

Protective Interventions to Prevent Aflatoxin-Induced Carcinogenesis in Developing Countries

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Abstract

The public health impact of aflatoxin exposure is pervasive in economically developing countries; consequently, we need to design intervention strategies for prevention that are practicable for these high-risk populations. The adverse health consequences of aflatoxins in populations are quite varied, eliciting acute effects, such as rapid death, and chronic outcomes, such as hepatocellular carcinoma. Furthermore, a number of epidemiological studies describe a variety of general adverse health effects associated with aflatoxin, such as impaired growth in children. Thus, the magnitude of the problem is disseminated across the entire spectrum of age, gender, and health status in the population. The aflatoxins multiplicatively increase the risk of liver cancer in people chronically infected with hepatitis B virus (HBV), which illustrates the deleterious impact that even low toxin levels in the diet can pose for human health. Thus other aflatoxin interactions, which likely contribute to the disease burden, still remain to be identified. Therefore, many diverse and appropriate strategies for disease prevention are needed to decrease the incidence of aflatoxin carcinogenesis in developing countries.

HCC:

hepatocellular carcinoma

HBV: hepatitis B virus

INTRODUCTION

The aflatoxins are ubiquitous contaminants of the human food supply throughout the economically developing world (13). The adverse toxicological consequences of these compounds in populations are quite varied owing to a wide range of exposures that lead to acute effects, including rapid death (2), and chronic outcomes, such as hepatocellular carcinoma (HCC) (60, 63). Furthermore, several studies describe a variety of general adverse health effects associated with aflatoxin, such as impaired growth in children (22). Aflatoxin exposures multiplicatively increase the risk of liver cancer in people chronically infected with hepatitis B virus (HBV), which illustrates the deleterious impact that even low toxin levels in the diet can have on human health (60, 63, 78). Collectively, the public health impact of aflatoxin exposure is pervasive; consequently, we need to design intervention strategies that are practicable for the high-risk populations in economically developing countries.

Mycotoxins, of which aflatoxins are one class, are fungal secondary metabolites of enormous chemical diversity that occur naturally on a wide variety of cereal grains, oilseeds, and tree nuts consumed by humans. The three major genera of mycotoxin-producing fungi of concern for human health are *Aspergillus*, *Fusarium*, and *Penicillium*, and this field has been extensively and more concisely reviewed by Bennett & Klich (3) and Etzel (18), respectively. The potential production of mycotoxins is invidious because fungal growth occurs both prior to and after grain harvest. Ecological conditions such as drought or damage to seeds by insects or mechanical harvesting can enhance mycotoxin production during both cultivation and storage. Mycotoxin production also occurs over a wide range of moisture content, relative humidity, and temperature, and all major human food crops such as corn (maize), peanuts (groundnuts), cotton, wheat, rice, and the processed food derived from these commodities

can be affected (34). Because the requirements for aflatoxin production are relatively nonspecific, commodities can become contaminated at final concentrations varying from $<1 \mu\text{g/kg}$ (1 ppb) to $>12000 \mu\text{g/kg}$ (12 ppm) (13). In addition, contamination of, for example, maize kernels or a batch of peanuts is highly heterogeneous; consequently, measurement of human exposure to aflatoxin by sampling foodstuffs or by dietary questionnaires is extremely imprecise. Thus aflatoxin biomarkers have great potential for accurate assessment of exposure (28).

Following the discovery of the carcinogenic aflatoxins nearly 50 years ago, the search for mycotoxins has led to the identification of more than 100 toxigenic fungi and greater than 300 mycotoxins worldwide, and the metabolism and genetics of these fungal metabolites have been recently updated (35). Most of these mycotoxins have not been definitively linked to any toxic syndromes in animals or people, but in addition to aflatoxin, certain trichothecenes, fumonisins, and ochratoxins have been implicated in highly lethal episodic outbreaks of mold poisoning or chronic diseases in exposed animals and/or human populations (83, 86). Mycotoxins with carcinogenic potency in experimental animal models include aflatoxins, sterigmatocystin, ochratoxin, fumonisin, patulin, and penicillic acid. Of these agents, aflatoxin B₁ has been classified as a Group 1 human carcinogen by the International Agency for Research on Cancer (32). Finally, because these mycotoxins co-occur in the developing world, the likelihood is high for additive and multiplicative toxic effects in people from this complex set of compounds.

DEFINING BIOMARKERS FOR PROTECTIVE INTERVENTIONS

In wealthy grain-producing countries of the world, economic resources exist to ensure that regulations to limit aflatoxin exposure in the food supply are implemented. Furthermore, in the grain commodities markets, the

prices of corn and groundnuts are often dictated by aflatoxin content, which contributes to much lower levels of exposure in wealthy countries (89). Thus, a result of these regulations and market forces is that people in economically developing countries are exposed to far higher levels of aflatoxins in the diet. This situation strongly motivates a number of groups to develop both effective and affordable intervention strategies. Biomarkers to assess the efficacy of these intervention strategies are becoming ever more important to validate and characterize these interventions in populations.

Biomarkers of exposure, internal dose, and the biologically effective dose of aflatoxin are the most commonly used for assessing intervention studies. These biomarker classifications are based on the classical paradigm for molecular epidemiology (82, 87). A biomarker of exposure refers to measurement of the specific agent of interest, its metabolite(s), or its specific interactive products in a body compartment or fluid and indicates the presence and magnitude of current and past exposures. Biomarkers of an internal dose and a biologically effective dose for aflatoxins are generally the oxidative metabolites and DNA and protein adducts formed by the critical aflatoxin-epoxide derivative. These biomarkers are illustrated in **Figure 1**.

One can find a marked difference between the ability to measure a particular biomarker in a human biological sample employing high-quality analytical approaches and the ability to interpret that information on the basis of thorough validation of the biomarker. The validation step involves the careful characterization of the relationship between the biomarker and, for example, environmental exposure to the agent of interest or to the consequent progression of disease. This process of biomarker validation is well served by parallel experimental and human studies (29).

As shown in **Figure 2**, a conceptually appropriate animal model is used to determine the associative or causal role of the biomarker on the disease pathway and to establish rela-

tions between dose (exposure) and response. The putative biomarker can then be validated in pilot human studies, where sensitivity, specificity, accuracy, and reliability parameters can be established. Data obtained in these studies are used to assess intra- or interindividual variability, background levels, relationships of the biomarker to external dose or to disease status, and feasibility for use in larger population-based studies. For a full interpretation of the information that the biomarker provides, prospective epidemiological studies may be necessary to demonstrate the role of the biomarker in the overall pathogenesis of the disease. Finally, these biomarkers can be employed as efficacy endpoints in interventions in both experimental models and high-risk human populations.

AFLATOXINS: CHEMISTRY, OCCURRENCE, AND HUMAN TOXICOLOGY

The aflatoxins were discovered in the early 1960s as the causative agent of turkey X disease, which resulted in the death of thousands of turkey poults, ducklings, and chicks fed a contaminated peanut meal (13). Chemically, the aflatoxins are a highly substituted coumarin structure containing a fused dihydrofuran moiety. Four major aflatoxins designated B₁, B₂, G₁, and G₂ are produced by *A. flavus* and *A. parasiticus*. The B series were named because of their strong blue fluorescence under UV light, whereas the G series fluoresced greenish-yellow.

Widespread concern regarding the toxic effects of aflatoxins in humans and animals and possible transfer of residues from animal tissues and milk to humans has led to regulatory actions governing the interstate as well as global transport and consumption of aflatoxin-contaminated food and feed commodities. The United States Food and Drug Administration (FDA) has set action levels for aflatoxins in commodities. For feeding mature nonlactating animals, the action level is 100 ppb; for commodities destined for human

consumption and interstate commerce, 20 ppb; and for milk, 0.5 ppb (13). The European Union has promulgated up to tenfold lower standards for aflatoxin content in foods (31).

The regulatory standards for aflatoxin originally promulgated in the 1960s were not based on toxicology data but were driven by the analytical method of the day: thin-layer chromatography. Thus, it was still unclear if ppb levels of aflatoxin posed a toxicological hazard. This issue was experimentally addressed by feeding aflatoxin to rats at levels of 1, 5, 15, 50, and 100 ppb. These levels induced liver tumors at incidences of 4.5%, 9%, 19%, 80%, and 100%, respectively (88). Therefore, even $\mu\text{g-per-day}$ doses of aflatoxin could induce liver cancer, and this result provided a context for human cancer investigations exploring the linkage between aflatoxin and HCC.

HCC is a major cause of cancer morbidity and mortality in many parts of the world, including Asia and sub-Saharan Africa, where there are upwards of 600,000 new cases each year and more than 200,000 deaths annually in the People's Republic of China alone (46, 79). Furthermore, the median age of diagnosis and death from HCC is between 45 and 55 years of age (45). The map in **Figure 3**, based on the IARC cancer database, illustrates the unequal distribution of this disease (<http://www-dep.iarc.fr/>) (20). Upwards of 80% of the burden of HCC is manifest in the developing world. Because the occurrence of HCC is coincident with regions where aflatoxin exposure is high, efforts started in the 1960s to investigate this possible association. As in all ecologic investigations, the work was hindered by the lack of adequate data on aflatoxin intake, excretion, and metabolism in people; by the underlying susceptibility factors such as diet and viral exposure; and by the incomplete statistics on worldwide cancer morbidity and mortality. Despite these deficiencies, early studies did provide data illustrating that increasing HCC rates corresponded to increasing levels of dietary afla-

toxin exposure (6). The molecular basis for the toxicology of aflatoxin has been recently reviewed by Wild & Turner (85).

Although investigators have focused extensively on the role of aflatoxin exposure in HCC, over the years a number of cases of acute aflatoxicosis in humans have been reported in regions of some economically developing countries (67). The clinical manifestations of aflatoxicosis were vomiting, abdominal pain, pulmonary edema, and fatty infiltration and necrosis of the liver. In the 1970s data showed putative aflatoxin poisoning in western India in conjunction with consumption of heavily molded corn. Officials reported at least 97 fatalities, and these deaths occurred only in households where the contaminated corn was consumed (47). Histopathology of liver specimens revealed extensive bile duct proliferation, a lesion often noted in experimental animals after acute aflatoxin exposure (5, 30). An incident of acute aflatoxicosis in Kenya in the early 1980s was also associated with consumption of maize highly contaminated with aflatoxin (55). Reports showed 20 hospital admissions with 20% mortality. In a more recent report in 1995 (52), the consumption of aflatoxin-contaminated noodles resulted in acute hepatic encephalopathy in children in Malaysia. Up to 3 mg of aflatoxin was present in a single serving of contaminated noodles.

In April 2004, one of the largest documented aflatoxicosis outbreaks occurred in rural Kenya, resulting in 317 cases and 125 deaths. Aflatoxin-contaminated homegrown maize was the major source of the outbreak. In a survey of 65 markets and 243 maize vendors, 350 maize products were collected from the most affected districts. Fifty-five percent of maize products had aflatoxin levels greater than the Kenyan regulatory limit of 20 ppb, 35% had levels >100 ppb, and 7% had levels >1000 ppb. Makueni, the district with the most aflatoxicosis case-patients, had significantly higher market maize aflatoxin than did Thika, the study district with fewest case-patients (geometric mean aflatoxin

= 52.91 ppb vs. 7.52 ppb, $p = 0.0004$). Maize obtained from local farms in the affected area was significantly more likely to have aflatoxin levels >20 ppb compared with maize bought from other regions of Kenya or from other countries (odds ratio = 2.71; 95% confidence interval, 1.12–6.59). In addition to the market survey for aflatoxin exposure, this outbreak in 2004 marked the first time that aflatoxin-albumin adducts independently confirmed the exposure in individuals (2, 49, 56, 59, 69).

Aflatoxin biomarkers of internal and biologically effective doses have been integral to establishing the etiologic role of this toxin in human HCC (45, 50). These biomarker investigations revealed the multiplicative interaction of lifetime exposure to high dietary levels of aflatoxins and infection in early life with HBV in the promulgation of this disease (30). The use of these biomarkers in etiologic investigations has been extended to preventive interventions in high-risk populations because experimental studies have established both quantitative and temporal linkages between modulation of aflatoxin biomarkers and changing disease risk.

DEVELOPMENT OF METHODOLOGIES FOR MEASURING BIOMARKERS

The chemical structures of the major aflatoxin DNA and protein adducts were identified (17, 64), and extensive research has been conducted to validate these structures for biomarker applications. Early experimental studies around 1980 demonstrated that the major aflatoxin-nucleic acid adduct, AFB₁-N⁷-Gua, was excreted exclusively in the urine of exposed rats (4, 15). The serum aflatoxin-albumin adduct was also examined as a biomarker of exposure because the longer half-life of albumin would be expected to integrate exposures over longer time periods, i.e., months instead of days. Studies in experimental models found that the formation of aflatoxin-DNA adducts in liver, urinary excre-

tion of the aflatoxin-nucleic acid adduct, and formation of the serum albumin adduct were highly correlated events (24). These investigations provided the rationale for exploring the application of these biomarkers in human studies.

An immunoaffinity clean-up/HPLC procedure was developed for aflatoxin metabolites in urine samples (15, 26). With this approach, initial validation studies investigated the dose-dependent excretion of urinary aflatoxin biomarkers in rats after a single aflatoxin B₁ (AFB₁) exposure. Investigators found a linear relationship between AFB₁ dose and excretion of the AFB₁-N⁷-Gua adduct in urine over the initial 24-h period (26). Subsequent studies in rodents that assessed the formation of aflatoxin macromolecular adducts after chronic administration also supported the use of DNA and protein adducts as molecular measures of exposure (14, 37, 82). For example, in rats treated with relatively low doses of AFB₁ (3.5 µg) twice daily for 24 days there was an accumulation of aflatoxin binding to peripheral blood albumin followed by steady-state levels, which illustrated the potential for this biomarker (aflatoxin-albumin adduct) to integrate exposure over time (14, 82).

Many different analytical methods are now available for quantitation of chemical adducts in biological samples (58, 65, 66, 87), each with unique specificity and sensitivity. An area of considerable importance, which has received far less attention than it should, is internal standard development. All quantitative measurements require the use of an internal standard to account for sample-to-sample variations in the analyte recoveries. In the case of mass spectrometry, internal standards generally employ an isotopically labeled material that is identical to the chemical being measured, albeit of different molecular mass. In the case of immunoassays, internal standards pose a different challenge because the addition of an internal standard recognized by an antibody results in an incremental contribution to the positive value. The dynamic

range is usually less than 100 in immunoassays, and therefore great care must be taken to spike a sample with an internal standard to obtain a valid result. In contrast, most chromatographic methods result in dynamic ranges of analyses that can be over a 10,000-fold range. Many of the aflatoxin studies used different analytical methods, and therefore the quantitative comparison of different data sets has been extremely problematic. However, a recent study compared methods of ELISA and mass spectrometry for aflatoxin-albumin adducts and found high correlation between these two ($r = 0.856$, $p < 0.0001$) (66).

RELATIONSHIP OF AFLATOXIN BIOMARKERS TO EXPOSURE AND DISEASE RISK IN EXPERIMENTAL ANIMALS

Coincident with the development of aflatoxin biomarkers, studies were underway to identify effective chemoprevention strategies for aflatoxin carcinogenesis. The hypothesis tested in these investigations was that reduction of aflatoxin-DNA adduct levels by chemopreventive agents would be predictive of cancer-preventive efficacy. Preliminary data with a variety of established chemopreventive agents demonstrated that following a single dose of aflatoxin, levels of DNA adducts were reduced (39). A more comprehensive study using multiple doses of aflatoxin and the chemopreventive agent ethoxyquin reduced the area and volume of liver occupied by presumptive preneoplastic foci by >95% and dramatically reduced binding of AFB₁ to hepatic DNA, with a 90% initial reduction and a 70% reduction at the end of a 2-week dosing period (38). An earlier study using a similar dosing protocol had established that ethoxyquin prevented HCC development in rats (37). The experiment was then repeated with several different chemopreventive agents, and in all cases, aflatoxin-derived DNA and protein adducts were reduced; however, even under optimal conditions, the reduction in the macromolecular

adducts always underrepresented the effect on tumor burden. Therefore, these macromolecular adducts can track with disease outcome on a population level, but in the multistage process of cancer the absolute level of adduct provides only a necessary but insufficient measure of tumor risk.

Using the chemopreventive agent oltipraz, Roebuck et al. (62) established correlations between reductions in levels of AFB₁-N⁷-Gua excreted in urine and incidence of HCC in aflatoxin-exposed rats. Overall, reduction in biomarker levels reflected protection against carcinogenesis, but these studies did not address the quantitative relationship between biomarker levels and individual risk. Thus, in a follow-up study, rats dosed with AFB₁ daily for five weeks were randomized into three groups: no intervention, delayed-transient intervention with oltipraz during weeks two and three of exposure, and persistent intervention with oltipraz for all five weeks of dosing (41). Serial blood samples were collected from each animal at weekly intervals throughout aflatoxin exposure for measurement of aflatoxin-albumin adducts. The integrated level of aflatoxin-albumin adducts over the exposure period decreased 20% and 39% in the delayed-transient and persistent oltipraz intervention groups, respectively, as compared with no intervention. Similarly, the total incidence of HCC dropped significantly from 83% to 60% and 48% in these groups. Overall, integrated biomarker level and risk of HCC were significantly associated ($p = 0.01$). When the predictive value of aflatoxin-serum albumin adducts was assessed within treatment groups, however, there was no association between integrated biomarker levels and risk of HCC ($p = 0.56$). These data clearly demonstrated that levels of the aflatoxin-albumin adducts could predict population-based changes in disease risk but did not have the power to identify individual rats destined to develop HCC. Such limitations in the utility of these biomarkers must be recognized in human biomonitoring.

VALIDATION OF AFLATOXIN BIOMARKERS IN CROSS-SECTIONAL STUDIES IN HUMAN POPULATIONS

Initial studies of aflatoxin biomarkers in human populations began in the Philippines, where investigators demonstrated that an oxidative metabolite of aflatoxin, AFM₁, could be measured in urine as an internal dose marker (9). Subsequent work conducted in the People's Republic of China and the Gambia, West Africa, areas with high incidences of HCC, determined that the levels of urinary aflatoxin biomarkers followed a dose-dependent relationship with aflatoxin intake (25, 27). However, as in the earlier experimental studies, this relationship was dependent on the specific urinary marker under study; for example, AFB₁-N⁷-Gua and AFM₁ showed strong correlations with intake, whereas urinary AFP₁, a different oxidative metabolite, showed no such link. Similarly Gan et al. (21) and Wild et al. (85) measured levels of aflatoxin-albumin adducts and observed a highly significant association between intake of aflatoxin and level of adduct. This type of study, to measure dietary aflatoxin intake and biomarkers at the individual level, is crucial to validate a biomarker for exposure assessment and is often overlooked in molecular epidemiology. Of particular interest in the Gambia was the observation that urinary aflatoxin metabolites reflected day-to-day variations in aflatoxin intake, whereas the aflatoxin-albumin adducts integrated exposure over the week-long study (85).

Data from these initial cross-sectional biomarker studies demonstrated short-term dose-response relationships for a number of the aflatoxin metabolites, including the major nucleic acid adduct, serum aflatoxin-albumin adduct, and AFM₁. This supported the validity of these exposure biomarkers for use in epidemiological studies, including investigations of intervention strategies and studies of the mechanisms underlying susceptibility.

INTERVENTION TRIALS FOR REDUCING AFLATOXIN EXPOSURE AND DOSE

Protective interventions against aflatoxin exposures in developing countries can take many forms (Table 1). It is axiomatic that as economic development increases, the ability of a population to afford a more diverse diet also increases. This dietary diversity usually leads to a reduction in aflatoxin exposures because the episodic events, such as those described in Kenya (69), become less frequent. Obviously, the economic development of a country can proceed at a very slow rate, and intervention strategies are needed during the interim times to lower risks associated with aflatoxin exposures. Any intervention strategies should be rigorously tested and validated using clinical trial designs with biomarkers serving as objective endpoints.

Clinical trials and other interventions are designed to translate findings from human and experimental investigations to public health prevention. Both primary (to reduce exposure) and secondary (to alter metabolism and deposition) interventions can use specific biomarkers as endpoints of efficacy. Such biomarkers can also be applied to the preselection of exposed individuals for study cohorts, thereby reducing study size requirements for validation trials. They can also serve as short-term modifiable endpoints (43). In a primary prevention trial, the goal is to reduce exposure to aflatoxins in the diet. A range of interventions includes planting pest-resistant varieties of staple crops, attempting to lower mold growth in harvested crops, improving storage methods following harvest, and using trapping agents that block the uptake of unavoidably ingested aflatoxins. In secondary prevention trials, one goal is to modulate the metabolism of ingested aflatoxin to enhance detoxification processes, thereby reducing internal dose and subsequent risk. These approaches have been previously summarized in terms of their application at the individual or community level (80, 81, 84).

Table 1 Strategies for reducing exposure and risk from aflatoxin in developing countries

<i>Intervention Type</i>	<i>Approach</i>	<i>Outcome</i>	<i>References</i>
Primary	Post-harvest storage conditions	Reduction in aflatoxin exposure measured by decreased aflatoxin-albumin adduct levels	92
Primary	Trapping agents (NovaSil)	Reduction in aflatoxin exposure in animal models and clinical trials to begin	1, 57, 74
Primary	Chlorophyllin	Efficacy demonstrated in experimental models and 55% reduction in urinary aflatoxin-DNA adducts in human clinical trial	7, 8, 10, 14, 68, 76
Secondary	Green tea polyphenols	Cancer prevention shown in rat models and reduction of oxidative DNA damage biomarkers found in clinical trial	16, 54, 61, 90, 91
Secondary	Dithiolethiones (Oltipraz)	Efficacy to prevent liver tumors in rat model demonstrated and modulation of aflatoxin biomarkers predictive of protection shown in human clinical trial	12, 40, 42, 48, 51, 77
Secondary	Broccoli sprouts (Sulforaphane)	Cancer prevention shown in rodent models. Dose-related reduction of urinary aflatoxin-DNA adducts in human clinical trial	33, 71, 72

There is active research in the development of mold-resistant strains of grains and in the genetic manipulation of molds to lower aflatoxin production (53, 92). Detailed descriptions of these types of interventions are beyond the scope of this article, and the following sections will focus on the use of aflatoxin biomarkers to develop and validate both primary and secondary prevention strategies in developing countries.

Primary Prevention

Post-harvest intervention. The use of aflatoxin biomarkers as efficacy endpoints in primary prevention trials has been recently reported (75). This work was built on earlier research that established high aflatoxin exposure in Guinea, West Africa, due to consumption of groundnuts as a dietary staple (11, 70, 74) and revealed that postharvest storage was correlated with increases in exposure (74). On the basis of these observations, a community-based intervention study was conducted among subsistence farmers in the

Kindia region of Guinea, West Africa. The intervention comprised a package of educational materials about postharvest measures to limit aflatoxin contamination of the groundnut crop delivered by local agricultural support workers (75). Farms from twenty villages were included in the design; ten were assigned to each of the intervention and control arms of the study. The intervention practices were all common in the region but were not systematically applied by the rural farmers. In the control villages, farmers were left to follow their normal postharvest practices, which therefore occasionally included one or more of the elements of the intervention strategy. Altered trajectories of aflatoxin-albumin adduct levels (**Figure 1**) were used to assess the success of the intervention, and this biomarker was measured in 600 subjects over a 5-month period.

In the control villages, the aflatoxin-albumin level increased postharvest, whereas in the intervention villages the level after five months of storage was similar to that immediately postharvest. Mean levels at this time were 60% lower in intervention compared

with control villages. The number of subjects with nondetectable aflatoxin-albumin at the time of harvest was approximately 30%, but this number decreased to 2% 5 months later in control villages. In contrast, almost 20% of individuals in the intervention group had nondetectable levels at the same time. The mean level of AFB₁ in groundnuts in household stores in intervention and control villages mirrored the pattern seen for aflatoxin-albumin adducts. The effectiveness of this intervention suggests that significant reductions in exposure can be achieved by using low-technology approaches at the subsistence farm level in sub-Saharan Africa.

Trapping agents. For many years sodium calcium aluminosilicate marketed as NovaSil (NS) clay, a common anticaking agent in animal feeds, has worked to adsorb aflatoxins in the gastrointestinal tract of animals and diminish the bioavailability and adverse effects of these toxins (57, 86). Animal model studies using male and female Sprague-Dawley rats fed diets containing 0, 0.25, 0.5, 1.0, or 2.0% (w/w) levels of NS for 28 weeks found no adverse health effects. Thus clay-based enterosorption of aflatoxins may be a useful strategy for the prevention of aflatoxicosis in humans. These experimental findings, in combination with a larger body of literature (57, 86), supported the exploration of this agent for intervention studies in human populations at high risk for aflatoxicosis (1).

An initial study reported on the safety and tolerance of NS in humans and established baseline protocols for long-term efficacy studies. In this work a randomized and double-blinded phase I clinical trial was conducted with 50 volunteers who were randomly divided into 2 groups: The low-dose group received 9 capsules containing 1.5 g NS/day, and the high-dose group received 9 capsules containing 3.0 g/day for a period of 2 weeks. Compliance was outstanding, and by the end of the study there were no statistically significant differences between the two groups for adverse health complaints nor were there dif-

ferences in any of the clinical chemistries or any other health parameter measured. Thus these findings provide an initial step for the translation of NS from use in the protection of animals from the adverse health effects of aflatoxins to a potential application for use in human populations at high risk for aflatoxicosis (76).

Chlorophyllin. The anticarcinogenic properties of chlorophyllin, a water-soluble derivative of chlorophyll, have been demonstrated in a number of animal models (7, 10). The initial characterization of chlorophyllin as an anticarcinogen arose from inhibition of HCC development in aflatoxin-treated trout (7). A major mode of action of this agent is thought to be the complexation of aflatoxin by chlorophyllin in a 1:1 ratio (8). Although the primary mode of action of chlorophyllin appears to be sequestration of aflatoxin, experimental data have characterized enzyme-inducing properties that may also contribute to its mechanism of action (19). Most recently chlorophyll was protective against aflatoxin-induced cancer in the rat, and the aflatoxin biomarkers tracked with the protective efficacy (68).

Residents of Qidong, People's Republic of China, are at high risk for development of HCC, in part from consumption of foods contaminated with aflatoxins. In a randomized, double-blind, placebo-controlled chemoprevention trial, chlorophyllin was tested to determine if it could alter the disposition of aflatoxin. 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. The primary endpoint was modulation of levels of urinary aflatoxin-N⁷-guanine adducts collected three months into the intervention. Adherence to the study protocol was outstanding, and no adverse events were reported. 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

GTP: green tea polyphenols

compared with those subjects taking placebo. Thus, prophylactic interventions with chlorophyllin or supplementation of diets with foods rich in chlorophylls may represent practical means to prevent the development of HCC or other environmentally induced cancers (16).

Secondary Prevention

Green tea polyphenols. Many studies have demonstrated that green tea polyphenols (GTP) inhibit various chemically induced cancers in experimental animals and epidemiological studies also point to the potential benefit of these compounds (54, 91). Qin et al. (61) studied the effects of GTP in drinking water for two or four weeks to protect against the development of AFB₁-induced hepatocarcinogenesis in the rat. Data from this investigation revealed that AFB₁-DNA binding in the liver was significantly inhibited by ~20%–30% in animals pretreated with green tea and that the burden of preneoplastic lesions was significantly inhibited by 60%–70%.

The experimental data on GTP provided the impetus to translate this strategy to human clinical trials. In the only study reported to date in an aflatoxin-exposed high-risk group in Guangxi, People's Republic of China, the effects of GTP on the oxidative DNA damage biomarker, 8-hydroxydeoxyguanosine (8-OHdG), was assessed in urine samples collected from a randomized, double-blinded, and placebo-controlled phase IIa chemoprevention trial. 8-OHdG increased following aflatoxin exposure and is associated with HCC risk (90). All participants tested positive for aflatoxin-albumin adducts and took GTP capsules daily at doses of 500 mg, 1000 mg, or a placebo for 3 months. Twenty-four hour urine samples were collected before the intervention and at the first and third month of the study. At the end of the 3-month intervention, 8-OHdG levels decreased significantly in both GTP-treated groups, with medians of 2.02, 1.03, and 1.15 ng/mg-creatinine for the placebo, 500-mg, and 1000-mg groups, respectively ($p = 0.007$). Thus chemopreven-

tion with GTP is effective in diminishing oxidative DNA damage; however, future studies will have to examine the efficacy of GTP to modulate aflatoxin-specific biomarkers (51).

Dithiolethiones (oltipraz). One successful strategy for cancer chemoprevention is modulation of drug-metabolizing enzymes, leading to a facilitated elimination of endogenous and environmental carcinogens. Inducers of conjugating enzymes such as dithiolethiones and sulforaphane inhibit tumorigenesis of environmental carcinogens in various animal models (26, 92). Increasing lines of evidence show that the Keap1-Nrf2 complex is a key molecular target of chemopreventive enzyme inducers. The transcription factor Nrf2 is a member of the basic leucine-zipper NF-E2 family and interacts with the antioxidant response element (ARE) in the promoter region of phase 2 detoxifying enzymes. A cytoplasmic actin-binding protein, Keap1, is an inhibitor of Nrf2 that sequesters it in the cytoplasm. Inducers dissociate this complex, allowing Nrf2 to translocate to the nucleus (12). Disruption of the *nrf2* gene in mice leads to enhanced sensitivity to carcinogens and the loss of chemopreventive efficacy by inducers (40, 48).

Aflatoxin biomarkers were used as intermediate endpoints in a phase IIa chemoprevention trial of oltipraz in Qidong, People's Republic of China (44, 77). This was a placebo-controlled, double-blind study in which participants were randomized to receive placebo or 125 mg oltipraz daily or 500 mg oltipraz weekly. Urinary AFM₁ levels were reduced by 51% compared with the placebo group in persons receiving the 500 mg weekly dose. No significant differences were seen in urinary AFM₁ levels in the 125-mg group compared with placebo. This effect was thought to be due to inhibition of cytochrome P450 1A2 activity. Median levels of AFB₁-mercapturic acid (a glutathione conjugate derivative) were elevated sixfold in the 125-mg group, but were unchanged in the 500-mg group. Increased AFB₁-mercapturic

acid reflects induction of aflatoxin conjugation through the actions of glutathione S-transferases. The apparent lack of induction in the 500-mg group probably reflects masking caused by diminished AFB₁,8,9-epoxide formation for conjugation through the inhibition of CYP1A2 seen in this group. This initial study demonstrated for the first time that aflatoxin biomarkers could be modulated in humans in a manner that would predict decreased disease risk by affecting the Nrf2-Keap1 signaling pathway.

Sulforaphane. Although the oltipraz clinical trial demonstrated the proof of principle for increasing pathways leading to aflatoxin detoxication in humans, the practicality of using a drug-based method for prevention in the economically developing world is limited. Not only is there a potential for adverse health effects from any long-term exposure to a drug, the expense of this type of intervention may make the intervention cost-prohibitive for these populations. Fortunately, oltipraz is not the only agent that affects enzyme changes through the Nrf2-Keap1 pathway. Many foods have high levels of these enzyme inducers (71, 72).

Drinking hot water infusions of 3-day-old broccoli sprouts, containing defined concentrations of glucosinolates as a stable precursor of the anticarcinogen sulforaphane, was recently evaluated for its ability to alter the disposition of aflatoxin. Sulforaphane has been extensively examined for its chemopreventive properties and is a potent activator of the Nrf2-Keap1 pathway (12, 33). In this study, 200 healthy adults drank infusions containing either 400 or <3 μ mole glucoraphanin nightly for 2 weeks. Urinary levels of AFB₁-N⁷-Gua were similar between the two intervention arms ($p = 0.68$). However, measurement of urinary levels of dithiocarbamates (sulforaphane metabolites) indicated striking interindividual differences in bioavailability. There were individual differences in the rates of hydrolysis of glucoraphanin into sulforaphane by the intestinal microflora

of the study participants. Accounting for this variability, an inverse association was observed for excretion of dithiocarbamates and AFB₁-N⁷-Gua adducts ($r = 0.31$; $p = 0.002$) in individuals receiving broccoli sprout glucosinolates (36). This preliminary study illustrates the potential use of an inexpensive, easily implemented food-based method for secondary prevention in a population at high risk for aflatoxin exposures.

SUMMARY AND PERSPECTIVES FOR THE FUTURE

Over the past 25 years, the development and application of molecular biomarkers reflecting events from exposure to manifestation of clinical disease have rapidly expanded our knowledge of the mechanisms of disease pathogenesis. These biomarkers have increasing potential for early detection, treatment, interventions, and prevention.

The molecular epidemiology and prevention studies of aflatoxin and HCC represent probably one of the most extensive data sets in the field, and this work may serve as a template for future studies of the role of other environmental agents in human diseases with chronic, multifactorial etiologies. The development of these biomarkers was founded on knowledge of the biochemistry and toxicology of aflatoxins, which was gleaned from both experimental and human studies. The biomarkers have been subsequently utilized in experimental models to provide data on their modulation under different situations of disease risk. This systematic approach provides encouragement for design and successful implementation of preventive interventions in exposed human populations.

Over the next 50 years, much of the population growth in the world is going to occur in countries that have intrinsically high aflatoxin exposure, in addition to many other chemical and biological agents. The observation that aflatoxin exposure may affect child growth and susceptibility to infection (23), as well HCC risk, serves only to emphasize the public

health need for development and implementation of interventions in these countries. Over the past 15 years, aflatoxin biomarkers have permitted the exploration of not just the etiology of aflatoxin-related disease but also the efficacy of various intervention strategies. One element in the appropriate application of prevention strategies is the targeting to various stages of the disease progression using knowledge of the underlying mechanisms of action (see **Figure 4**). In addition, investigators need to consider the temporal relationship between exposure and disease. For example, it is now well established that aflatoxin exposure begins in utero and increases markedly as a child is weaned onto solid family foods (80). This early exposure, at the time the child is also first exposed to numerous infections including HBV, suggests that there may be particular merit in intervening to reduce exposure during this critical window. Finally, different interventions may be appropri-

ate for different exposure scenarios. For example, the aflatoxicosis cases in Kenya discussed above occurred on a background of food insufficiency, where avoidance of exposure through primary prevention approaches was not feasible. However, some rapidly deployed secondary prevention may have provided benefits. Alternatively, in subsistence farming communities where dietary staples are frequently subject to aflatoxin contamination year after year, the introduction, through education of primary prevention approaches, may provide longer-term solutions of both health and economic benefit (73). Overall, the availability of a range of intervention approaches will help combat the adverse health effects of these common environmental contaminants. As the interventions are applied in longer-term studies the impact not only on exposure, but also on health endpoints will allow a more precise understanding of their benefits to public health.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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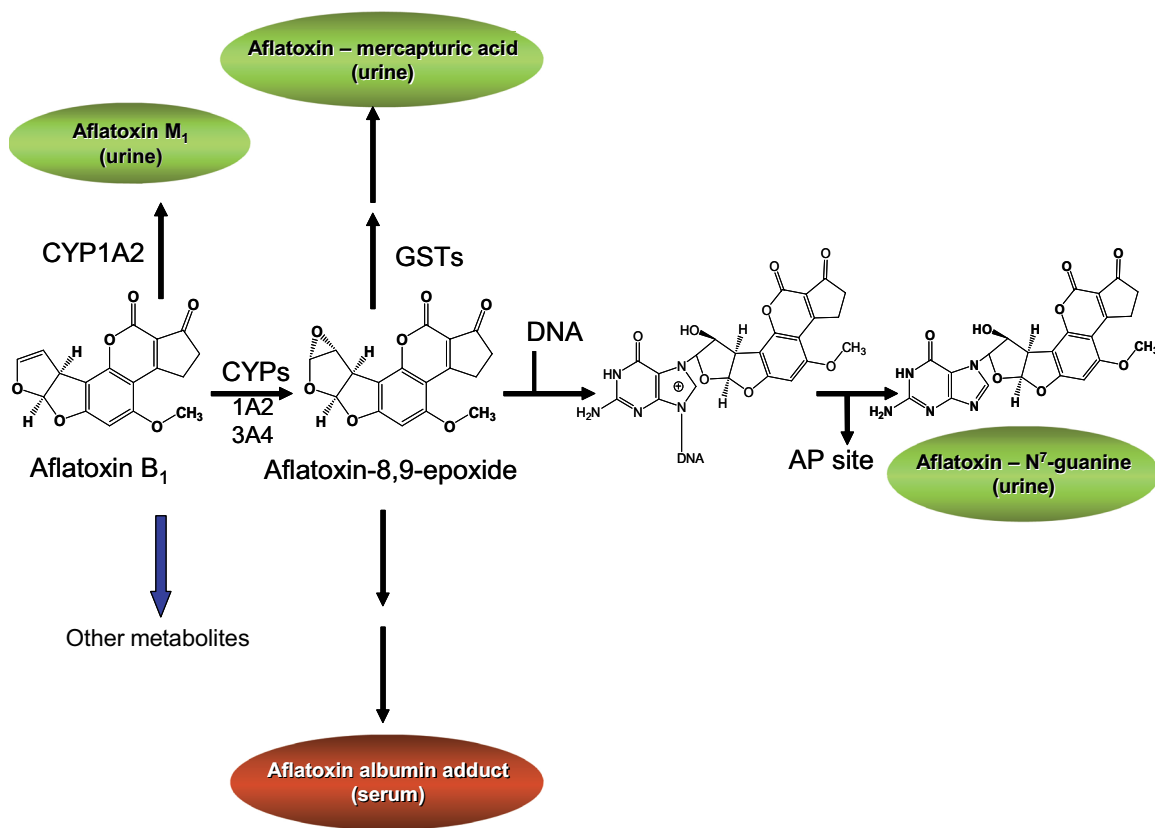


Figure 1

Biomarkers of aflatoxin exposure in an internal dose and a biologically effective dose. Biomarkers of exposure include aflatoxin M₁, the internal dose includes the aflatoxin-mercaptopuric acid and aflatoxin-albumin adduct, and the biologically effective dose is reflected by the excretion of the aflatoxin-N⁷-guanine adduct formed by depurination leading to an apurinic (AP) site in DNA.

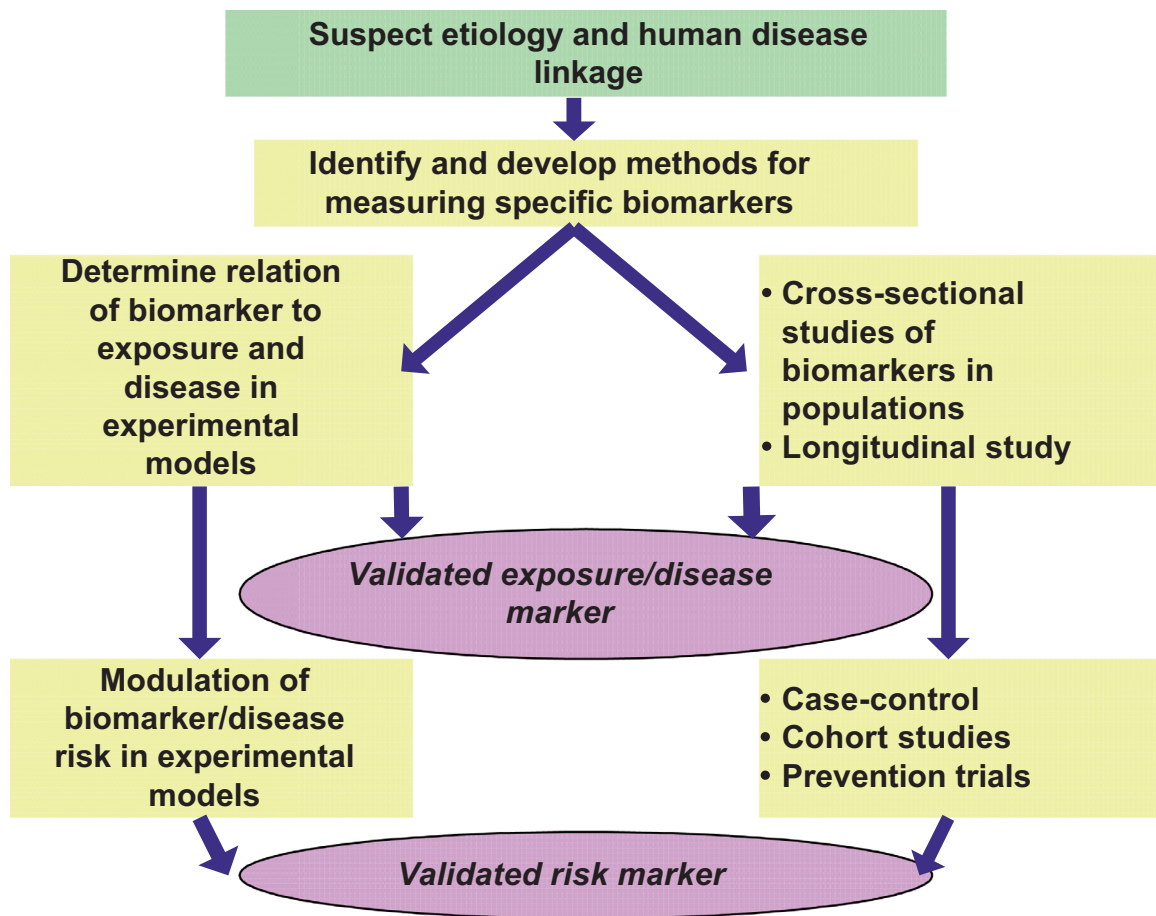


Figure 2

Biomarker validation strategy.

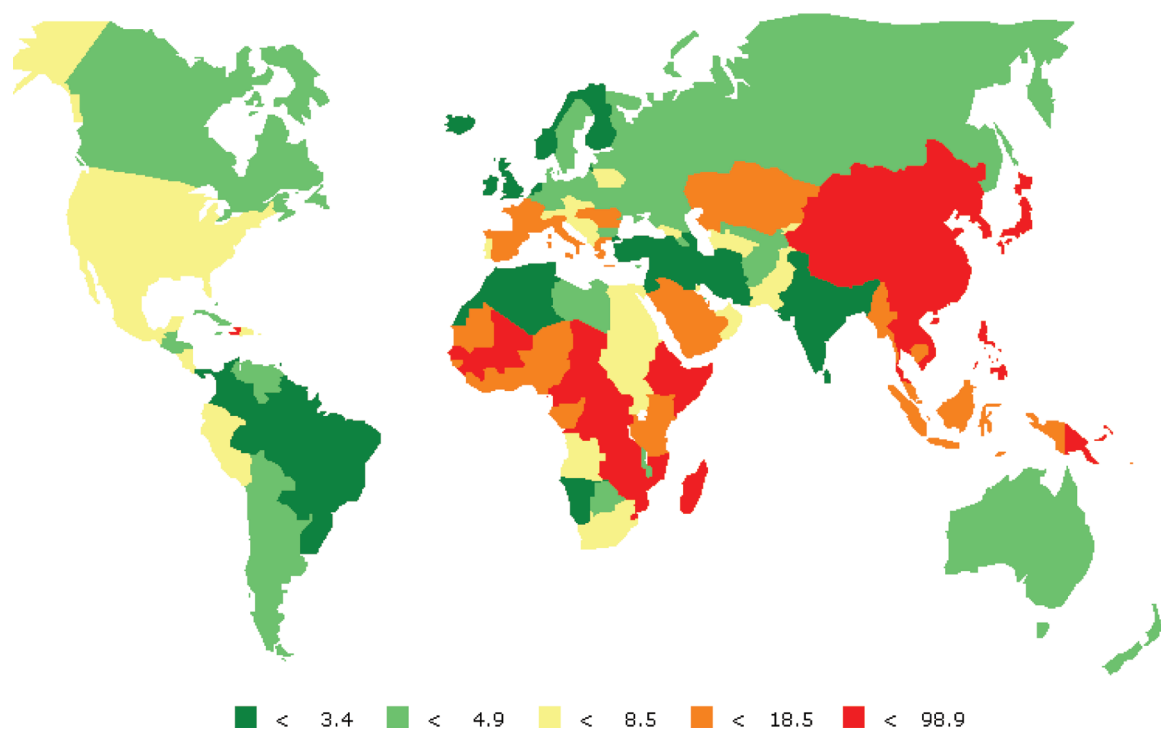


Figure 3

Age-standardized incidence rate of liver cancer per 100,000 men worldwide. From Reference 20.

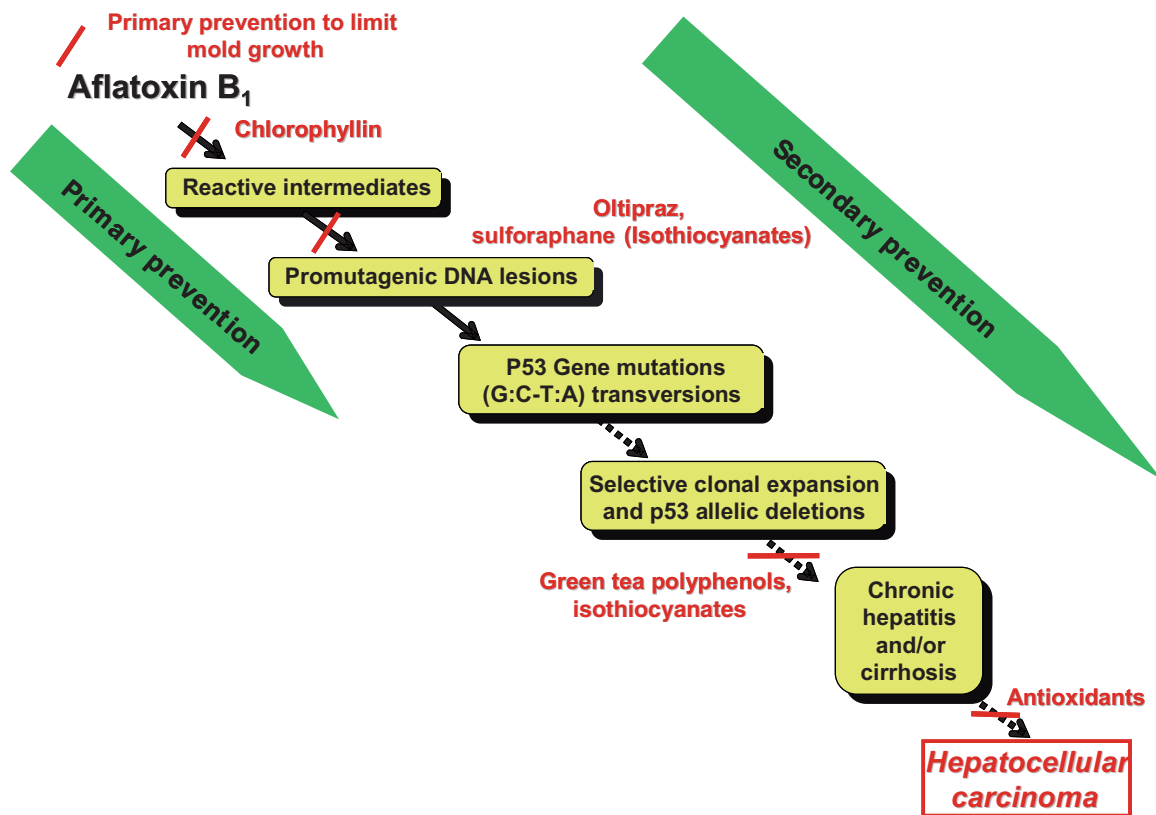


Figure 4

Targets for intervention in populations at high risk for liver cancer.