of turkey X disease marked a new era for the intensive research on the toxicology and biochemistry of mycotoxins.

The aflatoxins species B and G are designated according to their fluorescence, e.g. B species (B_1, B_2) show blue fluorescence and G species (G_1 and G_2) show greenish-yellow fluorescence under UV light. Chemical synthesis demonstrated that aflatoxins are a highly substituted coumarin structure containing a dihydrofurofuran moiety (Figure

2.1). The structural characterization and total synthesis of aflatoxins were accomplished in 1963 [16], which greatly enhanced mechanistic research of their toxicity and carcinogenicity.

Extensive assays were conducted in a wide range of animal models to assess the acute and chronic toxicity of aflatoxins starting from the early 1960s [18, 19]. Among the naturally occurring aflatoxins, aflatoxin B_1 (AFB_1) is the most potent in terms of acute toxicity, mutagenicity, and carcinogenicity [1]. Acute exposure to a single large dose of AFB_1 leads to massive liver damage, including edema, periportal necrosis and bile duct proliferation, and death in rodents. Along with the histological changes, altered liver enzyme activities (ALT, AST) and induction of altered foci of hepatocytes (preneoplastic lesion) are frequent outcomes within a few days to several weeks after administration [20, 21]. Chronic studies demonstrated that aflatoxins are complete carcinogens and that increasing the dose decreases the latency for tumor development [22]. An early study showed that the effective carcinogenic dose of AFB_1 is less than 0.5 mg per rat [23]. Repeated low dose exposure is more efficient than a single large exposure, which is consistent with the property of a complete carcinogen. A metabolism study led to identification of the major AFB_1-DNA adducts in 1977 [24]. Dose-dependent AFB_1-DNA adduct formation in rat liver occurs over a range of doses up to several orders of magnitude. The early development of analytical methods for quantifying aflatoxins from foods represents extensive international collaborations. These physical, chemical, and immunologic analytical methods [10] greatly facilitated the regulation, monitoring, and reducing of aflatoxins contamination in foods. Based on these methodologies, many epidemiologic studies have been conducted to evaluate the association of aflatoxin intake

and the development of hepatocellular carcinoma (HCC) in human populations [4]. The development and validation of aflatoxin biomarkers, including AFB_1-DNA adduct, serum aflatoxin-albumin adduct, aflatoxin M_1, and aflatoxin B_1 mercapturic acid, have provided the tools to evaluate the molecular epidemiology of aflatoxin exposure and risk to individuals in China and sub-Saharan Africa [25]. These lines of investigation are still being conducted in current efforts to develop effective intervention strategies to mitigate the health impacts of aflatoxin exposures. The human data from such studies and accumulated experimental evidence provided the basis for the classification of AFB_1 and its mixtures as a human carcinogen by the International Agency for Research on Cancer (IARC) in 1993 [1]. The time line for key events in the discovery, toxicological evaluation, molecular epidemiology, and regulation of aflatoxins has been nicely shown in a recent review (Figure 2.2) [4].

Aflatoxin exposure remains a largely ignored global issue. It is estimated that approximately 4.6-28.2% of global annual HCC cases are attributed to aflatoxin exposure alone [3]. In the developing world, more than 40% of all disease burden are estimated to be associated with aflatoxin exposure [2]. These chronic health effects together with the growth impairment in children [5] point to the urgent needs for effective intervention strategies to reduce the risks associated with aflatoxins. After the severe outbreak of aflatoxicosis in Kenya [26], an international workshop has initiated public health strategies for reducing aflatoxins exposure in the developing countries [27]. Intervention strategies, which aim to block, retard, reverse the exposure and risk of aflatoxins, have been a focus of research that is ongoing. The approaches of preventions that have been studied include improving of postharvest storage conditions, using trapping agent (e.g., clay) to reduce

bioavailability or agents (e.g., green tea polyphenol, chlorophyllin, and dithiolethiones) to alter the metabolic pathway to reduce risk caused by aflatoxins exposure [2, 28, 29].

The strategy to define the risks from aflatoxins exposure and to design approaches to prevent their effects serves as a template for the development, validation and application of molecular biomarkers for other environmental agents or their resulting chronic diseases. This strategy also provides a model for the design of future studies for risk assessment of exposure to other environmental agents. Key elements of such a strategy has been summarized by Kensler et al. [4], which include: the use of animal models to mimic the human disease; designing observational studies associating exposure with disease; development of mechanism-based molecular biomarkers in animal models; validation of these biomarkers in animals by dose-response studies; validation of transitional human studies; and association of these biomarkers with disease outcome in prospective studies.

Toxicokinetics of Aflatoxin B_1

Absorption

The rate of absorption of AFB_1 may be affected substantially by the route of exposure. Being a low molecular, lipophilic molecule, the absorption of AFB_1 from the gastrointestinal (GI) tract is fast and efficient. The distribution of radioactivity after oral or intraperitoneal (ip) injection of [^{14}C] AFB_1 in Male F344 rats is similar [30], suggesting efficient absorption after ingestion. Both species and strain differences play a role in the absorption of AFB_1 through oral administration. For example, the rate of stomach emptying in rat is slower than that in mouse [31], which may affect the rate of absorption through the GI tract. Within 24 h of an oral administration of [^{14}C]AFB_1, up to 20 to 60%

of the radioactivity was eliminated in feces in F344 rats [30], whereas approximately 10-30% of total radioactivity was excreted in the bile in Wistar rats [32].

A previous study showed that the rate of AFB_1 uptake in intestinal tissue is proportional to AFB_1 concentration [33], suggesting a mode of passive diffusion; intestinal absorption of aflatoxin is more efficient in suckling than older rats. Therefore, lipophilicity of aflatoxins and the composition of the intestinal epithelium may be important determinants of aflatoxin absorption. The aflatoxin metabolism that occurs in the GI mucosa represents a major AFB_1 detoxification site as approximately 20-25% of the ingested dose can reach the liver [34]. This is supported by a report that cytochrome P450 (CYP) 3A4 protein content is 3 times higher in the enterocyte homogenates than in the liver homogenates [35]. Reabsorption of AFB_1 conjugates through enterohepatic recirculation was found especially at higher doses (0.5-2.5 mg/kg) but not at low dose (0.05 mg/kg) [32]. A study of AFB_1 absorption in lactating Holstein dairy cows showed that after a single bolus of [^{3}H]AFB_1 (4.89 mg), plasma AFM_1 can be detectable at 5 min (10.4 ng/L) after treatment, which peaked at 25 min (136.3 ng/L) after treatment [36]. The author suggested that AFB_1 uptake and metabolism may occur in mouth or oesophageal mucous membranes, before the rumen compartment. This study also demonstrated that vaginal implant of AFB_1 lead to plasma detectable AFB_1 and AFM_1 at 30 min after treatment, suggesting an absorption mediated by passive diffusion.

Human exposures to aflatoxins occur mainly through ingestion of contaminated foods, including corn, peanuts, cottonseed, tree nuts and pepper. This issue is most serious in developing countries that lack proper processing procedures, storage facilities, and food-safety monitoring [37, 38]. Infants or young children can be exposed to AFM_1

through ingestion of breast milk or dairy products that are contaminated by AFM_1. Occasionally, the meat products intended for human consumption can be contaminated by aflatoxins, which represent a much less common source of exposure [39].

Although dietary exposure to AFB_1 has been widely recognized, evidence has been accumulated to suggest potentially high risks of occupational exposure to AFB_1 through inhalation [40]. During the processing of foods such as rice or maize, workers are exposed to aflatoxin producing fungi through dust where aflatoxins have been isolated [41, 42]. When human sera were collected, the AFB_1-albumin adduct levels were detected to be higher in workers exposed to dust with detectable aflatoxins than workers who were not exposed [43]. It was reported that AFB_1 was present in the lungs of one textile and two agricultural workers who died from pulmonary interstitial fibrosis [44], and that AFB_1 was found in the lungs of chemical engineers who had worked for 3 months on a method for sterilizing Brazilian peanut meal contaminated with *Aspergillus flavus* and died of alveolar cell carcinoma [45]. Thus, inhalation is an important route for occupational exposure. Recently, airborne aflatoxins have been assessed for their levels during a grinding phase. When a batch of imported shelled peanuts imported from Vietnam was ground wet, the level of aflatoxins was shown to be negligible (0.11 pg/m^3) [46]. However, the contamination level in the peanuts or shells was not provided, which may compromise this conclusion.

Human data with regards to the rate of absorption of aflatoxins are not as abundant as that in animal species. Whether AFB_1 can penetrate isolated human epidermis (stratum corneum plus viable epidermis) was tested in vitro [47]. [^{14}C] AFB_1 (7.5 - 9.3 μg) was applied to the stratum corneum of epidermal disks mounted in Teflon

diffusion cells. [^{14}C]AFB_1 penetrated through the isolated epidermis in chemically unaltered form, at a slow rate under nonoculated conditions but at a rate that was approximately 40 times greater when the skin was occulated. The maximum velocity of penetration was 0.63 ± 0.71 and 27.31 ± 10.15 pmoL/h under conditions of exposure to ambient conditions and occlusion, respectively. Chloroform extractable radioactivity accounted for 82.5 ± 3.7% of the total penetrating radioactivity in the receptor fluid of the diffusion cells. More than 95% of the applied dose penetrated epidermis within 46 h. Total recovery was 98.6 ± 6.4%, as estimated by a percentage of the applied radioactivity. These results demonstrate the features of AFB_1 absorption through skin: slow and near complete, but fast under conditions of oculation which increase the hydration of the skin. Human volunteers that were orally give low doses of [^{14}C] AFB_1 (30 ng, 5 nCi) demonstrated a peak total radioactivity in plasma at 1 h after exposure [48], suggesting rapid absorption through the route of gastrointestinal (GI) tract.

Distribution

Different animal models had been used to compare the biological fate of free AFB_1 in blood. A single iv dose of [^{14}C]AFB_1 at 0.3, 1 or 1.5 mg/kg was given to the rhesus monkey, Sprague-Dawley rat and Swiss-Webster mouse, respectively, which are approximately 1/10 of the LD_{50} doses, respectively. The apparent volumes of distribution were calculated to be 114%, 47%, and 28% for the monkey, rat and mouse, respectively [49]. This ranking is consistent with the susceptibility of these species to AFB_1 toxicity. Liver is the major site for concentration of AFB_1, especially at low doses. The radioactivity in liver was 5-15 fold higher than other tissues at 30 min after administration of [^{14}C]AFB_1 [30]. Within a few hours, most of the AFB_1 in liver is bounded

to macromolecules. For instance, it was found that about 15% of the AFB_1 dose is retained in rat liver at 2 h after treatment, and 70% of the retained dose is covalently bound adducts [50].

The tissue distribution of AFB_1 appears to be species-specific. After 2 weeks administration of $[^{14}C]AFB_1$ to broiler chickens and 5 h after the last dose, about 96.64% of the ^{14}C was excreted. Of the ^{14}C retained, 11.04, 9.83, 4.30, 12.52, 31.66, and 30.63% were detected in the blood, liver, heart, gizzard, breast, and leg, respectively [51]. In a 35 days feeding trial with AFB_1 in swine, the highest concentrations of aflatoxins were found in kidney and liver, and substantially lower concentrations were found in muscle and adipose tissues [52]. In rats that were given ip doses of $[^{14}C]$ AFB_1, radioactivity was present at maximum levels in liver and kidney 0.5 h after administration, with small amounts present in other organs. The concentration of radioactivity was 5 to 15 times greater in liver than in other tissues, and at the end of 24 h, the liver contained an amount equal to the content of the remainder of the carcass [30]. This finding is associated with the relative tissue specificity of AFB_1 as a hepatotoxin in rats.

Accumulated experimental evidence has shown that aflatoxin or its metabolites can reach the developing fetuses. In mice that were treated with $[^{14}C]AFB_1$, radioactivity were found in the eyes of the fetuses although their livers did not accumulate radioactivity [53]. Similar results were found in pregnant mice, the fetuses showed distinct uptake of radioactivity in the pigment layer of the eyes and in the nasal mucosa, the liver had only small amounts of radioactivity although the liver in the mother had a very high concentration [54]. Transplacental transfer of aflatoxin in humans have been confirmed by many studies showing the presence of aflatoxin and/or its metabolites in the cord sera [55-

[58]. Although several metabolites have been identified in the cord sera, it is not clear whether they are formed in placenta or are of maternal origin. A recent study has reported that aflatoxicol is the only metabolite detected in both human placenta perfusion and *in vitro* incubation studies [57]. If aflatoxicol is in fact the only metabolite in placenta, then aflatoxin metabolites found in cord sera (e.g., aflatoxin-albumin adducts) may come from the maternal blood rather than being a product of placenta metabolism. Although less mutagenic than AFB_1, aflatoxicol still has higher mutagenicity than other metabolites [59], therefore, the placenta may not be considered as a significant protective barrier for fetal aflatoxin exposure.

Biotransformation

The intestinal mucosa serves as a primary site of metabolism, reducing the amount of aflatoxin available to the liver. Once absorbed, the liver is the major site for biotransformation and metabolism of aflatoxins. The aflatoxins undergo phase I and phase II metabolic process in liver. Phase I reactions include hydroxylation, epoxidation and demethylation of aflatoxins, producing aflatoxin M_1, Q_1, P_1. Different members (1A2, 3A4, 3A5 and 3A7) of the cytochrome P450 (CYP450) enzyme family have been found to be responsible for aflatoxin metabolism [60]. Compared with CYP 3A4, human liver microsomal CYP 1A2 is reported to have a higher affinity toward low AFB_1 concentrations that are more reflective of dietary exposures [61]. This differential metabolism of these two isoforms was also found in human bronchial cells [62]. On the other hand, a significant correlation between CYP 3A4 expression (but not CYP 1A2) and AFB_1-DNA adduct levels was found in adult human livers [63]. Using a hepatic abundance model that takes into account the specific kinetic parameters and the

expression levels of these CYP450s, Kamdem et al. [60] compared the relative production rates of the carcinogenic metabolite, AFB_1-8,9-epoxide, by different CYP450s. They found that CYP 3A4 is the most important determinant towards the epoxide formation, followed by CYP 3A5, 3A7, and 1A2 [60]. In addition, CYP450 specific metabolites have been found, for instance, 3A4 catalyzes hydroxylation of AFB_1 to produce AFQ_1 while 1A2 generates another hydroxylated metabolite, AFM_1. Taken together, CYP1A2 has a higher affinity but a lower capacity towards metabolic activation of AFB_1 and conversely CYP3A4, has a much larger capacity but a lower affinity. Unlike the reduced metabolite aflatoxicol that is excreted in urine without further metabolism or in feces after glucuronyl conjugation [64], AFM_1 can be further metabolized to form AFM_1-8,9-epoxide. This reactive epoxide binds to DNA producing AFM_1-N^7-guanine, which can be detected in the liver DNA and urine of tree shrew and rats 24 h after treatment with AFB_1 (400 μg/kg) [65]. Tree shrews produced twice as much AFM_1-N^7-guanine adducts compared to rats (0.74 ± 0.14 pmoL/mg DNA vs 0.37 ± 0.07 pmoL/mg DNA). However, in the rat liver, the ratio of AFB_1-DNA adducts to AFM_1-DNA adducts is about 20 (6.56/0.42 pmoL/mg DNA). This metabolic difference might account for the sensitivity of rat to AFB_1 hepatocarcinogensis.

The lung is also a target organ for aflatoxins. Tissues derived from various portions of the lung have been used as *in vitro* models to study AFB_1 metabolism and activation [66]. AFB_1 can be metabolically activated by intact human tracheal epithelium from the bronchus and in human epithelioid lung cells *in vitro*. 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 [66]. Inhalation of AFB_1 by rats for 20-120 min resulted in an increase in AFB_1-N^7-Gua adduct in a dose-dependent manner.

Of the metabolic products produced during phase I reactions, the most biologically important is the highly reactive AFB_1-8,9-epoxide [67]. This metabolite has been found to exist in 2 stereoisomers, of which the *exo* isomer is much less stable in water and approximately 500 times as mutagenic as the *endo* isomer. The increased mutagenicity of the *exo* isomer is attributed to its steric configuration that allows covalent binding of DNA, almost exclusively between the N^7 of guanine and the C^8 of the 8,9-*exo*-epoxide [68-71]. The rate of conjugation of glutathione (GSH) with AFB_1 *exo*-epoxide is an important factor in determining the species variation in risk to aflatoxin hepatocarcinogenicity. The *endo*-epoxide was found to be a good substrate for GSH conjugate formation in rat liver cytosol while mouse liver cytosol conjugated the *exo*-epoxide almost exclusively [72]. This difference may partly account for the resistance of mouse to the hepatocarcinogenicity of AFB_1.

The Phase II biotransformation leads to conjugation of phase I metabolites of aflatoxins forming less toxic or carcinogenic products than the parent compound. This reaction also increases the polarity and facilitates the excretion of the conjugates from urine and feces. The detoxification pathway of aflatoxin is shown in Figure 2.3 [73]. Liver cytosolic glutathione S transferase (GST) plays a critical role in the detoxification of AFB_1-8,9-epoxide by conjugating glutathione with the epoxide [74]. Glutathione conjugation is an important reaction that determines the susceptibility of different species to the toxicity of AFB_1, with a most striking difference observed between the mouse and the rat [75].

Genetic polymorphism of the metabolic enzymes also affects the susceptibility to the toxicity and carcinogenicity of aflatoxins in different animal species or possibly ethnic human groups [76]. CYP3A5, GST, and epoxide hydrolase (EH) polymorphisms are of biological significance in particular. CYP3A5 exhibits genetic polymorphism known as CYP3A5*3, CYP3A5*6, and CYP3A5*7 [77, 78]. It is estimated that approximately 90% Caucasians, 67% Japanese, and 30-45% African-Americans are CYP3A5 low expressors [77-79]. Consistently, Wojnowski et al. reported increased plasma levels of AFB_1-albumin adducts in CYP3A5 high expressors from Gambia, particularly in those with low concomitant CYP3A4 expression [80]. It is conceivable that the individual expression levels of CYP450s producing AFB_1-8,9-epoxide could affect the individual risk to develop HCC.

Detoxification pathways prevent formation of DNA adducts, which involve the glutathione conjugation of reactive metabolites by GST or hydrolysis by microsomal epoxide hydrolase (mEH) [81, 82]. There are two types of genetic polymorphic GSTs that are responsible for AFB_1 detoxification: GST-μ encoded by the *GSTM1* gene and GST-θ encoded by *GSTT1*. Homozygous deletion of part of these genes (null genotype) results in enzyme deficiency and might therefore lead to hampered detoxification [83]. The frequencies of homozygous *GSTM1* and *GSTT1* deletion carriers are very high (i.e., 20-50%) in most populations studied to date [78], and the risk conferred to individuals who carry homozygous deletions in *GSTM1* and *GSTT1* is increased when the interactions of these genotypes with other environmental carcinogens are involved (e.g., odds ratio increasing from less than 2 to 3-5) [84]. In several studies, it has been shown that only the *GSTM1* genotype carriers are at increased risk of HCC in populations exposed to

aflatoxins [76, 85-87], although GST-θ has also shown high efficiency for conjugation of glutathione to AFB_1-8,9-epoxide [88, 89]. It has been noted that the deletion genotypes are so common in the general population that the proportion of cancer attributable to these variants may be large, however, these genotypes may be less suited for individual cancer risk assessment because of their relatively small contribution to the absolute risk of cancer [84]. Besides carcinogen metabolizing enzymes, the polymorphism of DNA repair (*XRCC1*) enzyme has been shown to be related to the risk of HCC development in Gambia, West Africa [90] where aflatoxin exposure is high. In that hospital based case-control study (216 HCC cases and 408 controls) [90], a multivariable analysis (adjusting for demographic factors, hepatitis B virus, hepatitis C virus, and TP53 status) revealed that the heterozygote *XRCC1*-399 AG genotype were significantly associated with HCC (OR, 3.18; 95% CI, 1.35-7.51), although the prevalence of variant genotypes was generally low.

Human mEH catalyzes the conversion of a broad array of reactive epoxide metabolites to more polar trans-dihydrodiol derivatives. The gene has been shown to exhibit polymorphism, including variation in the coding region leading to amino acid substitutions at positions 113 (Y/H) and 139 (H/R) [91]. *In vitro* studies have shown that human mEH provides no statistically significant changes to the rate of hydrolysis of AFB_1, benzo[a]pyrene-4, 5-oxide, and cis-stilbene oxide [92, 93], compared to the rate of spontaneous hydrolysis or the variants of mEH proteins. Such evidence suggests that human mEH may not play an important role in detoxification of AFB_1.

Excretion

Previous studies showed that after an oral or iv dose of [^{3}H]AFB_1 in Sprague-Dawley rats, more than half of the total radioactivity is excreted into feces [94]. The major biliary metabolite was AFB_1-glutathione, accounting for 49-57% of the total biliary excretion, and AFP_1-glutathione accounts for 4-15% of total biliary radioactivity in Sprague-Dawley rats [50]. Urinary excretion represents another important route of excretion. Approximately 10-20% of a single dose of AFB_1 is excreted in urine 24 h after ip administration to the rat, and the major urinary metabolites are determined to be AFM_1, AFP_1, and AFB_1-N^7-guanine [95].

Using a monoclonal antibody specific for aflatoxins, Groopman et al. detected the urinary metabolites of AFB_1 in urine from AFB_1 treated rats and human subjects from Guangxi province of China where aflatoxin exposure is high [96], the major urinary metabolites are AFB_1-N^7-guanine, AFM_1 and AFP_1 in both rats and humans. The fecal and urinary excretion of 3 AFB_1 metabolites, AFM_1, AFQ_1, and AFB_1-N^7-guanine in humans has been assessed in another study [97]. Eighty-three young Chinese males were selected from 300 people based on detectable urinary AFM_1. The fecal excretion of AFQ_1 (median, 137 ng/g fresh weight) was approximately 60 times higher than that of AFM_1 (median, 2.3 ng/g). In urine, the median levels of AFQ_1, AFM_1, and AFB_1-N^7-guanine were 10.4, 0.04, and 0.38 ng/mL, respectively. A subgroup (n = 14) with HBV infection had significantly higher fecal concentrations of AFQ_1 ($P = 0.043$) and AFM_1 ($P = 0.001$) than those who were not HBV positive. That study demonstrated that AFQ_1 is excreted in urine and feces at higher levels than AFM_1, and feces are an important route of excretion

of these AFB_1 metabolites. However, whether AFQ_1 should be suitable to be used as a marker for exposure and risk of dietary aflatoxin intake remains unclear.

The nutrient status seems to affect the excretion of aflatoxins. Children with kwashiorkor (n = 5) and marasmic kwashiorkor (n = 7) were investigated for the urinary and fecal excretion of aflatoxins [98]. Aflatoxin-free diet consisting of maize meal and milk powder was provided, and urine and feces samples were collected on admission and day 4 and 10. The children with kwashiorkor excreted aflatoxins in stools for up to 9 days and ceased urinary excretion after 2 days. The children with marasmic kwashiorkor excreted aflatoxins in stools for 6 days and ceased urinary excretion after 4 days. In stools, AFB_1 was most frequently detected in kwashiorkor and least frequently in marasmic kwashiorkor.

Toxicity of Aflatoxin B_1

Acute Toxicity

The aflatoxins were discovered because of their acute toxicity in poultry. The acute toxicities of aflatoxins and their metabolites have been tested in different animal species. Overall, AFB_1 is the most toxic form among the naturally occurring aflatoxins, as determined in duck [99, 100] and rat [99]. The acute toxicity varies up to several orders with regards to the median lethal dose (LD_{50}), as shown in Table 2.1 [99, 101-104]. It can be seen from Table 2.1 that age is an important factor affecting the acute toxicity to AFB_1: young mouse and rat are more susceptible to the toxicity of acute AFB_1 exposure than their adults. The rank of potency in terms of acute toxicity from high to low is $B_1 > G_1 > B_2 > G_2$ in rat and duck [99]. Acute exposure to AFB_1 causes a series of symptoms, including abdominal pain, nausea, jaundice, bleeding in the GI tract, and even death (aflatoxicosis).

The histopathological effects and biochemical changes induced by AFB_1 exposure have been extensively studied in animal models [18, 19, 105]. However, only a few studies [20, 106] reported the sequential biochemical and histological changes, in which rats were given either one single large dose of 3 mg/kg body weight (BW) or 7 mg/kg BW. In particular, no studies have been done to explore the correlations between biochemical and histopathological alterations with specific molecular targets via single or repeated treatment protocols. Rat liver GST-P positive ($GST\text{-}P^+$) foci are a specific and reliable preneoplastic marker used widely in animal carcinogenesis models [107]. Short-term exposure to AFB_1 induces $GST\text{-}P^+$ hepatocytes or foci in rat liver both independently and synergistically with other toxins, such as fumonisin [108] and microcystin LR [109]. However, the sequential development of $GST\text{-}P^+$ foci caused by AFB_1 as well as the correlations with biochemical and histological changes in animal models remains largely unknown.

Human Aflatoxicoses

Historically, the outbreaks of human aflatoxicoses have been reported especially in the developing countries [2, 110]. These outbreaks have been frequently reported from India [111, 112], Kenya [113], and Malaysia [114, 115]. A case was also reported in Uganda [116]. These acute aflatoxicoses are as summarized in Table 2.2. During the first reported outbreak in India (1975), analysis of contaminated samples showed that affected people could have consumed between 2 and 6 mg of aflatoxin daily over a period of one month. Affected people (n = 397) showed jaundice, rapidly developing ascites, portal hypertension, and a high mortality rate. This outbreak was associated with consumption of heavily contaminated maize [111]. The second outbreak in India involved 994 cases who had consumed aflatoxins contaminated maize [112]. Willis et al. reported a case that a 25-

year-old woman who attempted suicide twice by ingesting mixtures containing 15-45% AFB_1. She ate 5.5 mg over 2 days and 6 months later 35 mg more over 2 weeks. Following the first exposure, she was admitted to hospital with a transient, nonpruritic, macular rash, nausea and headache; the second time she reported nausea only [117]. On both occasions, physical and laboratory examinations were normal and liver biopsies appeared normal by light microscopy. After 14 years follow-up examination, the women remained healthy and no any signs or symptoms of disease or lesions were revealed. These findings suggest that the hepatotoxicity of AFB_1 may be lower in well nourished persons than in experimental animals or a longer latent period than 14 years would be expected for tumor formation. The outbreak of aflatoxicosis in Kenya (2004 and 2005) represented the most severe case of aflatoxicosis that caused 125 deaths and 317 cases which resulted from the ingestion of heavily contaminated maize and maize product [118].

Reye Syndrome

Reye syndrome is characterized by sudden brain damage and liver function problems of unknown cause. Although the syndrome has occurred in children who have been given aspirin or phenothiazines when they have chicken pox or the flu [119], aflatoxins have also been suggested as a potential etiological factor in Reye syndrome because of the similar changes induced by aflatoxins, such as encephalopathy and fatty degeneration of viscera [120]. Concurrent aflatoxin exposure and Reye syndrome in children were reported in children in Czechoslovakia. Of the 26 children with Reye syndrome, all of them had detectable AFB_1 in liver and 15 children also possessed detectable AFM_1 [121]. None of the 25 control children had detectable AFB_1 or AFM_1 in the liver [122]. It was also reported in New Zealand that two children with Reye syndrome had detectable AFB_1 in

the liver tissues [123]. Several reports in the United States showed that AFB_1 is 100% detectable in blood or liver tissues of children with Reye syndrome [124, 125]. In Thailand, a case-control study showed that AFB_1 was more frequently detected in the liver, brain, and kidney tissues from children who died of Reye syndrome than the control children who died of other diseases [126]. An association between AFB_1 exposure and Reye syndrome is suggested by these case reports and studies. However, since AFB_1 is not regularly recoverable from cases of Reye syndrome at a high rate, the causal effect was questioned [127]. Therefore, the causal effect of AFB_1 remains to be confirmed in more carefully designed studies particularly with a prospective design.

Chronic Toxicity: Hepatocellular Carcinoma

Based on sufficient evidence from animal experiments and human epidemiological studies, aflatoxins have been established as a known human carcinogen [1]. Approximately 4.5 billion people worldwide are estimated to be chronically exposed to large amounts of aflatoxins [2]. Among the 550,000-600,000 new HCC cases worldwide each year, about 25,200-155,000 may be attributable to aflatoxin exposure, as estimated by Liu and Wu [3]. There is a broad ecologic association between areas of high aflatoxin exposure (such as China, Southeast Asia, and parts of Africa) and HBV, which is endemic in these areas; rates of HBV positivity in Gambia, China, and Guinea are reported to be 15% [128], 14–20% [129], and 10% [130], respectively. Independently, each factor significantly increases the relative risk of cancer, and most studies report them to be synergistic [131], increasing the risk of cancer 25 to 30-fold; there is, however, one epidemiologic study that contradicts those findings [132]. Aflatoxin exposure multiplicatively increases the risk for development of human HCC for people chronically

infected with hepatitis B virus, as demonstrated by several human studies that employed molecular biomarkers (e.g., urinary AFB_1 metabolites and serum aflatoxin-albumin adducts or codon 249 mutations) to improve individual risk assessment [133-136]. Recently, a systematic review and meta-analysis reported the population attributable risk (PAR) of aflatoxin-related HCC [137]. The PAR of aflatoxin-related HCC was estimated to be 17% overall, which increased to 23% when the one study that contributed most to heterogeneity in the analysis is excluded. The summarised odds ratios (OR) of HCC with 95% CIs are 73.0 (36.0-148.3) from the combined effects of aflatoxin and HBV, 11.3 (6.75-18.9) from HBV only, and 6.37 (3.74-10.86) from aflatoxin only. These data indicate that aflatoxin multiplicatively interacts with HBV to induce HCC and reducing aflatoxin exposure to non-detectable levels could reduce HCC cases in high-risk areas by about 23%. Whereas the importance of this synergy to cancer is well recognized [138, 139], its importance to other health issues is largely unknown and clearly should be established.

A number of possible mechanisms for the interaction have been suggested [140-142], which include, among others:

- Chronic HBV infection may induce the CYP450s that metabolize inactive AFB_1 to the mutagenic AFB_1-8,9-epoxide.
- Nuclear excision repair, which is normally responsible for removing AFB_1-DNA adducts, is inhibited by HBVx protein.
- HBVx protein may also increase the overall frequency of DNA mutations, including the *p53* 249^{ser} mutation.
- The fixation of AFB_1-induced mutations in the presence of liver regeneration and hyperplasia induced by chronic HBV infection.

- The predisposition of HBV-infected hepatocytes to aflatoxin-induced DNA damage.
- An increase in susceptibility to chronic HBV infection in aflatoxin-exposed individuals.
- Oxidative stress exacerbated by co-exposure to aflatoxins and chronic hepatitis infection.

These mentioned mechanisms remain to be confirmed and inconsistent results have been found, e.g., an in vitro study has recently showed that in HepaRG cells, AFB_1 exposure (up to 5 μM) decreases HBV replication, whereas AFB_1 induced DNA damage (AFB_1-DNA adduct formation) and subsequent *p53* alteration (mutation at codon 249) is not affected by the presence of the virus [143].

Immune Toxicity

Immune suppression in experimental animals has been a common outcome of aflatoxin exposure. AFB_1 causes immune suppression in diverse laboratory and domestic animal models, with the cell-mediated immunity being the most affected [144]. A considerable amount of evidence from both *in vivo* and *in vitro* studies has demonstrated that AFB_1 affects immune functions [144, 145]. AFB_1 decreases splenic helper T cells and antibody response to sheep red blood cells (SRBC) in C57BL/6 mice [146] and inhibits production of IL-2, IL-3, and IFN-γ by splenic macrophages [147]. Phagocytosis and production of nitric oxide and TNF-α by macrophages are inhibited by AFB_1 both *in vivo* and *in vitro* [148, 149]. In weaning rats, exposure to AFB_1 decreases spleen and thymus weights, inhibits phagocytic capacity of macrophages [150] and suppresses delayed type hypersensitivity response [151]. Dietary exposure of rats to AFB_1 (0.01-1.6 ppm) produces

inhibitory effects on splenic T and B lymphocytes as well as IL-1, IL-2, and IL-6 production by splenocytes [152]. In addition, chick and swine are also susceptible to AFB_1 induced alterations in cell-mediated immune response [153-155] and AFB_1 has been shown to stimulate the expression of pro-inflammatory cytokines such as TNF-α, IFN-γ, and IL-6 [156]. Besides dietary exposure, inhalation of aflatoxins is also capable of reducing suppression of pulmonary and systemic host defense in mice and rats [157].

The complement components, as part of the innate immune system, helps antibodies and phagocytic cells to clear pathogens from an organism. The activities of complements have been shown to be depressed due to aflatoxin exposure in various animal models [158-161], including chicken, Guinea pig, swine and cattle. The observed depressive effects on complement activity may be related to the ability of aflatoxins to interfere with protein synthesis. Early research had also shown that aflatoxin exposure reduces the resistance of several animal species to bacterial, viral, fungal, and parasitic infection [162, 163]. Noteworthy, beneficial effects of aflatoxin exposure to bacterial infection were also reported. A nonspecific humoral factor appeared to be affected in the aflatoxin-impaired acquired immunity to *P. multocida* in turkey poults because serum from normal or immunized turkeys was beneficial in overcoming the impairment [164]. Similarly, oral exposure to aflatoxin decreased bacterial counts in intestinal tract and mesenteric lymph nodes of hamsters that were infected with mycobacterium paratuberculosis, although aflatoxin treated hamsters showed inhibitory growth and liver lesions [165]. The effects of aflatoxins on the resistance of animals to infectious disease agents are somewhat variable, depending on the organism, toxin dose and formulation, species, and perhaps sensitivity of the test [164].

Only circumstantial evidence supports the causative effects of AFB_1 exposure to suppression of human immune functions. Of significance is the cohort study investigating the effects of aflatoxin exposure on the immune functions in Gambian children [166]. The level of secretory IgA (sIgA) in saliva was significantly lower in children with detectable levels of serum AFB_1 albumin adduct than those with non-detectable levels. Jiang et al. found an association between higher levels of serum AFB-Ly adducts and decreased $CD8^+$ cell function in human blood [167]. Prospective studies are needed to confirm this finding. The exact mechanisms underlying these immune toxic effects are still not clear and remain to be investigated.

Growth Impairment

Increasing evidence has shown the adverse health effects of aflatoxins on growth in humans [5]. A large cross-sectional study demonstrated strong negative associations between levels of serum aflatoxin albumin adduct and the growth parameters (height for age, weight for age, and weight for height) in young children (9 month to 5 years) recruited in Benin and Togo, West Africa [168]. A longitudinal study conducted in Benin assessed the effects of aflatoxin exposure on growth of young children [169]. A total of two hundred children (16–37 months of age) were recruited from four villages, two with high and two with low aflatoxin exposure. The major weaning food was a maize based porridge that is frequently contaminated with aflatoxins. A strong negative correlation was found between aflatoxin-albumin adducts in serum and height increase over the 8-month follow-up period. Compared to the lowest quartile of aflatoxin-albumin adducts, the highest quartile of biomarker was associated with a mean 1.7 cm reduction in growth. Maternal exposure to aflatoxins during pregnancy was shown to affect the growth of the

first year of life in a follow-up study in Gambia [56]. The lowered birth weight was also reported to be correlated with pregnancy aflatoxin exposure in the United Arab Emirates [170].

Childhood stunting is an important public health problem that contributes to approximately 1/5 of deaths among children under 5 years old in developing countries. More recently, an interest is raised on how aflatoxin exposure may affect the gut health and what is the role of aflatoxin exposure in childhood stunting. Aflatoxins that are orally ingested are mainly absorbed in the intestinal mucosa, where they may interact with intestinal epithelium and bacterial flora [171]. However, the significance of this possible interaction remains largely unknown. In the developing world, environmental enteropathy (EE) is quite prevalent among children and may mediate stunting. EE is a subclinical condition of the small intestine and characterized by reduced intestinal absorption capacity and increased permeability. Scientists now suspect that aflatoxin exposure may play a role in the etiology of EE [171]. However, current human evidence relevant to this issue remains scanty and further human studies as well as mechanistic studies using animal models are warranted.

Kwashiorkor

In 1935, Cicely D. Williams introduced into the scientific literature of nutritional diseases, the West African name kwashiorkor [172]. Williams described that the critical period for the development of this disease is the second year of life when other foods are introduced and breastmilk is gradually replaced by a diet of foods suitable for intake and digestion by young children. The clinical appearance of hypopigmentation, hyperpigmentation and desquamation was her main focus for the differential diagnosis,

and this view is maintained to the present day. Early work by Hendrickse and others have reported the occurrence of aflatoxins in the liver or blood of children with kwashiorkor [173-176]. In several tropical countries (e.g., Nigeria, Sudan, Durban), aflatoxins have been found more frequently and in higher concentration in liver specimens from children with kwashiorkor than in controls, as reviewed by Peraica et al. [110]. These clinical observations indicate an association between aflatoxins and kwashiorkor although the causal association cannot be established. The geographical and seasonal prevalence of aflatoxins in food and of kwashiorkor shows a remarkable similarity [177]. Clinical investigation of aflatoxin elimination in children with kwashiorkor and marasmic kwashiorkor, who were fed an aflatoxin-free diet, proved that aflatoxins in these children are slowly eliminated [98]. In several studies [173, 176, 178], aflatoxicol was found in the liver, lung, serum, urine or stools of children with kwashiorkor, whereas this metabolite was not found in control children. It is not clear whether this difference is causally related to kwashiorkor or is a consequence of the disease.

The effects of AFB_1 on the development of kwashiorkor in mice were investigated by Kocabas [179]. In that study, young male Swiss mice were fed either low or normal protein level diets supplemented with or without AFB_1 (0.5 mg/day). The authors found that mice given aflatoxin and a low protein diet showed decreased serum total protein and albumin levels that are a little more prominent but not statistically different from the group of mice that fed on low protein diet only, and that remarkable histopathological changes of the liver in the group fed a low protein diet and AFB_1 when compared with the group fed only a low protein diet. Their conclusion that AFB_1 could not have contributed to the development of kwashiorkor remains arguable. Krawinkel [180]

gave an alternative explanation of this finding, "Although this finding lacks statistical significance, it indicates that aflatoxins may play a specific or unspecific role in some children. If exposure to aflatoxins is not a necessary prerequisite for the occurrence of kwashiorkor, it may contribute to the associated liver disease." Besides the weak animal evidence, there also exists discrepancy in human data. For example, Household and Hundt [181] detected the urine excretion of aflatoxins in normal children from a periurban area and those hospitalized with kwashiorkor and marasmus. They found in no case was aflatoxin isolated from the urine and concluded that aflatoxin exposure is unusual in this population and that aflatoxins do not play a primary role in the pathogenesis of kwashiorkor. There are possibilities that these hospitalized children had withdrawn from aflatoxins contaminated foods and the time of urine collection could be critical for a positive finding because urinary excretion of aflatoxins usually had a short half life. Alternatively, a chance finding might be possible considering the high prevalence of aflatoxins exposure in food in these areas.

To sum up, aflatoxins can be detected more frequently and at higher mean concentrations in the sera of children with kwashiorkor than in marasmic or healthy children. Prevalence of kwashiorkor is temporally correlated with the seasonal production of aflatoxins in foods. Confirmation of the causal relationship of aflatoxins in the etiology of kwashioror will necessitate radical change in approaches to prevent and manage the disease. It has to be acknowledged that exposure to aflatoxins constitutes a greater risk to children with this disease than normal healthy children [177].

Biomarkers of AFB_1

Biomarkers are cellular, biochemical, or molecular alterations that can be objectively measured in biological media, such as human tissues, cells or fluids, to indicate the exposure, effect or susceptibility in individuals [182]. The framework of the relationship of biomarkers to exposure and disease was brought forth by the National Research Council committee on biological markers in 1987 [182]. This conceptual framework nicely summarizes the concept of biomarkers (Figure 2.4). Biomarkers represent a continuum of changes, and the classification of change may not always be distinct. Once exposure occurred, a continuum of biological alteration may be detected. These biological alterations serve as biomarkers of internal dose, biological effect dose, early biological effect, functional and structural changes and disease. A biomarker of susceptibility is a measurable indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure. Measures of these biomarkers have great utility in the following aspects: assessing the relationship between environmental exposure and development of clinical diseases; identifying individuals at high risk for certain disease; and evaluating efficacy of intervention tools.

The use of chemical-specific biomarkers for identifying stages in the development of environmental agents related adverse outcomes can provide important information for critical public health, clinical and regulatory problems [182-184]. Since the development of a paradigm for molecular biomarkers by a committee of the National Research Council [182], such chemical-specific biomarkers to environmental situations have been of great interest to scientists in different fields, in which the study of aflatoxins is a representative one. A great challenge with biomarker research is how to interpret the resulting data. The

interactions between genetic makeup and environmental factors that underlie most human diseases make the interpretation even more complex. Carefully devised strategies for validation, application, and dissemination of information about biomarkers to the public are essential for the molecular biomarker paradigm to guide public health issues. It is believed that biomarkers that indicate the mode of action of an environmental agent will be strong predictors of an individual's risk of disease. Besides, these biomarkers can more accurately classify the status of exposure of individuals, and decrease the chances of misclassification of exposure status, a problem usually encountered by epidemiological investigations. Furthermore, these biomarkers can be used to indicate the effectiveness of interventions to certain exposure or risk and clarify the interactions between chemical and other exposures such as viral infection. To sum up, the overall goals chemical-specific biomarkers development and validation are, as summarized by Groopman and Kensler [184]:

- to assess the ambient exposures in populations and individuals;
- to improve methods in risk assessment and estimation in populations and individuals;
- to improve classification of at-risk individuals, communities and populations;
- to improve methods for assessing exposure remediation and preventive interventions;
- to further define the mechanisms of exposure-disease linkages and underlying susceptibility factors;
- and to further define the interactions of multiple agents and multiple exposure to disease outcomes.

The development of AFB_1-related biomarkers is rooted in the uncovering of the metabolism and mechanism of aflatoxin-induced hepatocarcinogenesis in animal models [4]. AFB_1 is metabolized by CYP450 enzymes to its ultimate reactive metabolite, AFB_1-8,9-expoxide. This reaction is catalyzed by CYP1A2 and 3A4 in humans [61]. The reactive epoxide can interact with DNA to produce an AFB_1-N^7-guanine adduct, which can be readily detected in urine after exposure. Urinary AFB_1-N^7-guanine adduct is an elegant biomarker of the biologically effective dose because this biomarker is promutagenic and mechanistically linked to the carcinogenicity of AFB_1 [67]. The epoxide is unstable and undergoes Schiff base reaction to form products that react with albumin to form albumin adducts [185]. Aflatoxin B_1-lysine (AFB-Lys) adduct, which can be obtained after enzymatic digestion of adducted albumins, has a longer half-life and serves as a reliable biomarker of internal dose. Other oxidation metabolites, including AFM_1 and AFQ_1, are also formed. Levels of AFM_1 in urine correlate well with dietary intakes of aflatoxin in humans and serve as a biomarker of internal dose [186]. The conjugation of glutathione with phase I metabolites produces aflatoxin B_1-mercapturic acid (AFB-NAC), a biomarker of detoxification. The more detailed information on the major biomarkers of AFB_1 is provided below.

AFB-N^7-Guanine Adduct

AFB-N^7-guanine is the principal DNA adduct formed in rat liver after AFB_1 exposure, which derives from covalent bond formation between C^8 of AFB_1-8,9-epoxide and N^7 of guanine bases in DNA. It was initially identified in *in vitro* and *in vivo* experiments in 1977 [24] and 1978 [67], respectively. The urinary excretion of this DNA adduct was reported in rat following the structural characterization. An excellent dose-

dependent correlation was found between the urinary excretion of this biomarker and AFB_1 intake over a dose range of 0.125-1.0 mg/kg [67]. Approximately 1% of administered AFB_1 is bound covalently to DNA and this binding reaches a peak level at 2 h after dosing; a single dose of 25 mg AFB_1 to Fischer 344 rats resulted in a measurable level of AFB_1-DNA adducts that was more pronounced following a second daily dose [187]. Studies on the reaction of synthetic AFB_1-8,9-epoxide with DNA *in vitro* strongly indicated adduct formation is proceeded by a precovalent intercalation complex between double-stranded DNA and the highly electrophilic, unstable AFB_1-*exo*-8,9-epoxide isomer [188]. It was also 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 reaction with N^7 position of guanine [189]. The availability of monoclonal antibodies specific for AFB_1-DNA adducts facilitates the measurement of this biomarker [190]. 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 formation of which was correlated with the incidence of hepatic tumor in rat and trout [191]. Several human studies have validated the correlation between dietary exposure to AFB_1 and excretion of AFB_1-N^7-guanine adducts in urine. For dosimetry of aflatoxin exposure, 42 individuals in the Guanxi region of China were studied [192]. A portion of the actual food consumed was assayed for aflatoxin content and urinary output was assayed for excretion of AFB_1-N^7-guanine by both males and females. Through a linear regression analysis, the AFB_1-N^7-guanine excretion and the AFB_1 intake from the previous day showed a correlation coefficient of $r = 0.65$ and P less than 0.000001. Analysis of the total AFB_1-N^7-guanine excretion in the urine during the complete collection period plotted against

the total AFB_1 exposure in the diet for each of the individuals revealed a correlation coefficient of r = 0.80 and *P* less than 0.0000001. This urinary biomarker has been used to assess human liver cancer risk in high aflatoxin exposure areas [136], and HBV carriers have been found to have higher urinary excretion of this adduct in Taiwan [193].

AFM_1 in Urine and Milk

Holzapfel et al. isolated and characterized the AFM_1 and AFM_2 in sheep urine in 1966 [194]. Before their structural characterization, several studies had reported the occurrence of an AFB_1 related compound in milk [195] and urine [196]. AFM_1 was initially detected in human urine in the Philippines [197] and it was suggested as a potential marker to link aflatoxin exposure with human liver cancer. The good correlation between dietary AFB_1 levels and urinary AFM_1 excretion was demonstrated in a study of Guangxi Residents in China [198]. In that study, a total of 252 urine samples were collected and analyzed by an ELISA method and the dietary intake of AFB_1 was measured on a daily basis for 1 week. The correlation coefficient was found in the range of r = 0.82-0.86 (*P* = 0.0000) in male population. In female population, the correlation is not significant, with a correlation coefficient in the range of 0.2-0.3 (*P*-values in the range of 0.26-0.53). The extrapolated values of AFM_1 excreted in the urine was in the range of 1.23-2.18% of total AFB_1 consumed for the male population and 1.30-1.78% for the female population, respectively. Another study conducted in Guangxi, China also showed a good correlation between serum aflatoxin albumin adduct levels and AFM_1 excretion in human urine (r = 0.60, $P < 0.00003$) [186].

The urinary AFM_1 biomarker has been used to indicate the risk of liver cancer. In a cohort study in Shanghai, China, it was found that people with detectable levels of

urinary AFM_1 are more likely to develop liver cancer [136]. AFM_1 is of similar acute toxicity [199, 200] and about 10-fold less carcinogenic compared to AFB_1 [201, 202]. On the other hand, the risk of liver cancer associated with exposure to AFM_1 (usually through milk), as estimated by Henry et al., is so low as to be immeasurable in populations with a low hepatitis B incidence (such as European populations) even in heavy consumers of milk products [203]. This risk assessment was carried on with two maximal contamination levels of AFM_1 at 0.05 or 0.5 μg/kg. A similar finding has been reported in Taiwan [204]. A total of 144 milk samples of three main brands in Taiwan were collected twice a month over a 1-year period. Of these milk samples, only trace amounts of AFM_1 were found (1.17-54.7 ng/L) with only one sample slightly exceeding the regulatory limit of the European Union (50 ng/L). Using a World Health Organization method of evaluating risk of liver cancer, the group at greatest risk was 6- to 9-year-old girls (average, 12.2 additional cases per billion) and the group with the lowest risk was men of 45 to 64 years of age (average, 3.45 additional cases per billion). Based on these findings, the risk for liver cancer due to ingestion of milk contaminated with AFM_1 was estimated to be low in Taiwan.

Quantitative analysis from a previous animal study has shown that approximately 1-2% of a single dose of AFB_1 is excreted as AFM_1 in the milk in cow [205]. The detection of AFM_1 in human breast milk has been reported in many different populations since the early 1980s. In certain populations, more than 80% of human breast milk samples have detectable levels of AFM_1 [206-209]. Given the fact that AFM_1 is of similar acute toxicity compared to its parent compound AFB_1 [200], the presence of AFM_1 in breast milk has raised a significant health concern as infants may be more susceptible to aflatoxins than

adults. A preliminary study estimated a conversion ratio of 0.09-0.43% of AFB_1 in diet to AFM_1 in human breast milk [210].

AFB_1-Albumin Adduct and AFB_1-Lysine (AFB-Lys) Adduct

In Wistar rats that were orally exposed to a single dose of AFB_1, a linear dose-response relationship between AFB_1 dose and plasma protein binding was found in the dose range of 3.5 to 200 μg/kg body weight. Twenty-four hours after exposure, approximately 0.98-2.15% of the total dose was found to be bound to the plasma protein. In addition, a constant ratio between levels of AFB_1 binding to plasma and liver DNA was also found [211]. An earlier study showed that albumin is the major protein in serum that binds AFB_1 to a significant level in rat [212], and the isolation and characterization of the major serum albumin adduct formed by AFB_1 *in vivo* in rat was demonstrated by Sabbioni et al. in 1987 [185]. That study supported a structure in which the terminal dihydrofuran ring of AFB_1 has been converted to a pyrrolinone ring, and proposed that the initial adduct is formed by condensation of the dialdehyde tautomer of 8,9-dihydro-8,9-dihydroxy-AFB_1, with the ε-amino group of lysine, to form a Schiff base, and that the Schiff base undergoes an Amadori rearrangement to an α-amino ketone (Figure 2.5). An alternative structure of the lysine adduct was also proposed [213], which requires an alternate scheme because the formation of an amide is not particularly feasible following an Amadori rearrangement (Figure 2.6). The relative contribution of AFB_1-8,9-epoxide and AFB_1 dialdehyde in formation of lysine adduct has been discussed (Figure 2.7) [214]. It is noted that the apparent rate of reaction of AFB_1 dialdehyde with N^2-acetylLys is approximately 7 times greater than for the epoxide at pH 7.2. In addition, the rapid, irreversible reaction of the epoxide with H_2O ($k = 42\ min^{-1}$) suggests that most of the Lys

adducts formed might be from the reaction with the dialdehyde. Using the kinetic model (Figure 2.7), it is estimated that ~ 90% of the lysine adduct would arise from the dialdehyde and ~ 10% from the epoxide in the absence of other reactions at a concentration of 1 mM (N^2-acetyl) lysine.

The radioactivity associated with serum albumin following administration of [^{14}C]AFB_1 to rats was cleared with a half-life of 2.5 days [185]. Similarly, the half life of serum AFB-Lys adduct has been reported to be 55 h [211] and 2.6 days [215] in rat. The elimination of serum AFB-Lys followed first-order kinetics. Compared to the short half life of urinary aflatoxin biomarkers, the serum AFB_1 albumin adduct in humans has a biological half life of 21 days, making it a good biomarker for integrating human exposure for several weeks or even months [192]. Because the plasma protein binding requires metabolic activation, this adduct could be used as a biomarker of effective dose.

A good correlation of AFB-Lys adduct with AFB_1-DNA adduct has been reported in several animal species [211, 216], including rat, Guinea pig, hamster, and mouse. Wild et al. assessed the aflatoxin B_1 albumin adduct as a basis for comparative carcinogenesis between animals and humans [216]. Three strains of rat (Fischer 344, Wistar, and Sprague-Dawley), and one strain each of guinea pig (Hartley), hamster (Syrian golden), and mouse (C57BL) were treated by gavage with up to 14 daily doses of between 1 and 80 µg AFB_1/kg body weight. A dose response in both AFB_1-albumin and AFB_1-DNA adducts was seen for all species and strains with steady-state adduct levels at 14 days. The level of both adducts was in the following order: rat > guinea pig > hamster > mouse. The levels of AFB_1-albumin adduct also reflect at least qualitatively the relative susceptibility of the different species to AFB_1 carcinogenesis; the rat is sensitive and the hamster and

mouse are resistant. In addition, when the levels of the albumin and DNA adducts at 14 days were plotted against each other for all species and strains, a correlation was observed ($r = 0.83$, $P \leq 0.0001$; $n = 57$; two-tailed test), suggesting a constant relationship between the level of binding of AFB_1 to serum albumin and liver DNA. The level of AFB_1-albumin adduct formed as a function of a single dose of AFB_1 in rodents was compared to data from humans exposed environmentally to AFB_1. The yield (pg AFB-Lys equivalent/mg albumin/μg AFB_1/kg body weight) for the three rat strains after a single dose was between 0.3 and 0.51, and a value for the mouse of < 0.025. At human exposure level, a linear relationship between formation of AFB-Lys adduct and liver AFB-DNA adduct has been demonstrated in rats (0.16 ng/kg - 12.3 μg/kg) [217]. These data suggest that the level of serum AFB-Lys adducts is a useful biomarker for AFB_1 dosimetry and may reflect the DNA adduct levels in the target tissue. In a human study in Guangxi, China, serum AFB_1 albumin adducts were correlated with dietary AFB_1 intake and urinary excretion of AFM_1. Regression analysis revealed that 1.4-2.3% of ingested AFB_1 is covalently bound to serum albumin [186], which is similar to that found in rats. AFB-Lys adduct, under proper storage conditions, is stable in sera for more than 20 years [218]. In those populations at high risk for aflatoxin exposure such as southeast Asia and sub-Sahara Africa, usually more than 90% of the human populations have detectable AFB-Lys adducts in their sera. Our data from Guangxi, Burkina Faso, Ghana, and Uganda also support this finding.

This biomarker has been widely applied in epidemiological studies to assess the exposure to and risk of aflatoxin exposure in high-risk populations [134, 219] and to test the efficacy of different intervention approaches [220, 221]. During the 2004 outbreak of

aflatoxicosis in Kenya, this biomarker was used to establish the exposure to aflatoxins in affected individuals [118]. Currently, the most commonly used methods to measure this adduct are the ELISA [222] method, the immunoaffinity column or solid phase extraction (SPE) based HPLC method with fluorescence detection [223], although other methods are still available, such as isotope dilution mass spectrometry (IDMS) method [215, 224], and radioimmunoassay (RIA) [186, 219]. The results of antibody-based assays are influenced by the specificity and the sensitivity of the antibodies used. Since the major AFB-albumin adduct was identified as the AFB-Lys adduct [185, 213], the development of more specific monoclonal antibodies recognizing this adduct was initiated and became available in 2001 [225]. The detection limit of HPLC and IDMS method is comparable, that is, approximately 0.5 pg/mg albumin. The ELISA method usually gives higher value as compared to the IDMS method (2.6-fold difference) but the values are well correlated ($r = 0.88$, $P < 0.0001$) [226]. The result suggests that ELISA method may result in nonspecific binding of the antibody to other aflatoxin adducts other than AFB-Lys adduct. For this reason, the data generated from the same method are favored for comparative purposes.

AFB_1-Mercapturic Acid (AFB-NAC)

Biological effects of AFB_1 are principally induced by the metabolite, AFB_1-8,9-epoxide. Detoxification of the epoxide can be mediated in part by glutathione S transferase (GST) [72]. The enzymatic conjugation of the AFB_1-epoxide with glutathione is a mechanism of protection against its toxic effects. Under physiological conditions, the glutathione conjugates undergo further metabolic processing to form mercapturic acids, which are expected to be excreted in urine and have been tested as a biomarker of AFB_1. Scholl et al. [227] synthesized and characterized aflatoxin B_1-mecapturic acid (AFB-NAC)

and investigated the dose-response relationship between AFB_1 exposure and urinary excretion of this conjugate in rat. They found that approximately 1% of AFB_1 dose is excreted as AFB-NAC within 24 h exposure in F344 rats. This conjugate is excreted in urine as a linear function of dose over the range of doses 0.03-0.8 mg/kg body weight. The finding that AFB-NAC is dose-dependently excreted in urine provides the basis for investigating its applicability as a biomarker of GST induction in aflatoxin chemoprevention studies [228].

In a randomized, placebo-controlled, double-blind phase IIa chemoprevention trial, the chemopreventive effects of oltipraz was tested in residents of Qidong, People's Republic of China [229]. The results showed that a daily intervention with 125 mg oltiprza lead to a 2.6-fold increase in median AFB-NAC excretion compared to the placebo group. This finding highlights the feasibility of inducing phase II enzyme as a chemopreventive strategy in humans. In another study evaluating the efficacy of green tea polyphenols (GTPs) in modulating AFB_1 biomarkers, a total of 352 urine samples were collected from a 3-month chemoprevention trial with 500 mg GTPs, 1000 mg GTPs and a placebo treatment [220]. Significant elevations in median AFB-NAC levels were found in both 500 and 1000 mg groups compared with the placebo group at both 1 month ($P < 0.001$) and 3 months ($P < 0.001$) of GTPs intervention. This finding suggests that GTPs effectively modulate AFB_1 metabolism and enhances the metabolic detoxification of AFB_1 and holds promise as a potential intervention tool in human studies.

Mutation at Codon 249 of Tumor Suppressor Gene *p53*

The mutations in the *p53* tumor suppressor gene is more frequently detected in HCC cases residing in high AFB_1 exposure regions than those in low AFB_1 exposure

regions (50% vs 20%), and the spectrum of mutations is quite different [230-233]. Among those HCCs from high AFB_1 region, more than half of the tumors containe an AGG to AGT missense mutatition in codon 249 (AGG to AGT) of the *p53* gene, leading to the replacement of arginine by serine. High prevalence of mutations at codon 249 of the *p53* gene in HCCs was also found in Senegal [234], this typical mutation was detected in 10 of the 15 tumor tissues tested (67%). Although the number of cases is not large, this frequency of mutation in codon 249 of the p53 gene is the highest described. A study of Mexico HCC cases showed that 7 of 16 HCC cases (44%) were found to have AGG to AGT mutations at the exon 7 of *p53* gene codon 249 [235], in which the serum AFB_1-alubmin adduct levels were found in a range of 0.54 to 4.64 pmoL/mg albumin.

It has been demonstrated that AFB_1 induces the G to T transversions in the third position of codon 249 of the *p53* tumor suppressor gene in human HCC cells HepG2 [236]. Different cellular *in vitro* models that take into account species and tissue specificity have been developed to examine the contribution of different metabolic enzymes in inducing AFB_1 associated *p53* gene mutation. A non-tumorigenic SV40-immortalized human liver epithelial cell line (THLE cells) that retains most of the phase II enzymes, but has markedly reduced phase I activities was used for stable expression of the human CYP1A2, CYP2A6, CYP2B6 and CYP3A4 cDNA. The four genetically engineered cell lines (T5-1A2, T5-2A6, T5-2B6 and T5-3A4) were developed, which produced high levels of the specific CYP450 proteins with comparable or higher catalytic activities compared to that in human hepatocytes. At similar levels of total DNA adducts, both the T5-1A2 and T5-3A4 cells showed AGG to AGT transversions at codon 249 of the *p53* gene [237].

The mechanisms underlying AFB_1-induced G to T transversion in codon 249 of the *p53* gene mutation remains largely unknown. The cytosine residues of CpG sites in the coding region of the *p53* gene are known to be methylated in a variety of human tissues [238]. It has been hypothesized that mutational hot spots at methylated CpG sequences may represent preferential carcinogen targets [239]. An *in vitro* study was designed to test the role of cytosine methylation on the mutation spectrum using AFB_1 as the carcinogen. A wild type *p53* cDNA fragment was cloned into a pUC18 vector to generate a DNA template for AFB_1 treatment and the mutations were identified by DNA sequencing. The results showed that cytosine methylation enhances AFB_1-induced guanine mutations at CpG sites but no mutations were detected at codon 249 [240]. This finding suggests that the DNA sequence at the third base of codon 249 itself may be insufficient to be a hot spot target.

Biomarkers of exposure to AFB_1 in human populations

The currently favored method of measuring human exposure consists of the analysis of body fluids for the presence of aflatoxin biomarkers [241, 242]. Each biochemical process results in derivatives that have a characteristic half-life within the body, and thus the measurement of these derivatives can reflect the exposure over a period of days, weeks, or months. Recent exposure to aflatoxin is reflected in urine as directly excreted AFM_1 and other detoxification products, but only a small fraction of the dose is excreted in that way. Measurements of aflatoxin and its byproducts in urine have been found to be highly variable from day to day, which reflects the wide variability in the contamination of food samples, and, for this reason, the measurement of AFM_1 on a single day may not be a reliable indicator of a person's chronic exposure [241-243]. The aflatoxin-albumin

adduct that can be measured in peripheral blood and has a half-life 21 days in humans [185]. This biomarker could integrate the exposure over a longer period and hence is a more reliable indicator of a person's chronic exposure. However, it should be remembered that the fraction of the ingested aflatoxin processed into any particular metabolite is variable. A given concentration of any particular biomarker cannot be used to make assumptions about the total dose or the amounts directed into any other competing pathway, partially due to the individual phenotype in metabolic enzymes as discussed in biotransformation section. Human exposure data using biological markers as a measure are relatively rare, because the locations of such measurements are mainly in West Africa [128, 130, 168, 178, 244-246] and China [219]. However, there are increasing data showing a high detection rate of aflatoxin biomarkers in Malaysia [247], Thailand [248], Egypt [249], Haiti, Uganda, Burkina Faso (unpublished data), and the United States [250] (Table 2.3). These data also demonstrate major variations in seasonal exposure [128, 245], with summer months usually have higher exposure than winter months [208]. which reflects the natural development of contamination in storage. These biomarker data show that, regardless of food preparation practices, the human populations of these developing countries are widely and significantly exposed to aflatoxin. Given that little is done to decontaminate foods in many developing countries, it is likely that the prevalence of chronic exposure in these countries is similar to that measured in these studies with the use of biomarkers [2].

Intervention strategies for reducing aflatoxins exposure and risk

It is estimated that approximately 4.5 billion people in the developing world are at risk for chronic exposure to aflatoxins [2]. Intervention strategies for reducing aflatoxins exposure or risk have been the concerns of several reviews [2, 4, 27, 28]. These strategies

could be undertaken at different stages, including pre-harvest (reduce toxin production and bio-control), during food processing and storage (improve environmental setting, selection, and education), and after ingestion (reduce active metabolite formation and bioavailability). Vaccination of infants against HBV and HCV to remove the interaction with AFB_1 exposure is of priority to reduce human HCC risks in the long-term [141]. The evaluation of different intervention strategies has been defined as one of the four gaps (quantification of health effects and disease burden of aflatoxin exposure; evaluation of current intervention strategies and disseminate the result; disease surveillance, food monitoring, laboratory and public health responses; development of response protocols addressing aflatoxicosis) for future effort by the organized workshop that was held after the outbreaks of aflatoxicosis in Kenya [27].

Primary intervention strategies for reducing aflatoxins exposure include post-harvest intervention, using trapping agents to reduce absorption such as clay and chlorophyllin. A strategy of post-harvest intervention has been reported in a community-based study in Kindia region of Guinea, West Africa [251]. A 60% reduction of mean aflatoxin level was achieved after 5-month in the intervention group, compared to the control. Reduction of the percentage of persons with detectable level of aflatoxin albumin adducts were also found. In Haiti, results have been achieved to reduce aflatoxin contamination in market peanuts, through visual-tactile sorting of the shell, flotation of select kernels, immediate roasting and grinding, and monitoring [252]. The level of AFB_1 in peanuts showed a reduction from 412.5 ± 32.1 ppb in September 2006 to 125 ± 7.1 ppb in November 2006 and further to a level of 0.20 ± 0.10 ppb in January 2007. Therefore, primary intervention is a promising approach to reduce aflatoxin exposure, and several

primary intervention strategies in West Africa have been determined to be cost effective [253].

Chemoprevention is a strategy that involves the use of natural or synthetic agents to block, retard, reverse or modulate the carcinogenic process of environmental agents [254]. Aflatoxin biomarkers were first used as surrogate endpoints in a phase II chemoprevention trial in Qidong, China [229, 255, 256]. Individuals receiving 500 mg oltipraz once a week for 8 weeks had a significant longitudinal decline in aflatoxin-albumin biomarker levels beginning 1 month into the intervention and continuing for 1 month after treatment was stopped. An association was observed within participants in the 500 mg group between rate of decline of aflatoxin-albumin adducts and excretion levels of AFM_1. AFB_1-NAC levels were elevated 2.6-fold in the 125 mg group, compared to placebo control. Oltipraz seems to regulate both phase I and phase II metabolizing enzymes to modulate the aflatoxin metabolic pathways [257]. The transcription factor Nrf2 mediated signal pathway appears to be essential for the induction of phase 2 enzymes by oltipraz. While oltipraz significantly reduced multiplicity of gastric neoplasia in wild-type mice by 55%, there is no effect on tumor burden in *nrf2*-deficient mice [258]. Chlorophyllin, a water-soluble derivative of chlorophyll, has demonstrated its anticarcinogenic properties in a number of animal models [259, 260]. The primary mode of action of chlorophyllin appears to be sequestration of aflatoxin through complexation in a 1:1 ratio [261], although experimental data have characterized its enzyme-inducing properties that may also contribute to its mechanism of action [262]. The effect of chlorophyllin on disposition of aflatoxin has been assessed in a randomized, double-blind, placebo-controlled chemoprevention trial in residents of Qidong, China. One

hundred and eighty healthy adults were randomly assigned to ingest 100 mg of chlorophyllin or a placebo three times a day prior to each meal for 4 months. Urine samples were collected from both groups to test for the modulation of urinary aflatoxin N^7-guanine, a primary endpoint. Aflatoxin-N7-guanine could be detected in 105 of 169 available samples. Chlorophyllin consumption at each meal led to an overall 55% reduction ($P = 0.036$) in median urinary levels of this aflatoxin biomarker compared with those subjects taking placebo. This study provides evidence that dietary supplementation with chlorophylls or ingestion of foods rich in chlorophylls may represent practical means to prevent the development of HCC in people chronically exposed to aflatoxins.

The natural product NovaSil (NS) clay has been shown to be safe in animals and human subjects [263, 264]. When included in the diet, NS clay has been shown to prevent aflatoxicosis in a variety of animals and epidemiological studies have demonstrated the efficacy of NS clay in reducing the serum biomarkers of AFB_1 in populations exposed to aflatoxins through contaminated foods [29, 221]. Green tea polyphenols (GTPs) have demonstrated efficacy in cancer prevention in rat models and reduction of oxidative DNA damage biomarkers in clinical trials [265, 266]. The induction of phase II enzyme and scavanging of oxidative damage might account for the protective effects in aflatoxin exposed population [220, 266]. When reviewing the current research on NS clay and aflatoxins, Phillips et al. concluded that the aflatoxin sequestering clays should be rigorously evaluated *in vitro* and *in vivo* and several criteria should be met: favorable thermodynamic characteristics of mycotoxin sorption; tolerable levels of priority metals, dioxins/furans and other hazardous contaminants; safety and efficacy in multiple animal species; safety and efficacy in long-term studies; and negligible interactions with

vitamins, iron and zinc and other micronutrients [29]. Most of the recommended criteria could be conveniently applied to the research of other chemopreventive agents as well. The readily accessible aflatoxin biomarkers and analytical tools greatly facilitate the evaluation of different intervention strategies in both animal experiments and human epidemiological studies.

References

1. IARC. Aflatoxins. *IARC Monogr Eval Carcinog Risks Hum*. 1993;56:245-395
2. Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences, and interventions. *Am J Clin Nutr*. 2004;80:1106-1122
3. Liu Y, Wu F. Global burden of aflatoxin-induced hepatocellular carcinoma: A risk assessment. *Environ Health Perspect*. 2010;118:818-824
4. Kensler TW, Roebuck BD, Wogan GN, Groopman JD. Aflatoxin: A 50-year odyssey of mechanistic and translational toxicology. *Toxicol Sci*. 2011;120 Suppl 1:S28-48
5. Khlangwiset P, Shephard GS, Wu F. Aflatoxins and growth impairment: A review. *Crit Rev Toxicol*. 2011;41:740-755
6. Williams JH, Grubb JA, Davis JW, Wang JS, Jolly PE, Ankrah NA, Ellis WO, Afriyie-Gyawu E, Johnson NM, Robinson AG, Phillips TD. Hiv and hepatocellular and esophageal carcinomas related to consumption of mycotoxin-prone foods in sub-saharan africa. *Am J Clin Nutr*. 2010;92:154-160
7. Herzallah SM. Determination of aflatoxins in eggs, milk, meat and meat products using hplc fluorescent and uv detectors. *Food Chem*. 2009;114:1141-1146

8. Wang Y, Chai T, Lu G, Quan C, Duan H, Yao M, Zucker BA, Schlenker G. Simultaneous detection of airborne aflatoxin, ochratoxin and zearalenone in a poultry house by immunoaffinity clean-up and high-performance liquid chromatography. *Environ Res*. 2008;107:139-144

9. Viegas S, Veiga L, Malta-Vacas J, Sabino R, Figueredo P, Almeida A, Viegas C, Carolino E. Occupational exposure to aflatoxin (afb(1)) in poultry production. *J Toxicol Environ Health A*. 2012;75:1330-1340

10. Wang JS, Groopman JD. Physical/chemical/immunologic analytical methods. *IARC Sci Publ*. 2011:43-61

11. Blount WP. Turkey "x" disease. *J. Br. Turkey Fed.* 1961;9:55-58

12. Sargeant K, Sheridan A, Okelly J. Toxicity associated with certain samples of groundnuts - reply. *Nature*. 1961;192:1096-1097

13. Wannop CC. Histopathology of turkey x disease in great britain. *Avian Dis*. 1961;5:371-381

14. Nesbitt BF, Okelly J, Sheridan A, Sargeant K. Toxic metabolites of aspergillus flavus. *Nature*. 1962;195:1062

15. Spensley PC. Aflatoxin, active principle in turkey x disease. *Endeavour*. 1963;22:75-79

16. Asao T, Wogan GN, Chang SB, Buchi G, Wick EL, Abdelkad.Mm. Aflatoxins b and g. *J Am Chem Soc*. 1963;85:1706-1707

17. Paget GE. Exudative hepatitis in guinea-pigs. *J Pathol Bacteriol*. 1954;67:393-400

18. Clifford JI, Rees KR. The action of aflatoxin b1 on the rat liver. *Biochem J*. 1967;102:65-75

19. Shank RC, Wogan GN. Acute effects of aflatoxin b1 on liver composition and metabolism in rat and duckling. *Toxicol Appl Pharmacol*. 1966;9:468-476

20. Yin SJ, Kao MC, Lee SC. Sequential biochemical and histological changes in rats treated with aflatoxin b1. *Br J Cancer*. 1980;42:319-325

21. Dwivedi Y, Rastogi R, Mehrotra R, Garg NK, Dhawan BN. Picroliv protects against aflatoxin-b1 acute hepatotoxicity in rats. *Pharmacol Res*. 1993;27:189-199

22. Wogan GN, Paglialunga S, Newberne PM. Carcinogenic effects of low dietary levels of aflatoxin b1 in rats. *Food Cosmet Toxicol*. 1974;12:681-685

23. Carnaghan RB. Hepatic tumours and other chronic liver changes in rats following a single oral administration of aflatoxin. *Br J Cancer*. 1967;21:811-814

24. Essigmann JM, Croy RG, Nadzan AM, Busby WF, Jr., Reinhold VN, Buchi G, Wogan GN. Structural identification of the major DNA adduct formed by aflatoxin b1 in vitro. *Proc Natl Acad Sci U S A*. 1977;74:1870-1874

25. Groopman JD, Johnson D, Kensler TW. Aflatoxin and hepatitis b virus biomarkers: A paradigm for complex environmental exposures and cancer risk. *Cancer Biomark*. 2005;1:5-14

26. Lewis L, Onsongo M, Njapau H, Schurz-Rogers H, Luber G, Kieszak S, Nyamongo J, Backer L, Dahiye AM, Misore A, DeCock K, Rubin C, Kenya Aflatoxicosis Investigation G. Aflatoxin contamination of commercial maize

products during an outbreak of acute aflatoxicosis in eastern and central kenya. *Environ Health Perspect*. 2005;113:1763-1767

27. Strosnider H, Azziz-Baumgartner E, Banziger M, Bhat RV, Breiman R, Brune MN, DeCock K, Dilley A, Groopman J, Hell K, Henry SH, Jeffers D, Jolly C, Jolly P, Kibata GN, Lewis L, Liu X, Luber G, McCoy L, Mensah P, Miraglia M, Misore A, Njapau H, Ong CN, Onsongo MT, Page SW, Park D, Patel M, Phillips T, Pineiro M, Pronczuk J, Rogers HS, Rubin C, Sabino M, Schaafsma A, Shephard G, Stroka J, Wild C, Williams JT, Wilson D. Workgroup report: Public health strategies for reducing aflatoxin exposure in developing countries. *Environ Health Perspect*. 2006;114:1898-1903

28. Groopman JD, Kensler TW, Wild CP. Protective interventions to prevent aflatoxin-induced carcinogenesis in developing countries. *Annu Rev Public Health*. 2008;29:187-203

29. Phillips TD, Afriyie-Gyawu E, Williams J, Huebner H, Ankrah NA, Ofori-Adjei D, Jolly P, Johnson N, Taylor J, Marroquin-Cardona A, Xu L, Tang L, Wang JS. Reducing human exposure to aflatoxin through the use of clay: A review. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2008;25:134-145

30. Wogan GN, Edwards GS, Shank RC. Excretion and tissue distribution of radioactivity from aflatoxin b1-14-c in rats. *Cancer research*. 1967;27:1729-1736

31. Steyn M, Pitout MJ, Purchase IF. A comparative study on aflatoxin b 1 metabolism in mice and rats. *Br J Cancer*. 1971;25:291-297

32. Degen GH, Neumann HG. The major metabolite of aflatoxin b1 in the rat is a glutathione conjugate. *Chem Biol Interact*. 1978;22:239-255

33. Kumagai S. Intestinal absorption and excretion of aflatoxin in rats. *Toxicol Appl Pharmacol*. 1989;97:88-97

34. Dalezios JI, Hsieh DP, Wogan GN. Excretion and metabolism of orally administered aflatoxin b1 by rhesus monkeys. *Food Cosmet Toxicol*. 1973;11:605-616

35. von Richter O, Burk O, Fromm MF, Thon KP, Eichelbaum M, Kivisto KT. Cytochrome p450 3a4 and p-glycoprotein expression in human small intestinal enterocytes and hepatocytes: A comparative analysis in paired tissue specimens. *Clin Pharmacol Ther*. 2004;75:172-183

36. Gallo A, Moschini M, Masoero F. Aflatoxins absorption in the gastro-intestinal tract and in the vaginal mucosa in lactating dairy cows. *Ital J Anim Sci*. 2008;7:53-63

37. Van Egmond HP. Rationale for regulatory programmes for mycotoxins in human foods and animal feeds. *Food Addit Contam*. 1993;10:29-36

38. Ioannou-Kakouri E, Aletrari M, Christou E, Hadjioannou-Ralli A, Koliou A, Akkelidou D. Surveillance and control of aflatoxins b-1, b-2, g(1), g(2), and m-1 in foodstuffs in the republic of cyprus: 1992-1996. *J Aoac Int*. 1999;82:883-892

39. Perduri R, Gobba S. Unexpected aflatoxin exposure in a woman in northern italy: A case report. *Cases J*. 2009;2:7736

40. Selim MI. Significance of aflatoxins in rural and global health: Concern for agricultural workers. *N C Med J*. 2010;71:438-441

41. Desai MR, Ghosh S. Occupational exposure to airborne fungi among rice mill workers with special reference to aflatoxin producing a. Flavus strains. *Ann Agric Environ Med*. 2003;10:159-162

42. Brera C, Caputi R, Miraglia M, Iavicoli I, Salerno A, Carelli G. Exposure assessment to mycotoxins in workplaces: Aflatoxins and ochratoxin a occurrence in airborne dusts and human sera. *Microchem J*. 2002;73:167-173

43. Autrup JL, Schmidt J, Autrup H. Exposure to aflatoxin b1 in animal-feed production plant workers. *Environ Health Perspect*. 1993;99:195-197

44. Dvorackova I, Pichova V. Pulmonary interstitial fibrosis with evidence of aflatoxin b1 in lung tissue. *J Toxicol Environ Health*. 1986;18:153-157

45. Dvorackova I. Aflatoxin inhalation and alveolar cell carcinoma. *Br Med J*. 1976;1:691

46. Traverso A, Bassoli V, Cioe A, Anselmo S, Ferro M. Assessment of aflatoxin exposure of laboratory worker during food contamination analyses. Assessment of the procedures adopted by an a.R.P.A.L. Laboratory (liguria region environmental protection agency). *La Medicina del lavoro*. 2010;101:375-380

47. Riley RT, Kemppainen BW, Norred WP. Penetration of aflatoxins through isolated human epidermis. *J Toxicol Environ Health*. 1985;15:769-777

48. Jubert C, Mata J, Bench G, Dashwood R, Pereira C, Tracewell W, Turteltaub K, Williams D, Bailey G. Effects of chlorophyll and chlorophyllin on low-dose aflatoxin b(1) pharmacokinetics in human volunteers. *Cancer Prev Res (Phila)*. 2009;2:1015-1022

49. Wong ZA, Hsieh DP. The comparative metabolism and toxicokinetics of aflatoxin b1 in the monkey, rat, and mouse. *Toxicol Appl Pharmacol*. 1980;55:115-125

50. Holeski CJ, Eaton DL, Monroe DH, Bellamy GM. Effects of phenobarbital on the biliary excretion of aflatoxin p1-glucuronide and aflatoxin b1-s-glutathione in the rat. *Xenobiotica*. 1987;17:139-153

51. Mabee MS, Chipley JR. Tissue distribution and metabolism of aflatoxin b 1 - 14 c in broiler chickens. *Appl Microbiol*. 1973;25:763-769

52. Beaver RW, Wilson DM, James MA, Haydon KD, Colvin BM, Sangster LT, Pikul AH, Groopman JD. Distribution of aflatoxins in tissues of growing pigs fed an aflatoxin-contaminated diet amended with a high affinity aluminosilicate sorbent. *Vet Hum Toxicol*. 1990;32:16-18

53. Arora RG, Appelgren LE, Bergman A. Distribution of [14c]-labelled aflatoxin b1 in mice. *Acta Pharmacol Toxicol*. 1978;43:273-279

54. Appelgren LE, Arora RG. Distribution studies of 14c-labelled aflatoxin b1 and ochratoxin a in pregnant mice. *Vet Res Commun*. 1983;7:141-144

55. Denning DW, Allen R, Wilkinson AP, Morgan MR. Transplacental transfer of aflatoxin in humans. *Carcinogenesis*. 1990;11:1033-1035

56. Turner PC, Collinson AC, Cheung YB, Gong Y, Hall AJ, Prentice AM, Wild CP. Aflatoxin exposure in utero causes growth faltering in gambian infants. *Int J Epidemiol*. 2007;36:1119-1125

57. Partanen HA, El-Nezami HS, Leppanen JM, Myllynen PK, Woodhouse HJ, Vahakangas KH. Aflatoxin b1 transfer and metabolism in human placenta. *Toxicol Sci*. 2010;113:216-225

58. De Vries HR, Maxwell SM, Hendrickse RG. Foetal and neonatal exposure to aflatoxins. *Acta Paediatr Scand*. 1989;78:373-378

59. Wong JJ, Hsieh DP. Mutagenicity of aflatoxins related to their metabolism and carcinogenic potential. *Proc Natl Acad Sci U S A*. 1976;73:2241-2244

60. Kamdem LK, Meineke I, Godtel-Armbrust U, Brockmoller J, Wojnowski L. Dominant contribution of p450 3a4 to the hepatic carcinogenic activation of aflatoxin b1. *Chem Res Toxicol*. 2006;19:577-586

61. Gallagher EP, Kunze KL, Stapleton PL, Eaton DL. The kinetics of aflatoxin b1 oxidation by human cdna-expressed and human liver microsomal cytochromes p450 1a2 and 3a4. *Toxicol Appl Pharmacol*. 1996;141:595-606

62. Van Vleet TR, Mace K, Coulombe RA, Jr. Comparative aflatoxin b(1) activation and cytotoxicity in human bronchial cells expressing cytochromes p450 1a2 and 3a4. *Cancer Res*. 2002;62:105-112

63. Doi AM, Patterson PE, Gallagher EP. Variability in aflatoxin b(1)-macromolecular binding and relationship to biotransformation enzyme expression in human prenatal and adult liver. *Toxicol Appl Pharmacol*. 2002;181:48-59

64. Gorelick NJ. Risk assessment for aflatoxin: I. Metabolism of aflatoxin b1 by different species. *Risk Anal*. 1990;10:539-559

65. Egner PA, Yu X, Johnson JK, Nathasingh CK, Groopman JD, Kensler TW, Roebuck BD. Identification of aflatoxin m1-n7-guanine in liver and urine of tree shrews and rats following administration of aflatoxin b1. *Chem Res Toxicol*. 2003;16:1174-1180

66. Coulombe RA, Jr. Non-hepatic disposition and effects of aflatoxin b1. *in: D.L. Eaton, J.D. Groopman Eds., The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance,* Academic Press, San Diego, 1994, pp. 89–101. 1994

67. Croy RG, Essigmann JM, Reinhold VN, Wogan GN. Identification of the principal aflatoxin b1-DNA adduct formed in vivo in rat liver. *Proc Natl Acad Sci U S A*. 1978;75:1745-1749

68. Essigmann JM, Croy RG, Bennett RA, Wogan GN. Metabolic activation of aflatoxin b1: Patterns of DNA adduct formation, removal, and excretion in relation to carcinogenesis. *Drug Metab Rev*. 1982;13:581-602

69. Iyer RS, Coles BF, Raney KD, Thier R, Guengerich FP, Harris TM. DNA adduction by the potent carcinogen aflatoxin b-1 - mechanistic studies. *J Am Chem Soc*. 1994;116:1603-1609

70. Raney KD, Coles B, Guengerich FP, Harris TM. The endo-8,9-epoxide of aflatoxin b1: A new metabolite. *Chem Res Toxicol*. 1992;5:333-335

71. Raney VM, Harris TM, Stone MP. DNA conformation mediates aflatoxin b1-DNA binding and the formation of guanine n7 adducts by aflatoxin b1 8,9-exo-epoxide. *Chem Res Toxicol*. 1993;6:64-68

72. Raney KD, Meyer DJ, Ketterer B, Harris TM, Guengerich FP. Glutathione conjugation of aflatoxin b1 exo- and endo-epoxides by rat and human glutathione s-transferases. *Chem Res Toxicol*. 1992;5:470-478

73. Kensler TW, Qian GS, Chen JG, Groopman JD. Translational strategies for cancer prevention in liver. *Nat Rev Cancer*. 2003;3:321-329

74. Hayes JD, Judah DJ, McLellan LI, Neal GE. Contribution of the glutathione s-transferases to the mechanisms of resistance to aflatoxin b1. *Pharmacol Ther*. 1991;50:443-472

75. Quinn BA, Crane TL, Kocal TE, Best SJ, Cameron RG, Rushmore TH, Farber E, Hayes MA. Protective activity of different hepatic cytosolic glutathione s-transferases against DNA-binding metabolites of aflatoxin b1. *Toxicol Appl Pharmacol*. 1990;105:351-363

76. Tiemersma EW, Omer RE, Bunschoten A, van't Veer P, Kok FJ, Idris MO, Kadaru AM, Fedail SS, Kampman E. Role of genetic polymorphism of glutathione-s-transferase t1 and microsomal epoxide hydrolase in aflatoxin-associated hepatocellular carcinoma. *Cancer Epidemiol Biomarkers Prev*. 2001;10:785-791

77. Kuehl P, Zhang J, Lin Y, Lamba J, Assem M, Schuetz J, Watkins PB, Daly A, Wrighton SA, Hall SD, Maurel P, Relling M, Brimer C, Yasuda K, Venkataramanan R, Strom S, Thummel K, Boguski MS, Schuetz E. Sequence diversity in cyp3a promoters and characterization of the genetic basis of polymorphic cyp3a5 expression. *Nat Genet*. 2001;27:383-391

78. Hustert E, Haberl M, Burk O, Wolbold R, He YQ, Klein K, Nuessler AC, Neuhaus P, Klattig J, Eiselt R, Koch I, Zibat A, Brockmoller J, Halpert JR, Zanger UM, Wojnowski L. The genetic determinants of the cyp3a5 polymorphism. *Pharmacogenetics*. 2001;11:773-779

79. Tateishi T, Watanabe M, Moriya H, Yamaguchi S, Sato T, Kobayashi S. No ethnic difference between caucasian and japanese hepatic samples in the

expression frequency of cyp3a5 and cyp3a7 proteins. *Biochem Pharmacol*. 1999;57:935-939

80. Wojnowski L, Turner PC, Pedersen B, Hustert E, Brockmoller J, Mendy M, Whittle HC, Kirk G, Wild CP. Increased levels of aflatoxin-albumin adducts are associated with cyp3a5 polymorphisms in the gambia, west africa. *Pharmacogenetics*. 2004;14:691-700

81. Eaton DL, Ramsdell HS, Neal GE. Biotransformation of aflatoxins. In: D. L. Eaton and j. D. Groopman (eds.), the toxicology of aflatoxin: Human health, veterinary, and agricultural significance, pp. 45–71. San diego, ca: Academic press, inc., 1994. 1994

82. Guengerich FP, Johnson WW, Ueng YF, Yamazaki H, Shimada T. Involvement of cytochrome p450, glutathione s-transferase, and epoxide hydrolase in the metabolism of aflatoxin b1 and relevance to risk of human liver cancer. *Environ Health Perspect*. 1996;104 Suppl 3:557-562

83. Liu YH, Taylor J, Linko P, Lucier GW, Thompson CL. Glutathione s-transferase mu in human lymphocyte and liver: Role in modulating formation of carcinogen-derived DNA adducts. *Carcinogenesis*. 1991;12:2269-2275

84. Rebbeck TR. Molecular epidemiology of the human glutathione s-transferase genotypes gstm1 and gstt1 in cancer susceptibility. *Cancer Epidemiol Biomarkers Prev*. 1997;6:733-743

85. McGlynn KA, Rosvold EA, Lustbader ED, Hu Y, Clapper ML, Zhou T, Wild CP, Xia XL, Baffoe-Bonnie A, Ofori-Adjei D, et al. Susceptibility to hepatocellular

carcinoma is associated with genetic variation in the enzymatic detoxification of aflatoxin b1. *Proc Natl Acad Sci U S A*. 1995;92:2384-2387

86. Chen CJ, Yu MW, Liaw YF, Wang LW, Chiamprasert S, Matin F, Hirvonen A, Bell DA, Santella RM. Chronic hepatitis b carriers with null genotypes of glutathione s-transferase m1 and t1 polymorphisms who are exposed to aflatoxin are at increased risk of hepatocellular carcinoma. *Am J Human Genet*. 1996;59:128-134

87. Omer RE, Verhoef L, Van't Veer P, Idris MO, Kadaru AM, Kampman E, Bunschoten A, Kok FJ. Peanut butter intake, gstm1 genotype and hepatocellular carcinoma: A case-control study in sudan. *Cancer Causes Control*. 2001;12:23-32

88. Guengerich FP, Johnson WW, Shimada T, Ueng YF, Yamazaki H, Langouet S. Activation and detoxication of aflatoxin b1. *Mut Res*. 1998;402:121-128

89. Johnson WW, Ueng YF, Widersten M, Mannervik B, Hayes JD, Sherratt PJ, Ketterer B, Guengerich FP. Conjugation of highly reactive aflatoxin b1 exo-8,9-epoxide catalyzed by rat and human glutathione transferases: Estimation of kinetic parameters. *Biochemistry*. 1997;36:3056-3060

90. Kirk GD, Turner PC, Gong YY, Lesi OA, Mendy M, Goedert JJ, Halls AJ, Whittle H, Hainaut P, Montesano R, Wild CP. Hepatocellular carcinoma and polymorphisms in carcinogen-metabolizing and DNA repair enzymes in a population with aflatoxin exposure and hepatitis b virus endemicity. *Cancer Epidem Biomar*. 2005;14:373-379

91. Hassett C, Aicher L, Sidhu JS, Omiecinski CJ. Human microsomal epoxide hydrolase: Genetic polymorphism and functional expression in vitro of amino acid variants. *Hum Mol Genet*. 1994;3:421-428

92. Johnson WW, Yamazaki H, Shimada T, Ueng YF, Guengerich FP. Aflatoxin b1 8,9-epoxide hydrolysis in the presence of rat and human epoxide hydrolase. *Chem Res Toxicol*. 1997;10:672-676

93. Omiecinski CJ, Hassett C, Hosagrahara V. Epoxide hydrolase--polymorphism and role in toxicology. *Toxicol Lett*. 2000;112-113:365-370

94. Coulombe RA, Jr., Sharma RP. Clearance and excretion of intratracheally and orally administered aflatoxin b1 in the rat. *Food Chem Toxicol*. 1985;23:827-830

95. Raj HG, Lotlikar PD. Urinary excretion of thiol conjugates of aflatoxin b1 in rats and hamsters. *Cancer Lett*. 1984;22:125-133

96. Groopman JD, Donahue PR, Zhu JQ, Chen JS, Wogan GN. Aflatoxin metabolism in humans: Detection of metabolites and nucleic acid adducts in urine by affinity chromatography. *Proc Natl Acad Sci U S A*. 1985;82:6492-6496

97. Mykkanen H, Zhu HL, Salminen E, Juvonen RO, Ling WH, Ma J, Polychronaki N, Kemilainen H, Mykkanen O, Salminen S, El-Nezami H. Fecal and urinary excretion of aflatoxin b-1 metabolites (afq(1), afm(1) and afb-n-7-guanine) in young chinese males. *Int J Cancer*. 2005;115:879-884

98. de Vries HR, Maxwell SM, Hendrickse RG. Aflatoxin excretion in children with kwashiorkor or marasmic kwashiorkor--a clinical investigation. *Mycopathologia*. 1990;110:1-9

99. Wogan GN, Edwards GS, Newberne PM. Structure-activity relationships in toxicity and carcinogenicity of aflatoxins and analogs. *Cancer Res*. 1971;31:1936-1942

100. Carnaghan RB, Hartley RD, O'Kelly J. Toxicity and fluorescence properties of the aflatoxins. *Nature*. 1963;200:1101

101. Lijinsky W, Butler WH. Purification and toxicity of aflatoxin g1. *Proc Soc Exp Biol Med*. 1966;123:151-154

102. Butler WH. Acute toxicity of aflatoxin b-1 in rats. *Br J Cancer*. 1964;18:756-762

103. Newberne PM, Russo R, Wogan GN. Acute toxicity of aflatoxin b1 in the dog. *Pathol Vet*. 1966;3:331-340

104. McGuire RA. M.S. Thesis. Factors affecting the acute toxicity of aflatoxin b1 in the rat and mouse. Massachusetts institute of technology, cambridge. 1969

105. Rastogi R, Srivastava AK, Rastogi AK. Biochemical changes induced in liver and serum of aflatoxin b1-treated male wistar rats: Preventive effect of picroliv. *Pharmacol Toxicol*. 2001;88:53-58

106. Dwivedi Y, Rastogi R, Mehrotra R, Garg NK, Dhawan BN. Picroliv protects against aflatoxin b1 acute hepatotoxicity in rats. *Pharmacol Res*. 1993;27:189-199

107. Ito N, Hasegawa R, Imaida K, Masui T, Takahashi S, Shirai T. Pathological markers for non-genotoxic agent-associated carcinogenesis. *Toxicol Lett*. 1992;64-65 Spec No:613-620

108. Gelderblom WC, Marasas WF, Lebepe-Mazur S, Swanevelder S, Vessey CJ, Hall Pde L. Interaction of fumonisin b(1) and aflatoxin b(1) in a short-term carcinogenesis model in rat liver. *Toxicology*. 2002;171:161-173

109. Sekijima M, Tsutsumi T, Yoshida T, Harada T, Tashiro F, Chen G, Yu SZ, Ueno Y. Enhancement of glutathione s-transferase placental-form positive liver cell foci development by microcystin-lr in aflatoxin b1-initiated rats. *Carcinogenesis*. 1999;20:161-165

110. Peraica M, Radic B, Lucic A, Pavlovic M. Toxic effects of mycotoxins in humans. *Bull World Health Organ*. 1999;77:754-766

111. Krishnamachari KA, Bhat RV, Nagarajan V, Tilak TB. Hepatitis due to aflatoxicosis. An outbreak in western india. *Lancet*. 1975;1:1061-1063

112. Tandon BN, Krishnamurthy L, Koshy A, Tandon HD, Ramalingaswami V, Bhandari JR, Mathur MM, Mathur PD. Study of an epidemic of jaundice, presumably due to toxic hepatitis, in northwest india. *Gastroenterology*. 1977;72:488-494

113. Ngindu A, Johnson BK, Kenya PR, Ngira JA, Ocheng DM, Nandwa H, Omondi TN, Jansen AJ, Ngare W, Kaviti JN, Gatei D, Siongok TA. Outbreak of acute hepatitis caused by aflatoxin poisoning in kenya. *Lancet*. 1982;1:1346-1348

114. Cheng CT. Perak, malaysia, mass poisoning. Tale of the nine emperor gods and rat tail noodles. *Am J Forensic Med Pathol*. 1992;13:261-263

115. Lye MS, Ghazali AA, Mohan J, Alwin N, Nair RC. An outbreak of acute hepatic encephalopathy due to severe aflatoxicosis in malaysia. *Am J Trop Med Hyg*. 1995;53:68-72

116. Serck-Hanssen A. Aflatoxin-induced fatal hepatitis? A case report from uganda. *Arch Environ Health*. 1970;20:729-731

117. Willis RM, Mulvihill JJ, Hoofnagle JH. Attempted suicide with purified aflatoxin. *Lancet*. 1980;1:1198-1199

118. Azziz-Baumgartner E, Lindblade K, Gieseker K, Rogers HS, Kieszak S, Njapau H, Schleicher R, McCoy LF, Misore A, DeCock K, Rubin C, Slutsker L. Case-control study of an acute aflatoxicosis outbreak, kenya, 2004. *Environ Health Perspect*. 2005;113:1779-1783

119. Casteels-van Daele M, Eggermont E. Reye's syndrome. *BMJ*. 1994;308:919-920

120. Olson LC, Bourgeois CH, Jr., Cotton RB, Harikul S, Grossman RA, Smith TJ. Encephalopathy and fatty degeneratiof on the viscera in northeastern thailand. Clinical syndrome and epidemiology. *Pediatrics*. 1971;47:707-716

121. Dvorackova I, Kusak V, Vesely D, Vesela J, Nesnidal P. Aflatoxin and encephalopathy with fatty degeneration of viscera (reye). *Ann Nutr Alimen*. 1977;31:977-989

122. Dvorackova I, Vesely D, Kusak V. [aflatoxin in the pathology of early-age infants]. *Cesk Pediatr*. 1979;34:80-83

123. Becroft DM, Webster DR. Aflatoxins and reye's disease. *Br Med J*. 1972;4:117

124. Hogan GR, Ryan NJ, Hayes AW. Aflatoxin b1 and reye's syndrome. *Lancet*. 1978;1:561

125. Ryan NJ, Hogan GR, Hayes AW, Unger PD, Siraj MY. Aflatoxin b1; its role in the etiology of reye's syndrome. *Pediatrics*. 1979;64:71-75

126. Shank RC, Bourgeois CH, Keschamras N, Chandavimol P. Aflatoxins in autopsy specimens from thai children with an acute disease of unknown aetiology. *Food Cosmet Toxicol*. 1971;9:501-507

127. Rogan WJ, Yang GC, Kimbrough RD. Aflatoxin and reye's syndrome: A study of livers from deceased cases. *Arch Environ Health*. 1985;40:91-95

128. Allen SJ, Wild CP, Wheeler JG, Riley EM, Montesano R, Bennett S, Whittle HC, Hall AJ, Greenwood BM. Aflatoxin exposure, malaria and hepatitis b infection in rural gambian children. *Trans R Soc Trop Med Hyg*. 1992;86:426-430

129. Wang JS, Huang T, Su J, Liang F, Wei Z, Liang Y, Luo H, Kuang SY, Qian GS, Sun G, He X, Kensler TW, Groopman JD. Hepatocellular carcinoma and aflatoxin exposure in zhuqing village, fusui county, people's republic of china. *Cancer Epidemiol Biomarkers Prev*. 2001;10:143-146

130. Diallo MS, Sylla A, Sidibe K, Sylla BS, Trepo CR, Wild CP. Prevalence of exposure to aflatoxin and hepatitis b and c viruses in guinea, west africa. *Nat Toxins*. 1995;3:6-9

131. Sylla A, Diallo MS, Castegnaro J, Wild CP. Interactions between hepatitis b virus infection and exposure to aflatoxins in the development of hepatocellular carcinoma: A molecular epidemiological approach. *Mut Res*. 1999;428:187-196

132. Hatch MC, Chen CJ, Levin B, Ji BT, Yang GY, Hsu SW, Wang LW, Hsieh LL, Santella RM. Urinary aflatoxin levels, hepatitis-b virus infection and hepatocellular carcinoma in taiwan. *Int J Cancer.* 1993;54:931-934

133. Qian GS, Ross RK, Yu MC, Yuan JM, Gao YT, Henderson BE, Wogan GN, Groopman JD. A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in shanghai, people's republic of china. *Cancer Epidemiol Biomarkers Prev*. 1994;3:3-10

134. Wang LY, Hatch M, Chen CJ, Levin B, You SL, Lu SN, Wu MH, Wu WP, Wang LW, Wang Q, Huang GT, Yang PM, Lee HS, Santella RM. Aflatoxin exposure and risk of hepatocellular carcinoma in taiwan. *Int J Cancer*. 1996;67:620-625

135. Chen CJ, Wang LY, Lu SN, Wu MH, You SL, Zhang YJ, Wang LW, Santella RM. Elevated aflatoxin exposure and increased risk of hepatocellular carcinoma. *Hepatology*. 1996;24:38-42

136. Ross RK, Yuan JM, Yu MC, Wogan GN, Qian GS, Tu JT, Groopman JD, Gao YT, Henderson BE. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. *Lancet*. 1992;339:943-946

137. Liu Y, Chang CCH, Marsh GM, Wu F. Population attributable risk of aflatoxin-related liver cancer: Systematic review and meta-analysis. *Eur J Cancer*. 2012;48:2125-2136

138. Henry SH, Bosch FX, Bowers JC. Aflatoxin, hepatitis and worldwide liver cancer risks. *Adv Exp Med Biol*. 2002;504:229-233

139. Turner PC, Sylla A, Diallo MS, Castegnaro JJ, Hall AJ, Wild CP. The role of aflatoxins and hepatitis viruses in the etiopathogenesis of hepatocellular carcinoma: A basis for primary prevention in guinea-conakry, west africa. *J Gastroenterol Hepatol*. 2002;17 Suppl:S441-448

140. Kew MC. Synergistic interaction between aflatoxin b1 and hepatitis b virus in hepatocarcinogenesis. *Liver Int*. 2003;23:405-409

141. Wild CP, Montesano R. A model of interaction: Aflatoxins and hepatitis viruses in liver cancer aetiology and prevention. *Cancer Lett*. 2009;286:22-28

142. Wild CP, Turner PC. The toxicology of aflatoxins as a basis for public health decisions. *Mutagenesis*. 2002;17:471-481

143. Lereau M, Gouas D, Villar S, Besaratinia A, Hautefeuille A, Berthillon P, Martel-Planche G, Nogueira da Costa A, Ortiz-Cuaran S, Hantz O, Pfeifer GP, Hainaut P, Chemin I. Interactions between hepatitis b virus and aflatoxin b(1): Effects on p53 induction in heparg cells. *J Gen Virol*. 2012;93:640-650

144. Bondy GS, Pestka JJ. Immunomodulation by fungal toxins. *Journal of toxicology and environmental health. Part B, Crit Rev*. 2000;3:109-143

145. Sharma RP. Immunotoxicity of mycotoxins. *J Dairy Sci*. 1993;76:892-897

146. Hatori Y, Sharma RP, Warren RP. Resistance of c57bl/6 mice to immunosuppressive effects of aflatoxin b1 and relationship with neuroendocrine mechanisms. *Immunopharmacology*. 1991;22:127-136

147. Dugyala RR, Sharma RP. The effect of aflatoxin b1 on cytokine mrna and corresponding protein levels in peritoneal macrophages and splenic lymphocytes. *Int J Immunopharmacol*. 1996;18:599-608

148. Moon EY, Rhee DK, Pyo S. Inhibition of various functions in murine peritoneal macrophages by aflatoxin b1 exposure in vivo. *Int J Immunopharmacol*. 1999;21:47-58

149. Moon EY, Rhee DK, Pyo S. In vitro suppressive effect of aflatoxin b-1 on murine peritoneal macrophage functions. *Toxicology*. 1999;133:171-179

150. Raisuddin, Singh KP, Zaidi SI, Saxena AK, Ray PK. Effects of aflatoxin on lymphoid cells of weanling rat. *J Appl Toxicol*. 1990;10:245-250

151. Raisuddin S, Singh KP, Zaidi SI, Paul BN, Ray PK. Immunosuppressive effects of aflatoxin in growing rats. *Mycopathologia*. 1993;124:189-194

152. Hinton DM, Myers MJ, Raybourne RA, Francke-Carroll S, Sotomayor RE, Shaddock J, Warbritton A, Chou MW. Immunotoxicity of aflatoxin b1 in rats: Effects on lymphocytes and the inflammatory response in a chronic intermittent dosing study. *Toxicol Sci*. 2003;73:362-377

153. Marin DE, Taranu I, Bunaciu RP, Pascale F, Tudor DS, Avram N, Sarca M, Cureu I, Criste RD, Suta V, Oswald IP. Changes in performance, blood parameters, humoral and cellular immune responses in weanling piglets exposed to low doses of aflatoxin. *J Anim Sci*. 2002;80:1250-1257

154. Meissonnier GM, Martin, D.E., Galtier, P., Bertin, G., Oswald, I.P. Modulation of the immune response by a group of fungal food contaminant, the aflatoxins. In: Mengheri, E. Roselli, M., Britti, M.S., Finamore, A. (Eds.), *Nutrition and Immunity.* Research Signpost, Kerala. 2006:147-166.

155. Sirajudeen M, Gopi K, Tyagi JS, Moudgal RP, Mohan J, Singh R. Protective effects of melatonin in reduction of oxidative damage and immunosuppression induced by aflatoxin b1-contaminated diets in young chicks. *Environ Toxicol*. 2011;26:153-160

156. Meissonnier GM, Pinton P, Laffitte J, Cossalter AM, Gong YY, Wild CP, Bertin G, Galtier P, Oswald IP. Immunotoxicity of aflatoxin b1: Impairment of the cell-mediated response to vaccine antigen and modulation of cytokine expression. *Toxicol Appl Pharmacol*. 2008;231:142-149

157. Jakab GJ, Hmieleski RR, Zarba A, Hemenway DR, Groopman JD. Respiratory aflatoxicosis: Suppression of pulmonary and systemic host defenses in rats and mice. *Toxicol Appl Pharmacol*. 1994;125:198-205

158. Thurston JR, Graham CK, Richard JL, Cysewski SJ, Pier AC. Effect of aflatoxin on complement activity in guinea-pigs. *P Soc Exp Biol Med*. 1972;139:300-303

159. Stewart RG, Skeeles JK, Wyatt RD, Brown J, Page RK, Russell ID, Lukert PD. The effect of aflatoxin on complement activity in broiler chickens. *Poult Sci*. 1985;64:616-619

160. Richard JL. Mycotoxins as immunomodulators in animal systems. In: Bray GA, Ryan DH (eds) *Mycotoxins, Cancer, and Health. Pennington Nutrition Series,* Louisiana State Univ Press, Baton Rouge, LA, pp 197-220. 1991

161. Richard JL, Thurston JR, Deyoe BL, Booth GD. Effect of ochratoxin and aflatoxin on serum proteins, complement activity, and antibody production to brucella abortus in guinea pigs. *App Microbiol*. 1975;29:27-29

162. Michael GY, Thaxton P, Hamilton PB. Impairment of reticuloendothelial system of chickens during aflatoxicosis. *Poult Sci*. 1973;52:1206-1207

163. Richard JL, Pier AC, Cysewski SJ, Graham CK. Effect of aflatoxin and aspergillosis on turkey poults. *Avian Dis*. 1973;17:111-121

164. Robens JF, Richard JL. Aflatoxins in animal and human health. *Rev Environ Contam Toxicol*. 1992;127:69-94

165. Larsen AB, Cysewski SJ, Miller JM. Effect of aflatoxin on susceptibility of hamsters to mycobacterium paratuberculosis. *Am J Vet Res*. 1975;36:1545-1547

166. Turner PC, Moore SE, Hall AJ, Prentice AM, Wild CP. Modification of immune function through exposure to dietary aflatoxin in gambian children. *Environ Health Perspect*. 2003;111:217-220

167. Jiang Y, Jolly PE, Ellis WO, Wang JS, Phillips TD, Williams JH. Aflatoxin b1 albumin adduct levels and cellular immune status in ghanaians. *Int Immunol*. 2005;17:807-814

168. Gong YY, Cardwell K, Hounsa A, Egal S, Turner PC, Hall AJ, Wild CP. Dietary aflatoxin exposure and impaired growth in young children from benin and togo: Cross sectional study. *BMJ*. 2002;325:20-21

169. Gong Y, Hounsa A, Egal S, Turner PC, Sutcliffe AE, Hall AJ, Cardwell K, Wild CP. Postweaning exposure to aflatoxin results in impaired child growth: A longitudinal study in benin, west africa. *Environ Health Perspect*. 2004;112:1334-1338

170. Abdulrazzaq YM, Osman N, Ibrahim A. Fetal exposure to aflatoxins in the united arab emirates. *Ann Trop Paediatr*. 2002;22:3-9

171. Smith LE, Stoltzfus RJ, Prendergast A. Food chain mycotoxin exposure, gut health, and impaired growth: A conceptual framework. *Adv Nutr*. 2012;3:526-531

172. Williams CD. Kwashiorkor: A nutritional disease of children associated with a maize diet. *Lancet*. 1935:1151-1152

173. Lamplugh SM, Hendrickse RG. Aflatoxins in the livers of children with kwashiorkor. *Ann Trop Paediatr*. 1982;2:101-104

174. Hendrickse RG, Coulter JB, Lamplugh SM, Macfarlane SB, Williams TE, Omer MI, Suliman GI. Aflatoxins and kwashiorkor: A study in sudanese children. *Br Med J (Clin Res Ed)*. 1982;285:843-846

175. Ramjee G, Berjak P, Adhikari M, Dutton MF. Aflatoxins and kwashiorkor in durban, south africa. *Ann Trop Paediatr*. 1992;12:241-247

176. Hendrickse RG. The influence of aflatoxins on child health in the tropics with particular reference to kwashiorkor. *Trans R Soc Trop Med Hyg*. 1984;78:427-435

177. Hendrickse RG. Kwashiorkor - the hypothesis that incriminates aflatoxins. *Pediatrics*. 1991;88:376-379

178. Oyelami OA, Maxwell SM, Adelusola KA, Aladekoma TA, Oyelese AO. Aflatoxins in the lungs of children with kwashiorkor and children with miscellaneous diseases in nigeria. *J Toxicol Environ Health*. 1997;51:623-628

179. Kocabas CN, Coskun T, Yurdakok M, Haziroglu R. The effects of aflatoxin b1 on the development of kwashiorkor in mice. *Hum Exp Toxicol*. 2003;22:155-158

180. Krawinkel M. Kwashiorkor is still not fully understood. *Bull World Health Organ*. 2003;81:910-911

181. Household KC, Hundt HK. Aflatoxin exposure and its relationship to kwashiorkor in african children. *J Trop Pediatr*. 1991;37:300-302

182. Council NR. Committee: Biological markers in environmental health research. *Environ Health Perspect*. 1987;74:3-9

183. Wogan GN. Molecular epidemiology in cancer risk assessment and prevention: Recent progress and avenues for future research. *Environ Health Perspect*. 1992;98:167-178

184. Groopman JD, Kensler TW. The light at the end of the tunnel for chemical-specific biomarkers: Daylight or headlight? *Carcinogenesis*. 1999;20:1-11

185. Sabbioni G, Skipper PL, Buchi G, Tannenbaum SR. Isolation and characterization of the major serum albumin adduct formed by aflatoxin b1 in vivo in rats. *Carcinogenesis*. 1987;8:819-824

186. Gan LS, Skipper PL, Peng XC, Groopman JD, Chen JS, Wogan GN, Tannenbaum SR. Serum albumin adducts in the molecular epidemiology of aflatoxin carcinogenesis: Correlation with aflatoxin b1 intake and urinary excretion of aflatoxin m1. *Carcinogenesis*. 1988;9:1323-1325

187. Croy RG, Wogan GN. Temporal patterns of covalent DNA adducts in rat-liver after single and multiple doses of aflatoxin-b1. *Cancer Res*. 1981;41:197-203

188. Gopalakrishnan S, Harris TM, Stone MP. Intercalation of aflatoxin-b1 in 2 oligodeoxynucleotide adducts - comparative h-1-nmr analysis of d(atcafbgat).D(atcgat) and d(atafbgcat)2. *Biochemistry*. 1990;29:10438-10448

189. Johnson WW, Guengerich FP. Reaction of aflatoxin b-1 exo-8,9-epoxide with DNA: Kinetic analysis of covalent binding and DNA-induced hydrolysis. *Proc Natl Acad Sci U S A*. 1997;94:6121-6125

190. Groopman JD, Donahue KF. Aflatoxin, a human carcinogen: Determination in foods and biological samples by monoclonal antibody affinity chromatography. *J Assoc Off Anal Chem*. 1988;71:861-867

191. Wang JS, Groopman JD. DNA damage by mycotoxins. *Mut Res*. 1999;424:167-181

192. Groopman JD, Zhu JQ, Donahue PR, Pikul A, Zhang LS, Chen JS, Wogan GN. Molecular dosimetry of urinary aflatoxin-DNA adducts in people living in guangxi autonomous region, people's republic of china. *Cancer Res*. 1992;52:45-52

193. Yu MW, Lien JP, Chiu YH, Santella RM, Liaw YF, Chen CJ. Effect of aflatoxin metabolism and DNA adduct formation on hepatocellular carcinoma among chronic hepatitis b carriers in taiwan. *J Hepatol*. 1997;27:320-330

194. Holzapfel CW, Steyn PS, Purchase IF. Isolation and structure of aflatoxins m1 and m2. *Tetrahedron Lett*. 1966;25:2799-2803

195. Deiongh H, Vanpelt JG, Vles RO. Milk of mammals fed aflatoxin-containing diet. *Nature*. 1964;202:466-&

196. Allcroft R, Rogers H, Lewis G, Nabney J, Best PE. Metabolism of aflatoxin in sheep: Excretion of the "milk toxin". *Nature*. 1966;209:154-155

197. Campbell TC, Caedo JP, Jr., Bulatao-Jayme J, Salamat L, Engel RW. Aflatoxin m1 in human urine. *Nature*. 1970;227:403-404

198. Zhu JQ, Zhang LS, Hu X, Xiao Y, Chen JS, Xu YC, Fremy J, Chu FS. Correlation of dietary aflatoxin b1 levels with excretion of aflatoxin m1 in human urine. *Cancer Res*. 1987;47:1848-1852

199. Purchase IF. Acute toxicity of aflatoxins m1 and m2 in one-day-old ducklings. *Food Cosmet Toxicol*. 1967;5:339-342

200. Pong RS, Wogan GN. Toxicity and biochemical and fine structural effects of synthetic aflatoxins m 1 and b 1 in rat liver. *J Natl Cancer Inst*. 1971;47:585-592

201. Hsieh DP, Cullen JM, Ruebner BH. Comparative hepatocarcinogenicity of aflatoxins b1 and m1 in the rat. *Food Chem Toxicol*. 1984;22:1027-1028

202. Cullen JM, Ruebner BH, Hsieh LS, Hyde DM, Hsieh DP. Carcinogenicity of dietary aflatoxin m1 in male fischer rats compared to aflatoxin b1. *Cancer Res*. 1987;47:1913-1917

203. Henry SH, Pennington JA, Whitaker TB, Verger PJP, X. BF. Aflatoxin m1 and risks of liver cancer. *SRA Annual Meeting*. 2001; Abstract

204. Peng KY, Chen CY. Prevalence of aflatoxin m1 in milk and its potential liver cancer risk in taiwan. *J Food Prot*. 2009;72:1025-1029

205. Lafont P, Lafont J, Mousset S, Frayssinet C. [contamination of cow's milk during ingestion of small quantities of aflatoxin]. *Ann Nutr Alimen*. 1980;34:699-707

206. Abdulrazzaq YM, Osman N, Yousif ZM, Al-Falahi S. Aflatoxin m1 in breast-milk of uae women. *Ann Trop Paediatr*. 2003;23:173-179

207. Jonsyn FE, Maxwell SM, Hendrickse RG. Ochratoxin a and aflatoxins in breast milk samples from sierra leone. *Mycopathologia*. 1995;131:121-126

208. Polychronaki N, West RM, Turner PC, Amra H, Abdel-Wahhab M, Mykkanen H, El-Nezami H. A longitudinal assessment of aflatoxin m1 excretion in breast milk of selected egyptian mothers. *Food Chem Toxicol*. 2007;45:1210-1215

209. Gurbay A, Sabuncuoglu SA, Girgin G, Sahin G, Yigit S, Yurdakok M, Tekinalp G. Exposure of newborns to aflatoxin m1 and b1 from mothers' breast milk in ankara, turkey. *Food Chem Toxicol*. 2010;48:314-319

210. Zarba A, Wild CP, Hall AJ, Montesano R, Hudson GJ, Groopman JD. Aflatoxin m1 in human breast milk from the gambia, west africa, quantified by combined

monoclonal antibody immunoaffinity chromatography and hplc. *Carcinogenesis*. 1992;13:891-894

211. Wild CP, Garner RC, Montesano R, Tursi F. Aflatoxin b1 binding to plasma albumin and liver DNA upon chronic administration to rats. *Carcinogenesis*. 1986;7:853-858

212. Skipper PL, Hutchins DH, Turesky RJ, Sabbioni G, Tannenbaum SR. Carcinogen binding to serum-albumin. *P Am Assoc Canc Res*. 1985;26:90-90

213. Sabbioni G. Chemical and physical properties of the major serum albumin adduct of aflatoxin b1 and their implications for the quantification in biological samples. *Chem Biol Interact*. 1990;75:1-15

214. Guengerich FP, Arneson KO, Williams KM, Deng Z, Harris TM. Reaction of aflatoxin b(1) oxidation products with lysine. *Chem Res Toxicol*. 2002;15:780-792

215. Scholl PF, McCoy L, Kensler TW, Groopman JD. Quantitative analysis and chronic dosimetry of the aflatoxin b1 plasma albumin adduct lys-afb1 in rats by isotope dilution mass spectrometry. *Chem Res Toxicol*. 2006;19:44-49

216. Wild CP, Hasegawa R, Barraud L, Chutimataewin S, Chapot B, Ito N, Montesano R. Aflatoxin-albumin adducts: A basis for comparative carcinogenesis between animals and humans. *Cancer Epidemiol Biomarkers Prev*. 1996;5:179-189

217. Cupid BC, Lightfoot TJ, Russell D, Gant SJ, Turner PC, Dingley KH, Curtis KD, Leveson SH, Turteltaub KW, Garner RC. The formation of afb(1)-macromolecular adducts in rats and humans at dietary levels of exposure. *Food Chem Toxicol*. 2004;42:559-569

218. Scholl PF, Groopman JD. Long-term stability of human aflatoxin b1 albumin adducts assessed by isotope dilution mass spectrometry and high-performance liquid chromatography-fluorescence. *Cancer Epidemiol Biomarkers Prev*. 2008;17:1436-1439

219. Wang JS, Qian GS, Zarba A, He X, Zhu YR, Zhang BC, Jacobson L, Gange SJ, Munoz A, Kensler TW, et al. Temporal patterns of aflatoxin-albumin adducts in hepatitis b surface antigen-positive and antigen-negative residents of daxin, qidong county, people's republic of china. *Cancer Epidemiol Biomarkers Prev*. 1996;5:253-261

220. Tang L, Tang M, Xu L, Luo H, Huang T, Yu J, Zhang L, Gao W, Cox SB, Wang JS. Modulation of aflatoxin biomarkers in human blood and urine by green tea polyphenols intervention. *Carcinogenesis*. 2008;29:411-417

221. Wang P, Afriyie-Gyawu E, Tang Y, Johnson NM, Xu L, Tang L, Huebner HJ, Ankrah NA, Ofori-Adjei D, Ellis W, Jolly PE, Williams JH, Wang JS, Phillips TD. Novasil clay intervention in ghanaians at high risk for aflatoxicosis: Ii. Reduction in biomarkers of aflatoxin exposure in blood and urine. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2008;25:622-634

222. Wild CP, Jiang YZ, Sabbioni G, Chapot B, Montesano R. Evaluation of methods for quantitation of aflatoxin-albumin adducts and their application to human exposure assessment. *Cancer Res*. 1990;50:245-251

223. Qian G, Tang L, Liu W, Wang JS. Development of a non-antibody method for rapid detection of serum aflatoxin b1-lysine adduct. *Toxicologist*. 2010;114:248.

224. McCoy LF, Scholl PF, Schleicher RL, Groopman JD, Powers CD, Pfeiffer CM. Analysis of aflatoxin b1-lysine adduct in serum using isotope-dilution liquid chromatography/tandem mass spectrometry. *Rapid Comm Mass Spectrom*. 2005;19:2203-2210

225. Wang JS, Abubaker S, He X, Sun G, Strickland PT, Groopman JD. Development of aflatoxin b(1)-lysine adduct monoclonal antibody for human exposure studies. *Appl Environ Microbiol*. 2001;67:2712-2717

226. Scholl PF, Turner PC, Sutcliffe AE, Sylla A, Diallo MS, Friesen MD, Groopman JD, Wild CP. Quantitative comparison of aflatoxin b1 serum albumin adducts in humans by isotope dilution mass spectrometry and elisa. *Cancer Epidemiol Biomarkers Prev*. 2006;15:823-826

227. Scholl PF, Musser SM, Groopman JD. Synthesis and characterization of aflatoxin b1 mercapturic acids and their identification in rat urine. *Chemical Res Toxicol*. 1997;10:1144-1151

228. Kensler TW. Chemoprevention by inducers of carcinogen detoxication enzymes. *Environ Health Perspect*. 1997;105 Suppl 4:965-970

229. Wang JS, Shen X, He X, Zhu YR, Zhang BC, Wang JB, Qian GS, Kuang SY, Zarba A, Egner PA, Jacobson LP, Munoz A, Helzlsouer KJ, Groopman JD, Kensler TW. Protective alterations in phase 1 and 2 metabolism of aflatoxin b1 by oltipraz in residents of qidong, people's republic of china. *J Natl Cancer Inst*. 1999;91:347-354

230. Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature*. 1991;350:427-428

231. Bressac B, Kew M, Wands J, Ozturk M. Selective g to t mutations of p53 gene in hepatocellular carcinoma from southern africa. *Nature*. 1991;350:429-431

232. Murakami Y, Hayashi K, Hirohashi S, Sekiya T. Aberrations of the tumor suppressor p53 and retinoblastoma genes in human hepatocellular carcinomas. *Cancer Res*. 1991;51:5520-5525

233. Scorsone KA, Zhou YZ, Butel JS, Slagle BL. P53 mutations cluster at codon 249 in hepatitis b virus-positive hepatocellular carcinomas from china. *Cancer Res*. 1992;52:1635-1638

234. Coursaget P, Depril N, Chabaud M, Nandi R, Mayelo V, LeCann P, Yvonnet B. High prevalence of mutations at codon 249 of the p53 gene in hepatocellular carcinomas from senegal. *Br J Cancer*. 1993;67:1395-1397

235. Soini Y, Chia SC, Bennett WP, Groopman JD, Wang JS, DeBenedetti VM, Cawley H, Welsh JA, Hansen C, Bergasa NV, Jones EA, DiBisceglie AM, Trivers GE, Sandoval CA, Calderon IE, Munoz Espinosa LE, Harris CC. An aflatoxin-associated mutational hotspot at codon 249 in the p53 tumor suppressor gene occurs in hepatocellular carcinomas from mexico. *Carcinogenesis*. 1996;17:1007-1012

236. Aguilar F, Hussain SP, Cerutti P. Aflatoxin b1 induces the transversion of g-->t in codon 249 of the p53 tumor suppressor gene in human hepatocytes. *Proc Natl Acad Sci U S A*. 1993;90:8586-8590

237. Mace K, Aguilar F, Wang JS, Vautravers P, Gomez-Lechon M, Gonzalez FJ, Groopman J, Harris CC, Pfeifer AM. Aflatoxin b1-induced DNA adduct

formation and p53 mutations in cyp450-expressing human liver cell lines. *Carcinogenesis*. 1997;18:1291-1297

238. Tornaletti S, Pfeifer GP. Complete and tissue-independent methylation of cpg sites in the p53 gene: Implications for mutations in human cancers. *Oncogene*. 1995;10:1493-1499

239. Denissenko MF, Chen JX, Tang MS, Pfeifer GP. Cytosine methylation determines hot spots of DNA damage in the human p53 gene. *Proc Natl Acad Sci U S A*. 1997;94:3893-3898

240. Chan KT, Hsieh DP, Lung ML. In vitro aflatoxin b1-induced p53 mutations. *Cancer Lett*. 2003;199:1-7

241. Makarananda K, Pengpan U, Srisakulthong M, Yoovathaworn K, Sriwatanakul K. Monitoring of aflatoxin exposure by biomarkers. *J Toxicol Sci*. 1998;23 Suppl 2:155-159

242. Wild CP, Pisani P. Carcinogen DNA and protein adducts as biomarkers of human exposure in environmental cancer epidemiology. *Cancer Detect Prev*. 1998;22:273-283

243. Groopman JD. Molecular dosimetry methods for assessing human aflatoxin exposures. In: Eaton DL, Groopman JD, eds. *The toxicology of aflatoxins: human health, veterinary and agricultural significance*. 1993; London: Academic Press, 1993:259 –79.

244. Shuaib FM, Jolly PE, Ehiri JE, Yatich N, Jiang Y, Funkhouser E, Person SD, Wilson C, Ellis WO, Wang JS, Williams JH. Association between birth outcomes

and aflatoxin b1 biomarker blood levels in pregnant women in kumasi, ghana. *Trop Med Int Health.* 2010;15:160-167

245. Jonsyn-Ellis FE. Seasonal variation in exposure frequency and concentration levels of aflatoxins and ochratoxins in urine samples of boys and girls. *Mycopathologia*. 2001;152:35-40

246. Hendrickse RG, Maxwell SM. Aflatoxins and child health in the tropics. *J Toxicol-Toxin Rev*. 1989;8:31-41

247. Leong YH, Rosma A, Latiff AA, Izzah AN. Associations of serum aflatoxin b1-lysine adduct level with socio-demographic factors and aflatoxins intake from nuts and related nut products in malaysia. *Int J Hyg Environ Health*. 2012;215:368-372

248. Hollstein MC, Wild CP, Bleicher F, Chutimataewin S, Harris CC, Srivatanakul P, Montesano R. P53 mutations and aflatoxin b1 exposure in hepatocellular carcinoma patients from thailand. *Int J Cancer*. 1993;53:51-55

249. Tomerak RH, Shaban HH, Khalafallah OA, El Shazly MN. Assessment of exposure of egyptian infants to aflatoxin m1 through breast milk. *J Egyp Public Health Assoc*. 2011;86:51-55

250. Johnson NM, Qian G, Xu L, Tietze D, Marroquin-Cardona A, Robinson A, Rodriguez M, Kaufman L, Cunningham K, Wittmer J, Guerra F, Donnelly KC, Williams JH, Wang JS, Phillips TD. Aflatoxin and pah exposure biomarkers in a U.S. Population with a high incidence of hepatocellular carcinoma. *Sci Total Environ*. 2010;408:6027-6031

251. Turner PC, Sylla A, Gong YY, Diallo MS, Sutcliffe AE, Hall AJ, Wild CP. Reduction in exposure to carcinogenic aflatoxins by postharvest intervention measures in west africa: A community-based intervention study. *Lancet*. 2005;365:1950-1956

252. Brown D. Food chain mycotoxins 2010: Threats and solutions. *http://www.ansci.cornell.edu/cnconf/2010proceedings/CNC2010.7.Brown.pdf*. 2010

253. Wu F, Khlangwiset P. Health economic impacts and cost-effectiveness of aflatoxin-reduction strategies in africa: Case studies in biocontrol and post-harvest interventions. *Food Addit Contami Part A Chem Anal Control Expo Risk Assess*. 2010;27:496-509

254. Sporn MB, Suh N. Chemoprevention: An essential approach to controlling cancer. *Nat Rev Cancer*. 2002;2:537-543

255. Jacobson LP, Zhang BC, Zhu YR, Wang JB, Wu Y, Zhang QN, Yu LY, Qian GS, Kuang SY, Li YF, Fang X, Zarba A, Chen B, Enger C, Davidson NE, Gorman MB, Gordon GB, Prochaska HJ, Egner PA, Groopman JD, Munoz A, Helzlsouer KJ, Kensler TW. Oltipraz chemoprevention trial in qidong, people's republic of china: Study design and clinical outcomes. *Cancer Epidemiol Biomarkers Prev*. 1997;6:257-265

256. Kensler TW, He X, Otieno M, Egner PA, Jacobson LP, Chen B, Wang JS, Zhu YR, Zhang BC, Wang JB, Wu Y, Zhang QN, Qian GS, Kuang SY, Fang X, Li YF, Yu LY, Prochaska HJ, Davidson NE, Gordon GB, Gorman MB, Zarba A, Enger C, Munoz A, Helzlsouer KJ, et al. Oltipraz chemoprevention trial in

qidong, people's republic of china: Modulation of serum aflatoxin albumin adduct biomarkers. *Cancer Epidemiol Biomarkers Prev*. 1998;7:127-134

257. Scholl P, Musser SM, Kensler TW, Groopman JD. Inhibition of aflatoxin ml excretion in rat urine during dietary intervention with oltipraz. *Carcinogenesis*. 1996;17:1385-1388

258. Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, Kensler TW. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proc Natl Acad Sci U S A*. 2001;98:3410-3415

259. Breinholt V, Hendricks J, Pereira C, Arbogast D, Bailey G. Dietary chlorophyllin is a potent inhibitor of aflatoxin b1 hepatocarcinogenesis in rainbow trout. *Cancer Res*. 1995;55:57-62

260. Dashwood R, Negishi T, Hayatsu H, Breinholt V, Hendricks J, Bailey G. Chemopreventive properties of chlorophylls towards aflatoxin b1: A review of the antimutagenicity and anticarcinogenicity data in rainbow trout. *Mut Res*. 1998;399:245-253

261. Breinholt V, Schimerlik M, Dashwood R, Bailey G. Mechanisms of chlorophyllin anticarcinogenesis against aflatoxin b1: Complex formation with the carcinogen. *Chem Res Toxicol*. 1995;8:506-514

262. Fahey JW, Stephenson KK, Dinkova-Kostova AT, Egner PA, Kensler TW, Talalay P. Chlorophyll, chlorophyllin and related tetrapyrroles are significant inducers of mammalian phase 2 cytoprotective genes. *Carcinogenesis*. 2005;26:1247-1255

263. Afriyie-Gyawu E, Mackie J, Dash B, Wiles M, Taylor J, Huebner H, Tang L, Guan H, Wang JS, Phillips T. Chronic toxicological evaluation of dietary novasil clay in sprague-dawley rats. *Food Addit Contam*. 2005;22:259-269

264. Wang JS, Luo H, Billam M, Wang Z, Guan H, Tang L, Goldston T, Afriyie-Gyawu E, Lovett C, Griswold J, Brattin B, Taylor RJ, Huebner HJ, Phillips TD. Short-term safety evaluation of processed calcium montmorillonite clay (novasil) in humans. *Food Addit Contam*. 2005;22:270-279

265. Qin G, Gopalan-Kriczky P, Su J, Ning Y, Lotlikar PD. Inhibition of aflatoxin b1-induced initiation of hepatocarcinogenesis in the rat by green tea. *Cancer Lett*. 1997;112:149-154

266. Moyers SB, Kumar NB. Green tea polyphenols and cancer chemoprevention: Multiple mechanisms and endpoints for phase ii trials. *Nutr Rev*. 2004;62:204-211

Table 2.1. Acute toxicity of AFB_1.

Species	Strain	LD_{50} (mg/kg)	Sex	Age or weight	Route
Duck	Khaki-Campbell	0.36	M,F	1 day	P.O.
Duckling	Peking	0.34	M,F	1 day	P.O.
Chicken		18	M	21 days	P.O.
Turkey	Beltsville	3.2	M	15 days	P.O.
Trout	Mt. Shasta	0.81	M,F	9 months	I.P.
Catfish	Channel	11.5	M,F	9.3-0.5 kg	P.O.
Mouse	Swiss	1.5	M,F	newborn	P.O.
	Swiss	>150	M	30 days	I.P.
	Swiss	40	M	58 days	I.P.
	Swiss	12	M	100 days	I.P.
	CD-1	7.3	M	weaning	P.O.
Rat	Porton	6.25	M	42 days	P.O.
	Porton	18	F	43 days	P.O.
	Fischer	4.2	M	42 days	I.P.
	Fischer	1.1	M,F	2 days	S.C.
	Fischer	8	M,F	21 days	I.P.
Hamster	Syrian	12.8	M	30 days	P.O.
	Syrian	5.85	F	42 days	I.P.
Guinea Pig		1	M	56 days	P.O.
		1.8	F	56 days	P.O.
Rabbit	Dutch breed	0.3	M,F	90 days	I.P.
Cat	Mixed breed	0.55	M,F	adult	P.O.
Dog	Mixed breed	0.8	M,F	weaning	P.O.
Pig	Poland China	0.62	M	weaning	P.O.
Sheep	Cross breed	2	M	2 years	P.O.
Baboon	Wild	2.2	M	aduct	P.O.
Monkey					
	Cynomolgus	2.2	M	adult	P.O.
	Macaque	8	F	adult	P.O.

I.P., intraperitoneal; P.O., per oral. S.C., subcutaneous (References #99, 101-104)

Table 2.2. Acute aflatoxicoses in humans.

Time	Country	# of Subjects affected	Contaminated foods	Reference
1966	USA	1	purified mixtures containg	117
1970	Uganda	1	Cassava	116
1974	Western India	397	maize	111
1974	Northwest India	994	maize	112
1982	Kenya	20	maize	113
1988	Malaysia	13	a Chinese noodle, "loh see fun"	114
2004	Kenya	317	maize and maize products	118

Table 2.3. A compilation of data on biomarkers of exposure to aflatoxin in humans

Country	Exposure rate (%)	Range or test type	Reference
Benin	99	5–1064 pmol/mg albumin	Gong et al (168)
China (Guangxi province)	89	0.9–3569 pg/24-h urine	Wang et al (219)
Egypt	65.3	AFM_1 in brease milk, 0.2 to 19.0 ng/mL	Tomerak et al (249)
Gambia	95	0–720 pmol/mg albumin	Allen et al (128)
Ghana	90.7	0.44 - 286.73 pg/mg albumin	Shuaib et al (244)
Guinea	90	0–385 pmol/mg albumin	Diallo et al (130)
Malaysia	98	0.81 - 13.67 pg/mg albumin	Leong et al (247)
Nigeria	40-90	Lung autopsies	Oyelami et al (178)
Sierra Leone	95–99	Aflatoxin in urine	Jonsyn-Ellis (245)
Sudan, Zimbabwe, Ghana, Liberia, Kenya, Transkei	32	Aflatoxin in urine	Hendrickse et al (246)
	99	Liver biopsy of kwashiorkors	
Thailand	44	AFM_1 in breast milk	Hollstein et al (248)
United States (San Antonio and Lubbock)	15	0.52-16.01 pg/mg albumin	Johnson et al (250) and unpublished data
Burkina Faso	100	0.56 - 918.72 pg/mg albumin	unpublished data
Haiti	67.5	0.41 - 130.39 pg/mg albumin	unpublished data
Uganda	95.3	0.41 - 253.11 pg/mg albumin	unpublished data

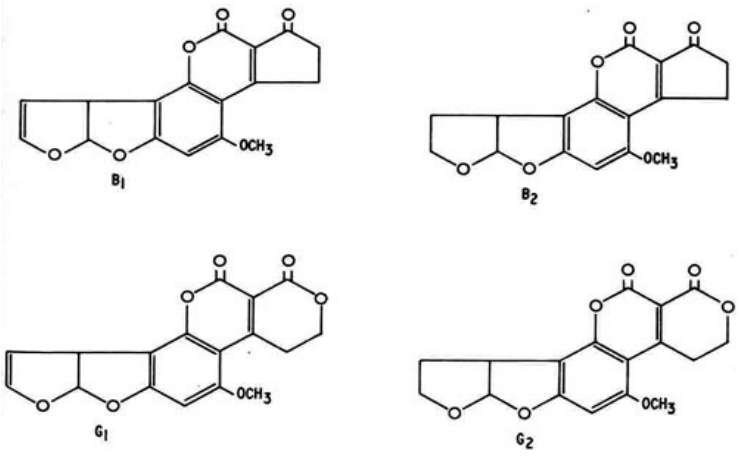

Figure 2.1. Structures of aflatoxin B_1, B_2, G_1, and G_2

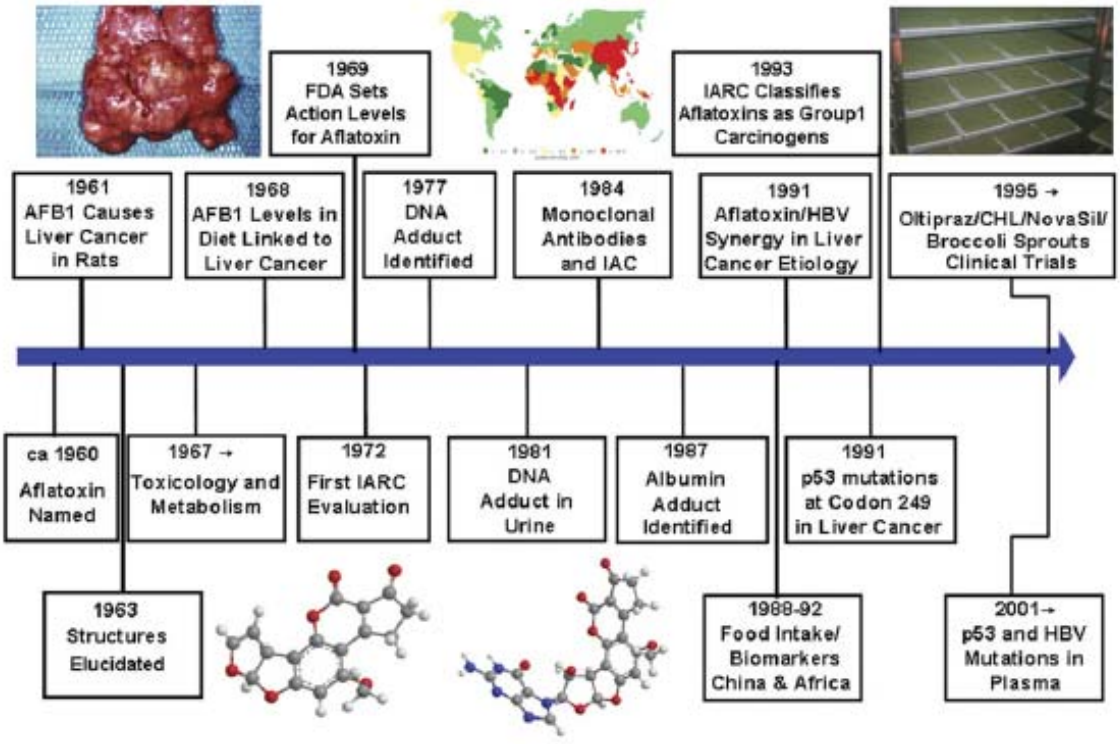

Figure 2.2. Time line for key events in aflatoxin research (Reference #4)

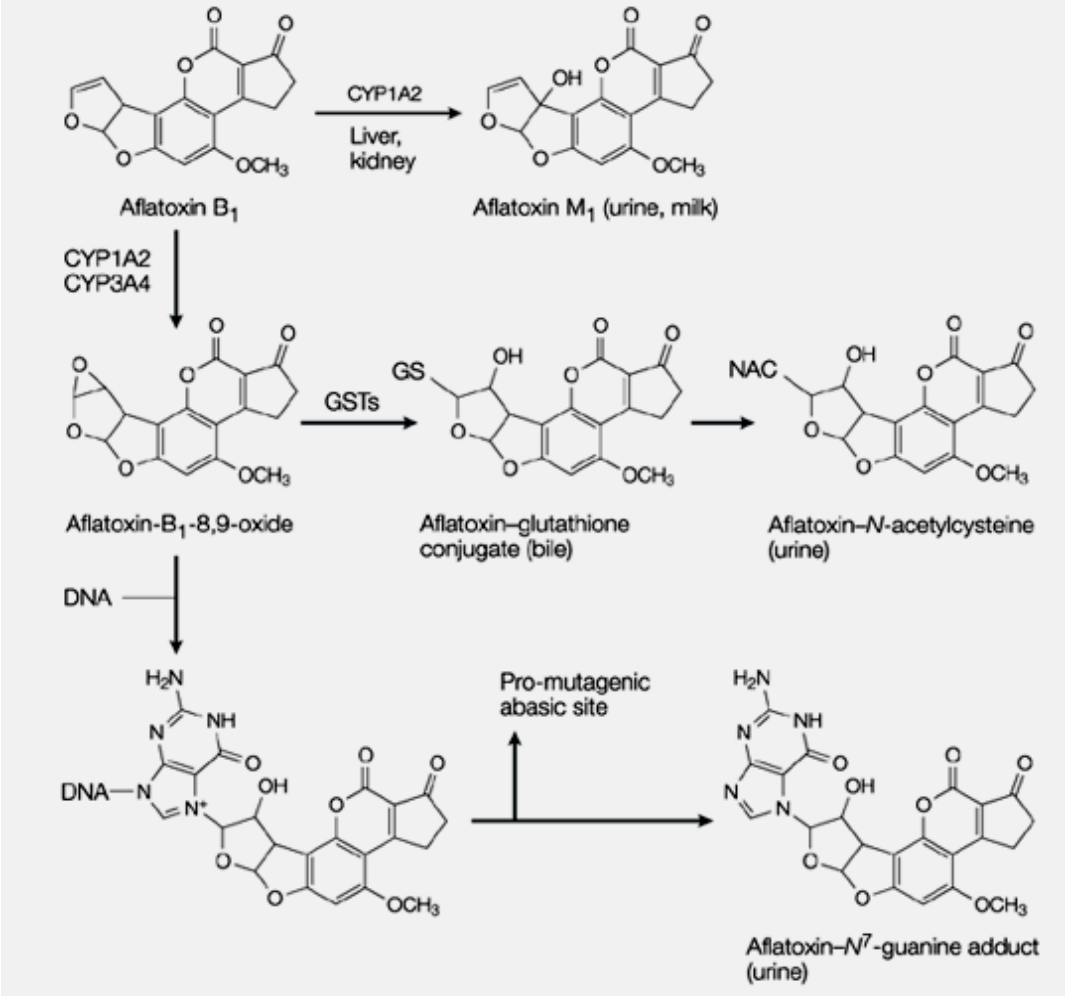

Figure 2.3. Detoxification pathways of aflatoxin B_1 (Reference #73)

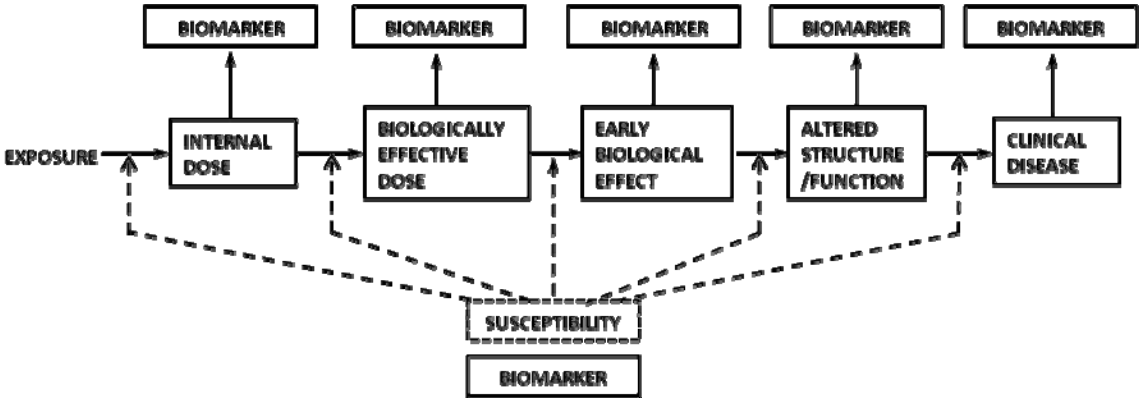

Figure 2.4. Biomarker paradigm (Reference #182)

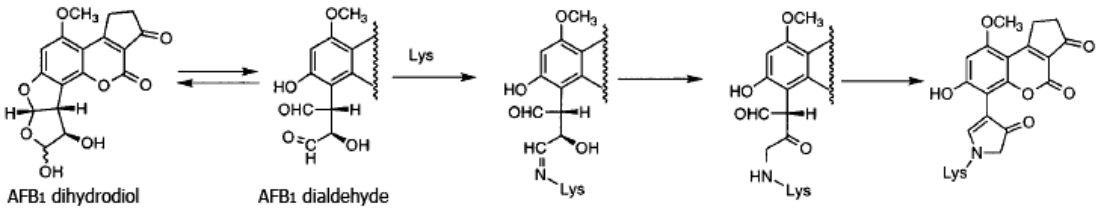

Figure 2.5. Proposed reaction of AFB_1 dialdehyde with lysine (Reference #213)

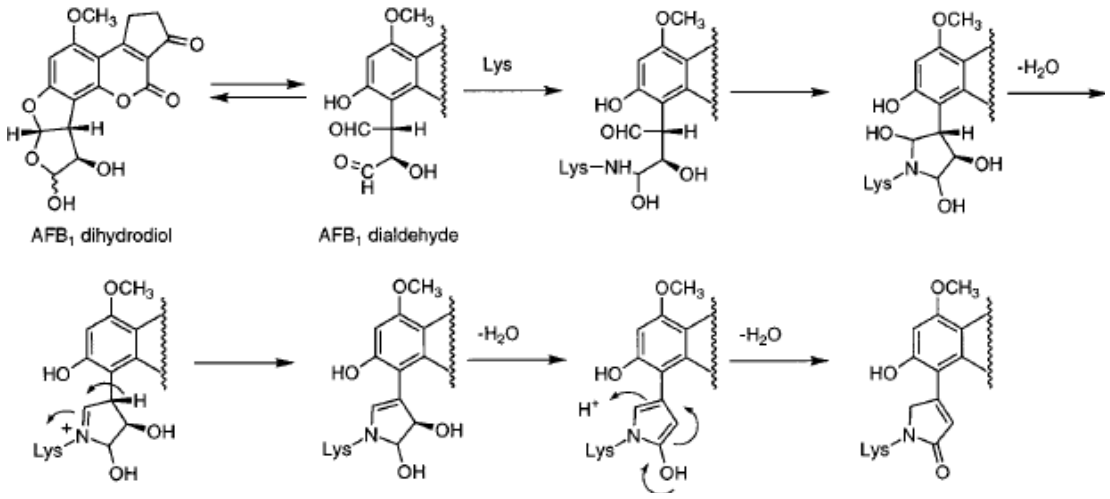

Figure 2.6. Alternative reaction of AFB_1 dialdehyde with lysine (Reference #214)

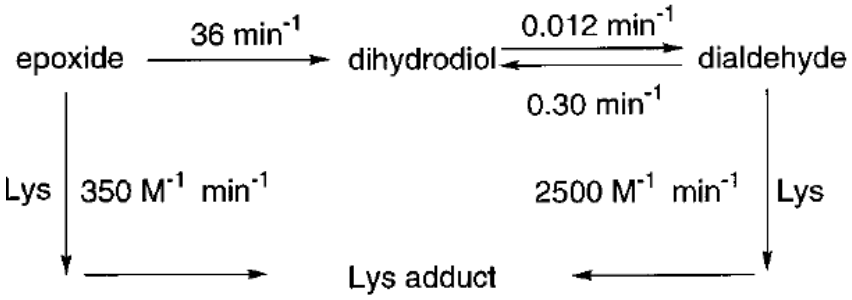

Figure 2.7. Proposed kinetic model for relative contribution of AFB_1-8,9-epoxide and AFB_1 dialdehyde in formation of lysine adduct (Reference #214)

CHAPTER 3

DEVELOPMENT OF A HPLC WITH FLUORESCENCE DETECTION METHOD FOR MEASURING SERUM AFLATOXIN B_1-LYSINE ADDUCT [1]

[1] Qian, G. Tang, L. Liu, W. and Wang, J.-S. To be submitted to *Toxicology*.

Abstract

Aflatoxins (AF) exposure is a major public health problem in the developing world, because acute aflatoxicosis occurs frequently and chronic exposure is a major risk factor for human liver cancer. Serum aflatoxin B_1-lysine adduct (AFB-Lys) is a reliable biomarker of AF exposure. The aim of this study is to develop a solid-phase extraction based high pressure liquid chromatography (HPLC) method with fluorescence detection for rapid detection of serum AFB-Lys. The protocols were extensively optimized, including conditions of enzyme digestion; comparison of various commercial solid-phase extraction cartridges, and for HPLC separation and quantification. The optimal level of AFB-Lys was detected in serum samples digested with pronase at 37°C for 3 h. Among various cartridges, OASIS MAX™ gave better recovery and efficiency, as compared to OASIS HLB™ and Sep-Pak™ cartridges. Calibration curves were linear for concentrations ranging from 0.01-5.0 ng/mL. The method was tested with AFB_1 treated rat serum and normal human serum spiked with synthesized authentic AFB-Lys from low to high concentrations. The imprecision based on 15 measurements on 3 separate days was 5.93% and 2.87% for low quality control (LQC) and high quality control (HQC) samples, respectively. The inaccuracy for repeated measurements was 4.97% and 4.44%, for LQC and HQC, respectively. Recoveries ranged 72% to 95% based on various adduct concentrations. Under the current procedures, AFB-Lys was stable for up to 12 h and decreased about 10% at 24 h at room temperature. Serum AFB-Lys levels had no

significant changes for up to 3 freeze-thaw cycles. The limit of detection was 0.4 pg/mg albumin.

Key words: aflatoxin; aflatoxin B_1 lysine; biomarker; HPLC.

Introduction

Aflatoxins are a large group of secondary fungal metabolites produced primarily by *Aspergillus flavus* and *Aspergillus parasiticus*, which occur naturally in foods such as corn, peanuts and oilseeds [1]. The human diseases caused by aflatoxins remain a largely ignored public health issue [2]. Human epidemiological studies have recognized aflatoxin exposure as a major risk factor for human hepatocellular carcinoma (HCC) in southeastern Asia and Sub-Sahara Africa [3]. It is estimated that approximately 4.5 billion people are affected by chronic exposure to aflatoxins in the developing world [4].

The development, validation and application of molecular chemical-specific biomarkers have the advantage of assessing internal human exposure to environmental agents on an individual basis, and therefore more accurate and precise than traditional food sampling survey [5-7]. Increasing knowledge of the toxicology and mechanisms of aflatoxins toxicity have provided insights into measurable biomarkers that have gained practical application in many human populations for assessment of internal human exposure and efficacy of intervention strategies [7-9].

Aflatoxin B_1 (AFB_1) is the most potent among naturally occurring aflatoxins, its mutagenicity and carcinogenicity, have been proven by numerous *in vitro* and *in vivo* test models and human studies as well [10]. AFB_1 is metabolized and activated by several cytochrome P450 (1A2, 2A6, 3A4, 3A5) to its most potent metabolite, *exo*-8,9-epoxide [11-13]. This active metabolite can bind to protein and DNA, and the ratio of hepatic protein to DNA adducts is constant even at very low dose [14]. The epoxide can readily attack

DNA to form the AFB_1-N^7-guanine adduct [15] if it has not been hydrolyzed or conjugated with glutathione [16]. The AFB_1-N^7-guanine adduct is excreted in urine and represents a short term effect marker with a half-life of about 7.5 hours in human [17-19]. Albumin is the only protein in serum that binds AFB_1 to a significant level in rats and about 1-3% of a single dose of AFB_1 is bound to albumin in rats [14, 20, 21]. AFB_1 albumin adduct represents a relative long-term exposure marker which has a half-life of 2.5 days in rats and 21 days in humans [22, 23]. The isolation and characterization of serum AFB-Lys adduct *in vivo* was reported by Sabbioni et al. [22], and further discussed by Guengerich et al. who examined the kinetics of reaction of AFB_1 dialdehyde and AFB_1-8,9-exposide with lysine *in vitro* [24]. Serum AFB-Lys has been employed as an exposure biomarker of AFB_1 in many human epidemiological studies [25-31].

Since the late 1980s, different analytical methods have been developed for quantification of AFB-Lys in human sera, including radioimmunoassay (RIA) [32, 33], enzyme linked immunosorbent assay (ELISA) [34], immnoaffinity column based high performance liquid chromatography (HPLC) method with fluorescence detection [27, 35]. The isotope-dilution mass spectrometry (IDMS) method has also been developed for measuring AFB-Lys adduct in human and rat sera [21, 36, 37]. However, the use of antibodies in sample purification or detection determines that the sensitivity and specificity of these methods are highly dependent on the antibodies selected. The prerequisite of LC/MS equipment and use of isotope in IDMS method makes it not particularly feasible in developing countries which lack such resources. In this study, we presented a non-

antibody based method for measuring serum AFB-Lys adducts using solid-phase extraction followed by HPLC separation with fluorescence detection.

Experimental Procedures

Chemicals and standards

Unlabeled AFB-Lys was synthesized using previously reported methods [22, 38]. Normal human serum, protein standard, ammonium phosphate monobasic, formic acid (FA) and phosphate-buffered saline (PBS) were obtained from Sigma Aldrich Chemical Co. (St. Lous, MO). Pronase (25 kU, nuclease-free) was purchased from Calbiochem (San Diego, CA). Albumin standard was obtained from Pointe Scientific Inc (Canton, MI). Methanol (MeOH), acetonitrile (ACN) and water were purchased from Mallinckrodt Baker Inc. (Phillipsburg NJ) as HPLC grade. Ammonium hydroxide was purchase from EMD Chemicals (Gibbstown, NJ). Protein assay dye reagent concentrate and protein standards were purchased from Bio-Rad Laboratories Inc. (Hercules, CA). Albumin reagent was purchased from Thermo Scientific Inc. (Waltham, MA). All other reagents or solvents are of highest grade available.

Equipment and Other materials

The Agilent 1100 series HPLC system (Santa Clara, CA) was used to analyze AFB-Lys and DU800 Series UV/Vis Spectrophotometer from Berkman Coulter Inc. (Fullerton, CA) was used to measure serum total protein and albumin content. Centrivap Concentrator and Centrivap coldtrap from Labconco Corporation (Kansas city, MO) were used for vaccum drying the purified eluates. VX-2500 multi-tube vortexer from VWR

International (West Chester, PA) were used to mix reconstituted samples. Oasis MAX™ 1cc (30 mg) extraction cartridge, Oasis HLB™ 3cc (60 mg) cartridge and Sep-Pak C18™ cartridge were purchased from Waters (Milford, MA). ZORBAX Eclipse XDB-C18 column (4.6×250 mm, 5 micron) was bought from Agilent Technologies Inc (Santa Clara, CA).

Quantification of Synthesized Standard

The synthesized AFB-Lys was quantified by measuring the absorbance at 400 nm wavelength in MeOH and the published molar extinction coefficient of 30866 ($Lmol^{-1}cm^{-1}$), as described in a previous study [38].

LC/MS/MS Confirmation of AFB-Lys Standard

The confirmation of synthetic AFB-Lys standard was done using a ThermoFinnigan HPLC system with surveyor autosampler and pump (Thermo Electron Corp. San Jose, CA, USA). The analytical column was a Thermo Betabasic-18 (150×2.1 mm, 5 μm). The mobile phases were: A, 10% ACN with 0.3% formic acid (FA); B, 90% ACN with 0.3% FA. The autosampler compartment was maintained at 4°C and column at 35°C. The mobile phase was run as a gradient at a flow rate of 0.2 mL/min. Initially, the mobile phase consisted of 100% A. Then 100% A was linearly decreased to 0% in 15 min. Isocratic elution was maintained for 10 min before changing back to the initial condition in 1 min and was the column was re-equilibrated to the initial condition for another 9 min. The single run-time was 35 min. The eluate was introduced into a ThermoFinnigan LCQ Advantage tandem mass spectrometer with electrospray

ionization. The positive ion mode was employed for selective reaction monitoring (SRM) m/z transition of 457 ($M+H^+$) to 394 for AFB-Lys [39]: capillary temperature 300°C, source voltage 4 kV, capillary voltage 7 V, the collision energy was set at 40 eV, sheath gas flow 60 units, auxiliary gas 20 units.

Binding Efficiency of Commercially Available Cartridges

MAX, HLB and Sep-Pak cartridges were compared for their binding efficiency, as shown in Figure 3.1. Briefly, the MAX cartridge was pre-conditioned with 1 mL of MeOH and equilibrated with 1 mL of water. Then 150 µL serum diluted with 500 µL DI water was loaded into the cartridge, followed by sequential washing with 1 mL water twice, 1 mL 70% MeOH, 1 mL 1% ammonium hydroxide in MeOH and 0.5 mL MeOH at a speed of approximately 1 mL/min. Vacuum was used to dry the cartridge after the last 0.5 mL MeOH washing. Elution of the AFB-Lys was done with 1 mL 2% formic acid in MeOH into a labeled 12×75 mm glass tube. Same procedure was used on both HLB™ and Sep-Pak™ cartridges. Briefly, the cartridges were conditioned with 1 mL MeOH and equilibrated with 1 mL water. Serum (150 µL) was diluted with 500 µL water which was passed through the cartridge. Then the cartridge was sequentially washed with 1 mL water, 1 mL 5% MeOH. Elution was made into a labeled 12×75 mm glass tube with 1 mL 100% MeOH. These eluates were further dried through a speed dryer at room temperature and reconstituted with 25% MeOH before analysis by HPLC with fluorescence detection.

Serum Sample Digestion Conditions

Diluted rat serum was used to determine the optimal conditions for enzymatic digestion and release of the AFB-Lys adduct such as incubation time, enzyme concentration used and temperature of incubation. A ratio of 1:4 (enzyme:total protein, w/w) was used for enzymatic digestion, as described previously [27]. Pronase was prepared in PBS (10mg/mL, pH 7.4) and 250 µL pronase was used to digest 150 µL serum by incubation at 37°C water bath for 3 h, and the mixture was immediately put onto ice to stop the reaction until solid-phase extraction.

Serum Sample Preparation

Aliquots of 150 µL human serum or diluted serum from AFB_1 treated rats were used for the method development. Solid-phase extraction with the Oasis Max™ cartridge was employed for the sample processing. The procedure for sample processing was same as that used in the Figure 3.1. The final eluates were dried under vacuum and reconstituted with 150 µL of 25% MeOH, mixed thoroughly using multi-tube vortexer for 5 min, and centrifuged at 5000 rpm for 10 min at 4°C. Finally, 125 µL of the supernatant was transferred into a HPLC vial for HPLC analysis.

HPLC Condition for AFB-Lys Analysis

The Agilent 1100 HPLC system with two mobile phases was used. Mobile phase A is 20 mM ammonium phosphate monobasic (pH 7.2) and B is 100% MeOH. A gradient was used for separation of AFB-Lys adduct and the run time was 25 min with a flow rate of 1.0 mL/min. Initial condition consisted of 70% A and 30% B and was

maintained for 5 min, then mobile phase A was linearly decreased to 35% in 15 min, retained for 3 min, then changed back to 70% in 2 min. The column was re-equilibrated for 5 min before the next run. A volume of 100 μL of the sample was injected into a reverse phase C18 column. The maximum excitation and emission wavelengths at 405 nm and 470 nm, respectively, were selected for the fluorescence detection.

Calibration Curve

Purified AFB-Lys was used to prepare standards which diluted in 25% MeOH for preparing calibration curves. Nine concentrations of AFB-Lys ranging from 10 to 5000 pg/mL were used to test the linearity on three separate days.

Limits of Detection

The known amount of AFB-Lys was added to human serum (20 pg/mg albumin) and then diluted with with normal human serum to determine the limit of detection (LOD). The limit of detection was defined as a S/N ratio of 2:1.

Accuracy and Precision

Accuracy was determined by diluting known amount of AFB-Lys with normal human serum to concentrations of 160 and 800 pg/mL. These diluted samples were analyzed with five replicates on three separate days. Intra-day precision was determined by the analysis of five replicates of low quality control (LQC, 160 pg/mL) and high quality control (HQC, 800 pg/mL) during a single run while inter-day precision was determined by analyzing five replicates of LQC and HQC for 3 separate analytical runs. The inaccuracy and imprecision were required to be within 15%.

Recovery Rate

Recovery rate was determined by adding known amounts of AFB-Lys adduct to normal human serum to produce triplicates at 50, 200 and 1000 pg/mL. These samples were incubated with 250 μL pronase (as described above) at 37℃ for 3 h. Sample preparation was done using the MAX™ cartridge as described above. After evaporation and reconstitution, the samples were analyzed in 25% MeOH. The peak area of standard without processing is represented as 100 percent. The recovery rate was calculated as the ratio between the mean peak areas of three processed samples and that of unprocessed standards.

Stability

Stability was determined using dilutions of serum obtained from AFB_1-treated rat. Room temperature stability up to 24 h was assessed by leaving the samples at room temperature for 12 h and 24 h followed by digestion and analysis. The stability of the AFB-Lys under conditions of repeated freezing and thawing was also examined for three cycles.

Serum Total Protein and Albumin Measurement

Total protein was measured before digestion of serum using a procedure modified from previous described method [33]. Content of AFB-Lys adduct in serum was adjusted by albumin content. The albumin content in each sample was determined by a brom-cresol purple dye binding method, which measures a stable blue-purple color complex between dye and albumin with maximum absorbance at 600 nm [40].

Statistics and Graphics

Concentrations of AFB-Lys adduct were expressed as mean ± SD and the ANOVA test was used to make comparisons among groups with SAS software (SAS Institute, Cary, NC, USA), followed by Tukey's test. *P*-values less than 0.05 were considered statistically significant.

Results

Quantification and Qualitative assessment of the Synthetic Standard

The synthetic standard was purified through fractional elution from the HPLC system and followed by evaporation and reconstitution in MeOH at 11.13 µg/mL as determined by maximal UV absorbance at 400 nm. This standard is further confirmed through LC/MS and the mass chromatography as shown in Figure 3.2. The standard synthesized was qualitatively assessed using LC/MS. The SRM scan showed a transition from m/z of 454.5 and 456.5 (AFB-Lys adduct mol. wt 455.43 [22]), to a m/z of 394.2 [22, 24], which agreed with previously described data, confirming that the only detectable compound was authentic AFB-Lys adduct.

Binding Efficiency of Different Solid-Phase Extraction Cartridges

Commercially available solid-phase extraction cartridges were compared for their binding efficiency of AFB-Lys after enzyme digestion, shown in Table 3.1. HLB™ cartridge showed significantly lower binding efficacy of AFB-Lys adducts. While both MAX™ and Sep-Pak™ demonstrated similar binding efficacy, MAX™ cartridge was

preferred because it was less time-consuming and required less solvent compared with Sep-Pak™ cartridge.

Enzymatic Digestion of Aflatoxin Albumin Adduct from Serum

Several incubation periods were chosen and tested for release of AFB-Lys adduct using same amount of pronase (250 μL). We found that the release of AFB-Lys adduct reached a peak at 3 h incubation, further elongation of incubation time did not produce higher amount (Figure 3.3), which was consistent with previous observations [36]. The common procedure of inactivation of serum by incubation at 56°C for 30 min in water bath did not affect the release of AFB-Lys adduct from serum (data not shown here).

Calibration Curve Using Pure Synthetic AFB-Lys

Figure 3.4 shows the calibration curve of purified synthetic AFB-Lys adduct in 25% MeOH analyzed by HPLC separation with fluorescence detection. A total of nine concentrations from 10 to 5000 pg/mL were analyzed on three separate days. The correlation coefficient of the curve is 0.999. The linear range of this method encompasses the expected variance based on known human exposure levels.

LOD and LOQ

Human samples with known concentrations of AFB-Lys adduct were diluted to determine the limit of detection and the chromatograms are shown in Figure 3.5. We determined the LOQ to be 0.4 pg/mg albumin. The absolute LOD was 0.01 ng/mL, or 1 pg on-column (equivalent to 0.2 pg/mg albumin).

Accuracy and Precision

The accuracy of the assay based on three days ranged from 93.32% to 96.25% for LQC samples and from 92.23% to 97.25% for HQC samples, as shown in Table 3.2. The within day imprecision was determined to be 5.93% (n = 15) for LQC (mean = 0.16 ng/mL; 3.20 pg/mg albumin) and 2.87% (n = 15) for HQC (mean = 0.80 ng/mL, 16.0 pg/mg albumin) after solid-phase extraction and reconstitution in 25% MeOH.

Recovery Rate

Three different concentrations of AFB-Lys adduct were carried through the solid-phase extraction, evaporation and reconstitution process and the recovery rate was determined (Table 3.3). The recovery rates ranges from 72% to 94.44%. The mean ± SD was 79.77 ± 12.71% at these three concentrations.

Stability

The stability of AFB-Lys was tested for up to 24 h at room temperature and 3 freeze-thaw cycles. Table 3.4 shows that there was no significant degradation of AFB-Lys adduct in diluted rat serum at 0.16 and 0.8 ng/mL concentrations (LQC and HQC) up to 12 h, the only significant decrease was observed at 0.8 ng/mL at 24 h. Table 3.5 shows that the three freeze-thaw cycles did no significantly affect the concentration.

Typical Chromatograms of Standard and Extracted AFB-Lys Adduct

Representative chromatograms from the synthetic standard and extracts from human serum are shown in Figure 3.6.

Discussion

Aflatoxin exposure remains an important food safety issue in that the Food and Agriculture Organization (FAO) estimates that 25% of the world's food crops are affected by mycotoxins, particularly aflatoxins. The worldwide burden of human hepatocellular carcinoma caused by long-term dietary exposure to aflatoxin is a public health concern [41]. However, less-costly and more accurate methods for measuring human AFB_1 exposure are not readily for use in undeveloped countries and antibody based methods often vary in their sensitivity, thus making the results reliable. Therefore, the development of a rapid method that doesn't require expensive mass spectrometer or antibodies or radioactive materials will allow developing countries to assess AFB_1 exposure without the need for major assistance from more developed countries. The purpose of the present study was to develop and validate such a method.

It is apparent that MAX™ and Sep-Pak™ cartridges are better than the HLB™ cartridge with regard to binding and recovery of AFB-Lys adduct in serum. However, we found that the standard procedure for the Sep-Pak™ cartridge took more time and had slightly higher variation compared with that of MAX™, thus the MAX™ cartridge was chosen for solid-phase extraction in this assay.

Inactivation of possible pathogens is a common procedure before analysis of biological samples and subjecting serum samples to 56℃ water bath for 30 min was tested and found to have no effect on the content of AFB-Lys in serum (data not shown). Enzyme content for digestion of treated serum was fixed at a ratio of 1:4 (w:w,

enzyme:total protein) based on total protein content present in serum [27]. Incubation in the 37°C water bath resulted in a peak release of AFB-Lys after 3 h which was adopted in this assay while 4 h was used in the IDMS method. Less serum (150 μL) was needed for analysis compared to 250 μL serum in the IDMS assay [36]. Thus, in terms of enzymatic digestion and volume of serum required, the method we have developed is similar to other validated methods.

The limit of detection in human serum was determined to be 0.4 pg/mg albumin, comparable to the reported 0.5 pg/mg albumin in the immune-affinity column based HPLC-fluorescence method [34] as well as in the isotope-dilution LC/MS/MS method [36]. The ELISA performed directly on intact serum had a LOD of 100 pg/mg albumin and after digestion, 5 pg/mg albumin [34]. The RIA method was reported to have a LOD of about 0.5 pmol (equivalent to 228 pg) [33]. Thus, the HPLC fluorescence method developed in this study compare favorably with other validated methods.

A previous immune-affinity column based HPLC method reported a recovery of 83 ± 6% [35]. The uncorrected recovery for the IDMS extraction procedure was reported as 78 ± 6.4% [36]. Our results showed that the recovery of AFB-Lys adduct ranged from 72-94.4% with mean ± SD of 79.77 ± 12.71%. Inaccuracy and imprecision were within a range of 10% and thus fulfilled the requirements for an acceptable analytical method [42], based on FDA guidelines.

Human sera from Texas subjects who participated in a collaborative project were analyzed using our method. The chromatograms displayed good separation of AFB-Lys

while commercial normal human serum showed less background interference at the same retention time. Taken together, this method for quantifying AFB-Lys in serum is fast and sensitive, which is amenable for application in large-scale population studies.

Acknowledgements

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References

1. Gourama H, Bullerman. LB. Aspergillus flavus and aspergillus parasiticus : Aflatoxigenic fungi of concern in foods and feeds : A review. *J Food Prot*. 1995;58:1395-1404

2. Wild CP, Gong YY. Mycotoxins and human disease: A largely ignored global health issue. *Carcinogenesis*. 2010;31:71-82

3. IARC. Aflatoxins. In: Evaluation of carcinogenic risks in humans. Lyon (France)*: IARC Sci Publ*. 2002;82:171-274

4. Roebuck BD, Johnson DN, Sutter CH, Egner PA, Scholl PF, Friesen MD, Baumgartner KJ, Ware NM, Bodreddigari S, Groopman JD, Kensler TW, Sutter TR. Transgenic expression of aflatoxin aldehyde reductase (akr7a1) modulates

aflatoxin b1 metabolism but not hepatic carcinogenesis in the rat. *Toxicol Sci*. 2009;109:41-49

5. Wild CP, Andersson C, O'Brien NM, Wilson L, Woods JA. A critical evaluation of the application of biomarkers in epidemiological studies on diet and health. *Br J Nutr*. 2001;86 Suppl 1:S37-53

6. Boffetta P. Biomarkers in cancer epidemiology: An integrative approach. *Carcinogenesis*. 2010;31:121-126

7. Kensler TW, Roebuck BD, Wogan GN, Groopman JD. Aflatoxin: A 50-year odyssey of mechanistic and translational toxicology. *Toxicol Sci*. 2011;120 Suppl 1:S28-48

8. Phillips TD, Afriyie-Gyawu E, Williams J, Huebner H, Ankrah NA, Ofori-Adjei D, Jolly P, Johnson N, Taylor J, Marroquin-Cardona A, Xu L, Tang L, Wang JS. Reducing human exposure to aflatoxin through the use of clay: A review. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2008;25:134-145

9. Tang L, Tang M, Xu L, Luo H, Huang T, Yu J, Zhang L, Gao W, Cox SB, Wang JS. Modulation of aflatoxin biomarkers in human blood and urine by green tea polyphenols intervention. *Carcinogenesis*. 2008;29:411-417

10. Wild CP, Turner PC. The toxicology of aflatoxins as a basis for public health decisions. *Mutagenesis*. 2002;17:471-481

11. Crespi CL, Penman BW, Steimel DT, Gelboin HV, Gonzalez FJ. The development of a human cell line stably expressing human cyp3a4: Role in the

metabolic activation of aflatoxin b1 and comparison to cyp1a2 and cyp2a3. *Carcinogenesis*. 1991;12:355-359

12. Forrester LM, Neal GE, Judah DJ, Glancey MJ, Wolf CR. Evidence for involvement of multiple forms of cytochrome p-450 in aflatoxin b1 metabolism in human liver. *Proc Natl Acad Sci U S A*. 1990;87:8306-8310

13. Kuehl P, Zhang J, Lin Y, Lamba J, Assem M, Schuetz J, Watkins PB, Daly A, Wrighton SA, Hall SD, Maurel P, Relling M, Brimer C, Yasuda K, Venkataramanan R, Strom S, Thummel K, Boguski MS, Schuetz E. Sequence diversity in cyp3a promoters and characterization of the genetic basis of polymorphic cyp3a5 expression. *Nat Genet*. 2001;27:383-391

14. Wild CP, Garner RC, Montesano R, Tursi F. Aflatoxin b1 binding to plasma albumin and liver DNA upon chronic administration to rats. *Carcinogenesis*. 1986;7:853-858

15. Essigmann JM, Croy RG, Nadzan AM, Busby WF, Jr., Reinhold VN, Buchi G, Wogan GN. Structural identification of the major DNA adduct formed by aflatoxin b1 in vitro. *Proc Natl Acad Sci U S A*. 1977;74:1870-1874

16. Ramsdell HS, Eaton DL. Mouse liver glutathione s-transferase isoenzyme activity toward aflatoxin b1-8,9-epoxide and benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide. *Toxicol Appl Pharmacol*. 1990;105:216-225

17. Groopman JD, Hall AJ, Whittle H, Hudson GJ, Wogan GN, Montesano R, Wild CP. Molecular dosimetry of aflatoxin-n7-guanine in human urine obtained in the gambia, west africa. *Cancer Epidemiol Biomarkers Prev*. 1992;1:221-227

18. Groopman JD, Hasler JA, Trudel LJ, Pikul A, Donahue PR, Wogan GN. Molecular dosimetry in rat urine of aflatoxin-n7-guanine and other aflatoxin metabolites by multiple monoclonal antibody affinity chromatography and immunoaffinity/high performance liquid chromatography. *Cancer Res*. 1992;52:267-274

19. Ross RK, Yuan JM, Yu MC, Wogan GN, Qian GS, Tu JT, Groopman JD, Gao YT, Henderson BE. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. *Lancet*. 1992;339:943-946

20. Skipper PL, Obiedzinski MW, Tannenbaum SR, Miller DW, Mitchum RK, Kadlubar FF. Identification of the major serum albumin adduct formed by 4-aminobiphenyl in vivo in rats. *Cancer Res*. 1985;45:5122-5127

21. Scholl PF, McCoy L, Kensler TW, Groopman JD. Quantitative analysis and chronic dosimetry of the aflatoxin b1 plasma albumin adduct lys-afb1 in rats by isotope dilution mass spectrometry. *Chem Res Toxicol*. 2006;19:44-49

22. Sabbioni G, Skipper PL, Buchi G, Tannenbaum SR. Isolation and characterization of the major serum albumin adduct formed by aflatoxin b1 in vivo in rats. *Carcinogenesis*. 1987;8:819-824

23. Groopman JD, Wogan GN, Roebuck BD, Kensler TW. Molecular biomarkers for aflatoxins and their application to human cancer prevention. *Cancer Res*. 1994;54:1907s-1911s

24. Guengerich FP, Arneson KO, Williams KM, Deng Z, Harris TM. Reaction of aflatoxin b(1) oxidation products with lysine. *Chem Res Toxicol*. 2002;15:780-792

25. Wild CP, Fortuin M, Donato F, Whittle HC, Hall AJ, Wolf CR, Montesano R. Aflatoxin, liver enzymes, and hepatitis b virus infection in gambian children. *Cancer Epidemiol Biomarkers Prev*. 1993;2:555-561

26. Diallo MS, Sylla A, Sidibe K, Sylla BS, Trepo CR, Wild CP. Prevalence of exposure to aflatoxin and hepatitis b and c viruses in guinea, west africa. *Nat Toxins*. 1995;3:6-9

27. Wang JS, Qian GS, Zarba A, He X, Zhu YR, Zhang BC, Jacobson L, Gange SJ, Munoz A, Kensler TW, et al. Temporal patterns of aflatoxin-albumin adducts in hepatitis b surface antigen-positive and antigen-negative residents of daxin, qidong county, people's republic of china. *Cancer Epidemiol Biomarkers Prev*. 1996;5:253-261

28. Soini Y, Chia SC, Bennett WP, Groopman JD, Wang JS, DeBenedetti VM, Cawley H, Welsh JA, Hansen C, Bergasa NV, Jones EA, DiBisceglie AM, Trivers GE, Sandoval CA, Calderon IE, Munoz Espinosa LE, Harris CC. An aflatoxin-associated mutational hotspot at codon 249 in the p53 tumor suppressor

gene occurs in hepatocellular carcinomas from mexico. *Carcinogenesis*. 1996;17:1007-1012

29. Kensler TW, He X, Otieno M, Egner PA, Jacobson LP, Chen B, Wang JS, Zhu YR, Zhang BC, Wang JB, Wu Y, Zhang QN, Qian GS, Kuang SY, Fang X, Li YF, Yu LY, Prochaska HJ, Davidson NE, Gordon GB, Gorman MB, Zarba A, Enger C, Munoz A, Helzlsouer KJ, et al. Oltipraz chemoprevention trial in qidong, people's republic of china: Modulation of serum aflatoxin albumin adduct biomarkers. *Cancer Epidemiol Biomarkers Prev*. 1998;7:127-134

30. Turner PC, Dingley KH, Coxhead J, Russell S, Garner CR. Detectable levels of serum aflatoxin b1-albumin adducts in the united kingdom population: Implications for aflatoxin-b1 exposure in the united kingdom. *Cancer Epidemiol Biomarkers Prev*. 1998;7:441-447

31. Azziz-Baumgartner E, Lindblade K, Gieseker K, Rogers HS, Kieszak S, Njapau H, Schleicher R, McCoy LF, Misore A, DeCock K, Rubin C, Slutsker L. Case-control study of an acute aflatoxicosis outbreak, kenya, 2004. *Environ Health Perspect*. 2005;113:1779-1783

32. Skipper PL, Tannenbaum SR. Protein adducts in the molecular dosimetry of chemical carcinogens. *Carcinogenesis*. 1990;11:507-518

33. Sheabar FZ, Groopman JD, Qian GS, Wogan GN. Quantitative analysis of aflatoxin-albumin adducts. *Carcinogenesis*. 1993;14:1203-1208

34. Wild CP, Jiang YZ, Sabbioni G, Chapot B, Montesano R. Evaluation of methods for quantitation of aflatoxin-albumin adducts and their application to human exposure assessment. *Cancer Res*. 1990;50:245-251

35. Sabbioni G, Ambs S, Wogan GN, Groopman JD. The aflatoxin-lysine adduct quantified by high-performance liquid chromatography from human serum albumin samples. *Carcinogenesis*. 1990;11:2063-2066

36. McCoy LF, Scholl PF, Schleicher RL, Groopman JD, Powers CD, Pfeiffer CM. Analysis of aflatoxin b1-lysine adduct in serum using isotope-dilution liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 2005;19:2203-2210

37. Scholl PF, Turner PC, Sutcliffe AE, Sylla A, Diallo MS, Friesen MD, Groopman JD, Wild CP. Quantitative comparison of aflatoxin b1 serum albumin adducts in humans by isotope dilution mass spectrometry and elisa. *Cancer Epidemiol Biomarkers Prev*. 2006;15:823-826

38. Sabbioni G. Chemical and physical properties of the major serum albumin adduct of aflatoxin b1 and their implications for the quantification in biological samples. *Chem Biol Interact*. 1990;75:1-15

39. Scholl PF, Groopman JD. Long-term stability of human aflatoxin b1 albumin adducts assessed by isotope dilution mass spectrometry and high-performance liquid chromatography-fluorescence. *Cancer Epidemiol Biomarkers Prev*. 2008;17:1436-1439

40. Hill PG, Wells TN. Bromocresol purple and the measurement of albumin. Falsely high plasma albumin concentrations eliminated by increased reagent ionic strength. *Ann Clin Biochem*. 1983;20 (Pt 5):264-270

41. Liu Y, Wu F. Global burden of aflatoxin-induced hepatocellular carcinoma: A risk assessment. *Environ Health Perspect*. 2010

42. FDA. Guidance for industry : Bioanalytical method validation. *http://www.labcompliance.com/info/links/methods/guidelines.aspx*. 2001

Table 3.1. Binding efficacy of different cartridges (n=3)

Concentration (ng/mL)	MAX	HLB	Sep-Pak
0.16	0.12±0.01	0.05±0.01*	0.11±0.00
0.8	0.58±0.01	0.31±0.00*	0.57±0.05

*: $P < 0.05$, compared with other cartridges at same concentration.

Table 3.2. Accuracy and precision rates using diluted rat serum (n=5)

	AFB-Lys (ng/mL)	Observed value (ng/mL)	Inaccuracy rate (%)	Imprecision rate (%)
Day 1	0.16*	0.15±0.01	3.75	5.55
	0.80	0.86±0.02	7.77	2.76
	AFB-Lys (ng/mL)	Observed value (ng/mL)	Inaccuracy rate (%)	Imprecision rate (%)
Day 2	0.16	0.16±0.01	4.48	8.60
	0.80	0.82±0.02	2.80	2.39
	AFB-Lys (ng/mL)	Observed value (ng/mL)	Inaccuracy rate (%)	Imprecision rate (%)
Day 3	0.16	0.15±0.01	6.68	3.65
	0.80	0.79±0.03	2.75	3.47

* 0.16 ng/mL and 0.80 ng/mL were the LQC and HQC samples, respectively.

Table 3.3. Recovery rate of AFB-Lys adduct (n=3)

AFB-Lys (ng/mL)	Peak area (LU)		Recovery rate (%)
	Processed	Unprocessed	
0.05	4.53±0.38	4.80±0.20	94.44
0.2	14.77±0.55	20.27±0.12	72.87
1	73.2±1.35	101.67±0.51	72

Table 3.4. RT stability of AFB-Lys in serum (n=3)

Concentration (ng/mL)	Time (hour)		
	0	12	24
0.16	0.16±0.00	0.15±0.01	0.15±0.00
0.8	0.80±0.01	0.78±0.01	0.71±0.01*

*: *P<0.05,* compared with 0 h at same concentration.

Table 3.5. Frozen-thawed stability of AFB-Lys in serum (n=3)

Concentration (ng/mL)	Frozen-thawed cycles			
	0	1	2	3
0.16	0.16±0.01	0.16±0.01	0.16±0.01	0.15±0.01
0.8	0.80±0.02	0.78±0.02	0.78±0.02	0.79±0.02

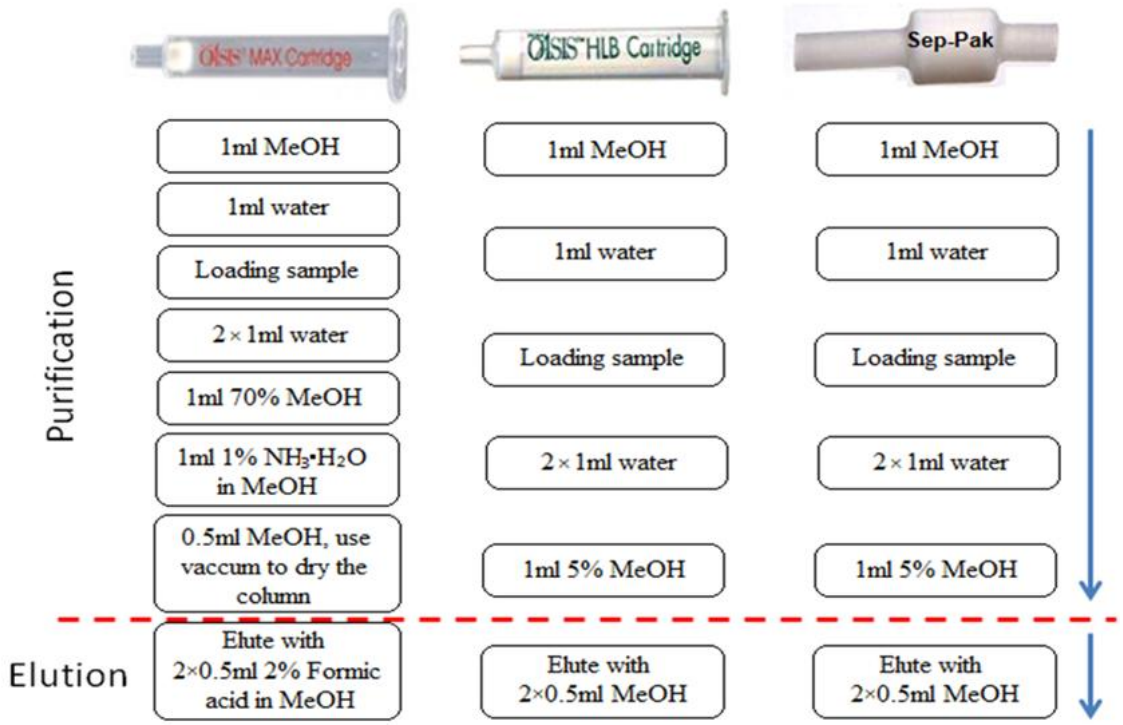

Figure 3.1. Flow chart of sample purification using different commercial cartridges.

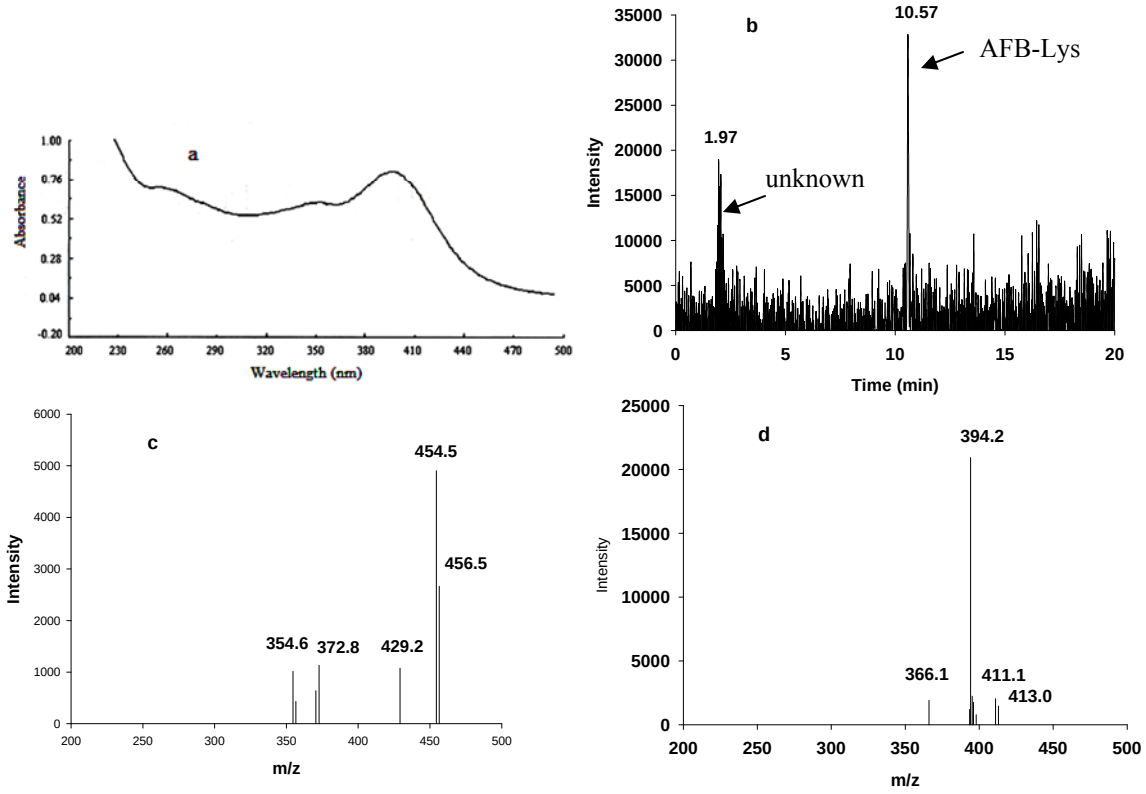

Figure 3.2. Chromatograms of synthetic AFB-Lys standard.

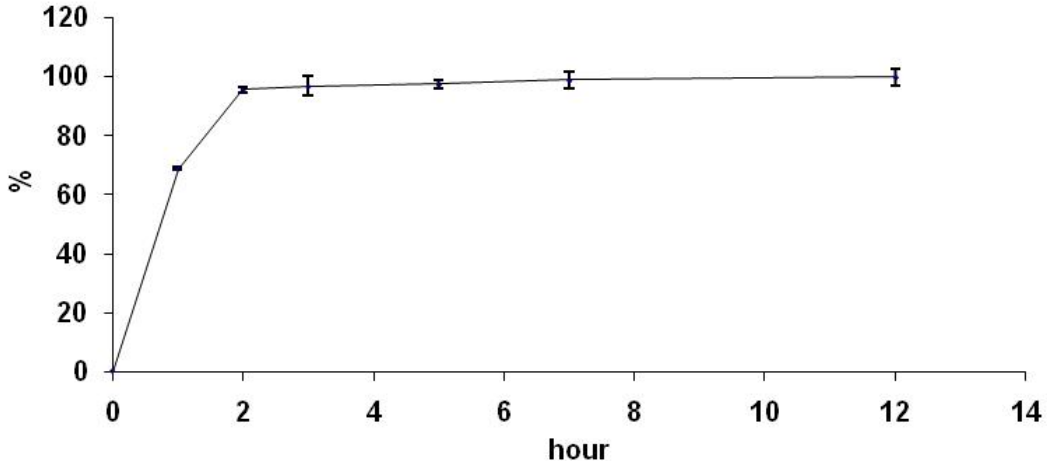

Figure 3.3. Effects of enzymatic incubation on the release of AFB-Lys from serum.

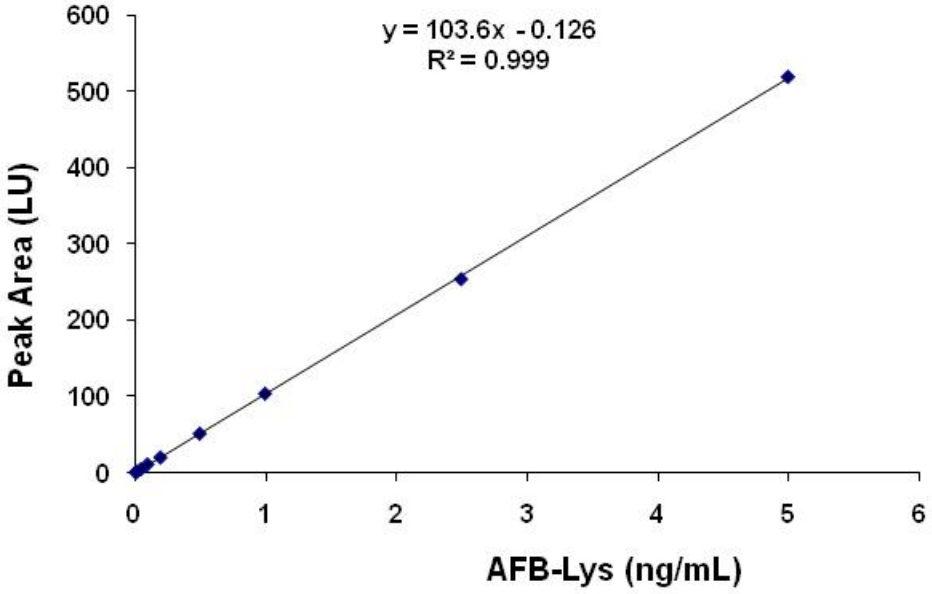

Figure 3.4. Calibration curve of AFB-Lys adduct.

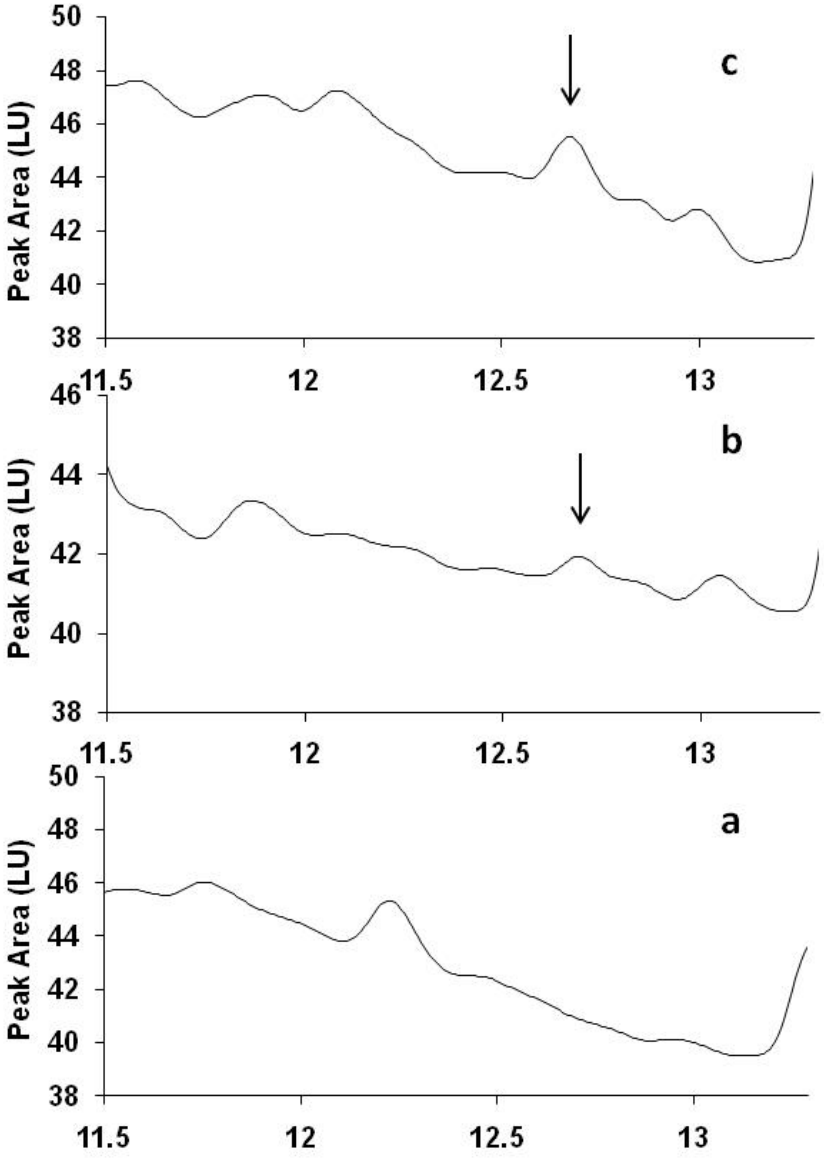

Figure 3.5. Detection limit of AFB-Lys adduct in human serum.

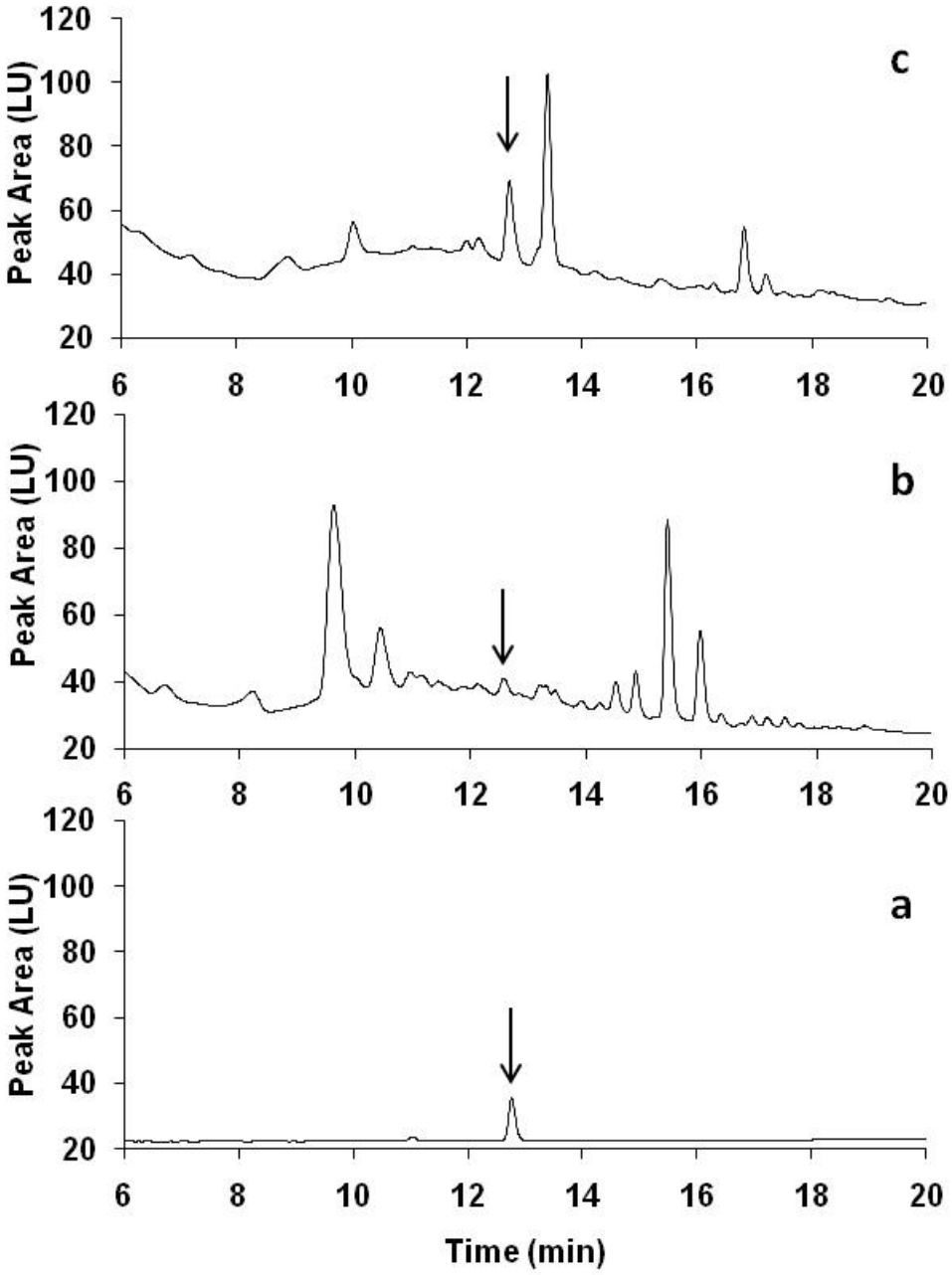

Figure 3.6. Typical chromatograms of synthetic and extracted AFB-Lys adduct

Figure Legends

Figure 3.1. Flow chart showing the steps used for sample purification using different commercial cartridges.

Figure 3.2. Purification of the AFB-Lys standard. a, UV/Vis spectrum of the AFB-Lys adduct showing maximal absorbance at 400 nm; b, chromatogram of the synthesized AFB-Lys adduct showing total ion current; c, mass spectra of the synthesized standard; d, collision-induced dissociation of the parent mass of 457 m/z scanning from m/z 200 to 500, the major fragment was 394.2 m/z, similar to that published by Scholl et al. (Reference #21).

Figure 3.3. Effects of length of enzymatic incubation on the release of AFB-Lys from serum.

Figure 3.4. Calibration curve of AFB-Lys adduct (10 to 5000 pg/mL) based on HPLC fluorescence detection as described in the methods.

Figure 3.5. Detection limit of AFB-Lys adduct in human serum. a), normal unexposed human serum; b), a processed sample containing 0.4 pg/mg albumin of AFB_1-Lys adduct; c) a processed sample containing 2.0 pg/mg albumin of AFB-Lys adduct. Retention time was about 12.7 min. Solid arrow indicated the peak for AFB-Lys adduct.

Figure 3.6. Typical chromatograms of synthetic and extracted AFB-Lys adduct a: 1 ng/mL calibration standard in 25% MeOH (equivalent to 20 pg/mg albumin); b: 1.5 pg/mg albumin AFB-Lys adduct in human serum; c: 30 pg/mg albumin in human serum. Retention time was about 12.7 min. Solid arrow indicated the peak for AFB-Lys adduct.

CHAPTER 4

PHYSIOLOGICALLY BASED TOXICOKINETICS OF SERUM AFLATOXIN B_1-LYSINE ADDUCT IN F344 RATS [1]

[1] Qian, G. Tang, L. Wang, F. Guo, X. Massey, M.E. Williams, J.H. Phillips, T.D. and Wang, J.-S. 2012. *Toxicology.* In print. Reprinted here with permission of the publisher.

Abstract

Aflatoxin B_1-lysine adduct (AFB-Lys) is a reliable biomarker for aflatoxin exposure; however, a systematic toxicokinetic evaluation has not been reported. In this study, male F344 rats were orally exposed to single, or repeated, doses of AFB_1 and the toxicokinetics of serum AFB-Lys that followed treatments were investigated. A single-dose of AFB_1 increased serum AFB-Lys levels rapidly peaking at 4 h, followed by first-order elimination, through which the half-life was estimated to be 2.31 days. A physiologically based pharmacokinetic model showed that approximately 3.00-3.90% and 1.12-1.98% of the administered AFB_1 doses were converted to serum AFB-Lys adducts at 2 h and 24 h post treatment, respectively. Repeated AFB_1 exposure at 5-25 μg/kg body weight linearly increased serum AFB-Lys levels for 5 weeks in animals, resulting in a 1-1.5 times higher AFB-Lys level overall. This indicates the potential of this adduct as a reliable biomarker for repeated low dose exposure. Higher dose exposure at 75 μg/kg increased the level of AFB-Lys to a maximum at 2 weeks, followed by a gradual decrease to near plateau level up to 5 weeks. In conclusion, this study systematically evaluated the toxicokinetics of serum AFB-Lys adduct in F344 rats using a physiologically based pharmacokinetic model and robust statistical modeling analysis and provided a firm and clear understanding of the toxicokinetics of this biomarker.

Keywords: aflatoxin B_1; aflatoxin B_1-lysine adduct; biomarker; toxicokinetics.

Introduction

Aflatoxins (AF) are ubiquitous food contaminants produced mainly by *Aspergillus flavus* and *A. parasiticus*. Humans exposed to AF contaminated food are subject to a wide range of health effects, including acute poisoning (aflatoxicosis), hepatocellular carcinoma (HCC) and stunting in children [1-4]. The uncovering of the etiological role of AF exposure is closely associated with the development and application of molecular markers relevant to AF exposure or risk [5, 6].

The toxicology of aflatoxin B_1 (AFB_1) has been detailed elsewhere [7]. AFB_1 is metabolically activated by liver cytochrome P450 (CYP450) 1A2 and 3A4 to AFB_1-8,9-epoxide which can readily bind DNA to form AFB_1-N^7-guanine adduct [8] or react with albumin to produce AFB_1-albumin adduct if not conjugated with glutathione [9]. It has been shown that albumin is the only protein in serum that binds AFB_1 to a significant level [10]. Lysine is the primary amino acid group for the covalent binding of AFB_1 to albumin. Under enzyme digestion, AFB_1-lysine (AFB-Lys) is released from the adducted albumin [9]. This biomarker has been widely used to indicate human exposure status [11, 12] and to assess human chemoprevention strategies [13, 14]. Several previous studies have reported the partial toxicokinetics of the AFB-Lys adduct in animal models [9, 15-17]. However, a complete study design relevant to human exposure (route, dose and duration) is lacking, and further studies are warranted to demonstrate the physiologically based toxicokinetics of the serum AFB-Lys biomarker.

Materials and Methods

Chemicals and Materials

Unlabelled aflatoxin B_1 (> 98% purity), dimethyl sulfoxide (DMSO), albumin determination reagent (bromocreosol purple), and normal rat serum were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). Pronase (25 kU, Nuclease-free) was purchased from Calbiochem (La Jolla, CA). Protein assay dye reagent concentrate and protein standards were purchased from Bio-Rad Laboratories Inc. (Hercules, CA). Mixed mode solid phase extraction (SPE) cartridges were purchased from the Waters Corp. (Milford, MA). Authentic AFB-Lys was synthesized as previously described [9]. All other chemicals and solvents were of highest grade and purity available.

Animals

Male F344 rats (120-130 g) were purchased from Harlan Laboratories Inc. (Indianapolis, IN) and maintained under controlled conditions of temperature (22-25°C), humidity (50-70%), and light/dark cycles (12 h/12 h). Animals were fed with AIN-76A diet (Teklad, Madison, WI) and acclimated for one week before treatment. Feed and water were provided *ad libitum*. Animal use and care and the experimental protocol were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia.

Experimental Design

For the single-dose study, rats (5 per group) were gavaged with 0, 50, 250, or 1,000 μg AFB_1/kg body weight (BW). AFB_1 was dissolved in DMSO with a gavage

volume of 50 μL/100g BW. Animals were anesthetized and blood was collected by cardiac puncture at 2, 4, 8 h, and 1, 3, 5, 7, 14, and 21 days after treatment. For the repeated-dose study, rats (25 per group) were gavaged daily with 0, 5, 10, 25, or 75 μg/kg BW for up to 5 weeks (5 days per week). Animals (5 per group) were anesthetized and blood samples were collected at weekly intervals and 24 h after the last dose. Serum samples were prepared and stored at -20°C until analysis.

Serum Sample Processing and Quantification of AFB-Lys Adducts

The method for serum processing and quantification of AFB-Lys adducts has been reported previously [18]. Albumin and total protein levels in serum were determined as previously described [11]. Aliquots of each serum sample (150 μL) were digested by pronase (pronase:total protein, 1:4, w:w) at 37°C for 3 h to release AFB-Lys adducts. The digests were purified using Waters MAX SPE cartridges. After priming with methanol and equilibration with water, the loaded cartridge was sequentially washed by water, 70% methanol, and 1% ammonium hydroxide in methanol at a flow rate of 1mL/min. The eluate in 2% formic acid in methanol was vacuum-dried with a Labconco Centrivap concentrator (Kansas City, MO) and reconstituted for HPLC-fluorescence detection. The average recovery rate was 90%.

The Agilent 1200 HPLC-fluorescence system (Santa Clara, CA) was used for quantification of the AFB-Lys adducts. The mobile phases consisted of buffer A (20 mM $NH_4H_2PO_4$, pH 7.2) and buffer B (100% Methanol). The Zorbax Eclipse XDB-C18 reverse phase column (5 micron, 4.6 × 250 mm) was used and 100 μL was injected at a

flow rate of 1 mL/min. A gradient was generated to separate the AFB-Lys adduct within 25 min and the retention time of AFB-Lys was equal to approximately 12.3 min. AFB-Lys adduct was detected by fluorescence at the excitation and emission wavelengths of 405 nm and 470 nm, respectively. Quality assurance and quality control procedures were taken during analyses, which included simultaneous analysis of one authentic standard and a quality control sample daily. The limit of detection was 0.4 pg/mg albumin. The serum AFB-Lys level of each sample was adjusted by its albumin content accordingly.

Statistical Analysis

The curves of serum AFB-Lys formation and elimination after a single dose were fitted with zero-, first-, and second-order kinetic functions, which were used to determine the appropriate model for AFB-Lys kinetics. The lowest-order model with acceptable residuals ($r^2 > 0.90$) was considered to optimally describe the elimination curve. All modeling and residual determinations were performed with Sigma Plot 10.0 software (San Jose, CA) using the least sum of squares method. The half-life was computed from the fitting of the model selected.

Results

Single Dose Study

Following a single-dose of AFB_1, the levels of AFB-Lys in serum rapidly increased at 2 h and reached a peak at 4 h, followed by a non-linear decrease (Figure 4.1). Linear dose-response relationships were found between AFB_1 doses and serum AFB-Lys levels at both 2 and 24 h post treatment (Figure 4.2A and B, $r^2 = 0.98$ and 0.99,

respectively). In addition, the elimination of serum AFB-Lys followed first-order kinetics for both 50 ($r^2 = 0.9829$, $p < 0.001$) and 250 μg/kg groups ($r^2 = 0.9745$, $p < 0.0001$) (Table 4.1). Non-linear decreases of serum AFB-Lys after 4 h were demonstrated based on model analysis for the 50 and 250 μg/kg groups (Figure 4.3A and B). Significant linear decreases were also observed with the log (AFB-Lys) over time for the 50 and 250 μg/kg groups. Half-lives of AFB-Lys were estimated to be 2.35 and 2.27 days from the curve fittings of the data from the 50 and 250 μg/kg groups, respectively. The half-life was estimated based on the integrated slope (λ, as the regression coefficient of the fitted model) of the log (AFB-Lys) over time curves, i.e., $t_{1/2} = 0.693/\lambda$ (Figures 4.3C and D). The conversion ratios of AFB_1 to AFB-Lys following a single dose treatment were determined to be 3.00-3.90% at 2 h or 1.12-1.98% at 24 h post treatment (Tables 4.2 and 4.3), as estimated by a physiologically based pharmacokinetic model.

Repeated Dose Study

Repeated dosing with AFB_1 increased serum AFB-Lys for up to 5 weeks at all doses (Figure 4.4). A dose-dependent temporal increase in the AFB-Lys level was found in animals that received 5-25 μg/kg AFB_1 over a 5-week study period. The level of AFB-Lys reached a maximum after 2 weeks, followed by a gradual decrease over time in the 75 μg/kg group. To further examine the kinetics of the serum AFB-Lys after the repeated-dose treatment, we conducted different model analyses and found that the linear regression model best fit the data for the 5 (Figure 4.5A, $r^2 = 0.96$, $p < 0.01$), 10 (Figure 4.5B, $r^2 = 0.97$, $p < 0.01$) and 25 μg/kg groups (Figure 4.5C, $r^2 = 0.98$, $p < 0.01$),

respectively. A Gaussian curve was the best fit for the 75 μg/kg group (Figure 4.5D, r^2 = 0.86, p = 0.1634). Linear dose-response relationships were also found between cumulative doses and serum AFB-Lys levels (Figure 4.6A, $r^2 = 0.99$, $p < 0.01$), as well as between the cumulative doses and the conversion ratio (AFB-Lys formation)/(AFB_1 dosed) after a 1-week treatment (Figure 4.6B, $r^2 = 0.99$, $p < 0.01$).

Discussion

In this study we evaluated the toxicokinetics of serum AFB-Lys adduct in F344 rats with special focus on doses relevant to acute (single) or chronic (repeated) human exposure. The peak concentration of serum AFB-Lys was found at 4 h in the current study. The mean AFB-Lys concentration of 2.56 ± 0.38 ng/mg albumin at 24 h after a single dose of 250 μg/kg was comparable to previously reported values in animals [19, 20]. The highest dose of 1000 μg/kg was selected to simulate the extreme condition in which human acute aflatoxicosis occurred, as found in 2004-2005 outbreaks in Kenya, where a mean serum level of 3.2 ng/mg albumin for this biomarker has been reported in cases of aflatoxin poisoning before death [12].

Our physiological model based estimation of the conversion ratio of AFB_1 to AFB-Lys at 24 h after a single dose (1.12-1.98%) was similar to previously reported values of 1-3% [9, 15]. The model generated half-life of serum AFB-Lys in this study (2.31 days, mean value of 2.35 and 2.27) was close to the previously reported values of 2.5 days [9], 55 h [15], and 2.6 days [16] in rats. The elimination kinetics of serum AFB-Lys

followed a first-order kinetic decay model, as determined by comparisons of different model fittings.

It is worth noting that the dose range selected in the repeated experiment (5-75 μg/kg BW) was relevant to the exposure levels in high-risk human populations, such as Kenya, Ghana, and Guangxi, China, where the mean AFB_1 levels were found to be 100-1000 μg/kg in corn [12, 21, 22]. We observed a linear increase of serum AFB-Lys for animals that received 5-25 μg AFB_1/kg daily, leading to a 1.0-1.5 times increase after 5 weeks compared to that after 1 week, which supports its potential use as a long-term biomarker. In addition, we observed that serum AFB-Lys reached a maximum after 2 weeks for animals that received a high daily dose of AFB_1 (75 μg/kg BW). The pattern observed for the 75 μg/kg group (Gaussian curve) may reflect a variation in the metabolic balance between AFB-epoxide formation and detoxification [23] or enzymatic induction of glutathione S transferase (GST) [24], and this coincided with the alternative signs of toxicity found at this dose.

It has to be pointed out that data for toxicokinetics presented in this study are different from previous studies in the following ways: (1) the current study employed a human relevant route of exposure (orally) rather than the i.p. injection adopted by Scholl et al [16], which could better mimic the toxicokinetics of the biomarker *in vivo*; (2) we exposed animals with unlabelled AFB_1 rather than ^3H- [15] or ^{14}C- labeled AFB_1 [9]; (3) we used a physiologically based model to estimate the conversion of AFB_1 doses to serum AFB-Lys adducts rather than expression of the conversion ratio as AFB-Lys level (ng/mg

albumin)/AFB_1 dose (μg/kg body weight). This conversion ratio as previously reported by Scholl et al. (2006) is not straightforward [16] given the different units involved; (4) the current study used both single and repeated treatment procedures with relatively longer duration as compared to previous repeated treatment protocols of 14 days [17] or 9 days [16]. One more highlight of this study is the use of robust statistical modeling analysis that greatly facilitates the understanding of the toxicokinetics of the AFB-Lys. This work also represents the first systematic evaluation of this biomarker using a complete design that incorporates relevant physiological basis into the toxicokinetic estimation. Data from this study provides a firm and clear understanding of the toxicokinetics of serum AFB-Lys adduct and will benefit future studies using this biomarker for linking to human health effects.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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References

1. Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences, and interventions. *Am J Clin Nutr*. 2004;80:1106-1122

2. Liu Y, Wu F. Global burden of aflatoxin-induced hepatocellular carcinoma: A risk assessment. *Environ Health Perspect*. 2010;118:818-824

3. Smith LE, Stoltzfus RJ, Prendergast A. Food chain mycotoxin exposure, gut health, and impaired growth: A conceptual framework. *Adv Nutr*. 2012;3:526-531

4. IARC. Aflatoxins. *IARC Monogr Eval Carcinog Risks Hum*. 1993;56:245-395

5. Qian GS, Ross RK, Yu MC, Yuan JM, Gao YT, Henderson BE, Wogan GN, Groopman JD. A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in shanghai, people's republic of china. *Cancer Epidemiol Biomarkers Prev*. 1994;3:3-10

6. Kensler TW, Roebuck BD, Wogan GN, Groopman JD. Aflatoxin: A 50-year odyssey of mechanistic and translational toxicology. *Toxicological Sci*. 2011;120 Suppl 1:S28-48

7. Eaton DL, Groopman JD. *The toxicology of aflatoxins: Human health, veterinary, and agricultural significance.*: San Diego: Academic Press. New York, NY 1994.

8. Essigmann JM, Croy RG, Nadzan AM, Busby WF, Jr., Reinhold VN, Buchi G, Wogan GN. Structural identification of the major DNA adduct formed by aflatoxin b1 in vitro. *Proc Natl Acad Sci U S A*. 1977;74:1870-1874

9. Sabbioni G, Skipper PL, Buchi G, Tannenbaum SR. Isolation and characterization of the major serum albumin adduct formed by aflatoxin b1 in vivo in rats. *Carcinogenesis*. 1987;8:819-824

10. Skipper PL, Hutchins DH, Turesky RJ, Sabbioni G, Tannenbaum SR. Carcinogen binding to serum-albumin. *P Am Assoc Canc Res*. 1985;26:90-90

11. Wang JS, Qian GS, Zarba A, He X, Zhu YR, Zhang BC, Jacobson L, Gange SJ, Munoz A, Kensler TW, et al. Temporal patterns of aflatoxin-albumin adducts in hepatitis b surface antigen-positive and antigen-negative residents of daxin, qidong county, people's republic of china. *Cancer Epidemiol Biomarkers Prev*. 1996;5:253-261

12. Azziz-Baumgartner E, Lindblade K, Gieseker K, Rogers HS, Kieszak S, Njapau H, Schleicher R, McCoy LF, Misore A, DeCock K, Rubin C, Slutsker L. Case-control study of an acute aflatoxicosis outbreak, kenya, 2004. *Environ Health Perspect*. 2005;113:1779-1783

13. Wang P, Afriyie-Gyawu E, Tang Y, Johnson NM, Xu L, Tang L, Huebner HJ, Ankrah NA, Ofori-Adjei D, Ellis W, Jolly PE, Williams JH, Wang JS, Phillips TD. Novasil clay intervention in ghanaians at high risk for aflatoxicosis: Ii. Reduction in biomarkers of aflatoxin exposure in blood and urine. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2008;25:622-634

14. Tang L, Tang M, Xu L, Luo H, Huang T, Yu J, Zhang L, Gao W, Cox SB, Wang JS. Modulation of aflatoxin biomarkers in human blood and urine by green tea polyphenols intervention. *Carcinogenesis*. 2008;29:411-417

15. Wild CP, Garner RC, Montesano R, Tursi F. Aflatoxin b1 binding to plasma albumin and liver DNA upon chronic administration to rats. *Carcinogenesis*. 1986;7:853-858

16. Scholl PF, McCoy L, Kensler TW, Groopman JD. Quantitative analysis and chronic dosimetry of the aflatoxin b1 plasma albumin adduct lys-afb1 in rats by isotope dilution mass spectrometry. *Chem Res Toxicol*. 2006;19:44-49

17. Wild CP, Hasegawa R, Barraud L, Chutimataewin S, Chapot B, Ito N, Montesano R. Aflatoxin-albumin adducts: A basis for comparative carcinogenesis between animals and humans. *Cancer Epidemiol Biomarkers Prev*. 1996;5:179-189

18. Qian G, Tang L, Liu W, Wang JS. Development of a non-antibody method for rapid detection of serum aflatoxin b1-lysine adduct. *Toxicologist*. 2010;114:248.

19. Vinitketkumnuen U, Chewonarin T, Dhumtanom P, Lertprasertsuk N, Wild CP. Aflatoxin-albumin adduct formation after single and multiple doses of aflatoxin b(1) in rats treated with thai medicinal plants. *Mut Res*. 1999;428:345-351

20. Wild CP, Jiang YZ, Sabbioni G, Chapot B, Montesano R. Evaluation of methods for quantitation of aflatoxin-albumin adducts and their application to human exposure assessment. *Cancer Res*. 1990;50:245-251

21. Groopman JD, Zhu JQ, Donahue PR, Pikul A, Zhang LS, Chen JS, Wogan GN. Molecular dosimetry of urinary aflatoxin-DNA adducts in people living in guangxi autonomous region, people's republic of china. *Cancer Res*. 1992;52:45-52

22. Tang L, Xu L, Afriyie-Gyawu E, Liu W, Wang P, Tang Y, Wang Z, Huebner HJ, Ankrah NA, Ofori-Adjei D, Williams JH, Wang JS, Phillips TD. Aflatoxin-albumin adducts and correlation with decreased serum levels of vitamins a and e in an adult ghanaian population. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2009;26:108-118

23. Knight LP, Primiano T, Groopman JD, Kensler TW, Sutter TR. Cdna cloning, expression and activity of a second human aflatoxin b1-metabolizing member of the aldo-keto reductase superfamily, akr7a3. *Carcinogenesis*. 1999;20:1215-1223

24. Strelic NJ, Saicic ZS, Magic ZM, Spasic MB, Trutic NV, Krtolica KV. Aflatoxin b1-induced changes of glutathione-s-transferase activity in the plasma and liver of the rat. *Vojnosanit Pregl*. 2003;60:415-420

Table 4.1. Elimination kinetics of AFB-Lys adduct after a single dose.

Dose (μg/kg)	Zero order		First order		Second order	
	r^2	*p*-value	r^2	*p*-value	r^2	*p*-value
50	0.4913	0.0528	0.9829	<0.0001	0.7779	0.0038
250	0.4696	0.0607	0.9745	<0.0001	0.7534	0.0052

Zero order kinetics tested by linear regression of serum AFB-Lys overtime; first order kinetics tested by ln(AFB-Lys) over time; second order kinetics tested by 1/AFB-Lys over time.

Table 4.2. Percentages of AFB_1 converted to serum AFB-Lys adduct 2 h after a single-dose treatment.

Dose (μg/kg)	Body weight (BW)	AFB_1 administrated (μg)	AFB-Lys measured (nM/mL)	Estimated parameters			Conversion ratio (%)
				Whole blood volume (mL)	Whole serum volume (mL)	AFB_1 dose in serum (nM)	
50	151.6	8.05	0.16	9.87	5.92	0.95	3.68
250	152.1	39.00	0.82	9.90	5.94	4.87	3.90
1000	150.8	148.00	2.42	9.82	5.89	14.25	3.00

Values are calculated from the average of 5 rats. Whole blood volume is calculated as (BW*0.06+ 0.77, mL); whole serum volume is calculated as (whole blood volume)*0.60; AFB_1 dose in serum (nM) is calculated as (AFB-Lys measured, nM/mL)*(whole serum volume, mL); conversion ratio is calculated as (AFB_1 dose in serum, nM) *(molecular weight of AFB_1, 312g/M)/1000/(AFB_1 administrated, μg))*100%. The calculation was based on assumptions that the binding of AFB_1 with proteins other than albumin is negligible in the blood stream and that serum AFB-Lys adducts are evenly distributed in the blood stream. Estimation of blood and serum volume was based on previous studies (*Lee, H.B., Blaufox, M.D., 1985. J. Nucl. Med. 26, 72-76* and *Everett, N.B., Simmons, B., Lasher, E.P., 1956. Circ. Res. 4, 419-424*).

Table 4.3. Percentages of AFB_1 converted to serum AFB-Lys adduct 24 h after a single-dose treatment.

Dose (μg/kg)	Body weight (BW)	AFB_1 administrated (μg)	AFB-Lys measured (nM/mL)	Estimated parameters			Conversion ratio (%)
				Whole blood volume (mL)	Whole serum volume (mL)	AFB_1 dose in serum (nM)	
50	161.39	8.05	0.05	10.45	6.27	0.31	1.20
250	156.36	39.00	0.23	10.15	6.09	1.40	1.12
1000	148.38	148.00	1.62	9.67	5.80	9.40	1.98

Values are calculated from the average of 5 rats. Whole blood volume is calculated as (BW*0.06+ 0.77, mL); whole serum volume is calculated as (whole blood volume)*0.60; AFB_1 dose in serum (nM) is calculated as (AFB-Lys measured, nM/mL)*(whole serum volume, mL); conversion ratio is calculated as (AFB_1 dose in serum, nM) *(molecular weight of AFB_1, 312g/M)/1000/(AFB_1 administrated, μg))*100%. The calculation was based on assumptions that the binding of AFB_1 with proteins other than albumin is negligible in the blood stream and that serum AFB-Lys adducts are evenly distributed in the blood stream. Estimation of blood and serum volume was based on previous studies (*Lee, H.B., Blaufox, M.D., 1985. J. Nucl. Med. 26, 72-76* and *Everett, N.B., Simmons, B., Lasher, E.P., 1956. Circ. Res. 4, 419-42.*).

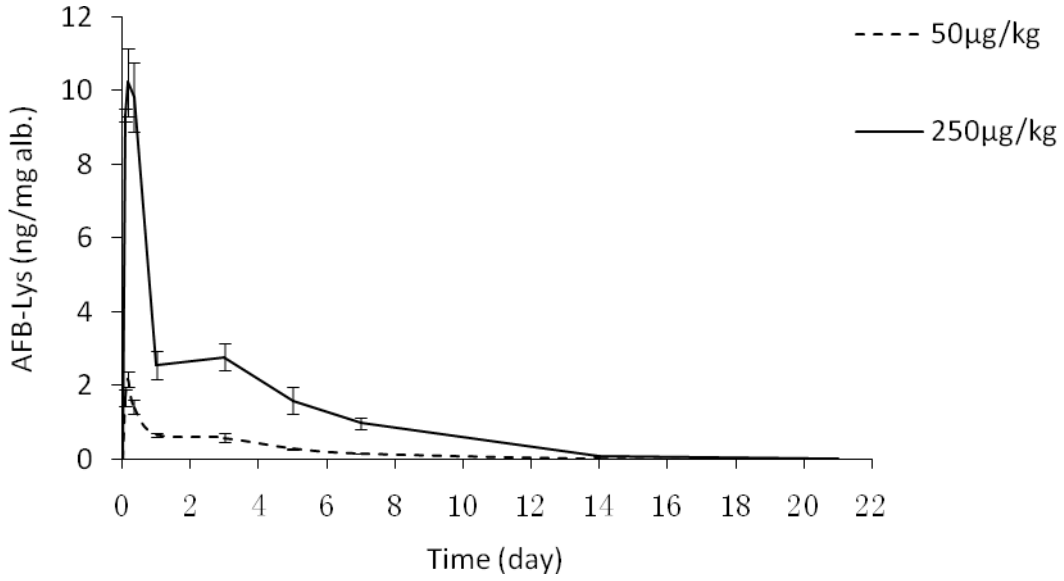

Figure 4.1. Serum concentrations of AFB-Lys adduct after a single-dose of AFB_1 exposure.

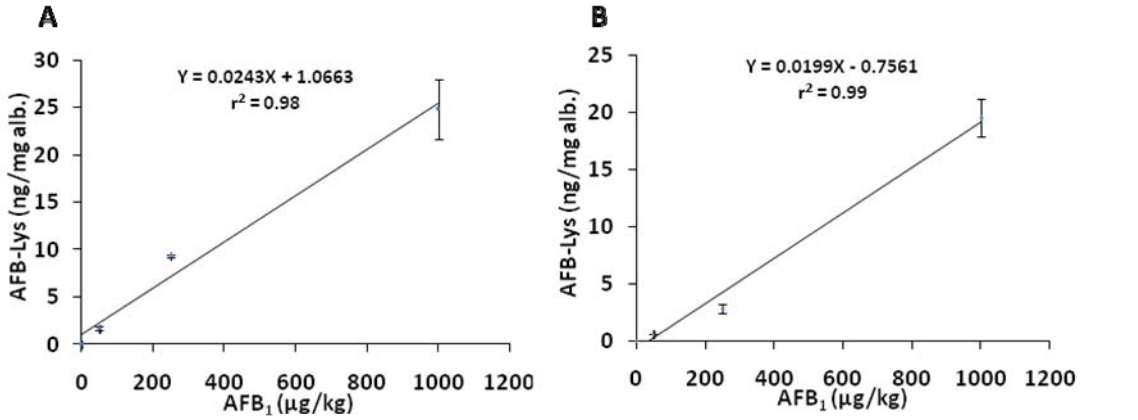

Figure 4.2. Linear relationships between AFB-Lys concentrations and AFB_1 doses after a single-dose treatment.

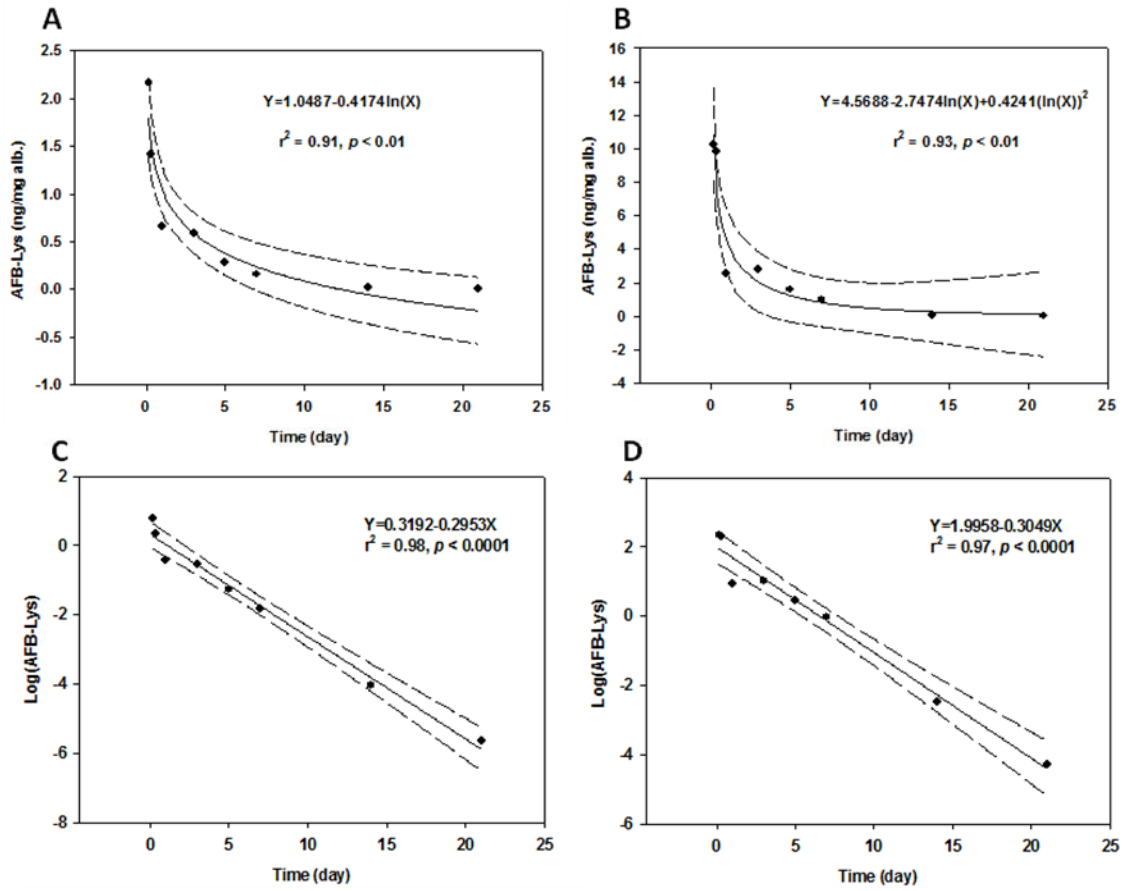

Figure 4.3. Elimination kinetics of serum AFB-Lys adduct after a single-dose of AFB_1 exposure.

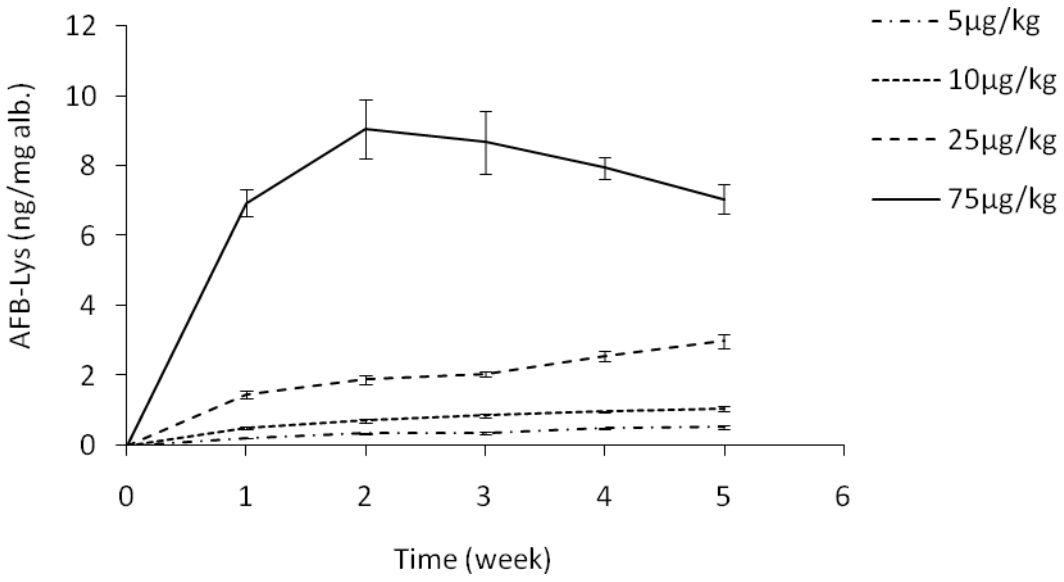

Figure 4.4. Serum concentrations of AFB-Lys adduct after repeated AFB_1 exposure.

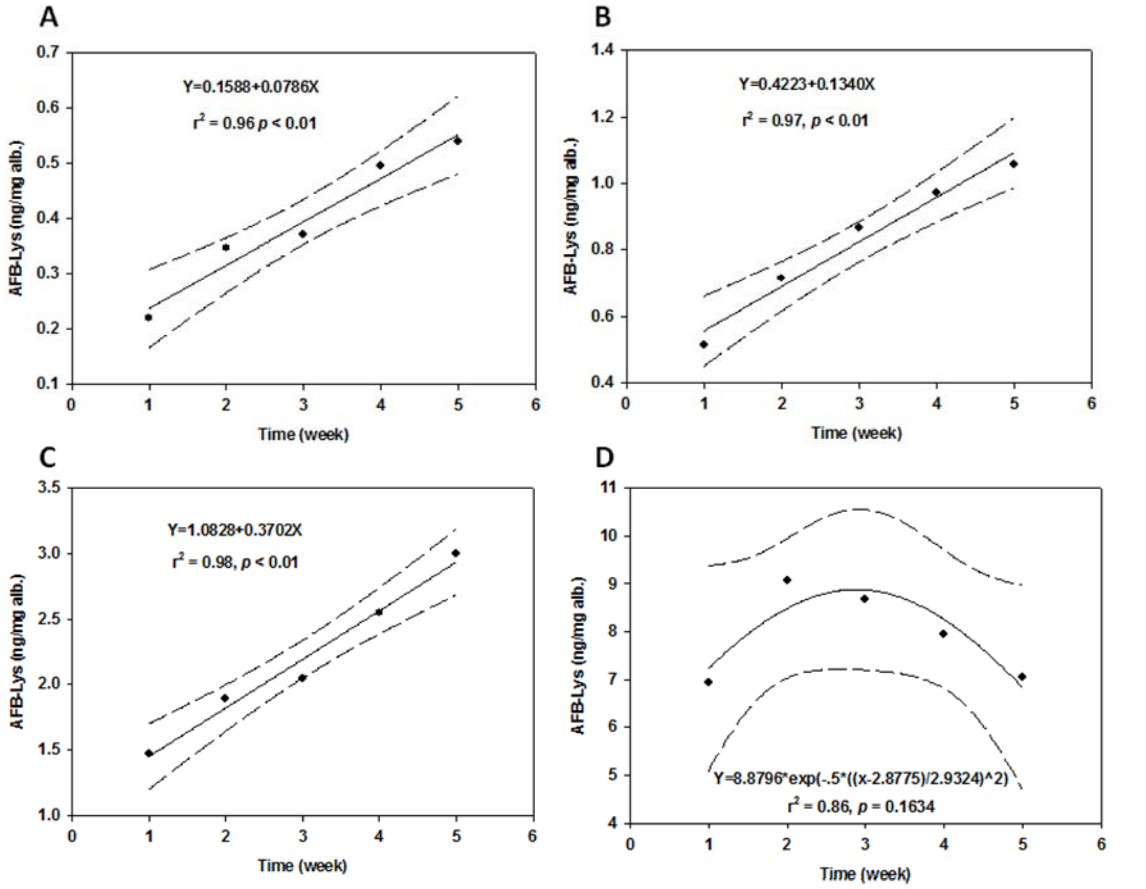

Figure 4.5. Regression analysis of serum concentrations of AFB-Lys adduct over repeated exposure.

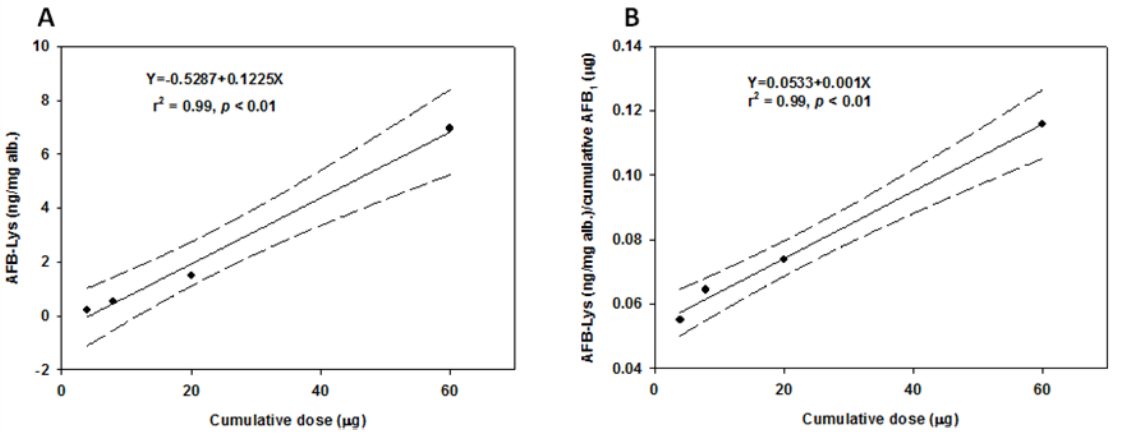

Figure 4.6. Dose-response and yield of AFB-Lys over cumulative AFB_1 doses.

Figure Legends

Figure 4.1. Serum concentrations of AFB-Lys adduct after a single-dose of AFB_1 exposure. The inset is an enlarged view of 0 to 24 h. Values shown are mean ± SD (n=5).

Figure 4.2. Linear relationships between AFB-Lys concentrations and AFB_1 doses after a single-dose treatment. (A) 2 h ($r^2 = 0.98$) and (B) 24 h ($r^2 = 0.99$).

Figure 4.3. Elimination kinetics of serum AFB-Lys adduct after a single-dose of AFB_1 exposure. Nonlinear curve fitting of AFB-Lys over time was tested for the 50 (A) and 250 μg/kg (B) groups. Linear curve fitting of the log (AFB-Lys) over time was tested for 50 (C) and 250 μg/kg (D) groups. The slopes generated from Figs. C and D were used for calculating the half-life for serum AFB-Lys. Dashed lines indicate 95% CI.

Figure 4.4. Serum concentrations of AFB-Lys adduct after repeated AFB_1 exposure. Values shown are mean ± SD (n=5).

Figure 4.5. Regression analysis of serum concentrations of AFB-Lys adduct over repeated exposure. (A), 5 μg/kg group; (B), 10 μg/kg group; (C), 25 μg/kg group and (D), 75 μg/kg group. Dashed lines indicate 95% CI.

Figure 4.6. Dose-response and yield of AFB-Lys over cumulative AFB_1 doses. Linear dose-response relationship between serum AFB-Lys concentrations and cumulative AFB_1 doses was fitted for 1 week after repeated-dose of AFB_1 ($r^2 = 0.99$, $p < 0.01$, A). Cumulative dose was calculated as dose (μg/kg/day) × day × body weight. Linear increase in yield of AFB-Lys over doses was also found ($r^2 = 0.99$, $p < 0.01$, B). Dashed lines indicate 95% CI.

CHAPTER 5

INTEGRATIVE TOXICOPATHOLOGICAL EVALUATION OF AFLATOXIN B_1 EXPOSURE IN F344 RATS [1]

[1] Qian, G. Wang, F. Tang, L. Massey, M.E. Mitchell, N.J. Su, J. Williams, J.H. Phillips, T.D. and Wang, J.-S. To be submitted to *Toxicologic Pathology*.

Abstract

In this study male F344 rats were orally exposed to a single-dose of aflatoxin B_1 (AFB_1) at 0, 50, 250 or 1000 μg/kg body weight (BW) or repeated-dose of 0, 5, 10, 25 or 75 μg/kg BW for up to 5 weeks. Biochemical and histological changes were assessed together with formation of AFB_1-lysine adduct (AFB-Lys) and liver foci positive for placental form glutathione S transferase (GST-P^+). In single-dose protocol, serum AST, ALT and ALP were dose-related elevated with maximal changes (> 100-fold) at 3-day after treatment. Animals, received 250 μg/kg AFB_1, showed concurrent bile duct proliferation, necrosis and GST-P^+ hepatocytes at 3-day, followed by liver GST-P^+ foci appearance at 1-week. In repeated-dose protocol, bile duct proliferation and liver GST-P^+ foci co-occurred after 3-week exposure to 75 μg/kg AFB_1, followed by proliferation foci formation after 4-week and dramatic ALT, AST and CK elevations after 5-week. Liver GST-P^+ foci were temporally and dose-related induced. Serum AFB-Lys increased temporally at low doses (5-25 μg/kg), and reached maximum after 2-week exposure at 75 μg/kg. This integrative study demonstrate that liver GST-P^+ cells and foci are sensitive biomarkers for AFB_1 toxic effect and correlated with bile duct proliferation and biochemical alterations in F344 rats.

Keywords: Aflatoxin B_1; aflatoxin B_1-lysine; placental form glutathione S transferase; histology; biochemistry.

Introduction

Aflatoxins (AF), especially aflatoxin B_1 (AFB_1), are common food contaminants in grains and groundnuts which represent a major group of harmful mycotoxins. The contamination of food by AF remains a significant public health problem worldwide, especially in the developing world where AF exposure has been reported to be associated with approximately 40% of all disease burden [1]. The acute poisoning of AF (aflatoxicosis) in Kenya (2004 and 2005) had caused 125 deaths and more cases which resulted from the ingestion of heavily contaminated maize and maize products [2, 3]. The adverse effects of AFB_1 on growth and development of children have become an increasing health concern [4, 5].

The histopathological effects and biochemical changes induced by AFB_1 exposure have been extensively studied in rat [6-8] and chick [9, 10]. However, only a few studies [11, 12] reported the sequential biochemical and histological changes, in which rats were given either one single large dose of 3 mg/kg body weight (BW) or 7 mg/kg BW. In particular, no studies have been done to explore the correlations between biochemical and histopathological alterations with specific molecular targets via single or repeated treatment protocol using human relevant doses. Such data could be useful for delineation of functional and microstructural changes in liver and for evaluation of various intervention strategies to diminish the acute and chronic health effects due to AFB_1 exposure.

AFB_1 is metabolically activated by cytochrome P450 enzymes in liver to its reactive metabolite, AFB_1-8,9-epoxide, which binds to macromolecules resulting in DNA

adduct formation and carcinogenesis [13]. AFB_1-8,9-epoxide can further be converted to AFB_1-8,9-diol that specifically binds to lysine in albumin and forms AFB_1-lysine adducts (AFB-Lys), which has been validated as a biomarker for indicating human exposure [14]. This adduct and other AFB_1 biomarkers (e.g., AFB_1-N^7-guanine adduct) have become critical tools for evaluation of chemopreventive agents in animals and humans [15].

Glutathione S transferase (GST) is a large family of metabolic enzymes comprised of several members, such as alpha, mu, pi, theta and others [16]. GST in the alpha and pi classes is abundant in rat livers and inducible by drugs. Because of the putative role in detoxifying carcinogens these two isoenzymes have been the molecular target for chemoprevention [17, 18]. The pi form of GST was initially found in preneoplastic hepatocytes; in rat it was named GST-placental form (GST-P) as this form is the only form abundant in rat placenta (Ito et al., 1992). Rat liver GST-P positive ($GST\text{-}P^+$) foci are a specific and reliable preneoplastic marker that have been widely used in animal carcinogenesis models [19, 20]. AFB_1 has been shown to induce $GST\text{-}P^+$ hepatocytes or foci in rat liver both independently and synergistically with other toxins, such as fumonisin and microcystin LR [21-23]. However, the sequential development of $GST\text{-}P^+$ foci caused by AFB_1 as well as the correlations with biochemical and histological changes in animal models remains largely unknown.

In this study we take an integrative approach to sequentially assess the toxic effects of AFB_1 in F344 rats following both single and repeated treatment protocols. The aims of this study are to validate the time course of development of liver $GST\text{-}P^+$ foci, to evaluate the potential correlations of this effect biomarker with other histological and

biochemical changes caused by AFB_1 exposure, and to provide toxicopathological information for future intervention studies.

Materials and Methods

Chemicals and Reagents

AFB_1 (≥ 98% purity), normal rat serum, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Inc (St. Louis, MO). AFB_1 was prepared in DMSO to a stock solution and was diluted freshly to use. Rabbit anti-glutathione S transferase pi antibody was purchased from Abbiotec (San Diego, CA). Vectastain Elite ABC Kit and diaminobenzidine (DAB) substrate kit for peroxidase were purchased from Vector Laboratories (Burlingame, CA). All solvents were of the highest grade commercially available.

Animals

Male F344 rats (100-120 g) were purchased from Harlan Laboratory (Indianapolis, IN). Animals were individually housed under controlled light/dark cycle (12 h/12 h) with temperature of 22±2°C and relative humidity of 50-70%. Purified AIH 76A diet and tap water were provided *ad libitum*. Animals were maintained on AIN 76A feed for 1 week before treatment. Animal husbandry and care, AFB_1 treatment, and biological samples processing were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia.

Experimental Design

For single dose treatment, a total of 140 male F344 rats were randomly divided into 4 groups, 0, 50, 250 and/or 1000 μg AFB_1/kg BW. Groups of 5 rats at each group were gavaged a single dose of AFB_1 and sacrificed at 2 h, 1, 3, 5, 7, 14, and 21 days after

treatment. For repeated-dose treatment, a total of 125 male F344 rats were randomly divided into 5 groups: 0, 5, 10, 25 and 75 μg AFB_1/kg BW. Animals were gavaged 5 days per week and 5 rats from each group were sacrificed at weekly intervals 24 h after the last dose. Gavage volume was set at 50 μL/100 g BW. Blood and liver tissues were collected at sacrifice for examination of serum biochemistry, liver histology, and the GST-P^+ foci formation. Animals were euthanized by cardiac puncture and exsanguination under isoflurane anesthesia.

Serum Biochemistry

Serum samples were collected by centrifuging the whole blood at 3000 rpm for 20 min at 4°C and stored at -20°C before analysis for serum biochemistry. All analysis was done with a Roche Hitachi 912 Chemistry Analyzer (Roche Diagnostics, Indianapolis, IN). The serum parameters measured were total serum protein (g/dL), albumin (g/dL), globulin (g/dL), albumin:globulin ratio (A/G ratio), serum calcium (mg/dL), phosphorus (mg/dL), glucose (mg/dl), BUN (mg/dL), creatinine (mg/dL), total bilirubin (mg/dl), alkaline phosphatase (ALP, U/L), creatine kinase (CK, U/L), alanine transaminase (ALT, U/L), aspartate transaminase (AST, U/L), g-glutamyl transpeptidase (GGT, U/L), amylase (U/L), cholesterol (mg/dl), serum sodium (mmol/L), potassium (mmol/L), Na/K ratio, chloride (mmol/L).

Serum AFB-Lys Adduct Analysis

The method for analysis of serum AFB-Lys has been described previously [24]. Briefly, aliquots of each serum sample (150 μL) was digested with pronase (pronase:total protein, 1:4, w:w) at 37°C for 3 h to release AFB-Lys. The digests were purified by Waters MAX cartridges which were preprimed with methanol and equilibrated with

water. The loaded cartridge was sequentially washed by water, 70% methanol, and 1% ammonium hydroxide in methanol at a flow rate of 1mL/min. The purified AFB-Lys adducts were eluted with 2% formic acid in methanol, dried in a Labconco Centrivap concentrator (Kansas City, MO) and reconstituted for HPLC-fluorescence detection. The adduct concentrations were adjusted by serum albumin content. The average recovery rate was 90% and detection limit was 0.4 pg/mg albumin.

Liver Histology

Liver tissues were always taken from the right portion of the median lobe. Liver tissue blocks of approximately 6 × 6 ×1.5 mm were routinely fixed in 10% neutral buffer formalin, dehydrated and embedded. Liver sections were cut at 5 μm and stained with hematoxylin and eosin for light microscopic examination. Photographs were taken on an Olympus XC30 microscope with an Olympus UC30 digital camera (Olympus America Inc., Center Valley, PA) linked to a Cellsense image analysis system (Celsense Inc., Pittsburgh, PA).

Liver GST-P Staining

Liver paraffin sections prepared as above were used for GST-P staining by the avidin-biotin-peroxidase complex (ABC) method [25]. Briefly, liver sections were routinely hydrated and antigen was retrieved by heat. Sections were sequentially treated with primary rabbit anti-GST-P antibody (1:800), secondary goat anti-rabbit IgG (1:200) and ABC. The staining was done by adding diaminobenzidine (DAB). These sections were finally counterstained with hematoxylin for light microscopic examination. A GST-P^+ focus was defined as a cluster of ≥ 5 hepatocytes positively stained with brown color.

Areas of each section were measured and the counting of GST-P^+ cells or foci was based on section area.

Statistical Analysis

Data on body weights, serum biochemical parameters and AFB-Lys adducts were expressed as mean ± standard deviation (SD). Statistical comparisons of these parameters were conducted using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison procedures. The raw data of body weight were used for statistical analysis while the biochemical parameters were analyzed following logarithmic transformation because of their right skewed distribution and non-equal variance. Correlation analysis was done through SigmaPlot 10.0 (San Jose, CA). A *p*-value of less than 0.05 was considered statistically significant.

Results

Body Weight Changes

Animals that received 1000 μg/kg AFB_1 showed decreased body weight starting from 1 day after treatment ($p < 0.05$), as compared to the control (Figure 5.1A). For the 250 μg/kg group, animals showed significantly decreased body weight gain at 3 and 5 days after treatment ($p < 0.05$). A dose-dependent decrease of body weight gain was found for animals that received repeated-dose treatment, however, significant decrease was found only in the 75 μg/kg group starting from the second week as compared to control group ($p < 0.05$, Figure 5.1B). Animals in the 1000 μg/kg group died within 7 days and the liver tissues were examined for histopathological changes and no animals died in any other group.

Serum Biochemistry after Single-Dose Treatment

Dose-dependent increases of serum AST and ALT activities were found at 1 and 3 days after single-dose treatment and these two enzymes were approximately 2- to7-fold higher at 250 μg/kg group and 20- to100-fold higher at 1000 μg/kg group, as compared to control group ($p < 0.05$, Table 5.1). AST and ALT were decreased after 1-week at 50 and 250 μg/kg groups ($p < 0.05$); ALT rather than AST returned to normal after 3-week. ALP activities were also elevated at 1000 μg/kg group at 1 and 3 days after treatment ($p < 0.05$, Table 5.1) while at lower doses ALP activities were found to be decreased starting at 3 days after treatment ($p < 0.05$). Serum CK was significantly decreased at 50 μg/kg group and elevated at 1000 μg/kg group at 1 day after treatment, as compared to control group ($p < 0.05$, Table 5.1). Starting from 3 days after treatment, CK was decreased in a dose-related manner and significantly lower than control group ($p < 0.05$). In addition, a dramatic elevation of total bilirubin was found at 1000 μg/kg group at 3 days after treatment with a mean ± SD value of 15.49 ± 0.26 mg/dL, compared to all other groups including controls that have < 0.1 mg/dL total bilirubin (Data not shown in Table 5.1). Amylase activities were dose-dependently decreased at 1 day after treatment, followed by gradual recovery to normal at 1 week after treatment. Total protein and albumin were decreased at 1 and 3 days after treatment in a dose-dependent manner, which returned to normal after 1 week. Serum glucose levels were decreased at high doses (≥ 250 μg/kg) but elevated at low dose (50 μg/kg) at 1 and 3 days after treatment. Other serum biochemical parameters were less affected and data are not shown here.

Serum Biochemistry after Repeated-Dose Treatment

There were no obvious changes in profiles of serum biochemical parameters in animals after 1- or 3-week treatment with AFB_1 (Data not shown). However, repeated-dose treatment with 75 μg/kg AFB_1 for 5-week significantly increased serum AST and ALT activities by 1.02 and 0.19-fold, respectively, as compared to control group ($p < 0.05$, Table 5.2). Serum CK activities were decreased at 25 μg/kg and were dramatically elevated (approximately 3-fold) at 75 μg/kg group after 5-week treatment ($p < 0.05$, Table 5.2). ALP activities were significantly decreased at 25 μg/kg group ($p < 0.05$). Amylase activities and phosphorus levels were significantly elevated at 75 μg/kg group ($p < 0.05$). Glucose levels were significantly elevated at low doses (5 and 25 μg/kg) but decreased at high dose (75 μg/kg, $p < 0.05$). Bilirubin levels were less than 0.1 mg/dL in all groups after 5-week treatment (data not shown).

Serum AFB-Lys Levels

The levels of serum AFB-Lys after single-dose treatment are shown in Table 5.3. Dose-dependent changes of serum AFB-Lys were present at all time points. A quick rise at 2 h followed by decrease was found after single-dose treatment. Repeated-dose treatment for 5-week increased the adduct levels by approximately 1-fold at low doses (5-25 μg/kg), however, at high dose (75 μg/kg), the levels of the adduct were increased from start to a peak after 2-week treatment, followed by slow decrease over the 5-week experimental period (Table 5.4).

Liver Histological Changes after Single-Dose Treatment

The histological changes in rat liver induced by AFB_1 occurred as a function of both dose and duration. Dramatic bile duct proliferation occurred at 250 and 1000 μg/kg

groups at 3 days after treatment; massive periportal necrosis foci with inflammatory cells infiltration, excessive red blood cells appearing around hepatocytes, and destruction of liver lobes occurred only at 1000 μg/kg group (Figure 5.2). Animals died in the 1000 μg/kg group showed more severe liver damage but these changes were not reported here. Bile duct proliferation and periportal necrosis persisted at 250 μg/kg group throughout the 3-week experimental period; however, no bile duct proliferation was found at 50 μg/kg group throughout the experimental period (data not shown).

Liver Histological Changes after Repeated-Dose Treatment

The major histological changes in rat liver after repeated-dose treatment with AFB_1 included bile duct proliferation, periportal necrosis, and proliferation foci formation (Figures 5.3 and 5.4). Bile duct proliferation first appeared at 75 μg/kg group after 3-week treatment, which progressed throughout the 5-week experimental period (Figure 5.3B). Bile duct proliferation was also found at 25 μg/kg group (Figure 5.3A) but not at lower doses after 5-week treatment (data not shown). Necrosis was present in the periportal zone at doses higher than 10 μg/kg group (Figure 5.3C and D). Proliferation foci were found only at 75 μg/kg group after 4- and 5-week treatment (Figure 5.4A and B).

Liver GST-P^+ Foci Formation after Single-Dose Treatment

Liver GST-P^+ cells and foci occurred in a dose- and time-dependent manner (Table 5.5 and Figure 5.5). Liver GST-P^+ foci were not found at 50 μg/kg group throughout the experimental period; however, single GST-P^+ cells were found starting from 1 week after a single-dose treatment. GST-P^+ cells appeared first at 3 days for the 250 μg/kg group, followed by foci formation at 1-week after treatment. The GST-P^+ foci

persisted throughout the experimental period of 3-week. Interestingly, the highest dose of AFB_1 (1000 μg/kg) did not induce the formation of GST-P^+ foci at 1-, 3- or 5-days after treatment. The liver GST-P^+ foci observed in this study consisted of clear cells.

Liver GST-P^+ Foci Formation after Repeated-Dose Treatment

The development of liver GST-P^+ foci after repeated-dose treatment with AFB_1 exhibited dose- and time-dependency (Table 5.6 and Figure 5.6). No liver GST-P^+ cells or foci were found in the control group at any time points examined; liver GST-P^+ cells appeared at 5 μg/kg group after 3-week treatment, at 10 μg/kg after 2-week treatment and at 25 and 75 μg/kg groups after 1-week treatment, respectively. Liver GST-P^+ foci appeared at 10 μg/kg group after 5-week treatment and at 25 and 75 μg/kg groups after 3-week treatment. There were no liver GST-P^+ foci found at 5 μg/kg group throughout the experimental period. A good correlation between serum AFB-Lys adduct levels and the numbers of GST-P^+ cells ($r = 0.99$, $p = 0.01$) or foci ($r = 0.97$, $p = 0.03$) after 5-week treatment was also found.

Discussion

In this integrative study we demonstrate the time course of development of biochemical, histological, and immunohistological changes and the connectivity between acute liver toxicity and carcinogenicity in both single-dose and repeated-dose treatment protocols (Figure 5.7). This is the first animal study to investigate the time course of development of liver GST-P^+ hepatocytes and foci caused by AFB_1 exposure. Liver AST and ALT activities are early and sensitive indicators of acute hepatocyte injury or necrosis, in this case injury caused by AFB_1, which preceded the appearance of bile duct proliferation and periportal necrosis. The occurrence of GST-P^+ hepatocytes was

temporally correlated with bile duct proliferation after single-dose treatment, while liver GST-P^+ foci formation was correlated with bile duct proliferation in rat liver upon repeated-dose exposure. Serum AFB-Lys adduct was demonstrated as a reliable exposure biomarker especially through repeated low does exposure. The doses of AFB_1 selected in this study were relevant to human exposure levels, which were found in cases of acute poisoning (such as in Kenya, up to 46.4 ppm AFB_1 in maize) [3] and high-risk populations with chronic dietary exposure in China (estimated AFB_1 daily intake of 48.4 μg/male and 77.4 μg/female) [26]. . An estimation of AFB_1 exposure, based on a 70 kg adult consuming 2 kg maize containing 1 ppm AFB_1, would be 2 kg × 1 ppm/70 kg = 26.7 μg/kg body weight. This is comparable to the selected doses in our study.

The transit rise of serum AFB-Lys at 2 h was in agreement with the quick absorption and metabolism of AFB_1 in rat liver [27]. Compared to other parameters (amylase, proteins and glucose), dramatic increases of serum enzymes (ALT, AST and ALP) were found at 3 days after single treatment. These changes were in agreement with the massive necrosis in liver as observed at 3 days after treatment. It has been reported that a transit elevation of ALT and AST occurs at 48 h after three i.p. doses of AFB_1 (150 μg/kg) in Sprague-Dawley rats [28]. The discrepancy is likely due to differences in animal species, route of exposure or examination period. Serum activities of AST and ALT returned to control group levels at 3-week for animals in the 50 but not 250 μg/kg group, indicating dose-related yet slow recovery process. The decreased CK after 1-day could be related to reduced muscle mass, evidenced by lowered body weight, and such finding has been reported in patients with alcoholic liver disease [29].

Repeated-dose treatment elevated serum AST and CK activities only after 5-week treatment with 75 μg/kg AFB_1, and these changes occurred after the appearance of bile duct proliferation, indicating that. these parameters are not sensitive indicators for repeated low dose AFB_1 exposure. The finding that CK was decreased at low dose (≤ 25 μg/kg) and elevated at high dose (75 μg/kg) may suggest different modes of action. In contrary, serum glucose levels were elevated at low doses and decreased at high dose, a finding similar to that in single-dose treatment. These outcomes could reflect a balance of different modes of actions, such as the decrease of glucose-6-phosphatase (G6P) activity [8] and the newly expressed G6P in proliferating bile duct-like cells [30].

Bile duct proliferation and periportal necrosis are characteristic of AFB_1-induced liver injuries [31, 32], these histological changes slowly reached a maximum in rat liver at 3 days after a single dose of 250 or 1000 μg/kg AFB_1 in this study. Animal received the highest dose (1000 μg/kg AFB_1) died within 7 days of treatment. The possible reason may involve the massive hemorrhagic necrosis and liver lobular dissolution that became irreversible. Bile duct proliferation was constantly found in animals received 250 μg/kg AFB_1 after 1 week but not at lower dose, indicating dose-related induction of this alteration. Among the three types of bile duct proliferation (typical, atypical and "oval cell"), the proliferation observed here is restricted to the periportal zone and usually has a well-defined lumen, which are characteristic of "typical" proliferation [33]. The distorted liver microstructure might cause biliary duct hypertension that has been thought as the initiating factor in a bile duct ligated rat model. [34]. Arguably, the newly produced bile duct could also be derived from a metaplasia of the periportal hepatocytes or from the

liver stem cells (or oval cells) [35]. Although many studies have reported AFB_1 induced bile duct proliferation, the exact mechanisms remain to be uncovered.

Similar histological changes were found in animals received repeated-dose of AFB_1 treatment, such as bile duct proliferation and necrosis. In contrast to the "typical" bile duct proliferation, repeated low dose treatment caused more of an "oval cell" type of proliferation. The proliferation induces the formation of disorganized tubular structures with a poorly defined duct lumen [33]. Since no specific staining has been done to distinguish these cells (bile duct epithelium, oval cell), it is likely that different types of proliferation may co-exist. The clear cell foci caused by repeated-dose treatment were early preneoplastic changes, which might return to normal or progress into hepatocellular carcinoma (HCC). For instance, HCC was reported in rats received 0.015 ppm AFB_1 for a period of 68-80 weeks with altered foci appearing within a few weeks' treatment [31].

Liver GST-P is a specific biomarker that is markedly and localized increased in preneoplastic foci in rat [16]. This enzyme can be specifically induced by a variety of carcinogens, such as diethylnitrosamine (DEN) and 2-acetylaminofluorene (2-AAF) [36, 37]. Both single hepatocytes heavily positive for GST-P and liver GST-P^+ foci have been used as markers of preneoplastic lesions in short-term carcinogenesis assays [19, 38]. In this study single GST-P^+ hepatocytes were induced as early as 3 days after a single dose of 250 μg/kg AFB_1, demonstrating the strong potency of AFB_1 in initiating rat hepatocytes. A previous study demonstrated an even earlier onset of GST-P^+ hepatocytes at 48 h after an i.p. injection of 2 mg/kg AFB_1 to young Fischer rats [23], and the differences were likely due to variance in animal age, dose and route of exposure. Liver GST-P^+ foci were present at 250 μg/kg but not 50 μg/kg groups, which was suggestive of a non-observed

effect level for liver GST-P$^+$ foci formation. Results from other studies on DEN and n-nitrosomorpholine [39, 40] were consistent with this finding, and the value of GST-P$^+$ foci in dose-dependent carcinogenic studies has been reviewed [41]. In contrast, liver GST-P$^+$ hepatocytes or foci were not induced in animals received 1000 μg/kg AFB_1. One possibility for the negative finding is the overwhelming of signaling pathways responsible for expressing GST-P, such as transforming growth factor β (TGF-β) [42] and mitochondrial oxidative stress [43] mediated signaling pathways.

The appearance of liver GST-P$^+$ hepatocytes was correlated with bile duct proliferation at 3 days after 250 μg/kg AFB_1 treatment, suggesting a synchronized onset. It was reported that 4-nitroquinoline 1-oxide induced placental GST expression was also correlated with cellular proliferation in a rat tongue carcinogenesis model [44]. Therefore, it appears that a temporal overlapping exists between the acute toxicity (bile duct proliferation in particular) and the preneoplastic changes (liver GST-P$^+$ cells and foci) at a non-lethal dose of AFB_1 exposure. While increased biliary pressure has been proposed as the initiating factor in bile duct proliferation [34] as mentioned above, the mechanisms for GST-P expression might also involve epigenetic mechanisms [45]. However, the exact mechanisms for these effects require more efforts to uncover. In addition, serum AFB-Lys adduct levels increased over the 5-week experimental period at low doses (≤ 25 μg/kg), evident of its value for indicating chronic exposure. Large dose exposure (75 μg/kg) produced a plateau of this adduct after 2-week which was consistent with the occurrence of bile duct proliferation and GST-P$^+$ foci at 3-week and may imply a variation of metabolic balance between its formation and detoxification.

Wild et al. have revealed the good correlation between serum AFB_1-albumin adducts and AFB_1-DNA adducts in multiple species including rats [46]. Their data provide evidence for the application of AFB_1-albumin adducts from a mechanistic standpoint of view. Our study highlights the correlation between AFB_1-lysine adduct levels and formation of preneoplastic lesions: a good correlation between serum AFB-Lys adduct levels and numbers of liver GST-P^+ cells or foci has been found after 5-week repeated exposure. It needs to mention that liver GST-P^+ foci formation and serum enzyme changes (e.g., ALT and AST) are not specific, although these changes are sensitive to and can be correlated with AFB_1 exposure.

In conclusion, we demonstrate AFB_1-induced time course of development of liver GST-P^+ foci and the correlations between formation of liver GST-P^+ foci and bile duct proliferation in F344 rats. Given the early formation and the temporal correlation of Liver GST-P^+ foci with bile duct proliferation after single or repeated AFB_1 treatment in F344 rats (as shown in this study), liver GST-P^+ foci may be used as an endpoint to evaluate the efficacy of interventions in animal experiments, e.g., we have been conducting an animal experiment to assess the efficacy of NovaSil clay in preventing or reducing the toxic effects of co-exposure to AFB_1 and FB_1 through a short-term carcinogenesis rat model. The simultaneous examination of serum AFB-Lys adducts and liver GST-P^+ positive foci might be useful for evaluation of chemopreventive agents in animal experiments.

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References

1. Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences, and interventions. *Am J Clin Nutr*. 2004;80:1106-1122
2. Azziz-Baumgartner E, Lindblade K, Gieseker K, Rogers HS, Kieszak S, Njapau H, Schleicher R, McCoy LF, Misore A, DeCock K, Rubin C, Slutsker L. Case-control study of an acute aflatoxicosis outbreak, kenya, 2004. *Environ Health Perspect*. 2005;113:1779-1783
3. Lewis L, Onsongo M, Njapau H, Schurz-Rogers H, Luber G, Kieszak S, Nyamongo J, Backer L, Dahiye AM, Misore A, DeCock K, Rubin C. Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central kenya. *Environ Health Perspect*. 2005;113:1763-1767
4. Khlangwiset P, Shephard GS, Wu F. Aflatoxins and growth impairment: A review. *Crit Rev Toxicol*. 2011;41:740-755
5. Smith LE, Stoltzfus RJ, Prendergast A. Food chain mycotoxin exposure, gut health, and impaired growth: A conceptual framework. *Adv Nutr*. 2012;3:526-531
6. Clifford JI, Rees KR. The action of aflatoxin b1 on the rat liver. *Biochem J*. 1967;102:65-75
7. Shank RC, Wogan GN. Acute effects of aflatoxin b1 on liver composition and metabolism in rat and duckling. *Toxicol Appl Pharmacol*. 1966;9:468-476

8. Rastogi R, Srivastava AK, Rastogi AK. Biochemical changes induced in liver and serum of aflatoxin b1-treated male wistar rats: Preventive effect of picroliv. *Pharmacol Toxicol*. 2001;88:53-58

9. Chattopadhyay SK, Taskar PK, Schwabe O, Das YT, Brown HD. Clinical and biochemical effects of aflatoxin in feed ration of chicks. *Cancer Biochem Biophys*. 1985;8:67-75

10. Ostrowski-Meissner HT. Biochemical and physiological responses of growing chickens and ducklings to dietary aflatoxins. *Comp Biochem Physiol C*. 1984;79:193-204

11. Yin SJ, Kao MC, Lee SC. Sequential biochemical and histological changes in rats treated with aflatoxin b1. *Br J Cancer*. 1980;42:319-325

12. Dwivedi Y, Rastogi R, Mehrotra R, Garg NK, Dhawan BN. Picroliv protects against aflatoxin b1 acute hepatotoxicity in rats. *Pharmacol Res*. 1993;27:189-199

13. Eaton DL, Groopman JD. *The toxicology of aflatoxins: Human health, veterinary, and agricultural significance.*: San Diego: Academic Press. New York, NY. 1994.

14. Sabbioni G, Skipper PL, Buchi G, Tannenbaum SR. Isolation and characterization of the major serum albumin adduct formed by aflatoxin b1 in vivo in rats. *Carcinogenesis*. 1987;8:819-824

15. Kensler TW, Roebuck BD, Wogan GN, Groopman JD. Aflatoxin: A 50-year odyssey of mechanistic and translational toxicology. *Toxicological Sci*. 2011;120 Suppl 1:S28-48

16. Ito N, Hasegawa R, Imaida K, Masui T, Takahashi S, Shirai T. Pathological markers for non-genotoxic agent-associated carcinogenesis. *Toxicology Lett*. 1992;64-65 Spec No:613-620

17. Chow HH, Hakim IA, Vining DR, Crowell JA, Tome ME, Ranger-Moore J, Cordova CA, Mikhael DM, Briehl MM, Alberts DS. Modulation of human glutathione s-transferases by polyphenon e intervention. *Cancer Epidemiol Biomarkers Prev*. 2007;16:1662-1666

18. Gross-Steinmeyer K, Eaton DL. Dietary modulation of the biotransformation and genotoxicity of aflatoxin b(1). *Toxicology*. 2012;299:69-79

19. Tatematsu M, Mera Y, Ito N, Satoh K, Sato K. Relative merits of immunohistochemical demonstrations of placental, a, b and c forms of glutathione s-transferase and histochemical demonstration of gamma-glutamyl transferase as markers of altered foci during liver carcinogenesis in rats. *Carcinogenesis*. 1985;6:1621-1626

20. Sato K. Glutathione transferases as markers of preneoplasia and neoplasia. *Adv Cancer Res*. 1989;52:205-255

21. Gelderblom WC, Marasas WF, Lebepe-Mazur S, Swanevelder S, Vessey CJ, Hall Pde L. Interaction of fumonisin b(1) and aflatoxin b(1) in a short-term carcinogenesis model in rat liver. *Toxicology*. 2002;171:161-173

22. Sekijima M, Tsutsumi T, Yoshida T, Harada T, Tashiro F, Chen G, Yu SZ, Ueno Y. Enhancement of glutathione s-transferase placental-form positive liver cell foci development by microcystin-lr in aflatoxin b1-initiated rats. *Carcinogenesis*. 1999;20:161-165

23. Tsuji K, Gopalan P, Lehmann K, Kimura M, Horiuchi A, Sato K, Lotlikar PD. Species and sex differences of aflatoxin b1-induced glutathione s-transferase placental form in single hepatocytes. *Cancer Lett*. 1992;66:249-254

24. Qian G, Tang L, Liu W, Wang JS. Development of a non-antibody method for rapid detection of serum aflatoxin b1-lysine adduct. *Toxicologist*. 2010;114:248.

25. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (abc) in immunoperoxidase techniques: A comparison between abc and unlabeled antibody (pap) procedures. *J Histochem Cytochem*. 1981;29:577-580

26. Groopman JD, Zhu JQ, Donahue PR, Pikul A, Zhang LS, Chen JS, Wogan GN. Molecular dosimetry of urinary aflatoxin-DNA adducts in people living in guangxi autonomous region, people's republic of china. *Cancer Res*. 1992;52:45-52

27. Butler WH, Clifford JI. Extraction of aflatoxin from rat liver. *Nature*. 1965;206:1045-1046

28. Kim YS, Kim YH, Noh JR, Cho ES, Park JH, Son HY. Protective effect of korean red ginseng against aflatoxin b-1-induced hepatotoxicity in rat. *J Ginseng Res*. 2011;35:243-249

29. Rosalki SB. Low serum creatine kinase activity. *Clinical chemistry*. 1998;44:905

30. Lenzi R, Liu MH, Lenzen R, Han T, Alpini G, Tavoloni N. Distribution of glucose-6-phosphatase activity in normal, hyperplastic, and preneoplastic rat liver. *Virchows Archiv B Cell Pathol Incl Mol Pathol*. 1991;61:279-287

31. Newberne PM, Wogan GN. Sequential morphologic changes in aflatoxin b carcinogenesis in the rat. *Cancer Res*. 1968;28:770-781

32. Kalengayi MM, Desmet VJ. Sequential histological and histochemical study of the rat liver during aflatoxin b1-induced carcinogenesis. *Cancer Res*. 1975;35:2845-2852

33. LeSage G, Glaser S, Alpini G. Regulation of cholangiocyte proliferation. *Liver*. 2001;21:73-80

34. Slott PA, Liu MH, Tavoloni N. Origin, pattern, and mechanism of bile duct proliferation following biliary obstruction in the rat. *Gastroenterology*. 1990;99:466-477

35. Forbes S, Vig P, Poulsom R, Thomas H, Alison M. Hepatic stem cells. *J Pathol*. 2002;197:510-518

36. Tiwawech D, Hasegawa R, Kurata Y, Tatematsu M, Shibata MA, Thamavit W, Ito N. Dose-dependent effects of 2-acetylaminofluorene on hepatic foci development and cell proliferation in rats. *Carcinogenesis*. 1991;12:985-990

37. Carthew P, Nolan BM, Smith AG, Edwards RE. Iron promotes den initiated gst-p foci in rat liver. *Carcinogenesis*. 1997;18:599-603

38. Moore MA, Nakagawa K, Satoh K, Ishikawa T, Sato K. Single gst-p positive liver cells--putative initiated hepatocytes. *Carcinogenesis*. 1987;8:483-486

39. Satoh K, Hatayama I, Tateoka N, Tamai K, Shimizu T, Tatematsu M, Ito N, Sato K. Transient induction of single gst-p positive hepatocytes by den. *Carcinogenesis*. 1989;10:2107-2111

40. Enzmann H, Zerban H, Kopp-Schneider A, Loser E, Bannach P. Effects of low doses of n-nitrosomorpholine on the development of early stages of hepatocarcinogenesis. *Carcinogenesis*. 1995;16:1513-1518

41. Tsuda H, Fukushima S, Wanibuchi H, Morimura K, Nakae D, Imaida K, Tatematsu M, Hirose M, Wakabayashi K, Moore MA. Value of gst-p positive preneoplastic hepatic foci in dose-response studies of hepatocarcinogenesis: Evidence for practical thresholds with both genotoxic and nongenotoxic carcinogens. A review of recent work. *Toxicol Pathol*. 2003;31:80-86

42. Ichimura R, Mizukami S, Takahashi M, Taniai E, Kemmochi S, Mitsumori K, Shibutani M. Disruption of smad-dependent signaling for growth of gst-p-positive lesions from the early stage in a rat two-stage hepatocarcinogenesis model. *Toxicol Appl Pharmacol*. 2010

43. Kakehashi A, Ishii N, Shibata T, Wei M, Okazaki E, Tachibana T, Fukushima S, Wanibuchi H. Mitochondrial prohibitins and septin 9 are implicated in the onset of rat hepatocarcinogenesis. *Toxicol Sci*. 2011;119:61-72

44. Silva RN, Ribeiro DA, Salvadori DM, Marques ME. Placental glutathione s-transferase correlates with cellular proliferation during rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. *Exp Toxicol Pathol*. 2007;59:61-68

45. Satoh K, Hatayama I. Anomalous elevation of glutathione s-transferase p-form (gst-p) in the elementary process of epigenetic initiation of chemical hepatocarcinogenesis in rats. *Carcinogenesis*. 2002;23:1193-1198

46. Wild CP, Hasegawa R, Barraud L, Chutimataewin S, Chapot B, Ito N, Montesano R. Aflatoxin-albumin adducts: A basis for comparative carcinogenesis between animals and humans. *Cancer Epidemiol Biomarkers Prev*. 1996;5:179-189

TABLE 5.1.—Selected alterations in serum biochemical parameters after single-dose treatment with AFB_1.

Time (day)	Dose (μg/kg)	AST (U/L)	ALT (U/L)	ALP (U/L)	CK (U/L)	Amylase (U/L)	Total Protein (g/dL)	Albumin (g/dL)	Glucose (mg/dL)
1	0	128.00±1.41[a]	54.50±0.71[a]	403.50±0.71[a]	829.50±31.82[a]	2261.00±18.38[a]	6.30±0.00[a]	4.35±0.07[a]	219.00±2.83[a]
	50	119.50±0.71[a]	48.00±2.00[a]	413.00±4.24[a]	720.00±8.49[b]	2233.50±2.12[a]	6.20±0.00[b]	4.30±0.00[a]	242.00±2.83[b]
	250	274.00±7.07[b]	174.50±7.78[b]	398.00±8.48[a]	828.00±2.83[a]	1915.00±21.21[b]	5.95±0.07[c]	4.25±0.07[a]	169.00±2.83[c]
	1000	2489.50±102.53[c]	1854.50±106.77[c]	485.50±3.53[b]	1122.50±6.36[c]	488.50±10.61[c]	5.25±0.07[d]	3.90±0.00[a]	93.00±1.41[d]
3	0	107.00±4.24[a]	49.50±3.54[a]	339.50±10.60[a]	638.50±17.68[a]	2415.00±110.31[a]	6.35±0.21[a]	4.45±0.07[a]	212.50±7.78[a]
	50	97.50±2.12[a]	48.00±1.41[a]	280.00±5.66[b]	485.00±9.90[b]	2483.00±21.21[a]	6.05±0.21[a,b]	4.25±0.07[a]	234.00±5.66[a]
	250	442.00±1.41[b]	355.00±1.41[b]	298.50±0.71[b]	569.50±6.36[c]	2523.00±29.70[a]	5.60±0.00[b]	3.80±0.00[b]	188.50±0.71[b]
	1000	10232.00±284.26[c]	6150.50±58.69[c]	639.50±6.36[c]	414.50±4.95[d]	1002.00±14.14[b]	3.75±0.07[c]	3.00±0.00[c]	60.00±1.41[c]
7	0	143.00±1.41[a]	65.00±3.00[a]	362.50±2.12[a]	851.50±2.12[a]	2510.50±23.33	6.10±0.00	4.20±0.00	200.50±0.71
	50	87.50±4.95[b]	38.00±2.83[b]	251.50±10.61[b]	505.50±16.26[b]	2161.00±83.44	5.85±0.21	4.10±0.14	204.50±6.36
	250	81.00±7.07[b]	42.50±0.71[b]	250.00±11.31[b]	383.50±51.62[b]	2384.00±124.45	6.10±0.14	4.20±0.00	213.00±15.56
21	0	98.00±2.83[a]	43.50±2.12	245.50±2.12[a]	618.50±6.36[a]	2663.00±14.14	6.35±0.07	4.30±0.00	182.00±1.41[a]
	50	87.50±6.36[a,b]	36.50±2.12	209.00±2.83[b]	443.50±57.28[a,b]	2340.50±23.33	6.25±0.07	4.35±0.07	198.00±12.73[a,b]
	250	74.00±4.24[b]	43.00±4.24	224.50±0.71[c]	327.00±32.53[b]	2636.00±141.42	6.35±0.07	4.40±0.00	224.00±9.90[b]

Note: values are means ± SD for 5 rats. Values that do not share a label (a letter or blank) are significantly different ($p < 0.05$).

TABLE 5.2.—Selected alterations in serum biochemical parameters after 5-week treatment with AFB_1.

Dose (μg/kg)	AST (U/L)	ALT (U/L)	ALP (U/L)	CK (U/L)	Amylase (U/L)	Glucose (mg/dL)	Phosphorus (mg/dL)
0	123.00±1.41[a]	57.00±0.71[a]	212.50±7.78[a,b]	872.00±31.11[a]	2644.00±73.54[a]	164.00±2.83[a]	10.27±0.36[a]
5	111.50±7.78[a]	47.50±0.71[b]	200.00±8.49[b,c]	819.00±35.36[a]	2817.00±98.99[a,b]	202.50±7.78[b,c]	10.90±0.46[a]
10	119.50±4.95[a]	52.00±1.41[a,b]	195.00±8.49[b,c]	797.50±36.06[a]	2735.00±96.17[a,b]	180.50±6.36[a,b]	9.86±0.47[a]
25	106.50±4.95[a]	50.50±0.71[b]	178.00±5.66[c]	429.00±14.14[b]	2676.00±82.02[a]	212.50±7.78[c]	9.62±0.21[a]
75	248.00±8.49[b]	68.00±2.83[c]	234.50±9.19[a]	2556.50±101.12[c]	3150.50±142.13[b,c]	87.00±2.83[d]	20.28±0.93[b]

Note: values are means ± SD for 5 rats. Values that do not share a label (a letter or blank) are significantly different ($p < 0.05$).

TABLE 5.3.—Serum concentrations of AFB-Lys adduct after single-dose treatment with AFB_1.

Dose (μg/kg)	AFB-Lys (ng/mg albumin)						
	2 h	1-day	3-day	5-day	1-week	2-week	3-week
0	ND	ND	ND	ND	ND	ND	ND
50	1.68±0.22	0.66±0.05	0.58±0.12	0.28±0.02	0.16±0.01	0.02±0.00	ND
250	9.34±0.18	2.56±0.38	2.78±0.37	1.59±0.37	0.98±0.16	0.08±0.01	0.01±0.00
1000	24.89±3.17	19.97±1.67	NA	NA	NA	NA	NA

Note: ND, not detectable; NA, not available.

TABLE 5.4.—Serum concentrations of AFB-Lys adduct after repeated-dose treatment with AFB_1.

Dose (μg/kg)	AFB-Lys (ng/mg albumin)				
	1-week	2-week	3-week	4-week	5-week
0	ND	ND	ND	ND	ND
5	0.22±0.01	0.35±0.02	0.37±0.04	0.50±0.03	0.54±0.05
10	0.51±0.03	0.71±0.07	0.87±0.06	0.97±0.03	1.06±0.08
25	1.48±0.12	1.89±0.12	2.05±0.08	2.55±0.15	3.00±0.20
75	6.94±0.38	9.06±0.84	8.67±0.90	7.96±0.31	7.06±0.43

Note: ND, not detectable.

TABLE 5.5.—Liver GST-P$^+$ cells and foci induced by single-dose treatment with AFB_1.

GST-P$^+$	Dose (μg/kg)	Time (days)					
		1	3	5	7	14	21
Cells	50	-	-	2.47±3.50 (3/5)	10.51±5.17 (5/5)	6.62±9.36 (4/5)	5.98±0.98 (5/5)
	250	-	22.72±17.25 (5/5)	90.37±23.17 (5/5)	189.80±102.78 (5/5)	142.23±53.24 (5/5)	92.62±32.29 (5/5)
Foci	50	-	-	-	-	-	-
	250	-	-	-	4.22±4.10 (3/5)	6.18±6.01 (5/5)	5.62±6.43 (5/5)

Note: data are mean values from 5 liver sections, each from one individual rat (No./cm^2). -, not found. Data in brackets are (No. of animals affected/No. of animals examined).

TABLE 5.6.—Liver GST-P$^+$ cells and foci induced by repeated-dose treatment with AFB_1.

GST-P$^+$	Dose (μg/kg)	Time (week)				
		1	2	3	4	5
Cells	5	-	-	14.99±12.55 (3/5)	18.83±10.28 (2/5)	16.74±13.20 (2/5)
	10	-	7.66±0.99 (5/5)	12.79±11.11 (4/5)	37.88±17.78 (5/5)	61.35±51.88 (5/5)
	25	20.97± 7.46 (5/5)	19.42±5.98 (5/5)	30.02±14.59 (5/5)	56.60±16.28 (5/5)	95.35±34.91 (5/5)
	75	21.47±11.62 (5/5)	63.36±11.57 (5/5)	245.42±92.78 (5/5)	203.65±82.77 (5/5)	259.80±41.19 (5/5)
Foci	5	-	-	-	-	-
	10	-	-	-	-	1.49±2.57 (2/5)
	25	-	-	1.36±1.22 (3/5)	1.12±1.94 (2/5)	9.24±12.78 (3/5)
	75	-	-	3.01±2.87 (3/5)	39.09±4.67 (5/5)	75.99±23.58 (5/5)

Note: data are mean values from 5 liver sections, each from an individual rat (No./cm^2). -, not found. Data in brackets are (No. of animals affected/No. of animals examined).

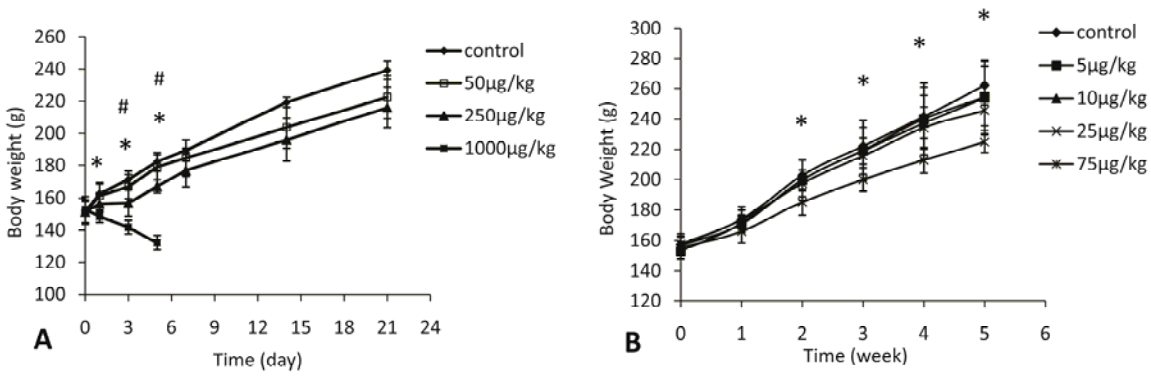

FIGURE 5.1.—Effects of AFB_1 on body weight gain after single-dose or repeated-dose treatment with AFB_1

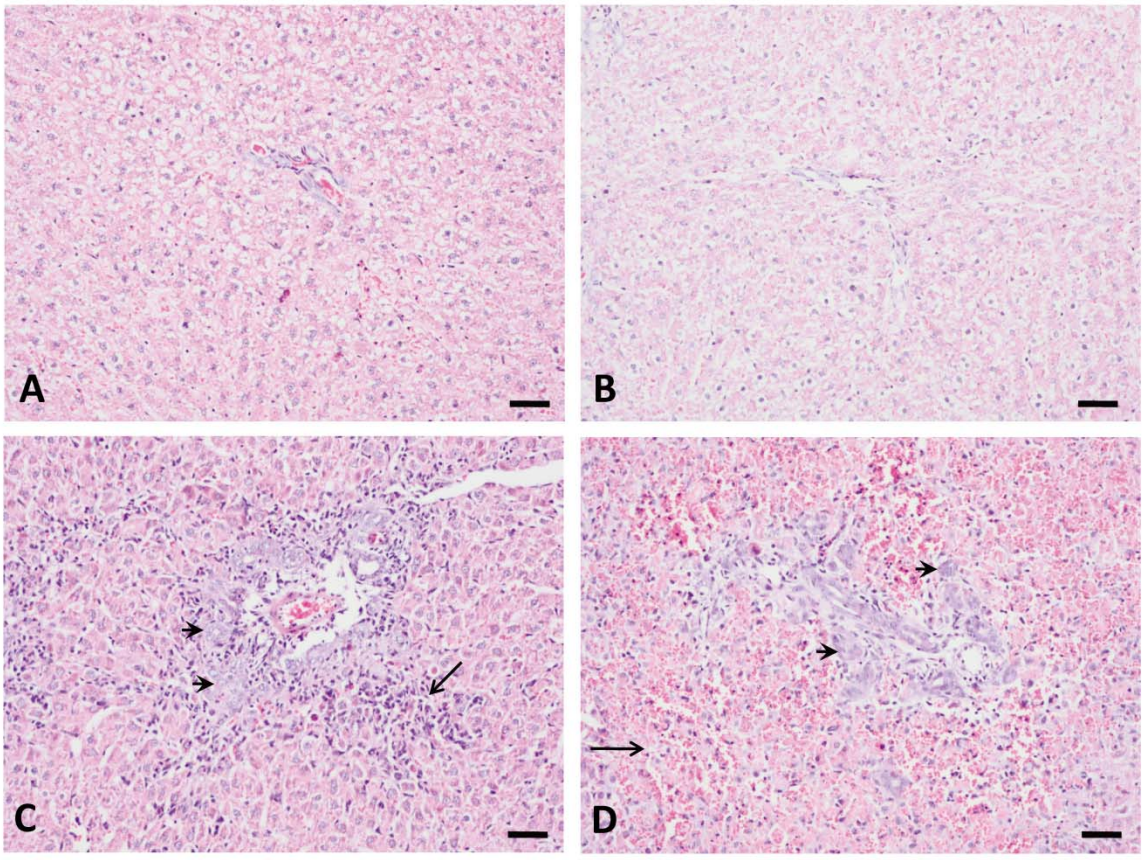

FIGURE 5.2.—Bile duct proliferation and periportal necrosis induced by AFB_1 at 3 days after single-dose treatment

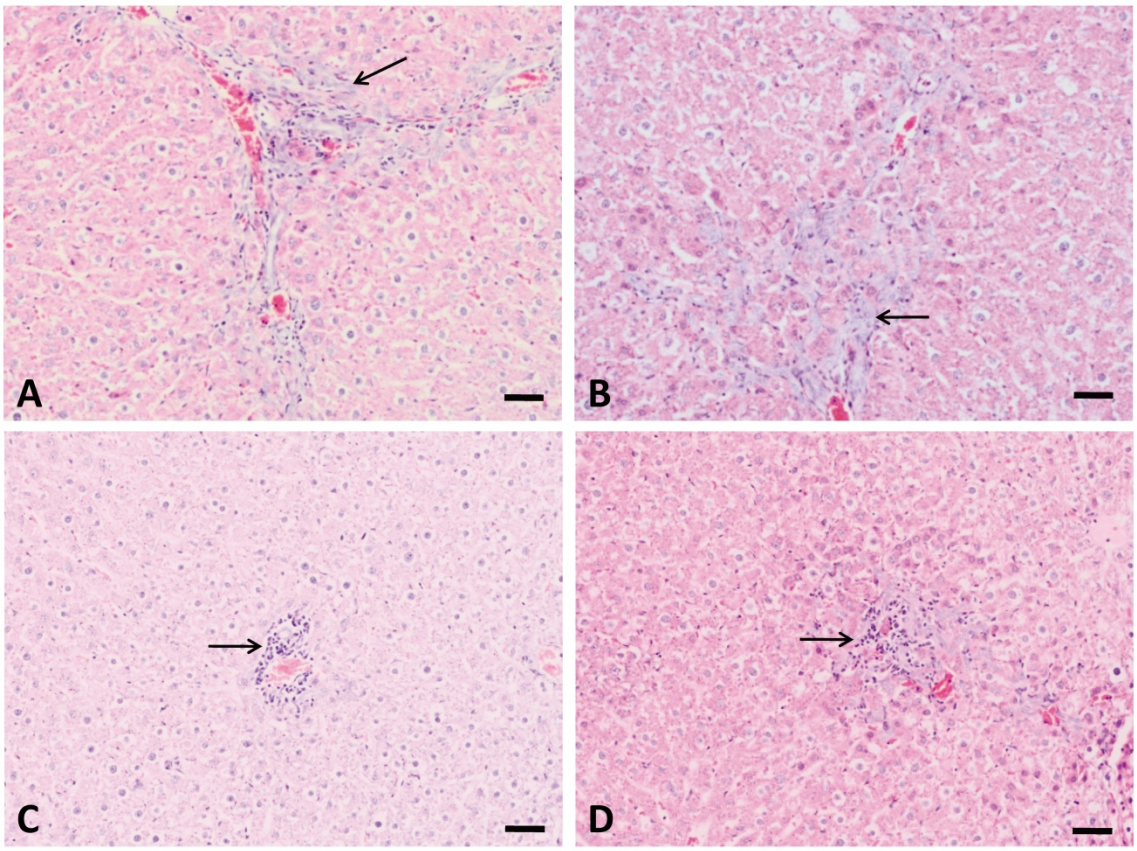

FIGURE 5.3.—Bile duct proliferation and periportl necrosis induced by 5-week treatment with AFB_1

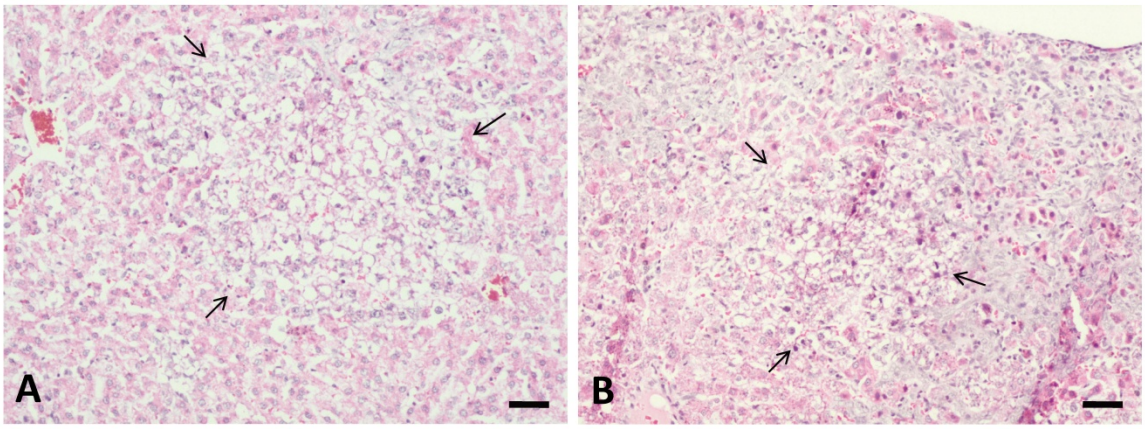

FIGURE 5.4.—AFB_1 induced proliferation foci in liver after repeated-dose treatment

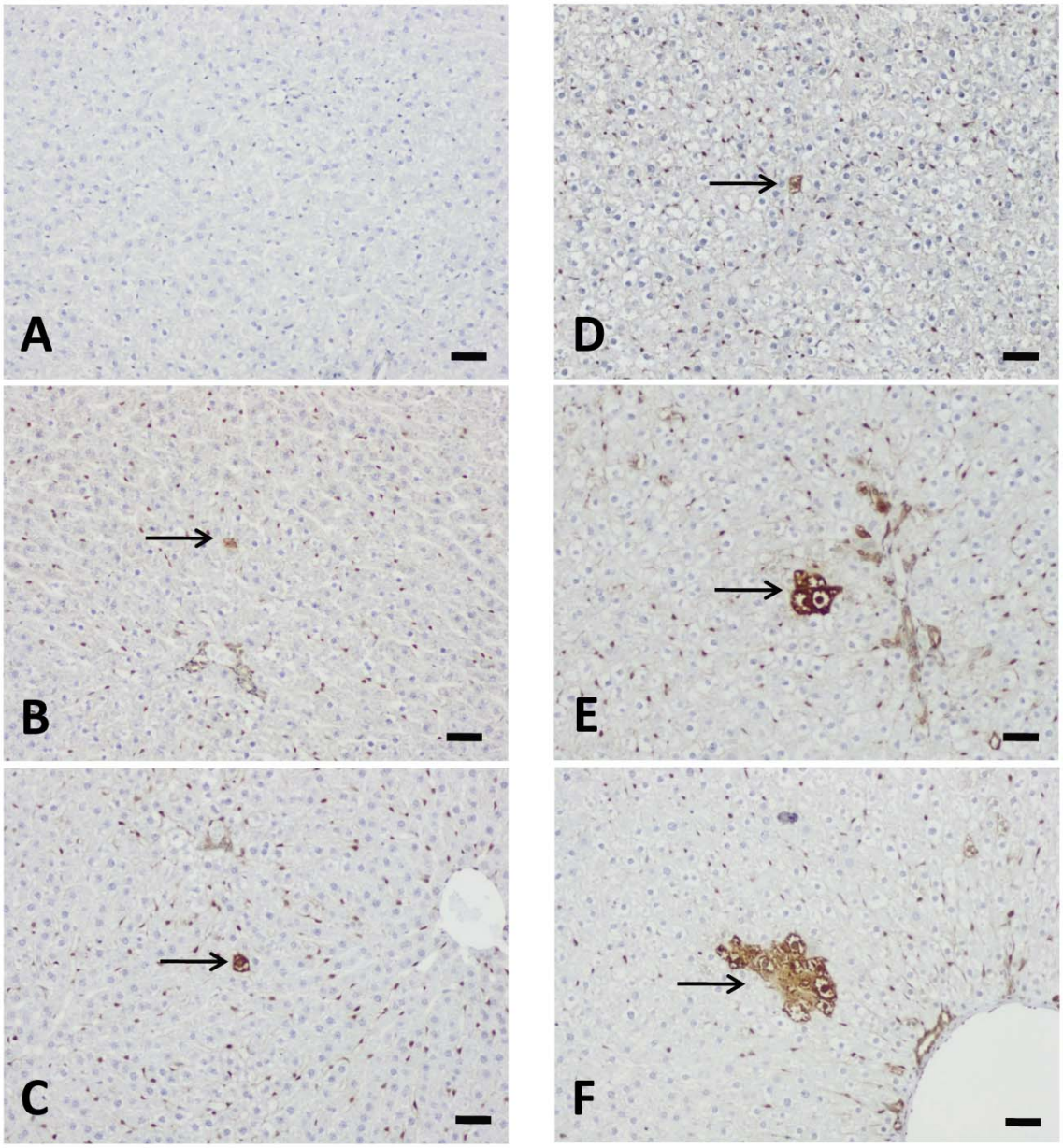

FIGURE 5.5.—AFB_1 induced liver GST-P^+ cells or foci formation after single-dose treatment with AFB_1

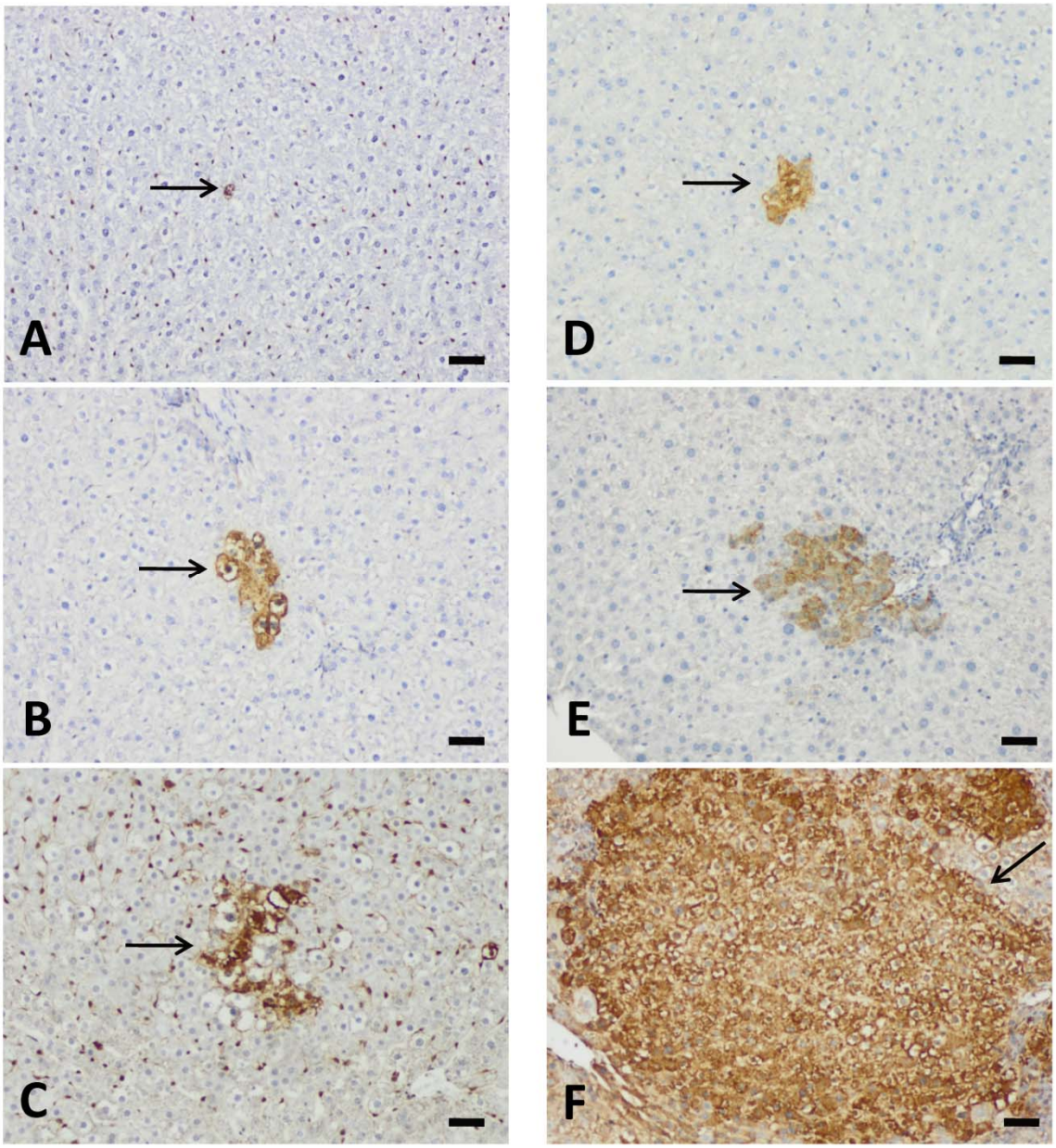

FIGURE 5.6.—AFB_1 induced liver GST-P^+ cells or foci formation after repeated-dose treatment with AFB_1

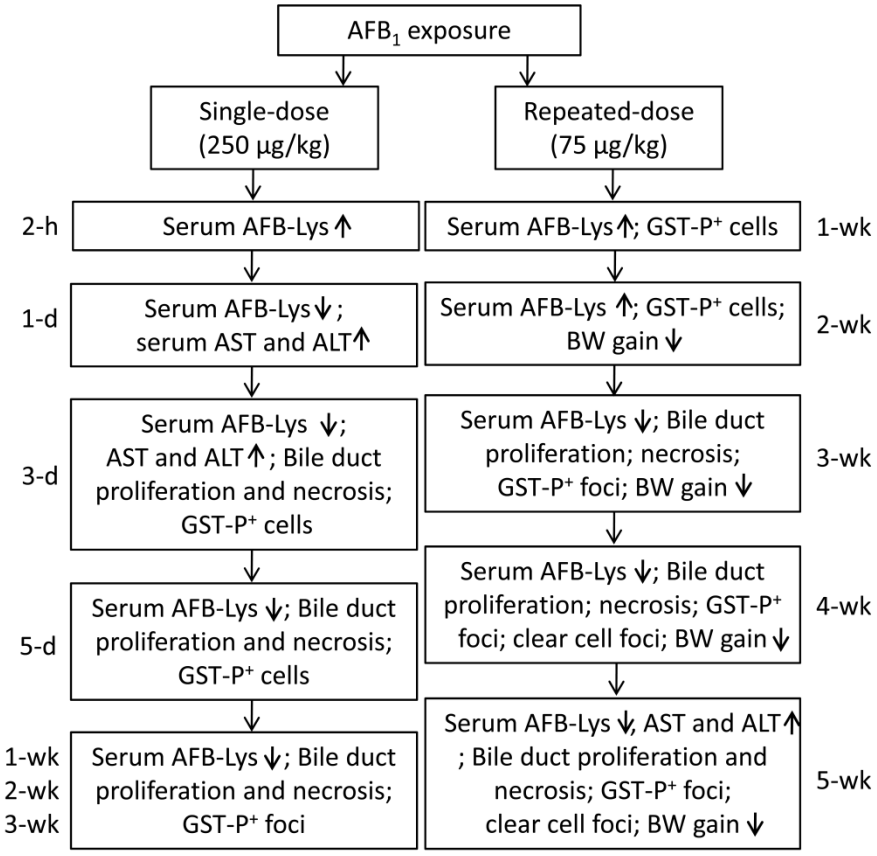

FIGURE 5.7.—Summary of the integrative toxicopathological findings in this study

Figure Legends

FIGURE. 5.1.—Effects of AFB_1 on body weight gain after single-dose or repeated-dose treatment with AFB_1. *, $p < 0.05$, the highest dose group vs control group; #, $p < 0.05$, the highest dose group vs 50 μg/kg group.

FIGURE 5.2.—Bile duct proliferation and periportal necrosis induced by AFB_1 at 3 days after single-dose treatment. No obvious histological changes occurred for control (A) and 50 μg/kg (B) groups. Dramatic bile duct proliferation (arrow head) and necrosis (arrow) were found at 250 μg/kg (C) and 1000 μg/kg (D) groups. Hemorrhagic necrosis occurred only at 1000 μg/kg (D, arrow). Bars represent 50 μm.

FIGURE 5.3.—Bile duct proliferation and periportl necrosis induced by 5-week treatment with AFB_1. Slight bile duct proliferation was found at 25 μg/kg group (A) and this change is more obvious at 75 μg/kg group (B). Periportal necrosis were also found at 10 (C) and 75 μg/kg (D) groups (arrow), necrosis at 25 μg/kg group was not shown here. Bars represent 50 μm.

FIGURE 5.4.—AFB_1 induced proliferation foci in liver after repeated-dose treatment. 75 μg/kg AFB_1 treatment induced formation of proliferation focus after 4-week (A) and 5-week (B) treatment (arrow). The altered foci were characteristic of clear cytoplasm and many cells have a centrally located nucleus (clear cell foci). Bars represent 50 μm.

FIGURE 5.5.—AFB_1 induced liver GST-P^+ cells or foci formation after single-dose treatment with AFB_1. GSP-P^+ hepatocytes were not present at 3-day (A) but 5-day (B) and 1-week (C) after single-dose treatment with 50 μg/kg AFB_1 (arrow). 250 μg/kg AFB_1

treatment induced GST-P^+ cells at 3-day (D), 5-day (E) and GST-P^+ foci at 1-week (F) after single-dose treatment (arrow). Bars represent 50 μm.

FIGURE 5.6.—AFB_1 induced liver GST-P^+ cells or foci formation after repeated-dose treatment with AFB_1. Three-week treatment induced liver GST-P^+ cells at 10 μg/kg group (A) and GST-P^+ foci at 25 (B) and 75 μg/kg (C) groups (arrow). Five-week treatment induced GST-P^+ foci at 10 (D), 25 (E), and 75 μg/kg (F) groups. Note the relative area of GST-P^+ foci. Bars represent 50 μm.

FIGURE 5.7.—Summary of the integrative toxicopathological findings in this study.

CHAPTER 6

AFLATOXIN B_1 MODULATES THE EXPRESSION OF PHENOTYPIC MARKERS AND CYTOKINES BY SPLENIC LYMPHOCYTES OF MALE F344 RATS [1]

[1] Qian, G. Wang, F. Tang, L. Massey, M.E. Mitchell, N.J. Su, J. Williams, J.H. Phillips, T.D. and Wang, J.-S. To be submitted to *Journal of Applied Toxicology*.

Abstract

Aflatoxin B_1 (AFB_1) is immunotoxic to animals and a suspicious immunosuppressant in humans. In this study we investigatedthe effects of AFB_1onsplenic lymphocyte phenotypes and the inflammatory cytokines expression in male F344 rats. In addition, serum AFB_1-lysine adducts level and liver and spleen histology were examined to correlate with possible immune toxicities. Exposure of animals to AFB_1 (5-75 μg/kg body weight) for 1-week showed dose-dependently decreased percentages of splenic $CD8^+$ T cells and $CD3^-CD8a^+$ NK cells. A general inhibition of the expressions of IL-4 and IFN-γ by $CD4^+$ T cells, IL-4 and IFN-γ by $CD8a^+$ cells, and TNF-α expression by NK cells was also found; however, no concurrent histological changes in liver or spleen tissues were present, suggesting acute immunosuppression without overt toxicity. Five-week exposure with AFB_1 significantly increased the percentages of $CD3^+$ and $CD8^+$ T cells, especially at low doses (≤ 25 μg/kg). AFB_1 treatment significantly decreased the anti-inflammatory cytokine IL-4 expression by $CD4^+$ T cells and significant increased the pro-inflammatory cytokine IFN-γ expression by $CD4^+$ T cells and TNF-α expression by NK cells. These results indicated that repeated AFB_1 exposure promotes inflammatory responses by regulating cytokines expression. Consistent with this finding, liver periportal necrosis and bile duct proliferation as well as splenic lymphocyte apoptosis were found, especially at the high dose (75 μg/kg). Our data provide novel insights into the mechanisms by which AFB_1 exposure differentially modulates the cell-mediated immune responses and suggest the involvement of inflammatory response upon repeated exposure.

Keywords: aflatoxin B_1; spleen; lymphocyte; cytokine; immunotoxicity.

Introduction

Aflatoxins (AFs) are produced predominately by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. AFs are frequent contaminants of food staples, such as maize and groundnuts, and AFs exposure is a significant public health issue in Southeast Asia and Sub-Saharan Africa [1, 2]. Aflatoxin B_1 (AFB_1) is the most potent aflatoxin that is activated in the liver to its reactive metabolite, aflatoxin B_1-8,9-epoxide. This metabolite can rapidly bind with biological macromolecules such as DNA and protein to exhibit adverse effects in animals and humans [3]. The immune toxic effects of AFB_1 have gained great attention due to chemical-viral interactions in the developing world where such co-exposure is common [4].

Mycotoxins, especially $AFB_1$and trichothecenes, have been shown to affect the immune system in domestic and laboratory animals [5]. A considerable amount of evidence from animal studies has demonstrated that AFB_1 possesses the immunosuppressive functions [5, 6]. Cell-mediated immunity is a particular target affected by AFB_1 exposure. AFB_1 decreases splenic helper T cells and antibody response to sheep red blood cells (SRBC) in C57BL/6 mice [7], and inhibits production of IL-2, IL-3, and IFN-γ by splenic macrophages [8]. Murine macrophages functions in phagocytosis and production of nitric oxide and TNF-α are inhibited by AFB_1 both *in vivo* and *in vitro* [9, 10]. Dietary exposure of rats to AFB_1 (0.01-1.6 ppm) produces inhibitory effects on splenic T and B lymphocytes as well as IL-1, IL-2, and IL-6 production by splenocytes [11]. The mechanisms behind the impaired cell-immunity have been proposed to involve the inhibition synthesis at the gene or protein level [8, 12] and the installment of the cell cycle at G_2/M [13].

The conclusion on AFB_1 induced inhibitory effects on cytokines in particular, however, deserves careful examination because of the inconsistent findings. For example, AFB_1 exposure has shown stimulating effects on the expression of pro-inflammatory cytokines such as TNF-α, IFN-γ, and IL-6 in pigs [14] while the inhibition of pro-inflammatory (IL-1β, TNF-α) and stimulating of anti-inflammatory (IL-10) cytokine mRNA expression by AFB_1 exposure have also been reported in pigs [15]. Even in a single feeding assay, AFB_1 containing diet has shown differential effects based on treatment duration and doses [11]. The model (spleen vs. white blood cells, e.g.) selection and differences in exposure duration or dose might play a role in the final outcomes.

Currently available animal data are mainly focused on the overall cytokine production from a cell mixture, the relevance to the cell-specific function is not known, due to the fact that these cytokines can be produced by various cells [16, 17]. There exists a lack of data on how AFB_1 affects cell-specific cytokine expression in animal studies. Whether AFB_1-induced histological changes in liver and spleen are related to modulatory effects on splenic lymphocyte subsets and cell-specific cytokines are largely unknown. Aims of this study are to demonstrate the modulatory effects of AFB_1 on splenic lymphocyte phenotypic markers and cell-specific cytokines, and their relevance to liver and spleen histological changes in F344 rats. This study also holds the potential to provide evidence for immunosuppressive effects for future human epidemiological studies.

Materials and Methods

Materials

Aflatoxin B_1 and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Inc (St. Louis, MO). Antibodies of CD28 and CD3 and fluorescence conjugated antibodies including CD3 phycoerythrin (PE) clone G4.18, CD4 allophycocyanin (APC) clone OX-35, CD8 fluorescein isothiocyanate (FITC) clone OX-8, CD8a peridinin chlorophyll protein complex (PERCP) clone OX-8, CD45RA phycoerythrincyanin 5 (PE-Cy5) clone OX-33, IL-4 PE clone OX-81 and INF-γ FITC clone DB-1 were purchased from BD Biosciences (San Jose, CA). Fix/perm and Perm/Wash Buffer were also purchased from BD Biosciences (San Jose, CA). TNF-α FITC clone TN3-19 antibody was purchased from Abcam (Cambridge, MA). Phorbol 12-myristate 13-acetate (PMA) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO). RPMI-1640 medium were purchased from ATCC (Manassas, VA). All other solvents were of the highest grade available.

Animals

Male F344 rats (5 weeks old, 120-130g) were purchased from Harlan Laboratory (Indianapolis, IN). Animals were individually housed under a 12h/12h light/dark cycle with temperature of 22 ± 2°C. Purified AIH 76A diet and tap water were provided *ad libitum*. Animals were acclimated for 1-week before treatment. Animal care and use and detailed animal protocol were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia.

Experimental Design

Male F344 rats were randomly divided into four groups: control, 5, 25, and 75 μg AFB_1/kg body weight (BW). Rats (5 per treatment group)were orally gavaged with AFB_1 in DMSO (50 μL/100 g BW) for duration of 1-or 5-week. Animals were sacrificed at 24 h following the last dose. Upon euthanasia, spleens were aseptically removed for preparing single cell suspension, which was used for cell surface marker and intracellular cytokine staining. The parameters tested in this study were shown in Table 6.1. The selection of these markers was based on previous studies [18, 19].

Serum Aflatoxin B_1-Lysine Adduct (AFB-Lys) Measurement

To monitor the AFB_1 exposure, the levels of serum AFB-Lys were measured from animals at each time point. The method was adopted as previously reported [20]. Briefly, 150 μL serum was digested with pronase (pronase:total protein, 1:4, w:w) for 3 h at 37°C. The digest was further purified by a solid phase cartridge, evaporated, reconstituted, and injected into an Agilent 1200 HPLC system for quantification by fluorescence signal. Excitation and emission wavelengths were 405 and 470 nm. The limit of detection is 0.4 pg/mg albumin. Serum AFB-Lys adduct level was adjusted by serum albumin content.

Single Splenic Cell Suspension Preparation

The procedure for preparing single splenic cell suspension was modified as previously reported [21]. Approximately one half of the spleen tissues taken at necropsy were minced in 1 mL cold complete RPMI-1640 medium (RPMI-1640 containing 2 mM L-glutamine (ATCC), supplied with 10% FBS and 100 IU/mL Penicillin and 100 IU/mL streptomycin), then gently mashed through a 70 nylon mesh cell strainer and collected into 5 mL cold complete RPMI-1640 medium. The collected cells were pelleted by

centrifuge at 400g for 5 min at 4°C and supernatants were discarded. Then red blood cells were removed by incubating with lysing buffer for 5 min at room temperature (RT). The remaining cells were washed once and collected into 3 mL complete RPMI-1640 medium. Cell count was done in a hemocytometer and was adjusted to 2×10^7/mL by volume. Cell viability was determined by Trypan Blue staining, which showed more than 90% viable cells.

Splenic Lymphocytes Phenotypic Markers Staining

Four splenic lymphocyte surface markers tested were CD3, CD4, CD8 and CD45RA. An aliquot of 1.0×10^6 splenic cells were first blocked in 100μL block buffer (CD32, 1:100) for 10 min at RT. Then cells were pelleted after centrifuging and supernatants were discarded. These cells were incubated with staining buffer (PBS including 10%FBS and 0.1%NaN_3) containing appropriately diluted fluorescence conjugated antibodies (CD3 PE: 1:200; CD4 APC: 1:100; CD8 FITC: 1:100; CD45RA PE-CY5: 1:200) for 30 min at 4°C in dark, 100 μL per tube. Single fluorescence antibody control was included. After incubation, the cell mixtures were washed once with staining buffer and then fixed in 250 μL cold 2% formaldehyde for 12 h. The fixed cell mixtures were pelleted and resuspended in 500 μL staining buffer in plastic tubes before flow cytometric analysis within 1 day.

Stimulation of Cytokine Expression

Duplicates of 1.0×10^6 cells for each sample were transferred to a 96-well flat bottom plate pre-coated with anti-CD3 antibody (1:100). The cells were pelleted by centrifuging at 250g for 4 min at 4°C and supernatants were discarded. 100 μL of stimulating buffer (complete RPMI-1640 medium supplied with anti-CD28 antibody

(1:100), brefeldin A (1:100), PMA (50 ng/mL), and ionomycin (500 ng/mL)) was added to stimulate cytokines production for 6 h in a humidified incubator (37°C, 5% CO_2).

Intracellular Cytokine Staining

To determine cell-specific intracellular cytokines expression, both surface markers and intracellular cytokines were stained. The combination of cell surface markers and cytokine markers were (1) CD4 APC + CD8a PERCP + IL-4 PE + INF-γ FITC, and (2) CD3 PE + CD8a PERCP + TNF-α FITC. The method was modified from a previous protocol [22]. Briefly, cells in each well were washed twice with 200 μL cold PBS after stimulation, then were resuspended in 100 μL staining buffer containing either CD4 APC (1:100) + CD8a PERCP (1:100) antibodies or PE CD3 (1:200) + CD8a PERCP (1:100) antibodies and incubated for 30 min at 4°C in dark. Cells were pelleted by centrifuging at 250g for 4 min at 4°C, washed once with 250 μL staining buffer. Next, 100 μL Fix/Perm buffer were added into each well, which was mixed quickly and thoroughly and incubated for 30 min at 4°C in dark. The cells were washed twice with 250 μL 1×Perm/Wash buffer (500g, 4°C, 5 min). Then intracellular cytokines staining was followed. Conjugated anti-cytokines were prepared in 1×Perm/Wash buffer as follows: IL-4 PE: 1:100 and IFN-γ FITC: 1:5; TNF-α FITC: 1:100. Cells were incubated in a mixture of IL-4 PE and IFN-γ FITC or TNF-α FITC for 30 min at 4°C in dark. After intracellular staining, cells were washed twice with 1×Perm/Wash buffer (500g 4°C, 5 min), followed by fixation with 200 μL cold 2% formaldehyde. Lastly, cells were washed once with staining buffer and resuspended in 500 μL staining buffer in plastic tubes before flow cytometric analysis within 1 day.

Flow Cytometric Analysis of Splenic Lymphocyte Phenotypes and Intracellular Cytokine Expressions

A total of 20,000 cells for either surface markers or different combinations of cytokine markers were counted on a HyperCyAn flow cytometer (Beckman Coulter Inc., Fullerton, CA) for analysis of splenic lymphocyte phenotype or cytokine expression and data were analyzed by FlowJo software (Tree Star Inc., Ashland, OR). Selective histograms and dot-plot images were shown in Figure 6.1.

H&E Staining of Liver Tissues

Dissected rat liver tissues were fixed in 4% neutral formalin and then embedded in paraffin. Tissue sections (5 μm) were cut and routinely processed for H.E. staining and examined under light microscopy. Photographs were taken on an Olympus XC30 microscope with an Olympus UC30 digital camera (Olympus America Inc., Center Valley, PA) linked to a Cellsense image analysis system (Celsense Inc., Pittsburgh, PA). The apoptotic cells in splenic tissue sections were defined by the presence of heterochromatic cell fragments or apoptotic bodies, as previously described [23].

Statistical Analysis

Serum levels of AFB-Lys adduct, the percentages of surface marker and cytokine expression were expressed as mean ± standard deviation (SD). Comparisons of these parameters between treated groups and the control group were conducted using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons. A *p*-value of less than 0.05 was considered statistically significant.

Results

Serum Levels of AFB-Lys Adducts

As shown in Table 6.2, serum levels of AFB-Lys adduct in animals were measured to reflect the internal doses. Dose-dependent increases of serum AFB-Lys adduct levels were found after 1- or 5-week treatment. The adduct levels increased by 134.95% and 98.41% after 5-week treatment for the 5 and 25 μg/kg groups, respectively. However, only slight change in the adduct level was found for the 75 μg/kg group compared to that after 1-week treatment (7.32 vs. 7.18 ng/mg albumin). The typical chromatograms were shown in Figure 6.2.

Effects of 1-week Exposure of AFB_1 on the Expression of Splenic Lymphocytes Phenotypic Markers and Intracellular Cytokines

The effects of AFB_1 on the percentage of splenic lymphocyte subsets after 1-week treatment are shown in Figure 6.3. Dose-dependent decreases in the percentages of cytotoxic T cells ($CD8^+$) and $CD3^-CD8a^+$ NK cells were found after 1-week treatment, and statistical significance was found for the 25 and 75 μg/kg groups as compared to the control ($p < 0.05$). No statistically significant effects on T lymphocytes ($CD3^+$), helper T lymphocytes ($CD4^+$), or B lymphocytes ($CD45RA^+$) were found after 1-week treatment (data not shown).

AFB_1 treatment produced a dose-related and significant reduction of IL-4 expression by $CD4^+$ T cells at all dose levels after *ex vivo* stimulation ($p < 0.05$, Figure 6.4A). Dose-dependent decrease of IFN-γ expression by $CD4^+$ T cells was also found with significant difference only at 75 μg/kg group ($p < 0.05$, Figure 6.4B). A significant decrease of IL-4 expression by $CD8a^+$ cells occurred at 25 and 75 μg/kg groups ($p <$

0.05), with 39% and 58% decrease, respectively (Figure 6.4C). Decreased IFN-γ expressions by $CD8a^+$ cells also appeared at 25 and 75 μg/kg groups ($p < 0.05$, Figure 6.4D). The effects of AFB_1 on IL-4 and IFN-γ expression by $CD8a^+$ cells also exhibited a dose-related change. Dose-dependent decrease in the expression of TNF-α by $CD3^-$ $CD8a^+$ NK cells was found with the statistically significant difference observed only at 75 μg/kg group ($p < 0.05$, Figure 6.4E).

Effects of 5-Week exposure of AFB_1 on the Expression of Splenic Lymphocytes Phenotypic Markers and Intracellular Cytokines

The effects of AFB_1 treatment on splenic lymphocyte phenotypes after 5-week treatment are shown in Figure 6.5. Significant increases in the percentages of $CD3^+$ and $CD8^+$ T lymphocytes were observed at 5 and 25μg/kg groups ($p < 0.05$) but not at 75 μg/kg group ($p > 0.05$). Other splenic lymphocyte subsets including $CD4^+$ T cells, B cells and NK cells were not significantly affected after 5-week treatment with AFB_1 (data not shown).

Effects of AFB_1 on cytokine expression are shown in Figure 6.5. Differential effects on IL-4 and IFN-γ expressions by $CD4^+$ T cells were found. Significant decreases in percentages of IL-4 expressing $CD4^+$ T cells were found at all dose levels ($p < 0.05$, Figure 6.6A) while a significant increase in IFN-γ expressing $CD4^+$ T cells (37.8%) occurred only at 25 μg/kg group ($p < 0.05$, Figure 6.6B). A significant decrease of IL-4 expressing $CD8a^+$ cells was also found at 25 μg/kg group ($p < 0.05$, Figure 6.6C). Although the inhibition at 75 μg/kg group was not statistically significant, the fluorescence intensity of IL-4 in $CD8a^+$ cells was significantly decreased by 58% ($p <$

0.05, data not shown). In addition, there was also a significant elevation of TNF-α expression by $CD3^{-}CD8a^{+}$ NK cells (85.9%) at 75 μg/kg group ($p < 0.05$, Figure 6.6D).

The pattern changes of splenic lymphocyte phenotypes and cell-specific cytokine expressions after 1-week and 5-week AFB_1 exposure are summarized in Table 6.3. IL-4 expression by $CD4^{+}$ T cells is consistently decreased following both 1-week and 5-week exposure; IFN-γ expression by $CD4^{+}$ T cells and TNF-α expression by NK cells experienced a temporal inversion from being decreased to elevation.

Effects of AFB_1 on Liver and Spleen Histology

One-week treatment with AFB_1 did not cause apparent histological changes at any dose group (data not shown). However, repeated treatment for 5-week induced dose-related periportal necrosis in the liver (Figure 6.7). In addition, bile duct proliferation was apparent at 25 and 75 μg/kg groups, with much larger area occupied by proliferated bile duct at 75 μg/kg group (Figures 6.7E and F); however, no bile duct proliferation was induced at 5 μg/kg group (Figure 6.7B).

No apparent histological changes of the splenic microstructures were found after 1-week or 5-week treatment with AFB_1 at all dose levels (Figure 6.8) except that treatment with 75 μg/kg AFB_1 caused an increased apoptosis of lymphocytes after 5-week (Figure 6.8D).

Discussion

Results of this study demonstrated that short-term exposure (1-week) to AFB_1could dose-dependently decrease the percentages of splenic $CD8^{+}$ lymphocytes and NK cells and the production of cytokines by $CD4^{+}$, $CD8^{+}$ lymphocytes and NK cells; prolonged exposure (5-week) to low levels of AFB_1 (≤ 25μg/kg) had stimulatory effects

on splenic lymphocytes but not at the high dose (75 μg/kg); prolonged 5-week exposure also decreased IL-4 expression while increasing IFN-γ and TNF-α expression. Splenic B lymphocytes were not significantly affected throughout the experimental period at all dose levels. Data from this study provide evidence of the modulatory effects of AFB_1 on splenic lymphocyte phenotypes and cell-specific cytokine expression. To our knowledge, this is the first animal study to investigate the effects of AFB_1 on lymphocyte specific functions in cytokine production. Results of this study demonstrate that both splenic lymphocyte phenotypes and cell specific cytokine production can be modulated by AFB_1, and prolonged exposure may play a role in chronic inflammation through regulating cytokine expression.

Doses selected in this study were comparable to human exposure which were approximately equivalent to 0.175, 0.875, and 2.625 ppm, respectively, in contaminated food. The doses herein are relevant to human exposure especially for populations in developing countries where food contamination is a common problem. For instance, the individual daily exposures can be expected to range from 1 to more than 50 mg AFB_1 in Southern China and Kenya [24, 25].

Serum AFB-Lys adduct is an established biomarker for AFB_1 exposure [26], and it was adopted to monitor the animal dosimetry for exposure in this study. Serum AFB-Lys adduct levels exhibited different patterns: at doses ≤ 25μg/kg, it increased by about 2 fold after 5-week treatment compared to that after 1-week treatment; nevertheless, there was no obvious change for the 75 μg/kg group. This observation may indicate a plateau reached for this biomarker or a damaged metabolic balance for liver enzyme functions in study animals. The periportal necrosis and bile duct proliferation found in liver at this

dose group supports that the liver injury influenced AFB_1 metabolic activation and adduct formation.

$CD4^+$ and $CD8^+$ T lymphocytes are important effector cells for cell-mediated immune responses [27]. NK cells serve as a defensive barrier to kill pathogens and foreign cells and participate in the innate and adaptive immune mechanisms [28]. $CD8^+$ T cells from spleen of normal rats almost exclusively express the alpha/beta isoform while NK cells carry only CD8 alpha chains (alpha/alpha) [18, 29]. Therefore, splenic $CD8a^+$cells could be a mixture of both $CD8^+$T cells and $CD3^-CD8a^+$NK cells, with the majority of which being $CD8^+$ cell. Due to the limited commercial availability of conjugated antibodies for rat, the $CD8a^+$ cells were chosen for assessing cytokine expression in this study. These lymphocytes secrete several different cytokines such as IL-4, INF-γ and TNF-α, which are important coordinators of immune and inflammatory responses [30]. In this study, we found that acute exposure to AFB_1 for 1-week decreased the percentages of $CD8^+$ lymphocytes and NK cells. The impaired cell-mediated immunity was similarly observed in other species such as mice [31] and chickens [32]. In agreement with this, B lymphocytes ($CD45RA^+$) were not significantly affected by AFB_1 after 1-week. Although the percentage of $CD4^+$ T lymphocytes was not reduced, the expression of IL-4 and IFN-γ by $CD4^+$ T lymphocytes was significantly decreased at the high dose (75 μg/kg). On the other hand, both the percentage of $CD8^+$ T lymphocytes and the intracellular expression of IL-4 and IFN-γ were significantly decreased after 1-week treatment, indicating that $CD8^+$ T lymphocytes might be more susceptible to AFB_1 exposure. TNF-α expression by NK cells was also found to be inhibited after 1-week exposure with AFB_1. Apparently these immune effects were not relevant to overt toxicity in liver or spleen tissues as

evidenced by histological results. Instead, the inhibitory effects could result from inhibited synthesis at either gene or protein level or restricted cell cycle at G_2/M , which has been reported by other studies [8, 12, 13].

Dose-dependent effects on splenic lymphocyte phenotypes were found after 5-week prolonged exposure, e.g., lower doses of AFB_1 (5 and 25 μg/kg) increased percentages of $CD3^+$ and $CD8^+$ T lymphocytes, while the highest dose (75 μg/kg) had no apparent effect. This finding was similar to deoxynivalenol (DON) induced immune modulations that low dose DON exposure upregulates cytokines and inflammatory genes with concurrent immune stimulation whereas high dose enhances leukocyte apoptosis with concurrent immune suppression[33]. Earlier studies have also demonstrated that repeated treatment with AFB_1 for 4-week could enhance protein and DNA synthesis in splenic lymphocytes at low dose (30 μg/kg body weight), but not at high doses (145 μg/kg body) [12], which is in agreement with our findings.

Contrary to the overall inhibition of cytokine production after 1-week exposure to AFB_1, significant inhibition of IL-4 expression by $CD4^+$ and $CD8^+$ cells and stimulation of IFN-γ expression by $CD4^+$ cells and TNF-α expression by NK cells were found after 5-week exposure to AFB_1. Stimulation of INF-γ production by $CD4^+$cells could be a direct response or a feedback caused by inhibition of IL-4 given the interaction between IL-4 and INF-γ [16]. The stimulation of TNF-α production by NK cells might take part in chronic inflammation, which is consistent with the elevated IL-6 production as reported previously [11] since TNF-α can stimulate splenic production of IL-6 in rat [34]. Given the anti-inflammatory effect of IL-4 and pro-inflammatory effects of INF-γ and TNF-α [35], the inhibition of IL-4 and stimulation of INF-γ and TNF-α might lead to a net result of

inflammatory responses. Data from in vitro studies also support this finding. Murine macrophages exposed to AFB_1 showed decreased secretion of anti-inflammatory IL-10 and increased pro-inflammatory IL-6 [36] and similar effects were found in fumonisinB_1 treated pig peripheral blood mononuclear cells which demonstrated an inhibitory expression of IL-4 and a stimulation of INF-γ at both protein and mRNA levels [37]. Considering the rather short turnover time of these cytokines [38], it seems unlikely that the observed pattern on cytokines are due to this natural turnover. However, further research is warranted to clarify the potential mechanisms involved and the complications associated with cytokine interactions, such as direct versus indirect effects.

In this study the dose-related induction of liver periportal necrosis and bile duct proliferation was indicative of inflammatory responses in liver and in agreement with the altered profiles of cytokines after 5-week exposure to AFB_1.Prolonged exposure also induced slightly increased apoptosis of splenic T lymphocytes at the high dose (75 μg/kg), and this alteration was consistent with the profile changes of cytokines, such as elevated TNF-α expression and INF-γ expression. Synergistic action of TNF-α and INF-γ in promoting apoptosis of pancreatic beta cells has been reported [39], and mode of action might be through activating JNK/SAPK and p53 pathway with the involvement of reactive oxygen species (ROS) production [40].

Findings in this study indicated that the functions of $CD4^+$ and $CD8^+$ cells were impaired by AFB_1, which supports the finding in humans that $CD8^+$ lymphocyte function was negatively correlated with AFB_1 exposure levels [4]; however, different effects were found in regard to expression of IFN-γ and IL-4 by $CD4^+$ cells. The variation might be due to different experimental design and exposure period as well as different comparison

groups (non-treated rats versus widespread low exposed humans). An i*n vitro* study has shown that IL-4 acts synergistically with IL-5 to increase IgA production by LPS activated murine B cells, while IFN-γ inhibits IL-5 mediated up-regulation of IgA secretion [41]. Therefore, the observed inhibition of IL-4 and stimulation of IFN-γ in this study supports the finding of decreased saliva IgA in Gambian children with high AFB_1 exposure [42].

To sum up findings in the current study, a flow chart was constructed as shown in Figure 6.9. Based on previous knowledge and our data, short-term exposure to AFB_1 may elicit immunosuppressive effects through inhibiting effects on gene or protein synthesis while prolonged exposure may upregulate cytokines and proinflammatory genes to enhance inflammation and apoptosis. The data provides novel insight into AFB_1 exposure induced alterations of cell-mediated immune responses. However, exact mechanisms for the complex regulation of cytokine production, interaction between liver and spleen changes, as well as for the transition from short-term to prolonged effects need further studies to clarify.

Conflict of interest statement

The authors declare no conflict of interest in this work.

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References

1. Kensler TW, Roebuck BD, Wogan GN, Groopman JD. Aflatoxin: A 50-year odyssey of mechanistic and translational toxicology. *Toxicol Sci*. 2011;120 Suppl 1:S28-48
2. Wogan GN, Kensler TW, Groopman JD. Present and future directions of translational research on aflatoxin and hepatocellular carcinoma. A review. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2012;29:249-257
3. IARC. Aflatoxins. *IARC Monogr Eval Carcinog Risks Hum*. 1993;56:245-395
4. Jiang Y, Jolly PE, Preko P, Wang JS, Ellis WO, Phillips TD, Williams JH. Aflatoxin-related immune dysfunction in health and in human immunodeficiency virus disease. *Clin Dev Immunol*. 2008;2008:790309
5. Bondy GS, Pestka JJ. Immunomodulation by fungal toxins. *J Toxicol Environ Health B Crit Rev*. 2000;3:109-143
6. Sharma RP. Immunotoxicity of mycotoxins. *J Dairy Sci*. 1993;76:892-897
7. Hatori Y, Sharma RP, Warren RP. Resistance of c57bl/6 mice to immunosuppressive effects of aflatoxin b1 and relationship with neuroendocrine mechanisms. *Immunopharmacology*. 1991;22:127-136
8. Dugyala RR, Sharma RP. The effect of aflatoxin b1 on cytokine mrna and corresponding protein levels in peritoneal macrophages and splenic lymphocytes. *Int J Immunopharmacol*. 1996;18:599-608
9. Moon EY, Rhee DK, Pyo S. Inhibition of various functions in murine peritoneal macrophages by aflatoxin b1 exposure in vivo. *Int J Immunopharmacol*. 1999;21:47-58

10. Moon EY, Rhee DK, Pyo S. In vitro suppressive effect of aflatoxin b1 on murine peritoneal macrophage functions. *Toxicology*. 1999;133:171-179

11. Hinton DM, Myers MJ, Raybourne RA, Francke-Carroll S, Sotomayor RE, Shaddock J, Warbritton A, Chou MW. Immunotoxicity of aflatoxin b1 in rats: Effects on lymphocytes and the inflammatory response in a chronic intermittent dosing study. *Toxicol Sci*. 2003;73:362-377

12. Reddy RV, Sharma RP. Effects of aflatoxin b1 on murine lymphocytic functions. *Toxicology*. 1989;54:31-44

13. Scott TR, Rowland SM, Rodgers RS, Bodine AB. Genetic selection for aflatoxin b1 resistance influences chicken t-cell and thymocyte proliferation. *Dev Comp Immunol*. 1991;15:383-391

14. Meissonnier GM, Pinton P, Laffitte J, Cossalter AM, Gong YY, Wild CP, Bertin G, Galtier P, Oswald IP. Immunotoxicity of aflatoxin b1: Impairment of the cell-mediated response to vaccine antigen and modulation of cytokine expression. *Toxicol Appl Pharmacol*. 2008;231:142-149

15. Marin DE, Taranu I, Bunaciu RP, Pascale F, Tudor DS, Avram N, Sarca M, Cureu I, Criste RD, Suta V, Oswald IP. Changes in performance, blood parameters, humoral and cellular immune responses in weanling piglets exposed to low doses of aflatoxin. *J Anim Sci*. 2002;80:1250-1257

16. Elser B, Lohoff M, Kock S, Giaisi M, Kirchhoff S, Krammer PH, Li-Weber M. Ifn-gamma represses il-4 expression via irf-1 and irf-2. *Immunity*. 2002;17:703-712

17. Kurokouchi K, Kambe F, Yasukawa K, Izumi R, Ishiguro N, Iwata H, Seo H. Tnf-alpha increases expression of il-6 and icam-1 genes through activation of nf-kappab in osteoblast-like ros17/2.8 cells. *J Bone Miner Res*. 1998;13:1290-1299

18. Liu X, Ryland L, Yang J, Liao A, Aliaga C, Watts R, Tan SF, Kaiser J, Shanmugavelandy SS, Rogers A, Loughran K, Petersen B, Yuen J, Meng F, Baab KT, Jarbadan NR, Broeg K, Zhang R, Liao J, Sayers TJ, Kester M, Loughran TP, Jr. Targeting of survivin by nanoliposomal ceramide induces complete remission in a rat model of nk-lgl leukemia. *Blood*. 2010;116:4192-4201

19. Morris DL, Komocsar WJ. Immunophenotyping analysis of peripheral blood, splenic, and thymic lymphocytes in male and female rats. *J Pharmacol Toxicol Methods*. 1997;37:37-46

20. Qian G, Tang L, Liu W, Wang JS. Development of a non-antibody method for rapid detection of serum aflatoxin b1-lysine adduct. *Toxicologist*. 2010;114:248.

21. Kizaki T, Kobayashi S, Ogasawara K, Day NK, Good RA, Onoe K. Immune suppression induced by protoscoleces of echinococcus multilocularis in mice. Evidence for the presence of cd8dull suppressor cells in spleens of mice intraperitoneally infected with e. Multilocularis. *J Immunol*. 1991;147:1659-1666

22. Yui MA. Introcellular cytokine detection. in: Diamond, R.A. DeMaggio, S. (Eds.), *In Living Color: Protocols in Flow Cytometry and Cell Sorting,* First ed. Springer, New York, pp. 552-559. 2000

23. Elmore SA. Enhanced histopathology of the spleen. *Toxicol Pathol*. 2006;34:648-655

24. Groopman JD, Zhu JQ, Donahue PR, Pikul A, Zhang LS, Chen JS, Wogan GN. Molecular dosimetry of urinary aflatoxin-DNA adducts in people living in guangxi autonomous region, people's republic of china. *Cancer Res*. 1992;52:45-52

25. Daniel JH, Lewis LW, Redwood YA, Kieszak S, Breiman RF, Flanders WD, Bell C, Mwihia J, Ogana G, Likimani S, Straetemans M, McGeehin MA. Comprehensive assessment of maize aflatoxin levels in eastern kenya, 2005-2007. *Environ Health Perspect*. 2011;119:1794-1799

26. Sabbioni G, Skipper PL, Buchi G, Tannenbaum SR. Isolation and characterization of the major serum albumin adduct formed by aflatoxin b1 in vivo in rats. *Carcinogenesis*. 1987;8:819-824

27. Jiang H, Chess L. The specific regulation of immune responses by cd8+ t cells restricted by the mhc class ib molecule, qa-1. *Annu Rev Immunol*. 2000;18:185-216

28. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, Yokoyama WM, Ugolini S. Innate or adaptive immunity? The example of natural killer cells. *Science*. 2011;331:44-49

29. Torres-Nagel N, Kraus E, Brown MH, Tiefenthaler G, Mitnacht R, Williams AF, Hunig T. Differential thymus dependence of rat cd8 isoform expression. *Eur J Immunol*. 1992;22:2841-2848

30. Arai KI, Lee F, Miyajima A, Miyatake S, Arai N, Yokota T. Cytokines: Coordinators of immune and inflammatory responses. *Annu Rev Biochem*. 1990;59:783-836

31. Reddy RV, Taylor MJ, Sharma RP. Studies of immune function of cd-1 mice exposed to aflatoxin b1. *Toxicology*. 1987;43:123-132

32. Thaxton JP, Tung HT, Hamilton PB. Immunosuppression in chickens by aflatoxin. *Poult Sci*. 1974;53:721-725

33. Pestka JJ. Mechanisms of deoxynivalenol-induced gene expression and apoptosis. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2008;25:1128-1140

34. Teti G, Mancuso G, Tomasello F. Cytokine appearance and effects of anti-tumor necrosis factor alpha antibodies in a neonatal rat model of group b streptococcal infection. *Infect Immun*. 1993;61:227-235

35. Niiro H, Otsuka T, Izuhara K, Yamaoka K, Ohshima K, Tanabe T, Hara S, Nemoto Y, Tanaka Y, Nakashima H, Niho Y. Regulation by interleukin-10 and interleukin-4 of cyclooxygenase-2 expression in human neutrophils. *Blood*. 1997;89:1621-1628

36. Bruneau JC, Stack E, O'Kennedy R, Loscher CE. Aflatoxins b(1), b(2) and g(1) modulate cytokine secretion and cell surface marker expression in j774a.1 murine macrophages. *Toxicol In Vitro*. 2012;26:686-693

37. Taranu I, Marin DE, Bouhet S, Pascale F, Bailly JD, Miller JD, Pinton P, Oswald IP. Mycotoxin fumonisin b1 alters the cytokine profile and decreases the vaccinal antibody titer in pigs. *Toxicol Sci*. 2005;84:301-307

38. Sander B, Hoiden I, Andersson U, Moller E, Abrams JS. Similar frequencies and kinetics of cytokine producing cells in murine peripheral blood and spleen.

Cytokine detection by immunoassay and intracellular immunostaining. *J Immunol Methods*. 1993;166:201-214

39. Suk K, Kim S, Kim YH, Kim KA, Chang I, Yagita H, Shong M, Lee MS. Ifn-gamma/tnf-alpha synergism as the final effector in autoimmune diabetes: A key role for stat1/ifn regulatory factor-1 pathway in pancreatic beta cell death. *J Immunol*. 2001;166:4481-4489

40. Kim WH, Lee JW, Gao B, Jung MH. Synergistic activation of jnk/sapk induced by tnf-alpha and ifn-gamma: Apoptosis of pancreatic beta-cells via the p53 and ros pathway. *Cell Signal*. 2005;17:1516-1532

41. Eckmann L, Morzycka-Wroblewska E, Smith JR, Kagnoff MF. Cytokine-induced differentiation of iga b cells: Studies using an iga expressing b-cell lymphoma. *Immunology*. 1992;76:235-241

42. Turner PC, Moore SE, Hall AJ, Prentice AM, Wild CP. Modification of immune function through exposure to dietary aflatoxin in gambian children. *Environ Health Perspect*. 2003;111:217-220

Table 6.1. Immune parameters examined.

Groups (cellular expression)	Immune parameters
T-lymphocytes	$CD3^+$
T cell subsets	$CD4^+$, $CD8^+$
B cells	$CD45RA^+$
NK cells	$CD3^-CD8a^+$
Cytokine expression	(IL-4-) expression by $CD4^+$ or $CD8a^+$ T cells, (IFN-γ-) expression by $CD4^+$ or $CD8a^+$ T cells, (TNF-α-) expression by $CD3^-CD8a^+$ NK cells

Table 6.2. Serum levels of AFB-Lys adduct.

	Serum AFB-Lys levels (ng/mg albumin)	
Dose (μg/kg)	1-week	5-week
0	ND	ND
5	0.22±0.01	$0.52±0.02^{c}$
25	$1.54±0.09^{a}$	$3.06±0.15^{a,c}$
75	$7.18±0.28^{a,b}$	$7.32±0.28^{a,b}$

Note. Values are mean ± SD for 5 rats. ND, not detectable. $^{a}p < 0.001$, compared to 5μg/kg in the same column. $^{b}p < 0.001$, compared to 25μg/kg in the same column. $^{c}p < 0.01$, compared to 1-week in the same row.

Table 6.3. Summary of the immune parameter changes.

	1-week (μg/kg)			5-week (μg/kg)		
Immune parameters (%)	5	25	75	5	25	75
$CD3^+$ T cells	-	-	-	↑	↑	-
$CD8^+$ T cells	-	↓	↓	↑	↑	-
$CD3^-D8a^+$ NK cells	-	↓	↓	-	-	-
$CD4^+IL\text{-}4^+$ T cells	↓	↓	↓	↓	↓	↓
$CD4^+IFN\text{-}\gamma^+$ T cells	-	-	↓	-	↑	-
$CD8a^+IL\text{-}4^+$ T cells	-	↓	↓	-	↓	-
$CD8a^+$ IFN-γ^+ T cells	-	↓	↓	-	-	-
$CD3^-CD8a^+TNF\text{-}\alpha^+$ NK cells	-	-	↓	-	-	↑

Note. ↑: $P < 0.05$, significant increase compared to control; ↓: $P < 0.05$, significant decrease compared to control; -: $P > 0.05$, no significant difference compared to control.

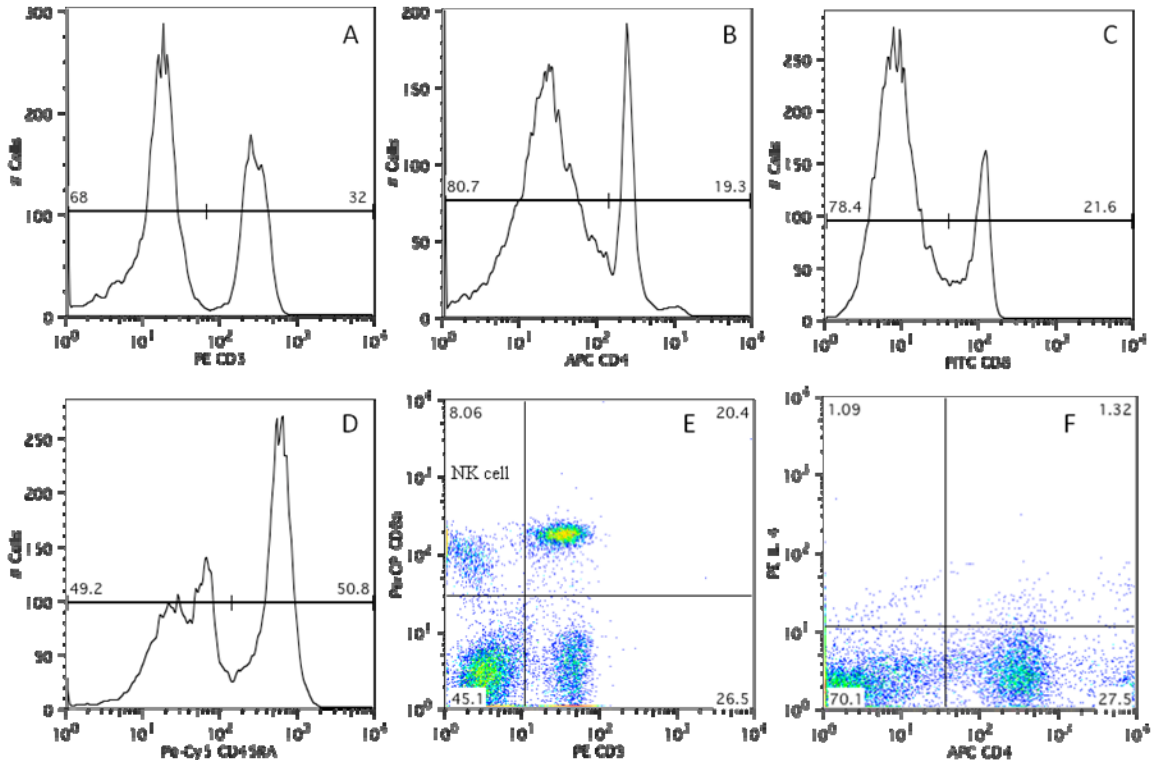

Figure 6.1. Selective typical histograms and two-parameter dot-plots.

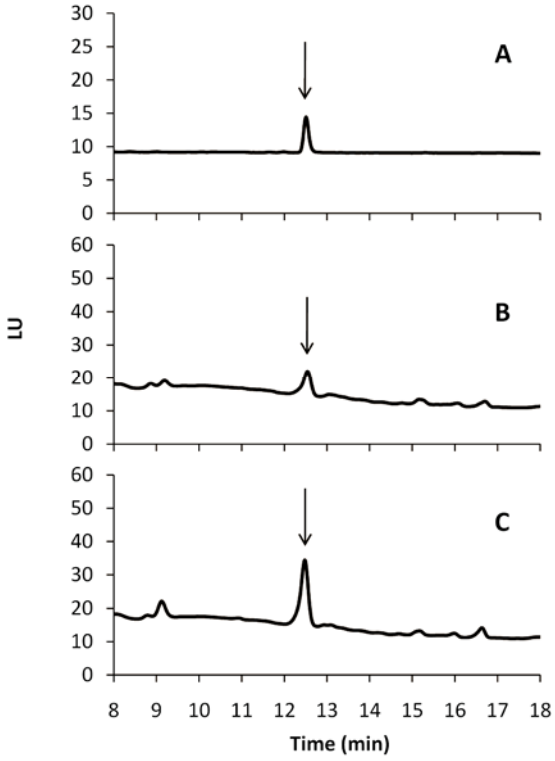

Figure 6.2. Typical chromatograms of AFB-Lys adduct.

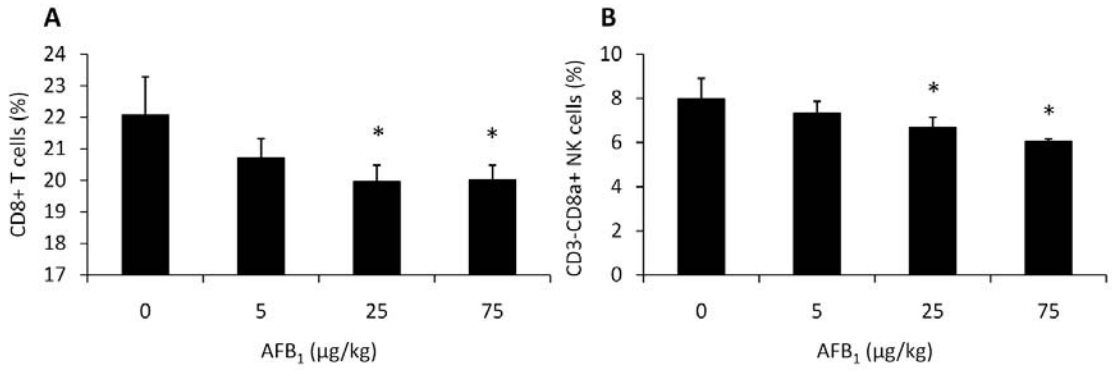

Figure 6.3. Effects of AFB_1 on splenic lymphocyte phenotypes after 1-week exposure.

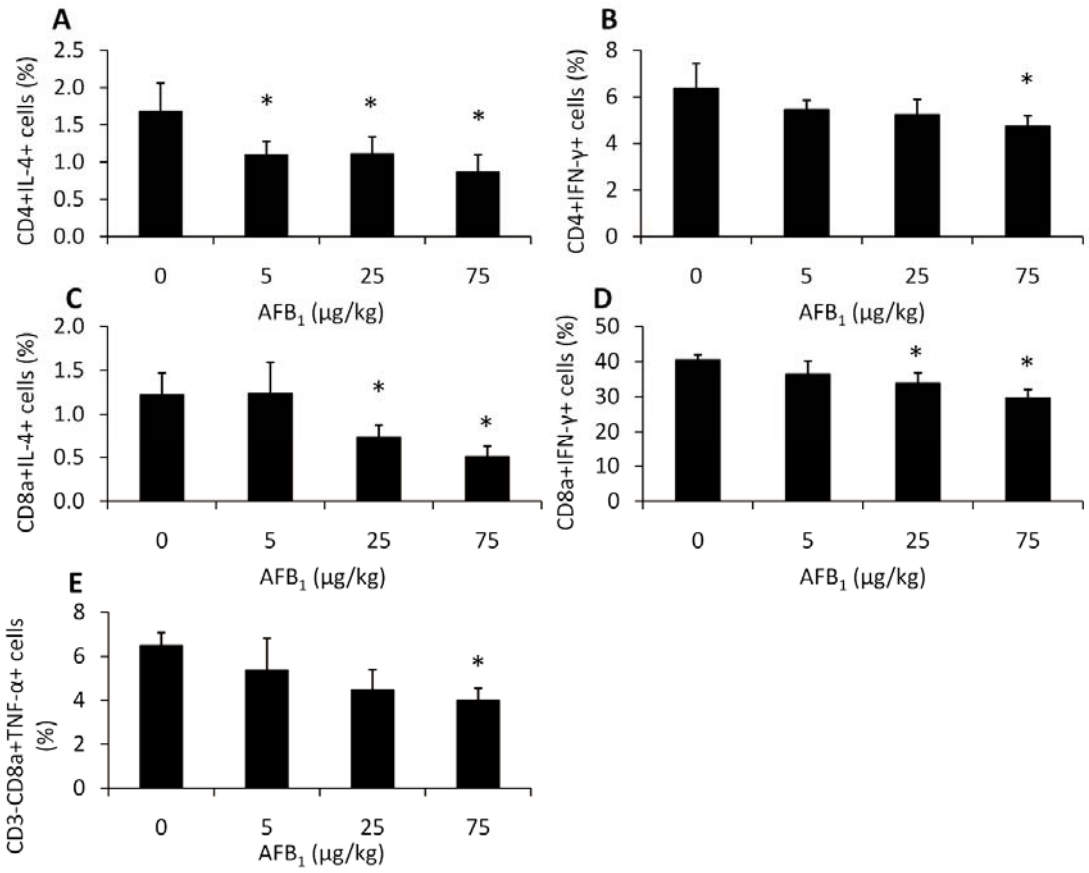

Figure 6.4. Effects of AFB_1 on cytokine expression in splenic lymphocytes after 1-week exposure.

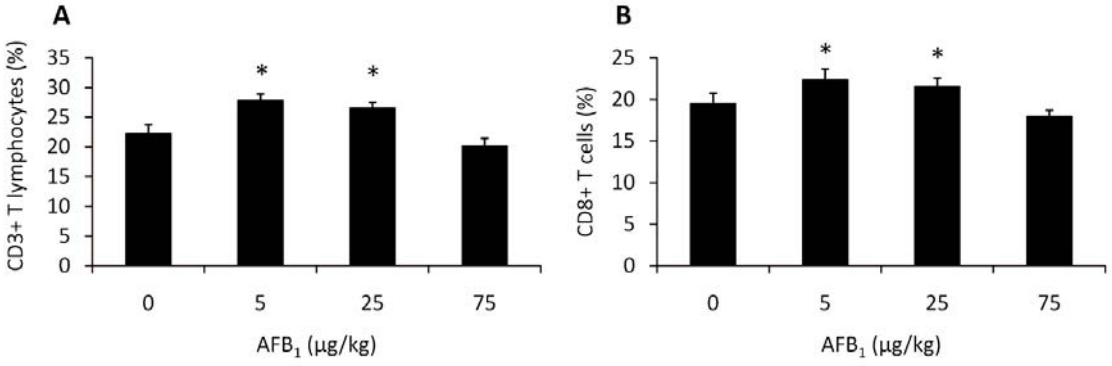

Figure 6.5. Effects of AFB_1 on splenic lymphocyte phenotypes after 5-week exposure.

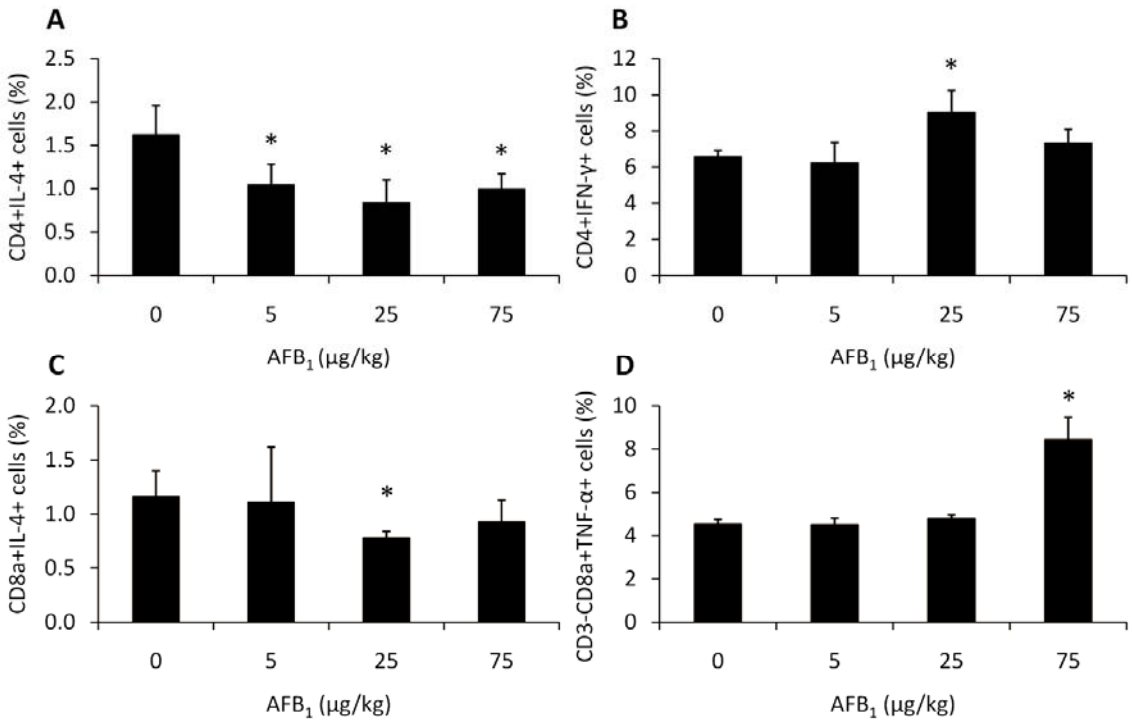

Figure 6.6. Effects of AFB_1 on cytokine expression in splenic lymphocytes after 5-week exposure.

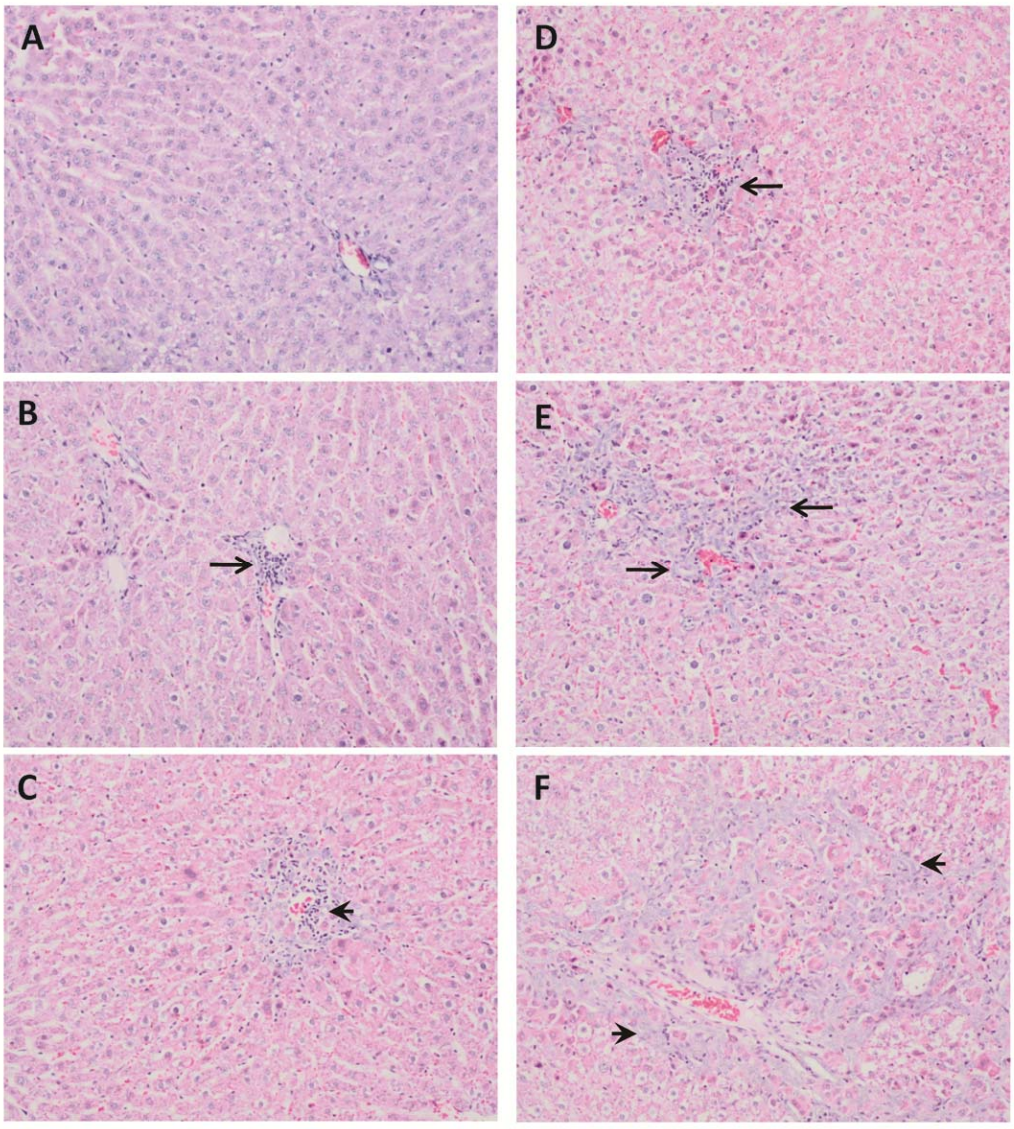

Figure 6.7. Histological effects of AFB_1 on liver after 5-week exposure.

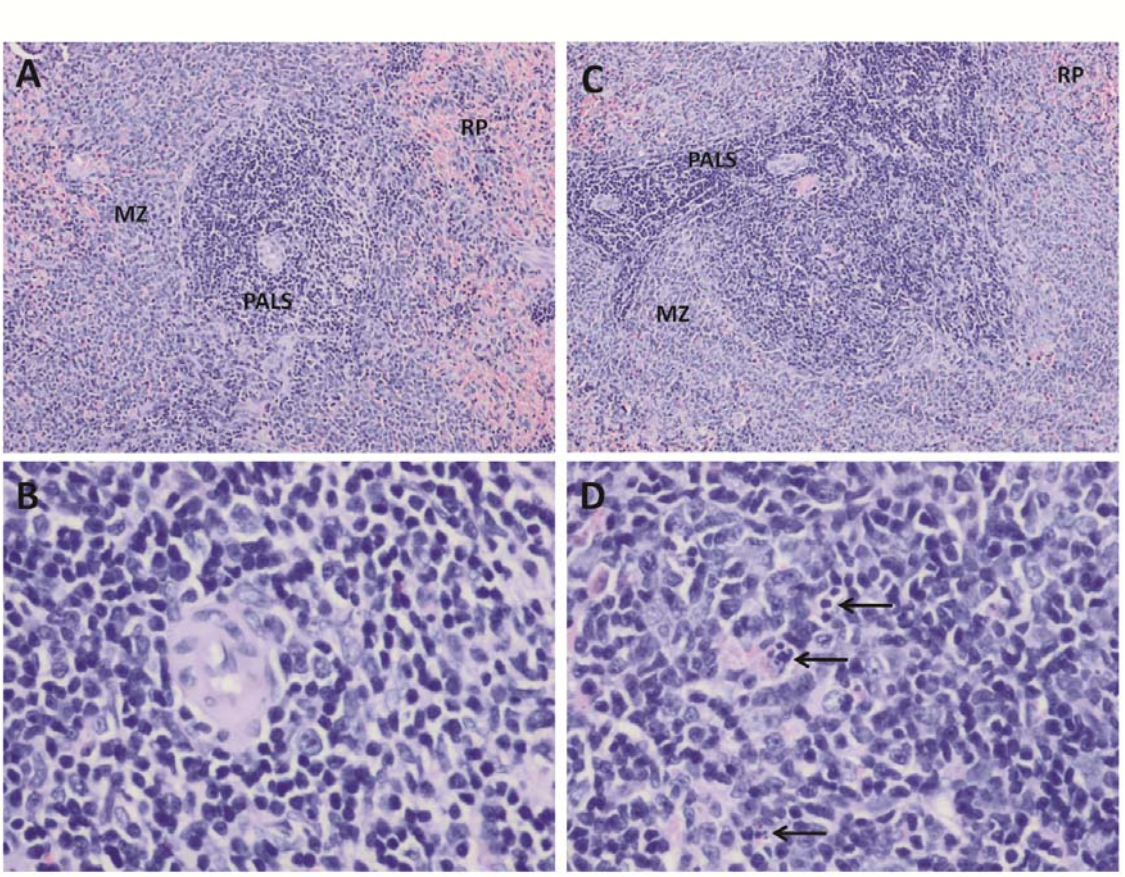

Figure 6.8. Histological effects of AFB_1 on spleen after 5-week exposure.

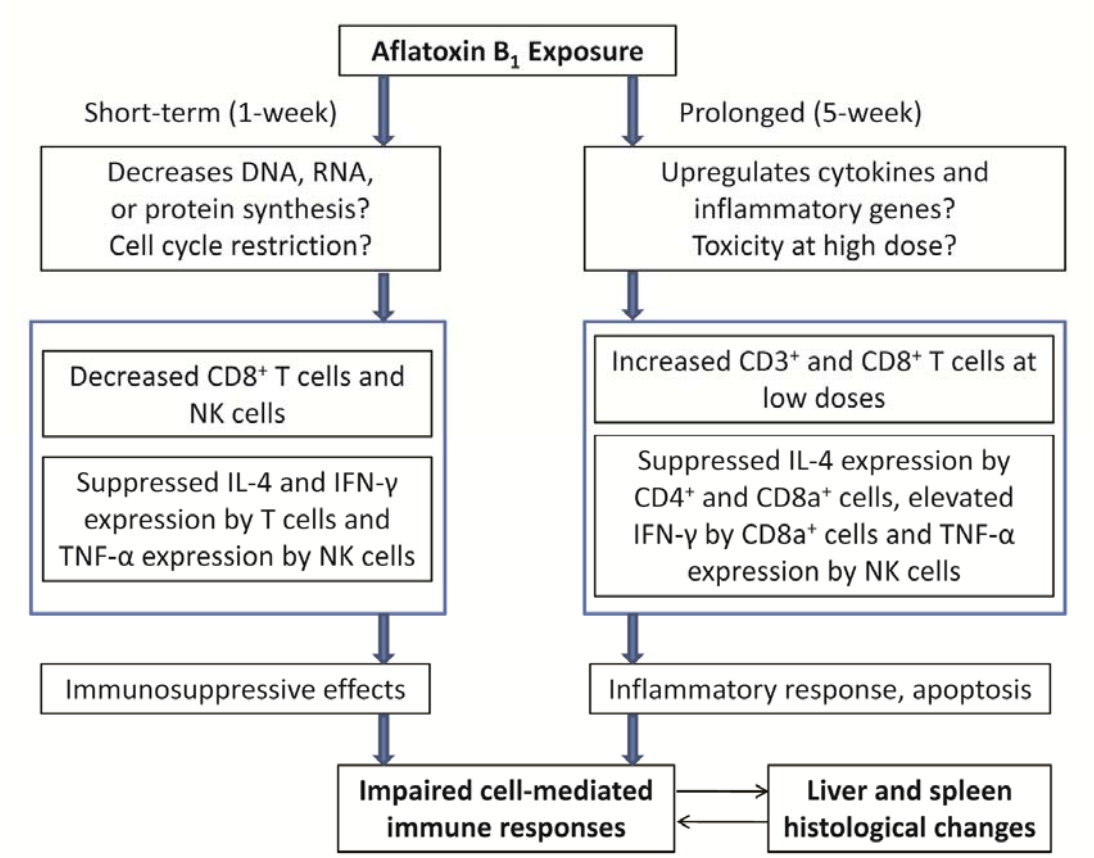

Figure 6.9. Possible mechanisms of AFB_1 immunotoxic effects in F344 rats.

Figure Legends

Figure 6.1. Selective typical histograms and two-parameter dot-plots. Histograms are PE-CD3 (A), APC-CD4 (B), FITC-CD8 (C), and Pe-Cy5-CD45RA (D). Two-parameter dot-plots show NK cells ($CD3^-CD8a^+$, E) and IL-4^+CD4^+ cells (F).

Figure 6.2. Typical chromatograms of AFB-Lys adduct. (A) An authentic standard, 1.0 ng/mL. (B) A diluted rat serum sample (5 fold) from 5 μg/kg AFB_1 exposure. (C) A diluted rat serum sample (10 fold) from 25 μg/kg AFB_1 exposure. Arrows indicate the peak for AFB-Lys adduct.

Figure 6.3. Effects of AFB_1 on splenic lymphocyte phenotypes after 1-week exposure. Dose-dependent decreases of the percentages of $CD8^+$ T cells (A) and $CD3^-CD8a^+$ NK cells (B) were found. *Indicates $p < 0.05$ compared with control group.

Figure 6.4. Effects of AFB_1 on cytokine expression in splenic lymphocytes after 1-week exposure. Short-term exposure significantly decreases the expressions of IL-4 (A) and IFN-γ (B) by $CD4^+$ T cells, IL-4 (C) and IFN-γ (D) by $CD8a^+$ T cells, and the TNF-α by $CD3^-CD8a^+$ NK cells (E). *Indicates $p < 0.05$ compared with control group.

Figure 6.5. Effects of AFB_1 on splenic lymphocyte phenotypes after 5-week exposure. Long-term exposure increased the percentages of $CD3^+$ T cells (A) and $CD8^+$ T cells (B), especially at low dose levels. *Indicates $p < 0.05$ compared with control group.

Figure 6.6. Effects of AFB_1 on cytokine expression in splenic lymphocytes after 5-week exposure. The expression of IL-4 by $CD4^+$ T cells was inhibited at all doses (A) while IFN-γ expression was elevated at 25 μg/kg group (B).The IL-4 expression by $CD8a^+$ T cells was inhibited at 25 μg/kg group (C) and TNF-α expression by$CD3^-CD8a^+$ NK cells was increased at 75 μg/kg group (D). *Indicates $p < 0.05$ compared with control group.

Figure 6.7. Histological effects of AFB_1 on liver after 5-week exposure. H&E-stained liver section showed normal microstructure in control group (A). Progressed periportal necrosis was found at 5 (B), 25 (C) and 75 (D) μg/kg groups (arrow). Bile duct proliferation occurred at 25 (E) and 75 (F) μg/kg groups (arrowhead). Note the larger area with bile duct proliferation at 75 μg/kg group (F) compared to 25 μg/kg group (E). Magnification, ×100.

Figure 6.8. Histological effects of AFB_1 on spleen after 5-week exposure. (A) and (C) are spleen tissues from control group at low (×100) and high (×400) magnifications. Normal spleen tissue is composed of two major parts: white pulp and red pulp (RP). The white pulp has three characteristic components: periarteriolar lymphoid sheath (PALS), follicle and marginal zone (MZ). (B) and (D) are spleen tissues from 75 μg/kg group at low (×100) and high (×400) magnifications. Increased apoptosis was found in the PALS region at 75 μg/kg AFB_1 treated spleen tissue, the arrows indicate small heterochromatic cell fragments, indicative of apoptotic bodies.

Figure 6.9. Possible mechanisms of AFB_1 immunotoxic effects in F344 rats.

CHAPTER 7

GLOBAL EXPOSURE OF AFLATOXIN B_1 IN DIFFERENT HUMAN POPULATIONS [1]

[1] Qian, G. Tang, L. Jauline, P.E. Nikiema, P.A. Rosma, A. Nkurunziza, P.M. Muwanika, R. Brown, D. Williams, J.H. Phillips, T.D. and Wang, J.-S. To be submitted to *Environmental Health Perspectives*.

Abstract

Aflatoxin exposure is a significant health issue worldwide. Assessing the current exposure status in different human populations is beneficial for effective chemoprevention strategies. OBJECTIVES: This study aims to reveal the current human exposure status to aflatoxins using the molecular biomarker, serum aflatoxin B_1-lysine (AFB-Lys) adducts. In this study we assessed the global human exposure to aflatoxins using a reliable exposure biomarker, AFB-Lys adduct in serum. We recruited serum samples from populations in West African countries (Ghana and Burkina Faso), an East African country (Uganda), Southeast Asian countries (China and Malaysia), a Caribbean country (Haiti) and the United States. The biomarker levels were determined by a non-antibody based HPLC method with fluorescence detection. Varied levels of AFB-Lys were found globally and the highest exposure was found in Burkina Faso (29.89 ± 74.16 pg/mg albumin) and the lowest in the United States (0.89 ± 2.08 pg/mg albumin). Consistent with this finding, the detection rate is highest in Burkina Faso (100%) and lowest in the United States (17.2%). The rank of exposure based on the biomarker levels from high to low is: West Africa countries > Southeast Countries > Uganda > Caribbean country > the United States. Currently AFB_1 exposure remains prevalent in parts of West Africa and Southeast Asia, which warrants urgent and effective interventions.

Key words: aflatoxin, exposure, aflatoxin B_1-lysine adduct, biomarker.

Introduction

Mycotoxins are estimated to contaminate 25% of cereal crops globally [1]. Among these mycotoxins, of biological significance are aflatoxins, fumonisins, deoxynivalenone, T-2, zearalenone and ochratoxins. Aflatoxins are frequent food contaminants produced mainly by *Aspergillus flavus* and *A. parasiticus* as secondary metabolites. Among the naturally occurring aflatoxins (B_1, B_2, G_1, G_2), aflatoxin B_1 (AFB_1) is most common and of predominant biological significance. The consumption of AFB_1 contaminated foods or dairy products can cause acute poisoning (aflatoxicosis) and chronic liver cancer in humans[2]. A quantitative risk assessment has estimated that approximately 4.8-25.2% of global annual HCC cases are attributed to aflatoxin exposure alone [3]. The ecological role of aflatoxins has also been associated with many other adverse health effects in humans, including impaired childhood growth [4] and immune functions [5]. In the developing world where aflatoxin exposure prevails, it was estimated that more than 40% of all disease burden is associated with aflatoxins [6].

Traditionally, questionnaires and food surveys are common approaches to assess human exposure to food contaminants. Through the years the aflatoxin contamination of food has been frequently reported especially in sub-Saharan Africa and southeast Asia[7]. These data provide important information about the environmental aflatoxin exposure. However, the heterogeneous distribution of aflatoxins in food limits the application of food survey based intake estimation as an effective exposure measurement. Besides, the individual exposure or risk cannot be adequately estimated by multiplying the contamination level with the amount of intake, considering the inter-individual genetic variation in the metabolism and detoxification enzymes that were shown to be associated

with different risks for HCC [8]. Instead, chemical-specific biomarkers from biological media provide an alternative measurement of individual exposure. The application of molecular biomarkers related to AFB_1 greatly facilitated the exposure assessment as well as efficacy evaluation of several chemoprevention strategies [9-11].

Accumulated knowledge from toxicological and biochemical studies has revealed several valuable biomarkers related to AFB_1 exposure or risk. In the human liver, cytochrome P450 enzymes (1A2 and 3A4) are responsible for the oxidation and activation of AFB_1 to its reactive metabolite, AFB_1-8, 9-epoxide [12]. This metabolite can readily bind to macromolecules such as protein or DNA to form albumin or DNA adducts. AFB_1-alubmin adducts are produced initially in liver and are transferred to the blood stream which release AFB_1-lysine (AFB-Lys) adducts under enzymatic digestion. AFB-Lys is shown to be a more specific biomarker of AFB_1 exposure compared to the albumin adducts as lysine in albumin is the only group of AFB_1 binding to a significant level [13]. The measure of this biomarker could reflect human chronic exposure to AFB_1 for up to several months [14, 15]. This adduct has been used to verify the etiological role of AFB_1 exposure during the outbreak of aflatoxicosis in Kenya in 2004 [16] and for indicating human exposure to AFB_1 in several cross-sectional [17-19] and case-control studies [16, 20]. Besides, urinary metabolite AFM_1, phase II metabolite AFB_1-mecapturic acid (AFB-NAC), and the DNA adduct AFB_1-N^7-guanine represent short-term biomarkers that correlate with AFB_1 exposure and have been used to indicate the efficacy of different intervention approaches [21]. These short-term biomarkers were also thought to be more appropriate in evaluating the adverse immune effects of aflatoxins given the short recovery process of the immune function changes [6].

Various methodologies have been developed as measurement tools for serum AFB-Lys adducts. Enzyme-linked immunosorbent assay (ELISA), immunoaffinity column based high performance liquid chromatography with fluorescence detection (HPLC-f), radioimmunoassay (RIA) and isotope dilution mass spectrometry (IDMS) method form the available techniques with an addition of the solid phase extraction based HPLC-f method [22-26]. Different methods tend to give different results, e.g. the ELISA method appears to measure some unspecific albumin adducts and produce higher estimation compared to the IDMS method [27]. The antibody based methods demand a high sensitivity of the antibody used and the results are prone to operating environment conditions. These different methodologies used to achieve the human exposure data restrict the direct comparison of the exposure data generated from different populations.

A global network for monitoring AFB_1 exposure based on the same method seems promising especially for comparison purpose. We have developed a non-antibody based method that has been validated in animal and humans studies [26]. Based on the method, we have measured the serum AFB-Lys adduct levels in different populations of the world with the aim of revealing the current AFB_1 exposure status, as well as providing evidence for future intervention strategies.

Materials and Methods

Study Populations

The study populations were recruited from different parts of the world, including West African countries (Ghana and Burkina Faso), an East African Country (Uganda), Southeast Asian countries (China and Malaysia), a Caribbean country (Haiti) and the United States. The distribution of these study populations were indicated in Figure 7.1. Burkina Faso study participants were recruited from three villages from 2001 to 2002. Ghana study populations were pregnant women recruited from two hospitals in Kumasi in 2006. Uganda study participants were from a cohort study conducted in the Medical Research Council and these samples were collected in 2011. Guangxi HCC cases and controls were recruited from Guangxi province, China, from 2004 to 2005. Malaysia study populations were healthy adults recruited from Penang, Malaysia in 2008. San Antonio participants were enrolled from three zip codes (with high liver cancer incidence) within the San Antonio metropolitan area of Bextar Co. from 2007 to 2008; Lubbock participants were local healthy volunteers that were recruited in 2005.

Serum Sample Processing

Thawed human serum samples were measured for albumin and total protein concentrations using procedures as previously described [23]. A portion of each serum sample (150 μL) was digested by pronase (pronase:total protein, 1:4, w:w) at 37°C for 3 h to release AFB-Lys adducts. AFB-Lys in digests were further extracted and purified by passing through a Waters MAX™ SPE cartridge, which was preprimed with methanol and equilibrated with water. The loaded cartridge was sequentially washed with water, 70% methanol, and 100% methanol containing 1% ammonium hydroxide at a flow rate

of 1 mL/min. Purified AFB-Lys was eluted with 2% formic acid in methanol. The eluate was vacuum-dried with a Labconco Centrivap concentrator (Kansas City, MO) and reconstituted for HPLC-fluorescence detection. The average recovery rate was 80%.

HPLC Conditions for Serum AFB-Lys Adduct Analysis

The analysis was conducted using an Agilent 1200 HPLC-fluorescence system (Santa Clara, CA). The mobile phases consisted of buffer A (20 mM $NH_4H_2PO_4$, pH 7.2) and buffer B (100% Methanol). The Zorbax Eclipse XDB-C18 reverse phase column (5 micron, 4.6 x 250 mm) equipped with a guard column was used. Column temperature was maintained at 25°C during analysis, and a volume of 100 µL was injected at a flow rate of 1 mL/min. A gradient was generated to separate the AFB-Lys adduct within 25 min of injection. The adduct was detected by fluorescence at maximum excitation and emission wavelengths of 405 nm and 470 nm, respectively. Calibration curves of authentic standard were generated weekly, and the standard AFB-Lys was eluted at approximately 12.3 min. Quality assurance and quality control procedures were taken during analyses, which included simultaneous analysis of one authentic standard and a quality control sample daily. The limit of detection was 0.4 pg/mg albumin. The serum AFB-Lys levels were adjusted by albumin content.

Statistical Analysis

AFB-Lys adducts were calculated for the mean ± SD, median, range, and the detection rate. Box-plot charts were made using the SigmaPlot 10.0 software from Systat Software Inc. (San Jose, CA). Two-sample comparisons were tested with Wilcoxon-Mann-Whitney test using the STAT 12 software from StataCorp LP (College station, TX). A *p*-value less than 0.05 was considered statistically significant.

Results

Demographic Characteristics of Study Populations

Demographic characteristics of different study populations are shown in Table 7.1. The study participants had a mean age ranging from 27 to 55. Because of the different focuses of each study design, the gender distribution varied largely. In the Guangxi case-control study, the majority of participants were male while the Ghana study enrolled exclusively young pregnant women. For the San Antonio study, fewer men were recruited than women because less male participants were available due to work committments. The gender distribution in Uganda, Burkina Faso and Lubbock was similar and approximately equal numbers of males and females were found.

Serum AFB-Lys Adduct Levels in Study Populations

The mean ± SD, median, range and detection rate of serum AFB-Lys adduct levels are summarized in Table 7.2. The levels of AFB-Lys varied in different study populations. West African (Burkina Faso and Ghana) and Southeast Asian countries (China and Malaysia) possessed the highest exposure, followed by the Middle African country (Uganda) and Haiti. The United States had the lowest exposure. The detection rate also supports this ranking, i.e. serum AFB-Lys adducts were most frequently detected in West African populations and least detected in American populations. Ghanaian pregnant women were found to have high levels of this adduct in their sera.

The box-plot chart of the distribution of the serum AFB-Lys adducts in different study populations is shown in Figure 7.2. It is obvious that high levels of AFB-Lys adducts were associated with more extreme values. The individual variations were most

prominent in West African countries and were least prominent in the United States populations.

Distribution Frequency of Serum AFB-Lys Adducts in Different Study Populations

The distribution frequencies of serum AFB-Lys adducts are shown in Figures 7.3a-g. Burkina Faso and Malaysia study participants had the most frequent exposure level at 5-10 pg/mg albumin. In addition, the highest exposure level (≥ 40 pg/mg albumin) was most frequently found in Burkina Faso and Ghana. Study participants from Guangxi, Ghana, Uganda and the United States had exposure levels mainly within 1-5 pg/mg albumin.

Gender Distribution of Serum AFB-Lys Adducts Levels

The level of AFB-Lys adducts varied between genders among different study populations (Table 7.3). The exposure between male and female study participants from the United States (San Antonio and Lubbock) and Uganda was comparable ($P > 0.05$). On the other hand, male participants in Guangxi, China and Burkina Faso seemed to have higher exposure compared to their corresponding female participants, although no significant differences were found ($P > 0.05$). However, the overall gender difference in the adduct levels from the pooled study participants of all study populations was statistically significant (Figure 7.4, $P < 0.0001$), with the male participants possessing higher level of the AFB-Lys adducts than female participants. The (mean ± SD) of AFB-Lys levels were (18.62 ± 56.72) pg/mg albumin for males and (10.62 ± 19.17) pg/mg albumin for females. (Malaysia and Haiti data were not included into this analysis.)

Discussion

This study represents a large scope, collaborative effort to assess the current AFB_1 exposures in global populations using the dosimetry of molecular biomarker, i.e. serum AFB-Lys adduct. One highlight of this study is that the all the analyses were conducted in the same laboratory which greatly facilitate the comparison of data from different populations. We demonstrate that the exposure level to AFB_1 varied greatly in different geographical areas and that a large inter-individual variance was present even in the same study population. The highest exposed populations were found in West Africa (Burkina Faso and Ghana) and Southeast Asia (Guangxi and Malaysia), followed by east Africa (Uganda) and Latin America (Haiti), and the least exposed population were found in the United States. The male participants had a mean AFB-Lys adduct level approximately 1.75-fold higher than that in female participants, as estimated from the pooled populations. The high level and the high detection rate of serum AFB-Lys adduct in West Africa and Southeast Asia strengthens the need for urgent intervention strategies especially considering the HCC risks associated with AFB_1 exposure.

The recruited study populations are mainly located between 40 N and 40 S 40 of the equator, as described by Williams et al [6]. The climate of this zone is featured by hot and dry weather (the dry season in Africa) and warm and humid (Southeast Asia and the rain season in Africa), which favor the growth of the toxicogenic fungi responsible for AFB_1 production either before harvest or during storage [28]. The increasingly warming and erratic climate adds to the uncertainty of how climate change will affect the growth of aflatoxin-producing fungi as well as the insect damage which may contribute to aflatoxin contamination [29]. The adverse health effects in humans in the high-risk areas

drive the efforts to formulate many epidemiological studies to assess the AFB_1 exposure status or to explore the causative effect of AFB_1 exposure in human diseases, such as HIV infection in Uganda (our collaborative project).

Evidence from human and animal studies has established AFB_1 exposure as a risk factor for human HCC development. In this study, the highest AFB-Lys levels were found in populations from Africa (West and Middle) and Southeast Asia (China and Malaysia). Distribution frequency analysis showed that the highest exposed populations also had larger proportions of people with higher concentrations of AFB-Lys adducts. Consistently, the highest global HCC incidences have been reported in these areas [30]. The endemic of several viral infections (HBV and HCV) in this zone makes these geographical areas more susceptible to human HCC, due to the synergistic effects of AFB_1 exposure and chronic HBV/HCV infection in increasing HCC risks [31].

The mechanisms of AFB_1 induced hepatocarcinogenesis have been increasingly understood. The active metabolite of AFB_1 can bind to DNA to form DNA adducts [15] and preferentially induce the transversion of G to T at codon 249 of the *p53* tumor suppressor gene in human hepatocytes [32]. In addition, it was demonstrated that AFB_1 produces mutations at codon 12 of the *c-ras* oncogenes using DNA extracted from AFB_1-induced transformed cell lines or primary liver tumors [33]. Besides the genotoxic effects, AFB_1 exposure has been shown to be associated with a global hypomethylation in HCC [34], suggesting a role of AFB_1 in inducing the epigenetic changes. Therefore, both genetic and epigenetic mechanisms may be involved during the development of HCC.

The issue of food contamination by AFB_1 in the above-mentioned high-risk areas has been frequently reported. The recent food surveys have reported that the estimated

level of AFB_1 in contaminated corn samples ranged 0.20-888.30 μg/kg in China (n = 279) [35] and 0.54-15.33 μg/kg (n = 95) in Malaysia [36]. The ranges of AFB_1 in contaminated corn were 3.4-636 μg/kg (n = 26) in Burkina Faso [37] and 0.4-490.6 μg/kg (n = 40) in Ghana [38], respectively. These food survey based estimations of AFB_1 exposure provide valuable information about the potential external exposure and on the estimation of daily intake. These reported high food contamination to AFB_1 strongly support our finding of high AFB-Lys adduct levels in populations from these countries. However, these estimations fail to take into account the biological and social factors important for AFB_1 metabolism, and thus may be inadequate for monitoring human risks.

Over the years, our laboratory has aimed to develop and validate molecular biomarkers associated with mycotoxin exposure and toxicity particularly for aflatoxin and fumonisin. The application of these biomarkers in epidemiological studies forms our ambitious goal of establishing a network for monitoring aflatoxin exposure. One highlight of this study is that the measure of AFB-Lys adducts was based on the same method which facilitates direct comparison. For the first time we reported the human AFB_1 exposure data in Burkina Faso, Uganda and Haiti. These preliminary data gave us an idea of the current human exposure status. Amongst the populations studied, people in Burkina Faso were most severely exposed to AFB_1, with the median serum AFB-Lys of 13.8 pg/mg albumin. Although relatively high levels of food contamination by AFB_1 have been found in Burkina Faso, Uganda, and Haiti, serum AFB-Lys levels varied largely among individual study participants in these countries as found in this study. The types of staple crops seem to play a role in the human exposure condition, e.g., the study participants in Uganda mainly eat plantains (a less frequent crop for AFB_1 contamination)

and corn and showed lower exposure compared to study participants in Burkina Faso whose staple crops are corn and peanuts, two known food media prone to AFB_1 contamination. In Haiti, AFB_1 contamination in market peanuts experienced a temporal reduction from 412.5 ± 32.1 ppb in September 2006 to 125 ± 7.1 ppb in November 2006 and further to a level of 0.20 ± 0.10 ppb in January 2007 [39]. The relatively low AFB-Lys level in Haitians may reflect the beneficial effect of these interventions to reduce AFB_1 contamination, including visual-tactile sort of the shell, flotation of select kernels, immediate roasting and grinding, and monitoring [39]. Certainly other factors might be involved, such as the season for blood drawing, the differences in gender distribution as well as in life styles such as smoking and drinking status.

Serum AFB-Lys adduct level indicates the individual exposure and reflects the outcome of genetic polymorphisms as well as dietary modulations. It is not surprising to find the large inter-individual variation of AFB-Lys levels even in the same population. Human genetic polymorphisms in AFB_1 metabolizing enzymes (CYP450 3A5, glutathione S transferase (GST) and epoxide hydrolase) are of biological significance in the carcinogenicity of AFB_1 [40, 41]. Other factors such as drugs and dietary components may also modify the formation of this adduct by induction of enzymes or simply binding with the toxin to reduce its bioavailability [42]. Ethnic factor seems to affect the AFB-Lys adduct levels, for example, Chinese and Indians are more likely to have higher levels of AFB-Lys than Malay. This rank was noted to be contrary to the nut consumption of these ethnic groups: Indians eat more nuts, followed by Malay and Chinese. As nuts are susceptible to high level of aflatoxins in Malaysia, the contradictory finding indicated a role of genetic variance in producing AFB-Lys adducts [19]. Local ethnic groups in Gambia

and Ghana also showed different levels of exposure to AFB_1, with the major reasons being the differences in diet staples and food processing [43, 44]. It seems that both genetic variance and dietary factors play an important role in determining the ethnic difference in AFB_1 exposure, or even the interaction of both. Less information on the AFB-Lys level is available in the U.S. populations, which restrict an estimation of possible ethnic difference.

There was no significant difference in serum AFB-Lys levels between males and females from each individual study population. However, when pooled, the overall AFB-Lys level is higher in males than females, and a significant difference (1.75 fold) was found ($P < 0.0001$). There is no simple answer to this question with regards to the metabolic enzymes. For example, the GSTM1-*nonnull* and GSTT1-*null* genotypes were found to be associated with a higher AFB_1-albumin adduct level in males than females in a Taiwanese population [45]. In addition, the current evidence suggests that CYP1A2 activity is higher in males than females and CYP3A activity seems to be higher in females [46]. Taken together, both the activities and the genetic polymorphism of metabolic enzymes need to be considered for explaining the gender difference in the AFB-Lys levels. Therefore, the current finding needs to be verified in more specific designs in human studies.

The current assessment of AFB_1 exposure was mainly based on cross-sectional studies. The feature of the study design limits the causative effect analysis of AFB_1 exposure in the etiology of human diseases. Cohort studies that use serum AFB-Lys adducts as molecular dosimetry of exposure are promising for delineating the ecological contribution of chronic AFB_1 exposure in human disease burdens, particularly in the

developing world. However, very limited information is available in this regard. Instead, our preliminary data could be used as the first step toward establishing a network for monitoring global AFB_1 exposure. This dynamic network, once established, could be used for exposure assessment, etiological studies in human diseases such as childhood development and efficacy evaluation of novel intervention strategies.

Our collective efforts show that AFB_1 exposure in West Africa and Southeast Asia remains quite common at present: more than 90% of study populations have various levels of AFB-Lys adducts. The fact that only a small fraction of AFB_1 ingested is converted to AFB-Lys adducts [13] means that the real world exposure could be underestimated. It is estimated that approximately 4.5 billion people in the developing world are at risk for chronic exposure to aflatoxins [6]. Therefore, an urgent need for effective intervention strategies is warranted. The intervention strategies for reducing aflatoxins exposure or risk have been the concerns of several reviews [6, 21, 42]. These strategies could be undertaken at different stages, including pre-harvest (reduce toxin production, and bio-control contamination), during food processing and storage (improve environmental setting, selection, and education), and after ingestion (reduce active metabolite formation and bioavailability). Vaccination of infants against HBV and HCV to remove the interaction with AFB_1 exposure is of priority to reduce human HCC risks in the long run [31]. The evaluation of different intervention strategies has been defined as one of the four gaps: quantification of health effects and disease burden of aflatoxin exposure; evaluation of current intervention strategies and disseminate the result; disease surveillance, food monitoring, laboratory and public health responses; and development of response protocols addressing aflatoxicosis [47].

In conclusion, this study demonstrates the current aflatoxin exposure status in different populations using serum AFB-Lys adduct as a molecular biomarker. The data presented in this study show that populations in West Africa and Southeast Asia represent the highest risk population for AFB_1 exposure at present, and highlight the task as well as the urgent need for effective interventions in these high-risk areas.

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References

1. CAST. Mycotoxins: Risks in plant, animal, and human systems. *Task Force Report* No. 139. Ames, IA: CAST. pp 13-85. 2003
2. IARC. Aflatoxins. *IARC Monogr Eval Carcinog Risks Hum*. 1993;56:245-395
3. Liu Y, Wu F. Global burden of aflatoxin-induced hepatocellular carcinoma: A risk assessment. *Environ Health Perspect*. 2010;118:818-824
4. Gong YY, Egal S, Hounsa A, Turner PC, Hall AJ, Cardwell KF, Wild CP. Determinants of aflatoxin exposure in young children from benin and togo, west africa: The critical role of weaning. *Int J Epidemiol*. 2003;32:556-562
5. Turner PC, Moore SE, Hall AJ, Prentice AM, Wild CP. Modification of immune function through exposure to dietary aflatoxin in gambian children. *Environ Health Perspect*. 2003;111:217-220

6. Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences, and interventions. *Am J Clin Nutr*. 2004;80:1106-1122

7. Wild CP, Gong YY. Mycotoxins and human disease: A largely ignored global health issue. *Carcinogenesis*. 2010;31:71-82

8. Kirk GD, Turner PC, Gong Y, Lesi OA, Mendy M, Goedert JJ, Hall AJ, Whittle H, Hainaut P, Montesano R, Wild CP. Hepatocellular carcinoma and polymorphisms in carcinogen-metabolizing and DNA repair enzymes in a population with aflatoxin exposure and hepatitis b virus endemicity. *Cancer Epidemiol Biomarkers Prev*. 2005;14:373-379

9. Tang L, Tang M, Xu L, Luo H, Huang T, Yu J, Zhang L, Gao W, Cox SB, Wang JS. Modulation of aflatoxin biomarkers in human blood and urine by green tea polyphenols intervention. *Carcinogenesis*. 2008;29:411-417

10. Kensler TW, Egner PA, Wang JB, Zhu YR, Zhang BC, Lu PX, Chen JG, Qian GS, Kuang SY, Jackson PE, Gange SJ, Jacobson LP, Munoz A, Groopman JD. Chemoprevention of hepatocellular carcinoma in aflatoxin endemic areas. *Gastroenterology*. 2004;127:S310-318

11. Ross RK, Yuan JM, Yu MC, Wogan GN, Qian GS, Tu JT, Groopman JD, Gao YT, Henderson BE. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. *Lancet*. 1992;339:943-946

12. Gallagher EP, Wienkers LC, Stapleton PL, Kunze KL, Eaton DL. Role of human microsomal and human complementary DNA-expressed cytochromes p4501a2 and p4503a4 in the bioactivation of aflatoxin b1. *Cancer Res*. 1994;54:101-108

13. Sabbioni G, Skipper PL, Buchi G, Tannenbaum SR. Isolation and characterization of the major serum albumin adduct formed by aflatoxin b1 in vivo in rats. *Carcinogenesis*. 1987;8:819-824

14. Wild CP, Hudson GJ, Sabbioni G, Chapot B, Hall AJ, Wogan GN, Whittle H, Montesano R, Groopman JD. Dietary intake of aflatoxins and the level of albumin-bound aflatoxin in peripheral blood in the gambia, west africa. *Cancer Epidemiol Biomarkers Prev*. 1992;1:229-234

15. Groopman JD, Zhu JQ, Donahue PR, Pikul A, Zhang LS, Chen JS, Wogan GN. Molecular dosimetry of urinary aflatoxin-DNA adducts in people living in guangxi autonomous region, people's republic of china. *Cancer Res*. 1992;52:45-52

16. Azziz-Baumgartner E, Lindblade K, Gieseker K, Rogers HS, Kieszak S, Njapau H, Schleicher R, McCoy LF, Misore A, DeCock K, Rubin C, Slutsker L. Case-control study of an acute aflatoxicosis outbreak, kenya, 2004. *Environ Health Perspect*. 2005;113:1779-1783

17. Shuaib FM, Jolly PE, Ehiri JE, Yatich N, Jiang Y, Funkhouser E, Person SD, Wilson C, Ellis WO, Wang JS, Williams JH. Association between birth outcomes and aflatoxin b1 biomarker blood levels in pregnant women in kumasi, ghana. *Trop Med Int Health*. 2010;15:160-167

18. Johnson NM, Qian G, Xu L, Tietze D, Marroquin-Cardona A, Robinson A, Rodriguez M, Kaufman L, Cunningham K, Wittmer J, Guerra F, Donnelly KC, Williams JH, Wang JS, Phillips TD. Aflatoxin and pah exposure biomarkers in a

u.S. Population with a high incidence of hepatocellular carcinoma. *Sci Total Environ*. 2010;408:6027-6031

19. Leong YH, Rosma A, Latiff AA, Izzah AN. Associations of serum aflatoxin b1-lysine adduct level with socio-demographic factors and aflatoxins intake from nuts and related nut products in malaysia. *Int J Hyg Environ Health*. 2012;215:368-372

20. Xu L, Qian G, Tang L, Su J, Wang JS. Genetic variations of hepatitis b virus and serum aflatoxin-lysine adduct on high risk of hepatocellular carcinoma in southern guangxi, china. *J Hepatol*. 2010;53:671-676

21. Kensler TW, Roebuck BD, Wogan GN, Groopman JD. Aflatoxin: A 50-year odyssey of mechanistic and translational toxicology. *Toxicol Sci*. 2011;120 Suppl 1:S28-48

22. Sabbioni G, Ambs S, Wogan GN, Groopman JD. The aflatoxin-lysine adduct quantified by high-performance liquid chromatography from human serum albumin samples. *Carcinogenesis*. 1990;11:2063-2066

23. Wang JS, Qian GS, Zarba A, He X, Zhu YR, Zhang BC, Jacobson L, Gange SJ, Munoz A, Kensler TW, et al. Temporal patterns of aflatoxin-albumin adducts in hepatitis b surface antigen-positive and antigen-negative residents of daxin, qidong county, people's republic of china. *Cancer Epidemiol Biomarkers Prev*. 1996;5:253-261

24. Wild CP, Jiang YZ, Sabbioni G, Chapot B, Montesano R. Evaluation of methods for quantitation of aflatoxin-albumin adducts and their application to human exposure assessment. *Cancer Res*. 1990;50:245-251

25. McCoy LF, Scholl PF, Schleicher RL, Groopman JD, Powers CD, Pfeiffer CM. Analysis of aflatoxin b1-lysine adduct in serum using isotope-dilution liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 2005;19:2203-2210

26. Qian G, Tang L, Liu W, Wang JS. Development of a non-antibody method for rapid detection of serum aflatoxin b1-lysine adduct. *Toxicologist*. 2010;114:248.

27. Scholl PF, Turner PC, Sutcliffe AE, Sylla A, Diallo MS, Friesen MD, Groopman JD, Wild CP. Quantitative comparison of aflatoxin b1 serum albumin adducts in humans by isotope dilution mass spectrometry and elisa. *Cancer Epidemiol Biomarkers Prev*. 2006;15:823-826

28. Cotty PJ, Jaime-Garcia R. Influences of climate on aflatoxin producing fungi and aflatoxin contamination. *Int J Food Microbiol*. 2007;119:109-115

29. Paterson RRM, Lima N. How will climate change affect mycotoxins in food? *Food Res Int*. 2010;43:1902-1914

30. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin*. 2011;61:69-90

31. Wild CP, Montesano R. A model of interaction: Aflatoxins and hepatitis viruses in liver cancer aetiology and prevention. *Cancer Lett*. 2009;286:22-28

32. Aguilar F, Hussain SP, Cerutti P. Aflatoxin b1 induces the transversion of g-->t in codon 249 of the p53 tumor suppressor gene in human hepatocytes. *Proc Natl Acad Sc U S A*. 1993;90:8586-8590

33. Eaton DL, Gallagher EP. Mechanisms of aflatoxin carcinogenesis. *Annu Rev Pharmacol Toxicol*. 1994;34:135-172

34. Zhang YJ, Wu HC, Yazici H, Yu MW, Lee PH, Santella RM. Global hypomethylation in hepatocellular carcinoma and its relationship to aflatoxin b(1) exposure. *World J Hepatol*. 2012;4:169-175

35. Gao X, Yin S, Zhang H, Han C, Zhao X, Ji R. [aflatoxin contamination of corn samples collected from six regions of china]. *J Hyg Res*. 2011;40:46-49

36. Reddy KR, Farhana NI, Salleh B. Occurrence of aspergillus spp. And aflatoxin b1 in malaysian foods used for human consumption. *J Food Sci*. 2011;76:T99-104

37. Warth B, Parich A, Atehnkeng J, Bandyopadhyay R, Schuhmacher R, Sulyok M, Krska R. Quantitation of mycotoxins in food and feed from burkina faso and mozambique using a modern lc-ms/ms multitoxin method. *J Agric Food Chem*. 2012;60:9352-9363

38. James B, Adda C, Cardwell K, Annang D, Hell K, Korie S, Edorh M, Gbeassor F, Nagatey K, Houenou G. Public information campaign on aflatoxin contamination of maize grains in market stores in benin, ghana and togo. *Food Addit Contam*. 2007;24:1283-1291

39. Brown D. Food chain mycotoxins 2010: Threats and solutions. *http://www.ansci.cornell.edu/cnconf/2010proceedings/CNC2010.7.Brown.pdf*. 2008

40. Tiemersma EW, Omer RE, Bunschoten A, van't Veer P, Kok FJ, Idris MO, Kadaru AM, Fedail SS, Kampman E. Role of genetic polymorphism of glutathione-s-transferase t1 and microsomal epoxide hydrolase in aflatoxin-associated hepatocellular carcinoma. *Cancer Epidemiol Biomarkers Prev*. 2001;10:785-791

41. Wojnowski L, Turner PC, Pedersen B, Hustert E, Brockmoller J, Mendy M, Whittle HC, Kirk G, Wild CP. Increased levels of aflatoxin-albumin adducts are associated with cyp3a5 polymorphisms in the gambia, west africa. *Pharmacogenetics*. 2004;14:691-700

42. Groopman JD, Kensler TW, Wild CP. Protective interventions to prevent aflatoxin-induced carcinogenesis in developing countries. *Annu Rev Public Health*. 2008;29:187-203

43. Allen SJ, Wild CP, Wheeler JG, Riley EM, Montesano R, Bennett S, Whittle HC, Hall AJ, Greenwood BM. Aflatoxin exposure, malaria and hepatitis b infection in rural gambian children. *Trans R Soc Trop Med Hyg*. 1992;86:426-430

44. Jolly P, Jiang Y, Ellis W, Awuah R, Nnedu O, Phillips T, Wang JS, Afriyie-Gyawu E, Tang L, Person S, Williams J, Jolly C. Determinants of aflatoxin levels in ghanaians: Sociodemographic factors, knowledge of aflatoxin and food handling and consumption practices. *Int J Hyg Environ Health*. 2006;209:345-358

45. Chen SY, Chen CJ, Tsai WY, Ahsan H, Liu TY, Lin JT, Santella RM. Associations of plasma aflatoxin b1-albumin adduct level with plasma selenium level and genetic polymorphisms of glutathione s-transferase m1 and t1. *Nutr Cancer*. 2000;38:179-185

46. Scandlyn MJ, Stuart EC, Rosengren RJ. Sex-specific differences in cyp450 isoforms in humans. *Exp Opin Drug Metab Toxicol*. 2008;4:413-424

47. Strosnider H, Azziz-Baumgartner E, Banziger M, Bhat RV, Breiman R, Brune MN, DeCock K, Dilley A, Groopman J, Hell K, Henry SH, Jeffers D, Jolly C, Jolly P, Kibata GN, Lewis L, Liu X, Luber G, McCoy L, Mensah P, Miraglia M,

Misore A, Njapau H, Ong CN, Onsongo MT, Page SW, Park D, Patel M, Phillips T, Pineiro M, Pronczuk J, Rogers HS, Rubin C, Sabino M, Schaafsma A, Shephard G, Stroka J, Wild C, Williams JT, Wilson D. Workgroup report: Public health strategies for reducing aflatoxin exposure in developing countries. *Environ Health Perspect*. 2006;114:1898-1903

Table 7.1. Demographic characteristics of study populations.

Factor	San Antonio	Lubbock	Guangxi Cases	Guangxi Controls	Malaysia	Ghana	Burkina Faso	Uganda
Sample size	151	70	68	136	170	755	474	127
Age (years)(mean±SD)	48 ± 15	31 ± 7	43 ± 10	43 ± 7	46 ± 17	27 ± 6	43 ± 10	55 ± 16
Gender [n(%)]								
male	43 (28.5)	32(45.7)	62 (91.2)	122 (89.7)	53 (31.2)	0 (0)	258 (54.4)	72 (56.6)
female	108 (71.5)	38(54.3)	6 (8.8)	14 (10.3)	117 (68.8)	755 (100)	216 (45.6)	55 (43.3)

Table 7.2. Global serum AFB-Lys levels in different populations (pg/mg albumin).

Parameters	San Antonio	Lubbock	Guangxi Cases	Guangxi Controls	Malaysia	Ghana	Burkina Faso	Uganda	Haiti
Numbers	151	54	68	136	175	755	474	127	193
Mean ± SD	4.44 ± 3.19	0.59 ± 0.33	10.60 ± 28.21	7.34 ± 16.56	6.31 ± 3.54	10.86 ± 19.01	26.73 ± 64.21	6.22 ± 24.44	6.12 ± 14.41
Median	3.44	0.63	4.97	3.69	6.08	5.01	13.8	2.21	2.41
Range	1.64 - 16.01	0.52-0.91	0.54 - 227.13	0.40 - 175.83	0.81 - 13.67	0.44 - 286.73	0.56 - 918.72	0.41 - 253.11	0.41 - 130.39
Detection Rate (%)	17.2	9.3	91.2	97.1	98	90.7	100	95.3	67.4

Table 7.3. Gender distribution of serum AFB-Lys levels in different populations (pg/mg albumin).

Gender	San Antonio	Lubbock	Guangxi Cases	Guangxi Controls	Ghana	Burkina Faso	Uganda
Male	4.15±2.76	0.69±0.20	11.30±29.46	6.30±8.35	NA	28.71±74.70	3.07±2.18
Female	4.63±3.51	0.73±0.21	3.45±2.59	4.19±3.69	10.86±19.01	24.37±48.88	4.02±5.56
p-value	0.5444	1	0.1247	0.6039		0.9952	0.7694

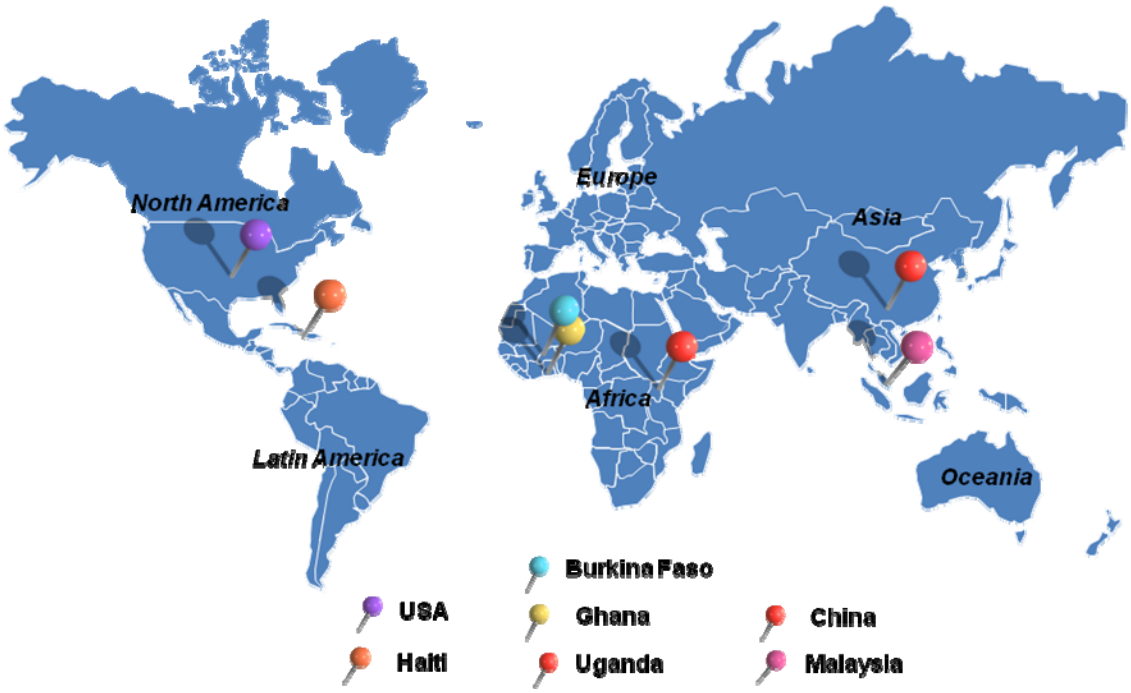

Figure 7.1. Geographic distribution of different study populations in this study.

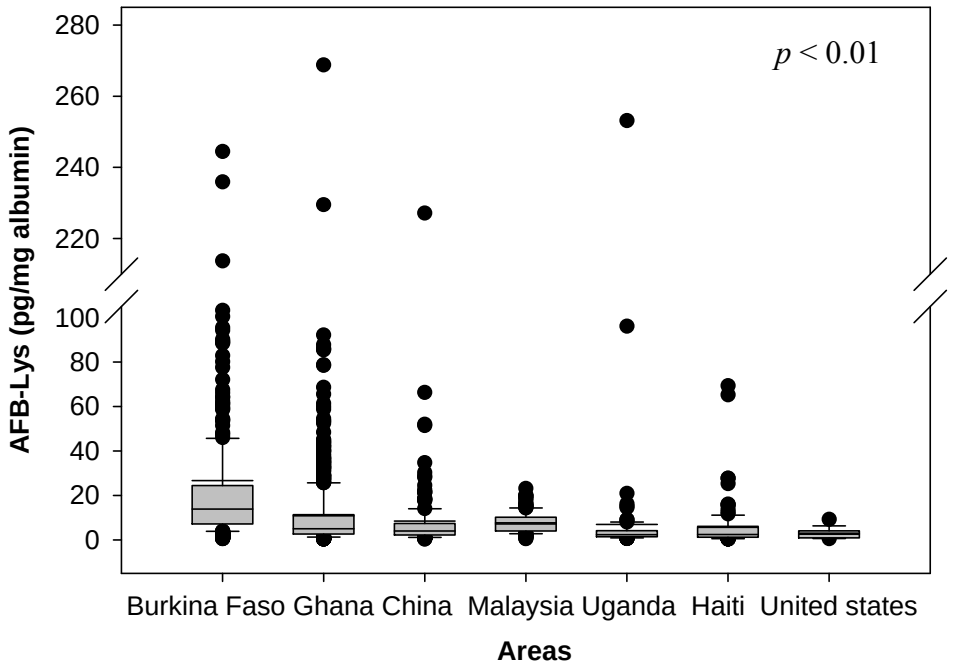

Figure 7.2. Box-plot of serum AFB-Lys levels in different study populations.

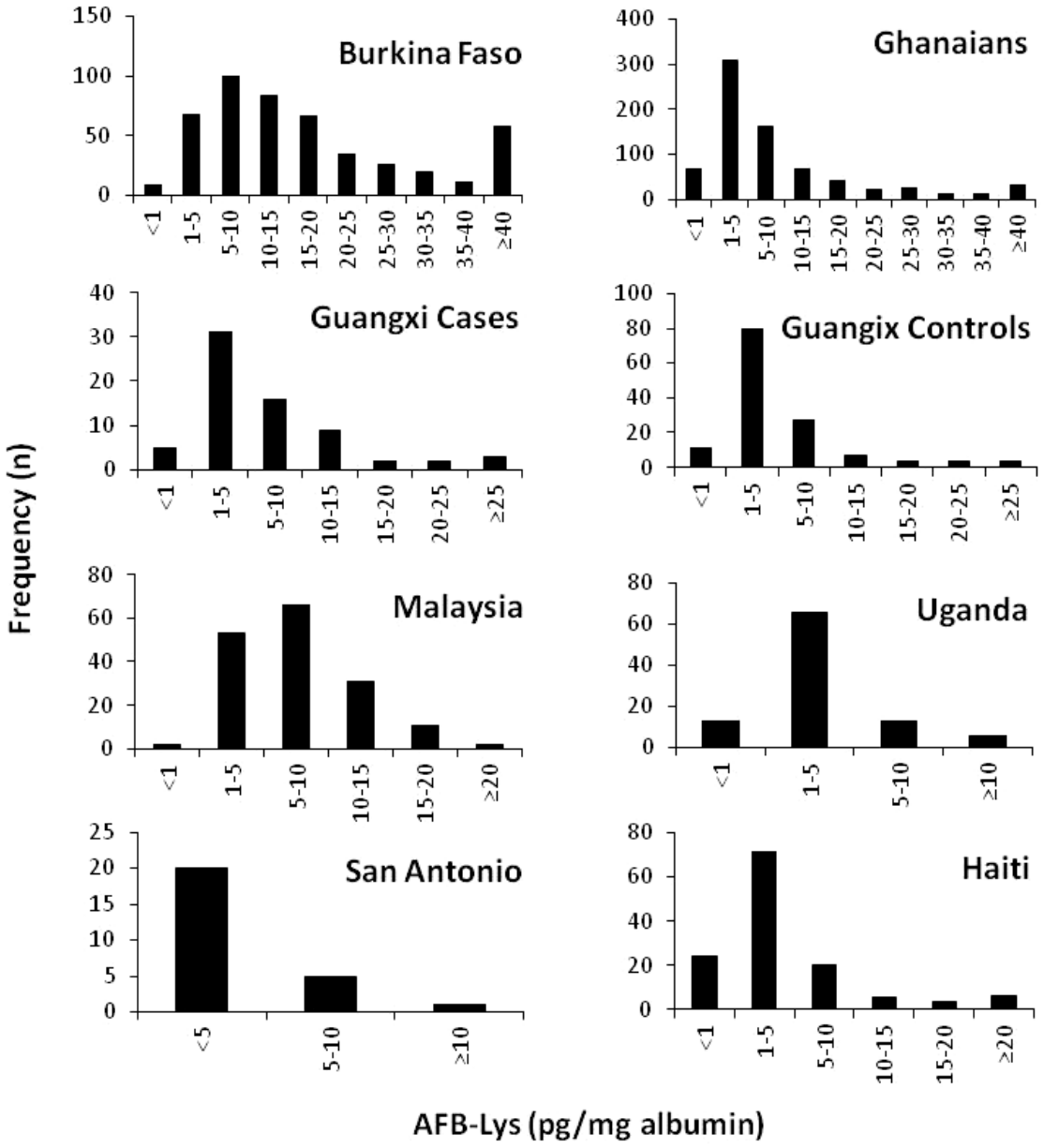

Figure 7.3. Distribution frequency of serum AFB-Lys levels in different study populations.

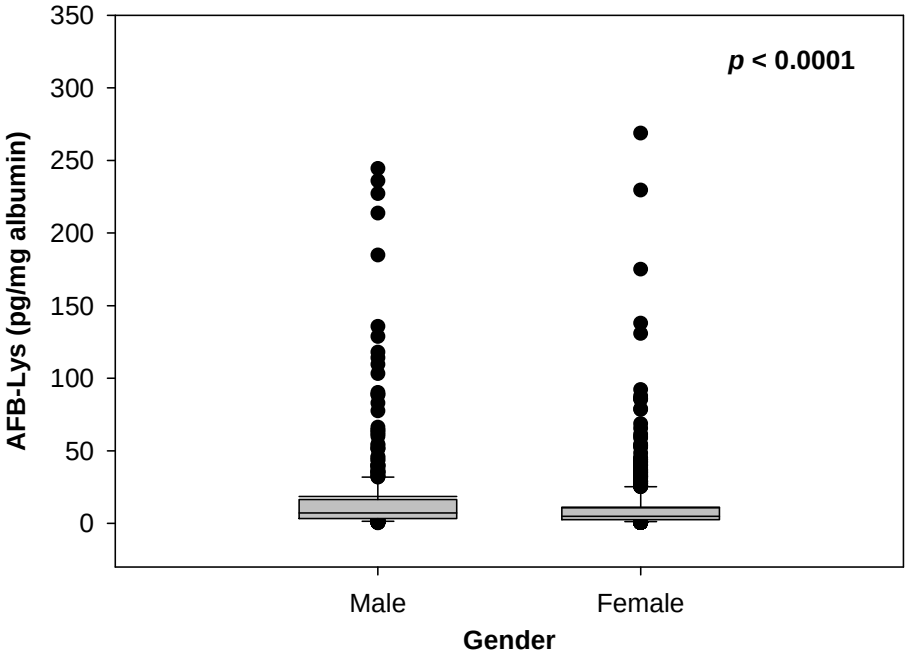

Figure 7.4. Overall gender distribution of serum AFB-Lys levels.

CHAPTER 8

CONCLUSION

This dissertation work was dedicated to the development of methods for detecting the human serum AFB-Lys adduct, a systematic evaluation of the toxicokinetics of this biomarker in rats, and the toxicity of AFB_1 in a sensitive rat model with special interest in the temporal histological, immunohistological, and the adverse immune effects. For the toxic effects evaluation, animal experiments that used both single-dose and repeated-dose approaches, were conducted. The development of a non-antibody AFB-Lys method facilitates assessment and comparison of human exposure to AFB_1 in many parts of the world, and serves as the first step towards the long-term goal of establishing a global network for studying global AFB_1 exposure and evaluating the efficacy of promising chemoprevention strategies. Overall, the advances and conclusions that have been accomplished by this dissertation work include:

1. Development and validation of a very sensitive solid-phase extraction based HPLC method with fluorescence detection. Among different test cartridges commercially available, the Water SPE MAX™ cartridge and Sep-Pak™ cartridge showed better recovery than the HLB™ cartridge. The MAX™ cartridge is chosen for the method development because it is more time efficient than the Sep-Pak™ cartridge. This non-antibody based method shows excellent sensitivity, with a detection limit of 0.4 pg/mg albumin. The inaccuracy and imprecision rates are less than 10% for intra- and inter-days analysis. This method is ready to be used in large-scale human serum samples analysis.

2. The single-dose treatment study in rats demonstrated that acute AFB_1 exposure produced a peak level of serum AFB-Lys at 4 h, followed by a rapid decrease. The pharmacokinetic analysis showed a half life of 2.31 days. Physiologically based pharmacologic model analysis showed that approximately 1.12-1.98% of AFB_1 dose is converted to serum AFB_1-Lys 24 h after exposure. Single-dose treatment elicited most pronounced histological changes (bile duct proliferation and periportal necrosis) and altered liver enzyme activities (e.g., serum ALT and AST) at 3 days after exposure.

3. Repeated-dose treatment in rats demonstrated that liver GST-P^+ cells and foci are dose and time dependently induced, supporting the potential use of this marker as a surrogate endpoint in chemoprevention studies. Liver enzyme activity changes (ALT and AST) in serum were not as sensitive as the development of GST-P^+ hepatocytes during repeated treatment. Repeated-dose study demonstrated that serum AFB-Lys levels linearly increases with time at low doses (5-25 μg/kg b.w.), indicative of the value of this biomarker to reflect chronic low dose exposure. Repeated AFB_1 exposure impaired the rat spleen lymphocyte function: short-term exposure (1-week) showed an overall inhibition of cytokine expression in $CD4^+$, $CD8^+$, and NK cells; long-term (5-week) exposure promoted IFN-γ and TNF-α secretion while suppressing IL-4 expression, suggesting the involvement of pro-inflammatory effects. The pro-inflammatory effects may not be reflective of histological changes and may imply other modes of action.

4. Human exposures to AFB_1 are highly variable among different populations and inter-individually. Populations from Sub-Saharan Africa and Southeast Asia areas were demonstrated to have much higher levels of serum AFB-Lys adduct and higher detection

rates compared to populations from the United States. The rank from high exposure to low is: sub-Sahara > Southeast Asia > Haiti > United States.

5. Collectively, our results demonstrate the validity of the non-antibody method in detecting the serum AFB_1-lysine adduct in both animal experiments and human populations.

CHAPTER 9

FUTURE DIRECTION

Aflatoxin research, started in the early 1960s, remains an active field of science at present. Through the literature, the trajectory of research on aflatoxins depicts intertwining lines of toxicological, agricultural, epidemiological, and regulatory efforts. There are good reasons for the continuing research on aflatoxins. The underlying economic cost, health effects and disease burdens, clinical implications, and mechanistic understanding of aflatoxin exposure cannot be ignored and stimulates research in many different fields.

Health costs and prevention

It has been estimated that worldwide, approximately 25% of crops are affected by mycotoxins annually [1], of which aflatoxins are a major problem. How to prevent the great economic loss due to aflatoxin contamination of world crops remains a huge task for industry and regulatory agencies. The relative cost-effectiveness of aflatoxin reduction strategies in Africa have been assessed by Wu's group [2, 3]. According to Wu's group [2, 3], both pre-harvest and post-harvest strategies have been proven cost-effective based on the World Health Organization (WHO) standard, and "public health interventions must be readily accepted by the public, and must have financial and infrastructural support to be feasible in the parts of the world where they are most needed". For the success of these public health intervention strategies (such as biocontrol, post-harvest package, NovaSil clay supplementation, and hepatitis B vaccination), public education, as well as financial and infrastructural support are critical especially in the less developed counties [4]. Future

work will be directed to advance the application of these intervention approaches as well as to assess the efficacy employing molecular biomarkers in a diversity of populations which are at high risk for aflatoxin exposure.

Clinical implications of aflatoxin exposure

Besides the etiological role of aflatoxins in human hepatocellular carcinoma [5], aflatoxin exposure has been associated with several adverse health effects, including kwashiorkor [6], Reye syndrome [7], growth impairment [8], and immune suppression in children [9]. Increasing evidence from epidemiological studies has suggested an association between aflatoxin exposure and those disease or adverse effects, however, a causal effect, if existing, warrants more specific studies especially in a prospective manner to verify. Still, other possible effects of continuous or intermittent dietary exposure to aflatoxins, which occurs widely in some developing countries, have received little study, such as the potential contribution of aflatoxin exposure to infectious diseases. Besides the well recognized interaction between aflatoxin and hepatitis B virus [5, 10-12], other viruses such as tuberculosis and HIV may interplay with aflatoxins, considering the co-existence of both environmental agents in the tropical areas and circumstantial evidence from human studies that suggest such an effect [13-16]. How to correlate the aflatoxin biomarkers with the risk or disease status remains a question to answer. To partially answer this question, the integration of data from clinical investigation and molecular biomarker measurement through the natural history of certain diseases might be useful to demonstrate such a link, which obviously will involve efforts from both doctors at the bed and scientists at the bench. Because most human diseases are an expression of the interaction between

individual genetic makeup and environmental agents [17], the individual phenotypes of the metabolic enzymes in particular, need to be known for an accurate risk analysis.

Mechanistic studies

Many human studies show an association between exposure to toxic agents and an increased risk of certain diseases. A mechanistic understanding of the relationship between exposure and disease depends on the availability of proper animal models and *in vitro* approaches. One difficulty associated with animal model development is the species difference in aflatoxin metabolism, which can sometimes be overcome by using transgenic animal models that specifically express human enzymes. Failure to demonstrate such an association in animal models sometimes may be related to the selection of endpoints, such as in the case of kwashiorkor [18]. In that study, serum total protein and albumin, and histopathological changes were used to indicate the development of kwashiorkor. Lack of specific endpoints to the disease may prevent the accurate assessment of such a causal relationship. Development of disease specific biomarkers may help to dissect the relationship between disease and exposure to aflatoxins. It is worth noting that, the assumption that "aflatoxin causes diseases" is so well accepted that other possibilities may be conveniently ignored. For instance, the disease of kwashiorkor is largely a problem of "being ignored", as described by Williams [19], that is, a problem of malnutrition.

Being lipophilic compounds, aflatoxins can penetrate cell membrane by passive diffusion [20]. They can also penetrate by active uptake [21]. AFB's lipophilicity favors its bioaccumulation through food chain. However, there is little, if any, evidence showing the concentration of aflatoxins in adipose tissues (the fast and efficient metabolism may

account for this). Several nutrition related diseases (such as kwashiorkor and environmental enteropathy) have been linked with aflatoxin exposure [22]. A common mechanism may involve the interference with the gut absorption, which can be mediated by affecting the normal gut microflora or increasing the permeability of gut membrane. The exact mechanisms deserve further research.

Interaction with or Confounding by Other Mycotoxins

Fumonisins are another group of mycotoxins that are frequently found in corn and animal feeds. Fumonisin B_1 (FB_1) has been shown to be associated with farm animal diseases and a potential etiological factor in human diseases [23]. The co-occurrence of aflatoxins and fumonisins in corn has been a common finding worldwide [24, 25]. Interaction of AFB_1 and FB_1 in acute toxicity and carcinogenicity has been demonstrated in animal models [26-28]. William et al. has also found an association between HIV infection, the incidence of AIDS developing from those HIV infections and the inclusion of maize in the diets of Africans [29]. This association, as noted by Brown [30], seems to be more closely related to the presence of fumonisins than aflatoxins. The potential interaction between AFB_1 and FB_1, and trichothecenes as well (e.g., T-2), in the etiology of human diseases remains largely unknown. Future work that directs risk assessment of mycotoxins should consider the co-existence of these mycotoxins.

In summary, efforts are needed to encourage the application of intervention strategies to reduce aflatoxin exposure and to advance a mechanistic understanding of the role of aflatoxin exposure in human health impairment. Integrative research incorporating both specific molecular biomarkers related to aflatoxin exposure and the adverse

outcomes is necessary if we are to develop a better mechanistic understanding of the role of aflatoxin in human diseases.

References

1. CAST. Mycotoxins: Risks in plant, animal and human systems. *Council for Agricultural Sciences and Technology,* Ames, Iowa, USA. 2003
2. Wu F, Khlangwiset P. Health economic impacts and cost-effectiveness of aflatoxin-reduction strategies in africa: Case studies in biocontrol and post-harvest interventions. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2010;27:496-509
3. Khlangwiset P, Wu F. Costs and efficacy of public health interventions to reduce aflatoxin-induced human disease. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2010;27:998-1014
4. Wu F, Khlangwiset P. Evaluating the technical feasibility of aflatoxin risk reduction strategies in africa. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2010;27:658-676
5. Ross RK, Yuan JM, Yu MC, Wogan GN, Qian GS, Tu JT, Groopman JD, Gao YT, Henderson BE. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. *Lancet*. 1992;339:943-946
6. Peraica M, Radic B, Lucic A, Pavlovic M. Toxic effects of mycotoxins in humans. *Bull World Health Organ*. 1999;77:754-766
7. Hogan GR, Ryan NJ, Hayes AW. Aflatoxin b1 and reye's syndrome. *Lancet*. 1978;1:561

8. Khlangwiset P, Shephard GS, Wu F. Aflatoxins and growth impairment: A review. *Crit Rev Toxicol*. 2011;41:740-755

9. Turner PC, Moore SE, Hall AJ, Prentice AM, Wild CP. Modification of immune function through exposure to dietary aflatoxin in gambian children. *Environ Health Perspect*. 2003;111:217-220

10. Wang LY, Hatch M, Chen CJ, Levin B, You SL, Lu SN, Wu MH, Wu WP, Wang LW, Wang Q, Huang GT, Yang PM, Lee HS, Santella RM. Aflatoxin exposure and risk of hepatocellular carcinoma in taiwan. *Int J Cancer.* 1996;67:620-625

11. Liu Y, Chang CCH, Marsh GM, Wu F. Population attributable risk of aflatoxin-related liver cancer: Systematic review and meta-analysis. *Eur J Cancer*. 2012;48:2125-2136

12. Qian GS, Ross RK, Yu MC, Yuan JM, Gao YT, Henderson BE, Wogan GN, Groopman JD. A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in shanghai, people's republic of china. *Cancer Epidemiol Biomarkers Prev*. 1994;3:3-10

13. Jiang Y, Jolly PE, Ellis WO, Wang JS, Phillips TD, Williams JH. Aflatoxin b1 albumin adduct levels and cellular immune status in ghanaians. *Int Immunol*. 2005;17:807-814

14. Jiang Y, Jolly PE, Preko P, Wang JS, Ellis WO, Phillips TD, Williams JH. Aflatoxin-related immune dysfunction in health and in human immunodeficiency virus disease. *Clin Dev Immunol*. 2008;2008:790309

15. Jolly PE, Shuaib FM, Jiang Y, Preko P, Baidoo J, Stiles JK, Wang JS, Phillips TD, Williams JH. Association of high viral load and abnormal liver function with

high aflatoxin b1-albumin adduct levels in hiv-positive ghanaians: Preliminary observations. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2011;28:1224-1234

16. Keenan J, Jolly p, Preko P, Baidoo J, Wang JS, Phillips TD, Williams JH, McGwin Jr G. Association between aflatoxin b1 albumin adduct levels and tuberculosis infection among hiv+ ghanaians. *Arch Clin Microbiol*. 2011;2:1-6
17. Hunter DJ. Gene-environment interactions in human diseases. *Nat Rev Genet*. 2005;6:287-298
18. Kocabas CN, Coskun T, Yurdakok M, Haziroglu R. The effects of aflatoxin b1 on the development of kwashiorkor in mice. *Hum Exp Toxicol*. 2003;22:155-158
19. Williams CD. Kwashiorkor: A nutritional disease of children associated with a maize diet. *Lancet*. 1935:1151-1152
20. Gallo A, Moschini M, Masoero F. Aflatoxins absorption in the gastro-intestinal tract and in the vaginal mucosa in lactating dairy cows. *Ital J Anim Sci*. 2008;7:53-63
21. Loe DW, Stewart RK, Massey TE, Deeley RG, Cole SP. Atp-dependent transport of aflatoxin b1 and its glutathione conjugates by the product of the multidrug resistance protein (mrp) gene. *Mol Pharmacol*. 1997;51:1034-1041
22. Smith LE, Stoltzfus RJ, Prendergast A. Food chain mycotoxin exposure, gut health, and impaired growth: A conceptual framework. *Adv Nutr*. 2012;3:526-531
23. WHO. Fumonisin b1. *Environmental Health Criteria*. 2000;219. World Health Organization, Geneva, Switzerland:1-134

24. Sun G, Wang S, Hu X, Su J, Zhang Y, Xie Y, Zhang H, Tang L, Wang JS. Co-contamination of aflatoxin b1 and fumonisin b1 in food and human dietary exposure in three areas of china. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2011;28:461-470

25. Wild CP, Gong YY. Mycotoxins and human disease: A largely ignored global health issue. *Carcinogenesis*. 2010;31:71-82

26. Gelderblom WC, Marasas WF, Lebepe-Mazur S, Swanevelder S, Vessey CJ, Hall Pde L. Interaction of fumonisin b(1) and aflatoxin b(1) in a short-term carcinogenesis model in rat liver. *Toxicology*. 2002;171:161-173

27. McKean C, Tang L, Tang M, Billam M, Wang Z, Theodorakis CW, Kendall RJ, Wang JS. Comparative acute and combinative toxicity of aflatoxin b1 and fumonisin b1 in animals and human cells. *Food Chem Toxicol*. 2006;44:868-876

28. Dilkin P, Zorzete P, Mallmann CA, Gomes JD, Utiyama CE, Oetting LL, Correa B. Toxicological effects of chronic low doses of aflatoxin b(1) and fumonisin b(1)-containing fusarium moniliforme culture material in weaned piglets. *Food Chem Toxicol*. 2003;41:1345-1353

29. Williams JH, Grubb JA, Davis JW, Wang JS, Jolly PE, Ankrah NA, Ellis WO, Afriyie-Gyawu E, Johnson NM, Robinson AG, Phillips TD. Hiv and hepatocellular and esophageal carcinomas related to consumption of mycotoxin-prone foods in sub-saharan africa. *Am J Clin Nutr*. 2010;92:154-160

30. Brown D. Food chain mycotoxins 2010: Threats and solutions. *http://www.ansci.cornell.edu/cnconf/2010proceedings/CNC2010.7.Brown.pdf*. 2010