Molecular Epidemiology of Aflatoxin Exposures: Validation of Aflatoxin-N7-Guanine Levels in Urine As a Biomarker in Experimental Rat Models and Humans

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Human epidemiology and experimental animal data have provided the statistical association and biological information necessary to propose that aflatoxins are risk factors for human liver cancer. As liver cancer causes at least 200,000 deaths per year, prevention measures must be developed to ameliorate this nearly always fatal disease. Preventive strategies will be facilitated by the identification of individuals at high risk. It is the goal of the molecular dosimetry field to provide facile and accurate biomarkers to identify people at high risk for carcinogen exposure and consequent adverse health effects. We have developed methods to detect the major aflatoxin DNA adduct, aflatoxin N7-guanine (AFB-N7-guanine), in urine, examined the dose–response characteristics in people living in China and The Gambia, and have found an excellent association of this biomarker with exposure. In addition to exposure studies in people, our laboratories have monitored AFB-N7-guanine excretion in the urine of rats whose risk for developing cancer has been modulated with dietary chemoprotective agents such that independent groups of animals receiving the same dosage of aflatoxin B1 were at either high or low risk for tumorigenesis. The production of DNA damage by aflatoxins is not the exclusive mechanism for liver cancer. Many other factors, including hepatitis B virus, cell proliferation, and nutritional status, can exert strong modification effects in human disease. Thus, molecular epidemiological investigations that examine only one biomarker may greatly underestimate or overestimate the risk for an individual. In our experimental studies, we have found the need to measure specific, biologically relevant metabolites in a urine sample. Our data show that the levels of the daily urinary excretion of total aflatoxin metabolites are unrelated to risk of aflatoxin-induced disease. Similar analyses using the human urine samples from China also revealed that total aflatoxin metabolites in urine do not reflect appropriate exposure assessments. In the rat model, AFB-N7-guanine is a minor, but biologically relevant, metabolite representing less than 1% of the excreted material in urine, which does reflect exposure in experimental models. Thus, the composite human and experimental data generated from our research indicates that a cautious interpretation of the relationship between any biomarker and tumor outcome must be employed. However, both experimental rat data and human studies have also found that the AFB-N7-guanine adduct in urine is a good, noninvasive, short-term biomarker for determining both aflatoxin exposure and risk of genetic damage in target organs.

Introduction

Human epidemiology and experimental animal data have provided the statistical association and biological information necessary to suggest that aflatoxins are risk factors for human liver cancer (1,2). The degree that aflatoxins contribute to this disease will be influenced by a number of human health factors, including hepatitis B virus infection, nutritional status, and age as well as the extent of aflatoxin exposure (2). As liver cancer causes at least 200,000 deaths worldwide per year, prevention measures must be developed to reduce the incidence of this largely fatal disease. Preventive strategies will be facilitated by the
identification of individuals at high risk. It is the goal of the molecular dosimetry field to provide reliable biomarkers to identify people at high risk for carcinogen exposure and consequent adverse health effects. Methods have been developed to detect the major aflatoxin-DNA adduct, aflatoxin-N7-guanine (AFB-N7-guanine), in urine and thus allowed the dose–response characteristics of this biomarker to be examined in humans (3–6). In addition, complementary approaches have been validated for the measurement of aflatoxin bound to peripheral blood albumin; the properties of this approach have been discussed elsewhere (7,8). The AFB-N7-guanine adduct has been monitored in the urine of rats whose risk for developing liver cancer has been modulated with dietary chemoprotective agents such that independent groups of animals receiving the same dose of aflatoxin B1 (AFB1) were at either high or low risk for tumorigenesis (9,10). These complementary human and experimental data have been used to evaluate AFB-N7-guanine excretion in urine as a noninvasive, short-term biomarker for determining both aflatoxin exposure and risk of genetic damage in target organs.

**AFB-N7-Guanine in Human Urine: Studies in China**

Liver cancer is the third leading cause of cancer-related deaths in China, and there are regions of the country where this disease is very prevalent. The Guanxi Autonomous Region is among the areas with the highest incidence of liver cancer. We initiated the following study to examine biomarkers for aflatoxin exposures. The dietary intake of aflatoxins was monitored for 1 week in a study group consisting of 30 males and 12 females, ages ranging from 25 to 64 years, living in Fusui county, Guangxi Autonomous Region, China (4,5). The vast majority of AFB1 exposure was from contaminated corn. The average male intake of AFB1 was 48.4 µg per day, giving a total mean exposure over the study period of 372.8 µg. The average female daily intake was 92.4 µg per day, resulting in a total average exposure over the 7-day period of 542.6 µg AFB1. The maximum intake for the male and female subjects over the 1-week collection was 963.9 and 1035 µg, respectively, and the minimum exposure for male and female subjects was 56.7 and 90 µg AFB1, respectively. Total 24-hr urine samples were collected starting on the fourth day as consecutive 12-hr fractions.

Total aflatoxin metabolites in the urine samples were measured by competitive radioimmunoassay using a monoclonal antibody that recognizes AFB1, aflatoxin P1 (AFP1) and aflatoxin M1 (AFM1) with equal affinity and cross-reacts with AFB-N7-guanine with 5- to 10-fold less affinity. A depiction of the statistical association between AFB1 intake per day and total aflatoxin metabolite excretion per day is shown in Figure 1, and the correlation coefficient of 0.6 only reaches a statistical significance level of 0.10. Thus, total metabolites in urine as measured by this monoclonal antibody do not provide data to indicate that total metabolites were an appropriate dosimeter measurement for exposure status.

These data prompted an analysis of the urine samples by combined preparative monoclonal antibody affinity chromatography/analytical HPLC to determine levels of individual aflatoxin metabolites. Nearly 550 individual analytical HPLCs were performed. AFB-N7-guanine, AFM1, AFP1, and AFB1 were the aflatoxins most commonly detected and quantified in the urine samples. Presented in Figure 2 are the linear regression analyses for the urinary levels of these individual aflatoxins compared to aflatoxin intake. The intake data represent the AFB1 exposure from the day before the specific urine collection. Figure 2A shows the correspondence between AFB-N7-guanine adduct excretion with intake, where a correlation coefficient of 0.65 with a p value of <0.000001 results. Figure 2B depicts the analysis for AFM1, in which a correlation coefficient of 0.55 with a p value of <0.00001 were determined. Figure 2C represents AFP1 excretion. There was no positive statistical association between exposure in the diet and AFP1 excretion in urine. Further, it should be noted that AFP1 contributes substantially to the overall levels of aflatoxins in the urine samples as detected by competitive radioimmunoassay (see Fig. 1), thereby masking the association between exposure and minor metabolites. However, the resolution of the total aflatoxin metabolite content in the urine permits the association of AFB-N7-guanine and AFM1 excretion as biomarkers of exposure to be revealed.

One objective of this study was to determine the number of samples required from an individual and the time frame for sample collection necessary to validate a biomarker as reflecting a biologically effective dose of AFB1 in humans. There was particular interest in characterizing the molecular dosimetry of AFB-N7-guanine because of the putative relationship of this metabolite with the cancer initiation process. Figure 3 shows total AFB-N7-guanine excretion in the urine of the male and female subjects over the complete urine collection period plotted against the total AFB1 exposure in the diet for each of the subjects. This analysis smooths the day-to-day variations in both intake and excretion of AFB-N7-guanine and reveals a correlation coefficient of 0.80 and a p value of <0.0000001. This analysis clearly demonstrates that a summation of excretion and exposure status provides a stronger association between exposure and a molecular dosimetry marker than was seen in prior statistical analyses (see Fig. 2A) and supports the concept that quantitation of the AFB-N7-guanine adduct in urine is a reliable biomarker for AFB1 exposures.
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**AFB-N7-Guanine in Human Urine: Studies in The Gambia, West Africa**

Multiple risk factors for human liver cancer exist, and the following study was undertaken to explore the relationship between two of these factors by examining dietary exposure to aflatoxins and excretion of AFB-N7-guanine in the urine of chronically exposed people who were either positive or negative for the surface antigen of hepatitis B virus (6,11). The diets of 20 individuals, 10 males and 10 females, ages 15–56 years, were monitored for 1 week and levels of daily aflatoxin intake determined. Starting on the fourth day, total 24-hr urines were collected consecutively for 4 days.

Preparative monoclonal antibody affinity chromatography/HPLC was done on each of the urine samples and the relation-

**Figure 2.** Linear regression analysis of daily (A) aflatoxin (AFB) N7-guanine, (B) AFM1, (C) AFP, and (D) AFB1 in urine compared with dietary aflatoxin intake from the previous day (5).

**Figure 3.** Linear regression analysis of the association between total aflatoxin-N7-guanine adduct excretion in urine from males and females with total dietary aflatoxin exposure during the study period (5).

**Figure 4.** Linear regression analysis of the association between total aflatoxin N7-guanine adduct excretion in urine and dietary aflatoxin intake during the study period (11).
ships between excretion of AFB-N7-guanine with aflatoxin intake determined. Figure 4 depicts the relationship between total AFB-N7-guanine excretion in the urine of the male and female subjects over the 4-day collection period with the total aflatoxin exposure in the diet. Linear regression analysis reveals a correlation coefficient of 0.82 and a p value of < 0.0000001. Given these data, it is clear that a summation of excretion and exposure status over the collection period, similar to the experience in China described above, provides strong evidence for the utility of AFB-N7-guanine in urine as an appropriate molecular dosimetry marker of exposure.

Interestingly, in this study, a polyclonal antibody used to measure total urinary metabolites showed a good correlation (r = 0.65) between this endpoint and aflatoxin intake (II), in contrast to the study in China. This can probably be explained by differing specificity, as discussed previously (II). The polyclonal antibody used in The Gambia binds AFP, poorly compared to the monoclonal antibody used in China. These sort of considerations complicate the quantitative aspects of this approach.

This investigation also provided an opportunity to examine the role of chronic hepatitis B virus infection in the metabolism of aflatoxins. Figure 5 shows the statistical analysis of the association of hepatitis B virus status and the excretion of the AFB-N7-guanine adduct in urine for all subjects. These data reveal that although considerable interindividual differences exist for the ability to convert AFB1 to its genotoxic metabolite, AFB-N7-guanine, this did not relate to HBV carrier status.

![AFB-N7-Guanine in Rat Urine: Single-Dose Studies](image)

The analysis of the kinetics of AFB-N7-guanine excretion in urine are complicated in humans because of the day-to-day fluctuations in exposures. Thus, studies in rats using a single exposure to AFB1 have been used to characterize these excretion kinetics. Urines were collected over 24 hr from 12 male F344 rats dosed orally at levels ranging from 0.030 to 1.00 mg AFB1/kg body weight (I3). Aliquots of each urine were preparatively purified by immunoaffinity chromatography. A HPLC profile from one of the rat urine samples is shown in Figure 6. In the upper left panel of Figure 6 is the UV-visible spectrum obtained from the diode-array detector for the AFB-N7-guanine adduct. AFB-N7-guanine, AFQ1, AFP1, AFM1, and AFB1 account for 7.5, 30, 31.5, 2.2, and 0.3% of the total aflatoxins injected on the HPLC, respectively. All of the other major peaks absorbing at 363 nm show characteristic UV-visible spectra of aflatoxins, but their structures have not yet been elucidated.

The relationship between AFB1 dose and the excretion of the AFB-N7-guanine adduct over the 24 hr following exposure was then determined (Fig. 7) with a correlation coefficient and r² value of 0.99 and 98.33%, respectively. This analysis demonstrates an excellent linear correspondence between oral

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0.075% w/w oltipraz for 4 weeks. In this study rats received 10 intragastric doses of AFB1 (25 μg/rat/day; days 8–12 and 15–19), and this 10-dose exposure to AFB1 produced an 11% incidence of hepatocellular carcinoma at 23 months, with an additional 9% of the rats exhibiting hyperplastic nodules in their livers. In contrast, feeding rats a diet supplemented with 0.075% oltipraz for a 4-week period near the time of AFB1 exposure afforded complete protection against AFB1-induced hepatocellular carcinomas and hyperplastic nodules. None of these lesions were observed in the oltipraz-fed, AFB1-treated animals. Further, no tumors were at secondary, extrahepatic sites for AFB1 carcinogenesis such as the colon and kidney. Thus, the chemoprotection model affords us an experimental system to answer basic questions about the relationships between levels of AFB1-N7-guanine in urine for high- and low-risk animals.

Structure–activity studies with dithiolethiones indicated that the cancer chemoprotective activity of oltipraz is exclusively embodied in the 1,2-dithiole-3-thione nucleus of the molecule. Recently completed experiments indicated that the unsubstituted congener of oltipraz, 1,2-dithiole-3-thione, is also an effective inhibitor of AFB1-induced tumorigenesis, as determined by analyses for preneoplastic foci expressing either γ-glutamyl-transpeptidase (+) or glutathione-S-transferase-π (15). These data justified an examination of the impact of chemoprotection by 1,2-dithiole-3-thione on the molecular dosimetry of AFB1-N7-guanine in urine and to compare the modulation of this biomarker with levels of aflatoxin–DNA adducts in the liver (10).

The effects of 1,2-dithiole-3-thione on the kinetics of hepatic aflatoxin–DNA adduct formation and removal in rats receiving 250 μg AFB1/kg by gavage on each of days 0–4 and 7–11 are shown in Figure 9. Maximal levels of carcinogen binding were achieved following the third dose in the control group and declined thereafter despite continued exposure to aflatoxin. This diminution of binding, particularly during the second dosing cycle, has been observed previously (16–18) and may be a consequence of the induction of glutathione S-transferases and/or other enzymes involved in AFB1 detoxication following chronic exposure to aflatoxin (15). Inclusion of 0.03% 1,2-dithiole-3-thione dose and excretion of a biologically relevant metabolite in urine. In contrast, other oxidative metabolites such as AFP1 revealed no linear excretion characteristics (data not shown). Finally, at 24 hr after dosing, the residual level of aflatoxin liver DNA adducts were determined and compared to AFB1-N7-guanine excretion in urine (Fig. 8). The correlation coefficient and r² values were 0.98 and 96.3%, respectively, supporting the concept that measurement of the AFB1-N7-guanine adduct in urine reflects DNA damage in the primary target organ.

**AFB-N7-Guanine in Rat Urine: Biomarker for Risk in Chronically Exposed Rats**

The risk for aflatoxin hepatocarcinogenesis can be modified in animals by using a number of chemoprotective agents, including phenolic antioxidants, ethoxyquin, and dithiolethiones. A particularly effective cancer chemoprotective agent for aflatoxin carcinogenesis is a substituted dithiolethione, oltipraz (14). Male F344 rats were fed a purified diet supplemented with

![FIGURE 7. Linear regression analysis of the excretion of aflatoxin-N7-guanine in urine over 24 hr compared with the dose of aflatoxin B1 (13).](image-url)

![FIGURE 8. Linear regression analysis comparing the excretion of aflatoxin-N7-guanine in urine over a 24-hr period with the residual aflatoxin–DNA adducts in the liver at 24 hr after dosing (13).](image-url)

![FIGURE 9. Effect of 1,2-dithiole-3-thione on levels of aflatoxin–DNA adducts in rat liver. DNA was isolated from the livers of rats fed either control or 1,2-dithiole-3-thione-supplemented diets after multiple administrations of aflatoxin B1 (AFB1); 250 μg of [3H]AFB1 were administered per os on days 0–4 and 7–11 as indicated by arrows. Rats were placed on 1,2-dithiole-3-thione-supplemented diet 1 week before initiation of AFB1 dosing and maintained on this diet for the duration of the experiment. (□) Control diet; (□) 0.03% 1,2-dithiole-3-thione-supplemented diet. Values are means of determinations done on two rats/group/day (10).](image-url)
in the diet, beginning 1 week before dosing with AFB1, resulted in substantially lower levels of hepatic aflatoxin–DNA adducts throughout the exposure period. Binding was reduced by 76% over the initial 18-day period.

Presented in Figure 10 are the levels of total aflatoxin equivalents in 24-hr urine samples collected over the 2-week exposure period. There are no remarkable differences in the levels of aflatoxin metabolites in rats fed the control AIN-76A diet compared to those fed the 1,2-dithiole-3-thione-supplemented diet. Urinary aflatoxin levels rise rapidly after dosing with AFB1 and drop equally quickly after cessation of dosing, reflecting the overall short in vivo half-life of this carcinogen. The lack of an effect by 1,2-dithiole-3-thione is not surprising given that exposures to AFB1 were identical in both dietary groups. However, a distinctly different pattern emerges when the urines were subjected to sequential monoclonal antibody immunoaffinity chromatography and HPLC. Shown in Figure 11 are the levels of AFB-N7-guanine in serial 24-hr urine samples collected from rats undergoing a chemoprotective intervention with 0.03% 1,2-dithiole-3-thione. The highest level of AFB-N7-guanine excretion occurred on day 2 in both groups after the third dose of AFB1. This outcome is identical to that observed with hepatic levels of aflatoxin–DNA adducts (Figure 9). Over the 15-day collection period in which AFB-N7-guanine adducts were detectable in the urine, feeding of 1,2-dithiole-3-thione produced an overall reduction of 62% in the elimination of this aflatoxin–DNA adduct excision product, mirroring the data on the overall levels of hepatic aflatoxin-DNA adducts depicted in Figure 9. The amount of AFB-N7-guanine in urine represents only 1% of the total aflatoxin metabolites in urine and accounts for the dramatic differences seen between treatment groups in AFB-N7-guanine levels, which are not reflected in the levels of total urinary aflatoxin metabolites shown in Figure 11.

**Discussion**

Prevention strategies for hepatocellular carcinoma offer the best hope to eventually lower liver cancer incidence. In recent years hepatitis B virus (HBV) vaccination programs have been started to achieve this goal; however, this intervention is effective only when given to an uninfected person. Because this virus commonly infects people before they are 2 years old, many years will be required before vaccinations of individuals would be expected to result in lower cancer rates. In addition, the lack of knowledge regarding the mechanism of HBV induction of primary hepatocellular carcinoma and possible interaction with aflatoxin has made it difficult to assess the role of aflatoxin in the etiology of the tumor. Thus, if exposure to dietary aflatoxins can be lowered, reduction in disease rates may occur earlier and to a greater extent. Interest in aflatoxin as a risk factor for human liver cancer has been heightened by recent reports which showed that one-half of the human liver tumors examined from China and Southern Africa had a hotspot guanine in thymine transversion mutation in the tumor suppressor gene, p53, at codon 249. Given previous experimental data showing that aflatoxin causes this type of mutation, the suggestion was raised that AFB1 could be an etiological agent for these tumors (19,20). Recent work from our laboratory has shown that aflatoxin-epoxide can bind to this particular codon 249 of p53 in a plasmid, providing further indirect evidence for a putative role of aflatoxin exposure in p53 mutagenesis (21). The biological importance of mutant p53 is being suggested for a wide number of human tumors (22), and the molecular dosimetry methods described in this paper for AFB-N7-guanine in urine may become useful in epidemiological investigations to examine the role of this gene in liver cancer.

The damage of DNA aflatoxin is only one of many potential factors involved in the etiopathogenesis of liver cancer. Many other factors, including, for example, recurrent cytotoxicity, cell proliferation, and nutritional status could strongly influence the disease process. Thus, molecular epidemiological investigations that examine only one biomarker may greatly under- or overestimate the risk for an individual.

In our experimental studies, we have found the need to measure specific, biologically relevant metabolites in a urine sample. This is illustrated by the data shown in Figure 10, which demonstrate that the levels of the daily urinary excretion of total aflatoxin metabolites are unrelated to risk of aflatoxin-induced disease. Similar analyses using the human urine samples from China also revealed that total aflatoxin metabolites in urine do not reflect appropriate exposure assessments. In the rat model it is a minor, but biologically relevant metabolite, AFB-N7-guanine, representing less than 1% of the excreted material in urine,
which does reflect exposure in experimental models. Thus, the composite human and experimental data generated from our research indicates that a cautious interpretation of the relationship between any biomarker and tumor outcome must be employed. In a recent report by Campbell et al. (23), no association between composite aflatoxin metabolites in human urine and liver cancer disease rates was found for the People’s Republic of China, which is consistent with our finding that these two outcomes are not biologically related.

A significant finding of all of these studies was the dose-dependent relationship between aflatoxin exposure and the excretion of the major DNA adduct, AFN-7-guanine, in urine. Using the data obtained from China, about 0.2% of the AFN 1 dose was excreted as AFN-7-guanine in urine. This value is similar to excretion of AFN-7-guanine in dosed rats, where 0.6% of AFN 1 is found as the DNA adduct in urine. Thus, work in the rat models appears to yield similar excretion patterns compared to humans.

Finally, the rapidly excreted AFN-7-guanine adduct only reflects relatively recent exposures to AFN 1. Temporal relationships between DNA damage and long-term risk from exposure in humans remains to be established. This potential association will be examined in future studies, in conjunction with other biomarkers that give an integration of exposure over a number of weeks such as aflatoxin-albumin adducts in serum, and will eventually be tested directly through prospective epidemiological investigations.

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