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Molecular Dosimetry of Urinary Aflatoxin-DNA Adducts in People Living in Guangxi Autonomous Region, People's Republic of China[1]

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ABSTRACT

Hepatocellular carcinoma is one of the five leading human cancers causing at least 250,000 deaths each year. One of the major risk factors for this disease is exposure to dietary aflatoxins, and the development of appropriate molecular dosimetry biomarkers would facilitate the identification of individuals at risk. This study was undertaken to explore the relationship between dietary intake of aflatoxins and the excretion of the major aflatoxin-DNA adduct and other metabolites into the urine of chronically exposed people. The following protocol was developed for this investigation in Guangxi Autonomous Region, People's Republic of China, where the diets of 30 males and 12 females (ages, 25–64 years) were monitored for 1 week and aflatoxin intake levels determined each day. Starting on the fourth day, total urine volumes were obtained in consecutive 12-h fractions for 3 or 4 days. High performance liquid chromatography and competitive radioimmunoassay analyses were done on each of the urine samples, and the relationships between excretion of total aflatoxin metabolites, aflatoxin-N^7-guanine, aflatoxin M_1, aflatoxin P_1, and aflatoxin B_1, and aflatoxin B_1 intake values were determined. The average intake of aflatoxin B_1 by men was 48.4 μg/day, giving a total mean exposure during the study period of 276.8 μg. The average daily intake by women was 77.4 μg/day, resulting in a total average exposure during the 7-day period of 542.6 μg aflatoxin B_1. Initial efforts to characterize aflatoxin metabolites in urine samples were with an analysis by competitive radioimmunoassay. The analysis by linear regression of the association between aflatoxin B_1 intake/day and total aflatoxin metabolite excretion/day showed a correlation coefficient of only 0.26. These findings stimulated the immunoaffinity/analytical high performance liquid chromatography analysis for individual metabolites. When the data were analyzed by linear regression analysis, the aflatoxin N^7-guanine excretion and aflatoxin B_1 intake from the previous day showed a correlation coefficient of 0.65 and $P < 0.000001$. Similar analysis for aflatoxin M_1 resulted in a correlation coefficient of 0.55 and $P < 0.00001$, whereas there was no positive statistical association between exposure in the diet and aflatoxin P_1 excretion, despite aflatoxin P_1 being quantitatively a major metabolite. Analysis of the total aflatoxin-N^7-guanine excretion in the urine during the complete collection period plotted against the total aflatoxin B_1 exposure in the diet for each of the individuals, smoothing the day to day variations, revealed a correlation coefficient of 0.80 and $P < 0.0000001$. Given this analysis, it is clear 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. These findings support the concept that quantitation of the aflatoxin-N^7-guanine adduct in urine is a good biomarker for aflatoxin B_1 exposures.

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INTRODUCTION

Primary liver cancer causes at least 250,000 deaths annually worldwide, and prevention measures to limit both exposure to and the adverse health effects from two major risk factors, aflatoxins and HBV[3], are important to lower the incidence of this nearly always fatal disease. The association between AFB_1 exposure and HBV status and human liver cancer has been established through epidemiological studies (1–3), although there is controversy about the relative importance of aflatoxins in this disease (4). While these epidemiological investigations have shown populations at risk, the molecular dosimetry field has developed in response to the limitations of traditional epidemiological methods of inquiry to assess the exposure status of an individual to an environmental chemical carcinogen. These evolving molecular dosimetry methods will help to identify individuals at high risk when multiple etiological agents exist for a human cancer, such as hepatocellular carcinoma.

Recent work in molecular dosimetry of aflatoxins has focused on methods for DNA and protein adduct analysis and is reviewed by Groopman *et al.* (5). AFB_1 is metabolized by cytochrome P-450 enzymes producing an unstable, highly reactive 8,9-epoxide metabolite which can covalently interact with many nucleophilic centers in cellular macromolecules, such as DNA and serum albumin. The two major macromolecular adducts identified are the AFB-N^7-gua adduct in DNA (6) and the lysine adduct in serum albumin (7), respectively. Detoxification of AFB_1 is accomplished by the enzymatic conjugation of oxidized metabolites to form conjugates which are excreted (8). In addition, the enzyme-catalyzed reaction of the 8,9-epoxide metabolite with glutathione occurs (9). Thus, a major factor for assessing the biological hazard to a cell or organism from exposure to AFB_1 is the integrated balance between the activation and detoxication reaction pathways.

The possible role for AFB-N^7-gua in the cancer initiation process provides the justification to study this agent as a molecular dosimeter in humans. Other molecular dosimetry methods for measuring aflatoxins have been developed using serum albumin adduct formation (10, 11). The data from the limited number of molecular dosimetry studies suggest that these methods are going to be more reliable than dietary surveys to assign exposure and risk for aflatoxins. It is well known that dietary surveys are difficult to perform and have high statistical coefficients of variation in analysis (12). Thus, the use of both DNA and protein adduct measurements is both mechanistically justified and in practical terms will be more feasible in population studies.

In this paper, we present the characterization of the AFB-N^7-gua adduct excretion and other aflatoxin metabolites in human urine and their relationship to dietary intake of AFB_1 The

[3] The abbreviations used are: HBV, hepatitis B virus; AFB_1, aflatoxin B_1; AFM_1, aflatoxin M_1; AFQ_1, aflatoxin Q_1; AFP_1, aflatoxin P_1; AFB-N^7-gua, aflatoxin-N^7-guanine; RIA, radioimmunoassay; HPLC, high performance liquid chromatography; AU, absorbance units.

population studied resides in Fusui county, Guangxi Autonomous Region, People's Republic of China, a region with a high incidence of liver cancer. These same individuals have also been assessed for aflatoxin-albumin adduct formation and AFM_1 in urine, results of which have been reported in other publications (10, 13). The molecular dosimetry studies described in this paper support the concept that measurement of the major, rapidly excised AFB-N^7-gua adduct in urine is a reliable dosimeter for estimating exposure status and possibly risk in individuals consuming this mycotoxin.

MATERIALS AND METHODS

Analysis of Aflatoxin Contents in Foods. Dietary exposure to AFB_1 from corn and peanut oil was measured. The vast majority of exposure was from the consumption of 350–500 g of corn/day. The aflatoxin levels in the corn samples were quantitated by thin layer chromatography following solvent extraction. Briefly, to analyze the corn samples, 20 g of corn were ground to a fine powder and extracted with 3 volumes of 55% methanol:water using vigorous shaking. The extract was filtered using paper filters, and the filtrate containing the aflatoxins was extracted into chloroform. The chloroform was evaporated to dryness under reduced pressure. The dried sample was redissolved in benzene:acetonitrile (98:2, v:v), and an aliquot was chromatographed using a silica thin layer chromatography system as described by the Association of Official Analytical Chemists (14). The aflatoxins were quantitated against authentic standards. In all corn samples, only AFB_1 and AFB_2 were detected, and the levels of AFB_2 were never greater than 10% of the total amount.

Human Sample Collection Design. A total of 42 people, 30 adult males and 12 adult females, were selected for this study. The age range of the subjects was 25 to 64 years, and the mean age of the male and female subjects was 46.6 and 44.8 years, respectively. The urine and food samples from men were collected from September 17 to 24, 1985, and the samples from women were obtained from October 5 to 13. Food samples and meal weights were ascertained and recorded each day during the collection period. Starting on the fourth day of the protocol period, 6–8 sequential 12-h (5 a.m. to 5 p.m. and 5 p.m. to 5 a.m.) urine collections were made. The total volume of urine was recorded, and then the urine was frozen and sent to Beijing for further processing. In total, there were 252 twelve-h urine samples collected.

Human Urine Analysis. After the human urine was collected, two aliquots of 25 ml each were loaded onto individual C18 Sep-Pak cartridges (Waters Associates, Milford, MA), which had been prepared by the sequential washing of 5 ml each of 5% methanol:water, 80% methanol:water, 100% methanol, and 5% methanol:water. The urine was applied to the Sep-Pak cartridge at a flow rate of 2–3 ml/min, and the effluent was discarded. The Sep-Pak cartridge was sequentially washed with 10 ml of 5% methanol:water and 10% methanol:water, the Sep-Pak cartridge was removed from the syringe, and the ends were sealed with Parafilm. The prepared Sep-Paks were shipped to Beijing and then placed on dry-ice and sent to the United States for analysis.

Prior to the elution of the aflatoxins from the Sep-Pak cartridge, the column was rewetted by using 5 ml 5% methanol:water. The aflatoxin metabolites were eluted from the column using 10 ml 80% methanol:water. The 80% methanol:water fraction was rotary evaporated to dryness under reduced pressure and reconstituted by using 0.3 ml 0.1 N HCl with heating at 50°C for 10 min. This ensured that the relatively insoluble aflatoxin-DNA adducts were in solution. The sample was cooled to room temperature, and 0.5 ml 1 M ammonium formate, pH 4.5, was added. The volume was adjusted to 10 ml with water and the sample applied to a 4-ml preparative monoclonal antibody affinity column at a flow rate of about 0.3 ml/min, as previously described (15, 16).

Analytical HPLC of Human Urine. Analytical reverse phase HPLC analysis used, in series, a Beckman model 160 fixed wavelength detector set at 365 nm, 0.001 AUFS, and a Hewlett-Packard model 1040A diode array detector to quantify aflatoxin metabolites in the immunoaffinity-purified human urine samples. The HPLC column was a C_{18} 5-μm, 25-cm Ultrasphere analytical column (Rainin Instruments Co., Woburn, MA), and chromatographic separation was obtained by elution for 20 min with 13% ethanol, followed by a 13–25% ethanol linear gradient generated over 25 min and then isocratic elution with 25% ethanol. All mobile phases were buffered with 0.01 M triethylammonium formate, pH 3.0, and the column temperature was maintained at 35°C. The flow rate was 1 ml/min. Authentic aflatoxin standards were used to determine chromatographic retention times.

Competitive RIA. Competitive RIAs were performed on the 12-h urine samples following cleanup by C_{18} Sep-Pak cartridge and preparative monoclonal antibody affinity chromatography. The samples used for competitive RIA were aliquots of the 100-μl HPLC samples described above. In general, 10 μl of this sample was taken and a serial 2-fold was dilution made. These samples were then quantified using a radioimmunoassay as previously described (15, 16). Five control human urine samples obtained from laboratory personnel were used as negative controls in these assays.

Statistical Analysis of Data: Box and Whisker Plots. Initial graphical data analysis was done by box and whisker plot analysis. The box and whisker plot represents a description of the data showing the 10th and 90th percentile of data (ends of the whiskers), the 25th and 75th percentile (ends of the box), and a line for the 50th percentile (line within the box). Any outlier points were depicted as individual values. This type of data analysis reveals useful distribution and outlier characteristics of the data set, which can be lost or suppressed if the information were calculated using standard mean and SE analysis. Other data analyses were done by standard linear regression methods.

RESULTS

Aflatoxin Exposure in Study Population. A preliminary report describing the application of preparative monoclonal antibody affinity chromatography to the cleanup of human urine from Fusui county, Guangxi Autonomous Region, People's Republic of China, for HPLC analysis (16, 17) demonstrated that AFB-N^7-gua and several oxidative metabolites could be quantitated in urine samples. The initial study consisted of 20 urine samples collected on a single day, and dietary AFB_1 intake values were only available for the day prior to the urine collection. To facilitate a better understanding of the relationship between exposure and excretion of AFB_1 and its metabolites in chronically exposed people, the following protocol was developed for an investigation in Guangxi Autonomous Region, People's Republic of China. The diets of 30 males and 12 females (ages, 25 to 64 years) were monitored for 1 week, and aflatoxin intake levels were determined each day. Starting on the fourth day, all excreted urine was obtained in consecutive 12-h fractions for 3 or 4 days. HPLC and competitive radioimmunoassay analyses were done on each of the urine samples, and the relationships between excretion of total aflatoxin metabolites, AFB-N^7-gua, AFM_1, AFP_1, and AFB_1, and AFB_1 intake values were determined.

Corn (daily intake, 350–500 g) was the primary dietary source of AFB_1 exposure for the subjects in this study. The box and whisker plot analyses for AFB_1 intakes for the males and females are depicted in Fig. 1. This investigation was timed for a period of the year when AFB_1 levels were historically high. The average intake of AFB_1 by men was 48.4 μg/day, giving a total mean exposure during the study period of 276.8 μg. The average daily intake by women was 77.4 μg/day, resulting in a total average exposure during the 7-day period of 542.6 μg AFB_1. The maximum and minimum values for the male subjects during the 1-week collection was 963.9 and 56.7 μg, respec-

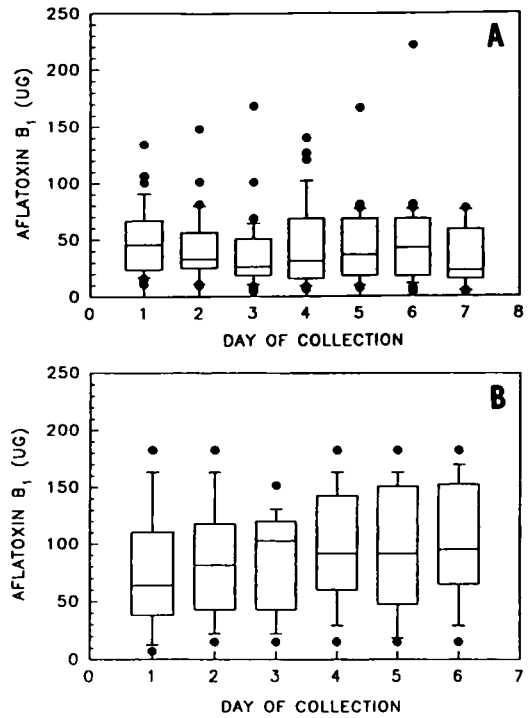

Fig. 1. Total dietary intake of aflatoxins during the 6- to 7-day study period. Each day is depicted by a box and whisker plot analysis. The box and whisker plot represents a description of the data showing the 10th and 90th percentiles of data (ends of the whiskers), the 25th and 75th percentiles (ends of the box), and a line for the 50th percentile (*line within the box*). Any outlier points were depicted as individual values. *A*, males; *B*, females.

tively. The maximum and minimum dietary exposures for the female subjects during the study period was 1035 and 90 μg AFB_1, respectively. As shown in Fig. 1, there was day-to-day variation for individual exposures, but the daily mean levels were fairly constant. The highest single daily exposure recorded in this study was 221.6 μg AFB_1 and the lowest was 3.8 μg AFB_1. Thus, even the lowest reported value in this study was comparable to high levels described in other human aflatoxin investigations (18). Finally, none of the male or female outlier samples from a given day were repeated on any other day during the collection.

Initial Analysis of the Urine Samples by Competitive RIA. Initial efforts to characterize aflatoxin metabolites in urine samples used an analysis by competitive RIA (16, 17). The monoclonal antibody used recognizes AFB_1, AFP_1, and AFM_1 with equal affinity and cross-reacts with the major aflatoxin-DNA adducts with 5- to 10-fold less affinity. *In vitro* spiking experiments with human urine samples indicated that the affinity differential for the DNA adducts did not pose significant problems for total aflatoxin metabolite quantitation, because the DNA adducts were minor constituents in the sample. In all experiments reported in this paper, the standard curve used for the RIA analysis was generated with AFB_1, and all values are reported in AFB_1 equivalents.

The concentrations of AFB_1 equivalents/ml urine for all samples obtained during the consecutive 12-h collections are depicted in Fig. 2. In all samples, outlier points were found for each of the collection time periods. The average concentration for all of the collections ranged from 1.5 to 2.3 ng AFB_1 equivalents/ml. The most extreme point was the 25 ng AFB_1/ml sample; however, most of the outlier points were in the 4- to 6-ng AFB_1/ml range. These initial findings indicated that aflatoxin metabolites were present in these urine samples and this encouraged further investigation.

The next step in the analysis was to convert the concentration data into total aflatoxin excretion/day. The range of urinary output was 500–4500 ml, and the average daily urinary volume for all subjects was 2388 ± 115 ml (mean ± SE). This value appears to be high, but given that all of the subjects were field workers, daily consumption of water would be expected to be high. When the total aflatoxin excretion for each 12-h sample period was calculated by multiplying the urine volume with the concentration of aflatoxin determined by competitive RIA, the data shown in Fig. 3 were generated. The average total aflatoxin metabolite excretion ranged from 3.3 to 6.6 μg aflatoxin metabolites/day. The average total aflatoxin metabolites in the urine during the collection period was 12.98 μg for men and 13.09 μg for women. Thus, a crude estimate of the average percentage of aflatoxin metabolite excretion during the 3-day urine collection period from the food intake data for the entire 6- to 7-day analysis was 4.7 and 2.4% for the male and female subjects, respectively. If the food intake values for the 3 or 4 days of urine collection were used to calculate the percentage of intake excreted in urine, values of 7.6 and 4.4% were derived for the male and female samples, respectively.

Results of the analysis by linear regression of the association between AFB_1 intake/day and total aflatoxin metabolite excretion/day are shown in Fig. 4. A regression line is drawn in the graph; however, the correlation coefficient of 0.26 only reaches a statistical significance level of 0.10. Thus, this analysis does not lead to the conclusion that total aflatoxin metabolite excretion into the urine, as measured by this composite competitive

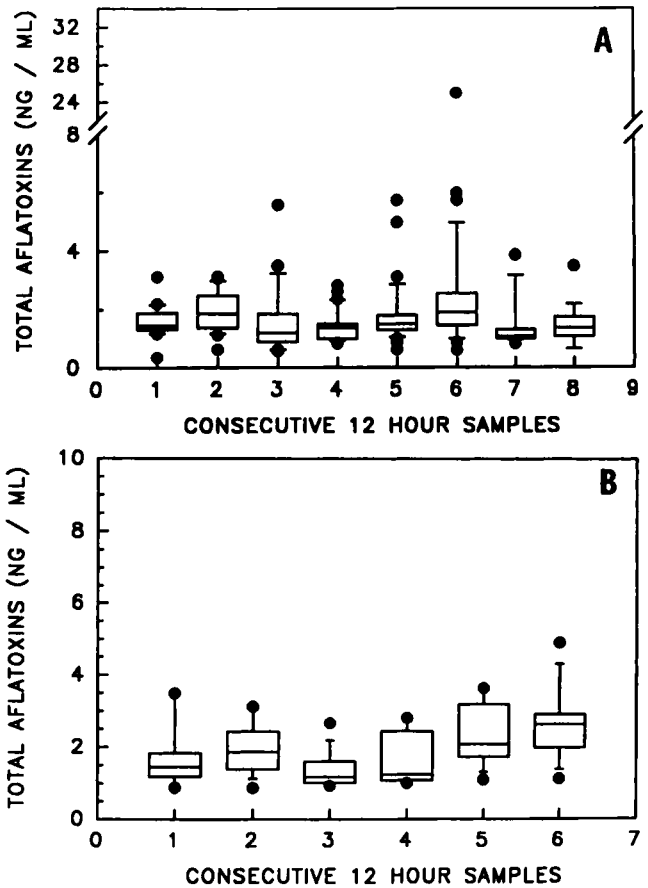

Fig. 2. Concentration of total aflatoxin metabolites in the urine samples depicted by box and whisker plot analysis. *A*, males; *B*, females.

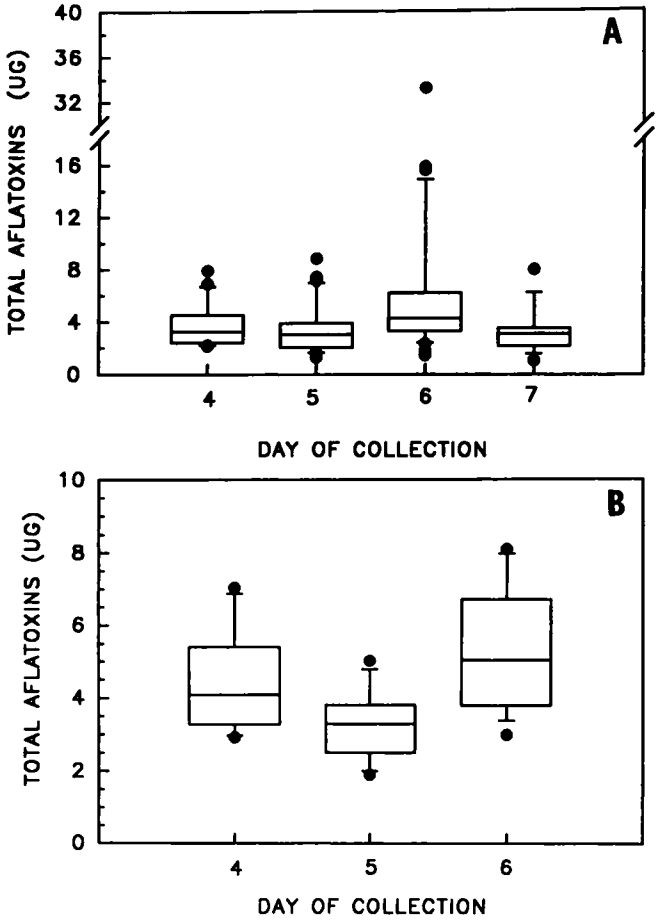

Fig. 3. Total aflatoxin metabolites excreted in urine depicted by box and whisker plot analysis. *A*, males; *B*, females.

radioimmunoassay, was an appropriate dosimeter measurement for exposure status. Because of this information, we performed the analytical HPLC analysis of individual urine samples to determine values for AFM_1, AFP_1, and the AFB-N^7-gua.

HPLC Separation of Aflatoxin Metabolites in Human Urine. The lack of a strong positive association between the total aflatoxin metabolite levels in the urine and the intake of AFB_1 in the diet indicated that a more detailed examination of the individual metabolites was required to reveal whether any aflatoxin derivative was an appropriate biomarker for exposure and potential risk assessment. In particular, AFB-N^7-gua was of interest because animal model work indicated that this rapidly repaired metabolite from DNA was exclusively excreted into urine (19). Since no antibody had the specificity to uniquely recognize only one of the aflatoxin metabolites, a separation method using HPLC was required to resolve the individual aflatoxin components in urine. Two separate 25-ml samples from each urine specimen were analyzed by the combined preparative monoclonal antibody affinity chromatography/HPLC method, and approximately 550 individual analytical HPLCs were run. To assure the validity of the method, four separate control human urine samples were spiked with 50 ng of AFB-N^7-gua, AFM_1, AFQ_1, and AFB_1. These samples were individually applied to the preparative monoclonal antibody column and then analyzed by analytical HPLC (data not shown). The recovery of each aflatoxin was >90% in these standards.

Four representative HPLCs following the preparative monoclonal antibody affinity procedure are depicted in Fig. 5. These chromatograms were selected because they illustrate the diversity of metabolite profiles obtained from the samples. Despite the high exposures of aflatoxins from the diet, and because of the large urine volumes in the samples, <100 ng (320 pmol) of total aflatoxins were generally injected on the HPLC. Most of the aflatoxin metabolite peaks were <3 mAU at 362 nm. AFB_1 and three oxidative metabolites, AFB-N^7-gua, AFM_1, and AFP_1, are depicted in Fig. 5*A*. Fig. 5*B* shows a urine profile where AFM_1 was the only significant peak detected. In Fig. 5*C*, AFB_1 was not detected, but AFQ_1, another hydroxylated metabolite, was found. Finally, Fig. 5*D* illustrates a urine sample obtained from the individual who had the highest aflatoxin concentration determined by competitive RIA (see Figs. 2*A* and 3*A*). In this profile, the major aflatoxin in the urine was AFB_1, although some AFM_1 was detected. In each of the panels, a UV-visible spectrum obtained from the diode array detector system is displayed in the upper left quadrant of the chromatogram; however, the overall quality of the spectra was diminished when <1.5 mAU was present at the absorbance maxima. Following HPLC separation, each of the aflatoxin peaks were integrated, and the amount of aflatoxin metabolite was quantified against calibration curves generated using authentic standards for each of the known metabolites and parent compound.

Analysis of AFB-N^7-Gua, AFM_1, AFP_1, and AFB_1 in Urine Samples. AFB-N^7-gua, AFM_1, AFP_1, and AFB_1 were the aflatoxins most commonly detected and quantified in these urine samples. In rare samples, AFQ_1 was also found. In Fig. 6 are the linear regression analyses of these individual aflatoxins. The intake data used in this analysis represent the AFB_1 exposure from the day prior to the specific urine collection; in a later analysis in this report a comparison of the total intake and AFB-N^7-gua excretion will be shown. The convention of using the intake from the prior day was adopted because the data obtained from previous experiments in rats showed that significant excretion of aflatoxin metabolites occurs in the first 24 h following exposure (20).

The correspondence between AFB-N^7-gua excretion and intake is shown in Fig. 6*A*. In this analysis of the AFB-N^7-gua excretion and AFB_1 intake from the previous day, a correlation coefficient of 0.65 with $P < 0.000001$ was found. Analysis for AFM_1 produced a correlation coefficient of 0.55 and $P < 0.00001$ (Fig. 6*B*). The AFP_1 excretion response is shown in Fig. 6*C*. There was no positive statistical association between

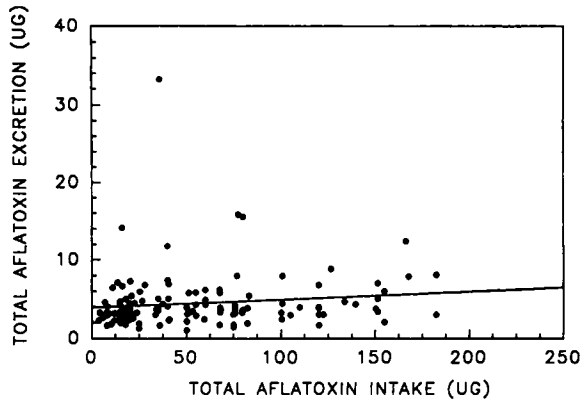

Fig. 4. Linear regression analysis of the total aflatoxins in urine of males and females measured by a monoclonal antibody-based radioimmunoassay compared with dietary aflatoxin intake/day.

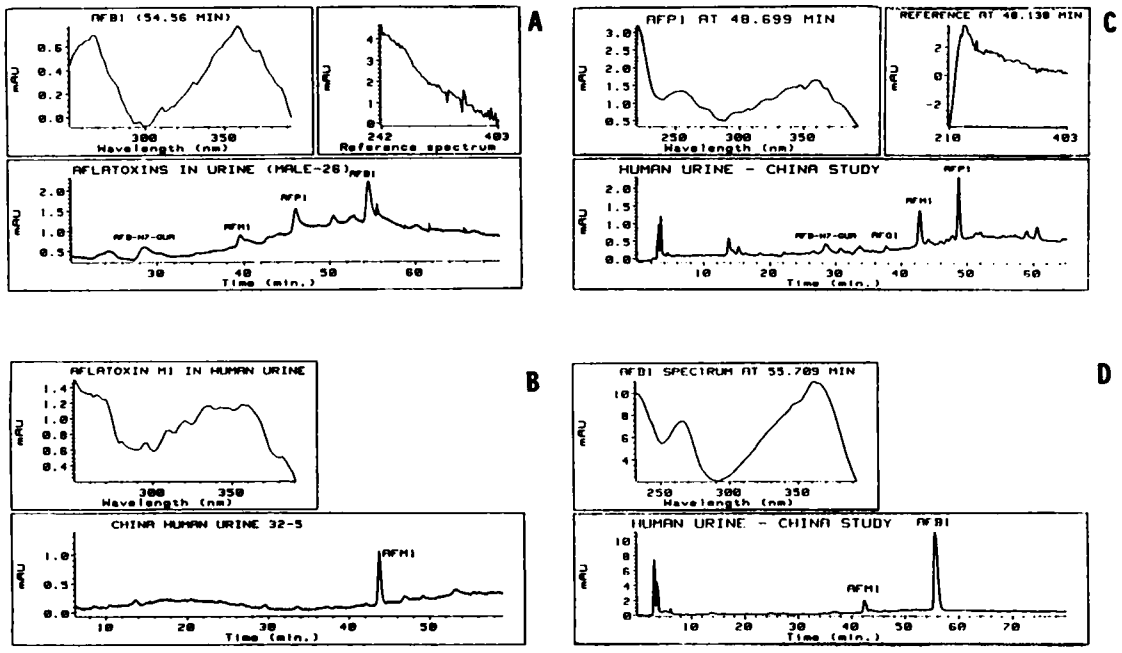

Fig. 5. Representative HPLC profiles showing different patterns of aflatoxins from human urine samples preparatively purified using a monoclonal antibody immunoaffinity column as described in "Materials and Methods."

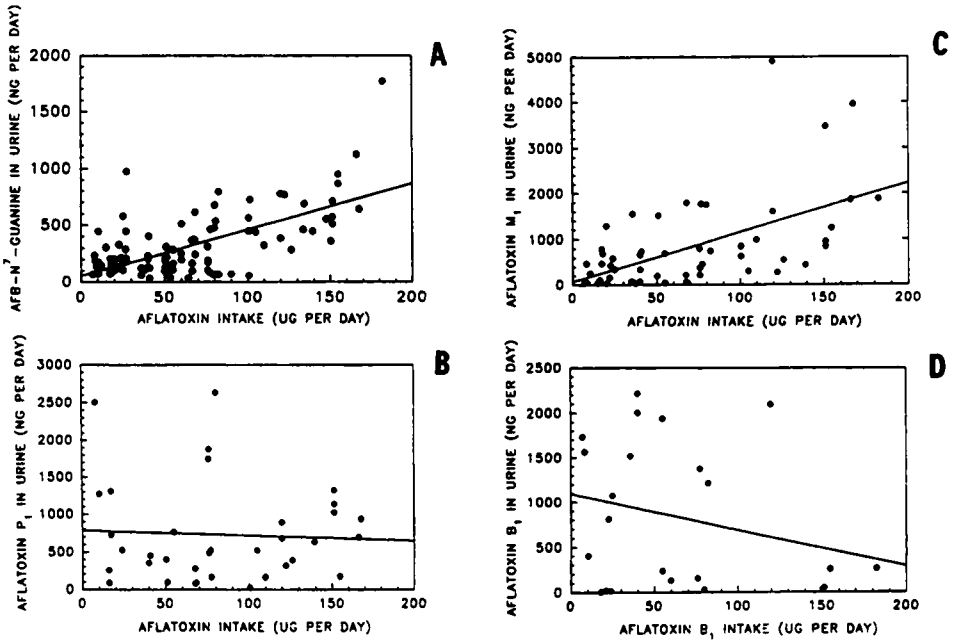

Fig. 6. Linear regression analysis of daily (*A*) aflatoxin N^7-guanine, (*B*) AFM_1, (*C*) AFP_1, and (*D*) AFB_1 in urine compared with dietary aflatoxin intake from the previous day.

exposure in the diet and AFP_1 excretion; however, the levels of AFP_1 measured in the 34 positive urine samples greatly contributed to the overall level of aflatoxin metabolite content detected by competitive RIA (see Fig. 4). In a similar manner, the 22 positive AFB_1 samples (Fig. 6*D*) are at levels which also affect the dose-response curve shown in Fig. 4. Taken together, it is the dissociation of the total aflatoxin metabolite content into individual metabolites that permits the association of AFB-N^7-gua and AFM_1 excretion as biomarkers of exposure to be determined.

Excretion of AFB-N^7-Gua as a Biomarker of Exposure. One of the objectives 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 reflecting a biologically effective dose of AFB_1 in people. Fig. 7 shows total AFB-N^7-gua excretion in the urine of the male and female subjects during the complete urine collection period plotted against the total AFB_1 exposure in the diet for each of the individuals. This analysis smooths the day-to-day variations in both intake and excretion of AFB-N^7-gua and reveals a correlation coefficient of 0.80 and $P < 0.0000001$. This analysis clearly shows 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. 6*A*). These findings support the concept that quantitation of the AFB-N^7-gua adduct in urine is a reliable biomarker for AFB_1 exposures.

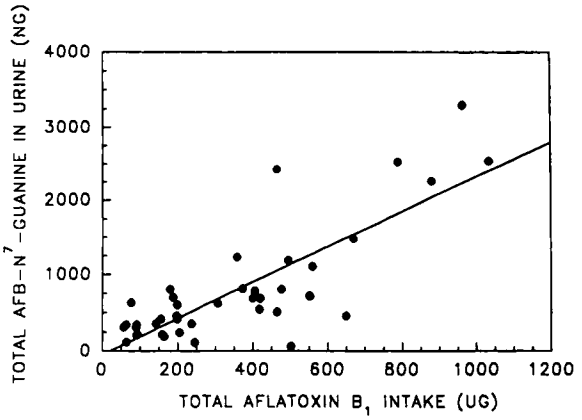

Fig. 7. Linear regression analysis of the association between total AFB-N^7-gua adduct excretion in urine from males and females and total dietary aflatoxin exposure during the study period.

DISCUSSION

Hepatocellular carcinoma is a nearly always fatal disease causing at least 250,000 deaths each year. The vast majority of these cancers occur in regions of Asia and Africa, where risk factors such as dietary aflatoxins and HBV infection are common. Prevention strategies for this disease offer the best hope for eventually lowering the incidence of liver cancer, and in recent years HBV vaccination programs have been started. HBV vaccination is presumably effective only when given to an uninfected person, and since this virus commonly infects people before the age of 2 years, many years will be required before vaccinations of individuals would be expected to result in lower cancer rates. Thus, if exposure to dietary aflatoxins can be lowered, then disease incidence may be lowered sooner.

One of the steps in the process of lowering aflatoxin exposure is the development of molecular dosimetry markers to assess biologically effective dosages in people. This field is rapidly expanding and the goal of these measurements is the assignment of risk to an individual from an exposure. In addition, the identification of high risk groups should permit the allocation of resources for prevention strategies. A number of molecular dosimetry markers of aflatoxin exposures have been investigated, and many studies have focused on the DNA and protein adducts formed by this chemical carcinogen (5). In relatively few studies has there been a systematic evaluation of these markers from the same individuals, together with a study of the relationships of these markers with dietary intakes of the carcinogen. This has been accomplished in this study population in Guangxi Autonomous Region for AFM_1 (13), in aflatoxin serum albumin adduct formation (10), and in the work reported here concerning AFB-N^7-gua excretion in urine.

The population in Fusui county, Guangxi Autonomous Region, has been actively investigated with respect to liver cancer risk factors. Yeh *et al.*(3) reported the roles of HBV and AFB_1 in the development of liver cancer. A cohort of men, aged 25 to 64 years, was studied, and 149 deaths were observed, 76 of which were due to liver cancer. Ninety-one % of liver cancer patients were hepatitis B surface antigen positive in contrast to 23% of all members of the study cohort. It was observed that a 3.5-fold difference in liver cancer mortality existed by place of residence, and when estimated AFB_1 levels in the subpopulations were plotted against the corresponding mortality rates of liver cancer, a positive and almost perfectly linear relationship was found. In contrast, no significant association was observed when the prevalence of hepatitis B surface antigen positivity in the subpopulations was compared with the corresponding rates of liver cancer deaths. These data suggest that aflatoxin exposure may be important in this population and that better measures of exposure will be critical for this risk assessment.

In contrast to the findings reported above, Campbell *et al.* (4) recently reported the nonassociation of aflatoxin exposure with primary liver cancer in the People's Republic of China. This study was a cross-sectional ecological survey of 48 different sites throughout the country. Urine samples were obtained from the people being surveyed, and pooled urine samples from each of the survey sites were analyzed for aflatoxin metabolite levels. The urine samples were analyzed by the same monoclonal antibody and competitive RIA described in this paper. This antibody cross-reacts with a large number of aflatoxins, and the data reported in this paper reveal no statistical association between total aflatoxin metabolite content in the urine and dietary exposure. In addition, HPLC analysis of the urine samples described herein showed that AFP_1 was a major metabolite but that the urinary concentration of this metabolite was not dose related to exposure. Since the monoclonal antibody used in the RIA had a high recognition for AFP_1, this may be the major factor in the lack of association between exposure and urine excretion. These findings illustrate the problems associated with measuring a sample by immunoassay in which multiple compounds recognized by the antibody are present. The lack of linearity in the excretion of this metabolite may be explained by use of the glucuronidation pathway of this aflatoxin metabolite. It is known that the glucurondiation pathway can become saturated, and at the two low doses of AFB_1, the AFP_1 may be predominately a glucuronide that is excreted in the bile (21). Only at the high dose, when saturation of this pathway has occurred, is the AFP_1 excreted as an unconjugated compound in urine. Thus, it is possible that an abundant aflatoxin metabolite, such as AFP_1, unrelated to the biological activity of AFB_1 contributed to the nonassociation of aflatoxin exposures and liver cancer in the Campbell *et al.* study (4). Further investigations using the aflatoxin-specific biomarkers will be required to identify biomarkers with the requisite specificity for risk assessments. These biomarkers will undoubtedly include short- and long-term exposure markers such as the DNA and albumin adducts, as well as other markers reflecting genetic damage.

A significant finding of this study is the dose-dependent relationship between aflatoxin exposure and the excretion of AFB-N^7-gua in urine. Using these data, we determined that about 0.2% of the AFB_1 dose was excreted as AFB-N^7-gua in urine. This value is similar to excretion of AFB-N^7-gua in AFB_2 dosed rats in which 0.6% of the dose is found as the DNA adduct in urine (20). Thus, the rat model work appears to provide similar excretion patterns compared to humans. Previous research has shown that AFB-N^7-gua is rapidly excised and exclusively excreted into the urine of experimental animals (19). The AFB-N^7-gua is also stable to urinary pH and long-term storage conditions. The utility of using measurements of AFB-N^7-gua in urine as a marker of exposure has been suggested by our studies (16, 17) and the work of Autrup *et al.* in Kenya (22, 23). In the Kenya study, synchronous fluorescence spectroscopy and HPLC were used for the analysis of AFB-N^7-gua in urine. More than 1000 urine samples were analyzed, and 12.6% were positive for this DNA adduct. In contrast to the work reported here, these researchers did not have individual dietary surveys available for correlational analyses. Nonethe-

less, these studies show that AFB_1 is metabolized to the highly reactive 8,9-epoxide by human cytochrome P-450, resulting in DNA damage and adduct removal. While the rapidly excreted adduct only reflects relatively recent exposures to the parent compound, it is still not understood what the temporal relationship is between DNA damage and long-term risk from exposure in people. This will be examined in future studies and eventually verified through prospective epidemiological investigations.

Aflatoxin metabolites other than the DNA adducts have also been measured in human urine. Early investigations by Campbell *et al.* (24) used thin layer chromatography to detect the presence of AFM_1 in human urine of people living in the Philippines. Recently, the availability of monoclonal antibodies has permitted the development of an enzyme-linked immunosorbent assay method to measure AFM_1 in human urine (13). This research group analyzed the same samples described in this paper and found a good correlation ($r = 0.66$) between total dietary aflatoxin intake and total AFM_1 excretion in urine. This result is very similar to the data reported here for AFM_1. Zhu *et al.* (13) calculated that between 1.2 and 2.2% of dietary aflatoxin was present as AFM_1 in urine. Similar calculations of AFM_1 excretion into urine were reported by Nyathi *et al.* (25) in a population survey in Zimbabwe. More than 1200 urine samples collected from different areas of Zimbabwe were analyzed for aflatoxin contamination. The urine samples were extracted with chloroform and the resultant aflatoxins quantified by thin layer chromatography and high performance liquid chromatography. The most commonly observed contaminant was AFM_1, at an average concentration of 4.2 ng/ml of urine. Although the national average of urine samples contaminated was 4.3%, there were areas in which up to 10% of the urine samples were contaminated. Finally, Sun and Chu (26) used a monoclonal antibody affinity column and HPLC approach to analyze AFM_1 in human urine. All of these findings provide support for the use of aflatoxin metabolites in urine as a molecular dosimeter and also unequivocally demonstrate that people can metabolize AFB_1 to DNA-damaging species and a metabolite, AFM_1, which is also carcinogenic in experimental animals (27).

The predominant mutation induced by activated AFB_1 in both bacterial and mammalian species is the guanine to thymine transversion (28, 29). Thus, when one-half of the human liver tumors examined from China and Southern Africa were found to have a hotspot guanine to thymine transversion mutation in the tumor suppressor gene, p53, at codon 249, the suggestion was raised that AFB_1 could be the etiological agent (30, 31). This supposition is solely based upon the fact that these liver tumors were isolated from regions of the world with high aflatoxin exposure. The biological importance of mutant p53 is suggested for a large number of human tumors (32), and the molecular dosimetry methods described in this paper for AFB-N^7-gua in urine may become useful in epidemiological investigations to examine the role of mutant tumor suppressor genes and oncogenes in the liver cancer process.

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