Dietary Intake of Aflatoxins and the Level of Albumin-bound Aflatoxin in Peripheral Blood in The Gambia, West Africa[1]

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Abstract

Aflatoxin is implicated as a risk factor for hepatocellular carcinoma in areas of the world with a high incidence of this tumor. The present study was designed to validate the use of aflatoxin-albumin adducts in peripheral blood as a measure of individual exposure to this carcinogen. Dietary intake of aflatoxin was measured at the individual level in 20 residents of Keneba, West Kiang, The Gambia, over a 7-day period and correlated with the level of aflatoxin bound to peripheral blood albumin at the beginning and end of the study. Complementary enzyme-linked immunosorbent assay and high-performance liquid chromatography-fluorescence techniques were used to assay the aflatoxin adducts. All subjects were exposed to aflatoxin originating from several food types, with an average daily intake of 1.4 μg/day. A significant correlation ($r = 0.55$; $P = <0.05$) was observed between the dietary intake and the level of albumin-bound aflatoxin at the end of the study. In addition, a good agreement was obtained with the two analytical techniques. A comparison of matched chronic hepatitis B surface antigen carriers with noncarriers did not reveal any difference in adduct formation for a given dietary intake of aflatoxin. These studies demonstrate the validity of aflatoxin-albumin adducts as a marker of human exposure to this carcinogen.

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[3] The abbreviations used are: AF, aflatoxin(s); HBV, hepatitis B virus; MRC, Medical Research Council, Cambridge; HB_sAg, hepatitis B surface antigen; HPLC, high-performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay.

Introduction

Molecular dosimetry markers of chemical carcinogen exposure may contribute substantially to the sensitivity and specificity of epidemiological studies aimed at the elucidation of the etiopathogenesis of human cancer. However, in order to provide reliable quantitative data that can be interpreted usefully, a considerable amount of characterization and validation of the specific marker is required. In the case of AF[3] (food contaminants that are produced by fungi and are carcinogenic in various animal species) a number of methods have been developed, including the measurement of AF bound to DNA and to peripheral blood albumin, and of excreted urinary metabolites and/or nucleic acid adducts (1-3). Aflatoxin binding to hemoglobin and peripheral blood cell DNA *in vivo* is low compared to albumin binding (see Ref. 1), and therefore use of these potential biomarkers has not been pursued. The methods used to measure AF-N^7-guanine in urine (2, 4) and AF bound to albumin (5-9) have been successful, however, in generating a significant database on human exposure. These methods should be of value in elucidating the contribution of AF and its possible interaction with HBV infection (7) to the incidence of hepatocellular carcinoma. The epidemiological data, although clearly pointing to AFB_1 as an etiological agent in liver cancer (10), were criticized based on the indirect assessment of exposure to this carcinogen through food contamination (11), and more recently the role of AFB_1 has been questioned (12). The limitations of these criticisms have been further discussed (13).

The study presented here was designed to test whether the level of AF-albumin adduct in peripheral blood was correlated with dietary intake of AF in individuals from a population chronically exposed to high levels of AF. In addition, we examined whether any differences in adduct formation occurred for a given dietary exposure to AF in chronic HB_sAg carriers compared to noncarriers. The same individuals have been assessed with respect to urinary excretion of AF-N^7-guanine (4).

Materials and Methods

Study Population

The study population was from the village of Keneba in the West Kiang region of The Gambia. Subjects were identified from medical records at the MRC clinic in Keneba as being chronic HB_sAg carriers or noncarriers. This was defined from HB_sAg data obtained in 1970, 1980, 1984, and in October 1988, the time of the present study; carriers were HB_sAg-positive at least in the tests in 1980, 1984, and at the time of study. In addition to matching subjects for age and sex, an attempt was made to find HB_sAg carriers and noncarriers who were eating from the same food bowl (*sinkiro*), in order to match the

pairs as closely as possible for food intake of aflatoxin. This was possible for females who were wives of the same husband but was more difficult for males, because it is rare to have adult males sharing the same bowl. In total, 20 subjects were recruited, 10 males and 10 females, with 5 paired HB_sAg carriers and noncarriers among the females and 4 such pairs among the males. The other two subjects were brothers, both noncarriers but who shared the same food. In addition, five of the women were lactating during the study period. Details of subjects are presented in Table 1. The subjects were spread throughout the village of Keneba in 12 different compounds of the total of 70 to be found in the village at the time of the study. All subjects were of the Mandinka tribe.

In order to perform the sample collection, one field worker was assigned to each pair of subjects. All subjects gave informed consent to participate in the study as approved by the joint MRC/Gambian government ethical committee.

Food Collection

In brief, samples were obtained from each meal for each subject over a period of 7 days. In cases of shared food, normally only one set of food samples was collected. Sauces and staple (normally rice) were sampled separately from the bowl immediately prior to the subject's eating the food. Sauces were sampled by taking five separate spoonfuls from different parts of the bowl, giving a total wet sample weight of about 10 g, representing one-tenth of the total sauce portion. Subjects ate almost exclusively 2 meals/day (midday and evening), and a total of 400 food samples were collected throughout the study. No attempt was made to collect food eaten between meals.

Table 1 Subject profiles

Subject	Sex	Age	HB_sAg	Sinkiro no.	Compound
A	F	29	+	118	25
B	F	21	−	118	25
C	F	28	+	125	26
D	F	29	−	125	26
E	F	33	+	128	29
F	F	38	−	128	29
G	F	46	+	60	13
H	F	30	−	60	13
I	F	54	+	198	52
J	F	56	−	198	52
K	M	43	+	35	6
L	M	22	−	37	6
M	M	54	−	100	23
N	M	60	−	100	23
O	M	39	+	81	15
S	M	15	−	85	17
P	M	28	+	140	32
T	M	28	−	140	32
Q	M	16	+	189	46
R	M	16	−	171	37

Food Analysis

The analysis of aflatoxin content of food samples was performed by preparative immunoaffinity clean-up and both HPLC and fluorescence quantification. These methods are described in detail elsewhere.[4] The detection limit for aflatoxin B_1 was 0.1 ng by HPLC and 0.05 ng by fluorescence measurement. Thus, the limit of detection from a food sample was dependent upon the sample size available for analysis.

Blood Collection

Blood samples were taken on days 1 and 8 of the study by venipuncture, except in the case of one subject where this proved unacceptable and a fingerprick blood sample was obtained. Blood was collected into heparinized tubes and centrifuged to obtain plasma. A 1-ml sample was frozen immediately for determination of HB_sAg. The remainder of the plasma was heated to 56°C for 45 min to inactivate human immunodeficiency virus, and the plasma was then separated into 1-ml portions and stored frozen at −30°C until shipment to Lyon on solid CO_2. The pellets remaining after plasma separation were stored frozen.

Hepatitis B Virus Markers

Blood samples were tested for HB_sAg by reverse-passive hemagglutination (Wellcotest; Welcome Diagnostics).

Aflatoxin-Albumin Adduct Analysis

ELISA. The assay of aflatoxin-albumin adducts by ELISA was performed as described in detail elsewhere (6, 14). In brief, albumin was extracted from 0.5 ml of serum by precipitation, and a 2-mg sample was hydrolyzed with proteinase K overnight. Aflatoxin residues were purified on a Sep-Pak C18 cartridge and quantified by competitive ELISA against an AFB_1-lysine standard. The limit of detection in ELISA was 5 pg AFB_1-lysine equivalent/mg albumin.

HPLC-Fluorescence. The albumin samples were assayed blind in a separate laboratory for the presence of the AFB_1-lysine adduct by HPLC with fluorescence detection (ex, 405 nm; em. 470 nm). In this case albumin samples were hydrolyzed with pronase and subjected to immunoaffinity purification prior to HPLC analysis (6, 8). The detection limit in this assay was 20 fmol AFB_1-lysine.

Results

Food Intake. The dietary sampling was complete for all subjects except individual P, who left Keneba village for 24 h during the study. AF analyses were made in 91% of all the sauces collected in the study, with >90% coverage of 13 subjects and 68–86% coverage of the other 7 subjects. At the time of year in which the study was conducted the diet of the subjects consisted predominantly of either a leaf sauce (*kucha*), a flour sauce (*bukolo*), or a groundnut sauce (*tia durango*) with boiled rice

[4] G. J. Hudson *et al.*, unpublished data.

Table 2 Frequency of food contamination by aflatoxin

Food type (no.)	Level of aflatoxin (ppb)[a]						
	ND[b]	1-5	6-15	16-25	26-50	51-100	>100
Kucha (87)	31	3	34	9	5	4	1
Bukolo (22)	9	1	8	0	3	0	1
Groundnut (47)	6	16	11	1	4	3	6
Other[c] (30)	22	3	2	1	2	0	0

[a] Level present in lyophilized sample.
[b] ND, not detected (<1 ppb).
[c] Includes *nyankantango* (rice, chili, and fish), oil stews (groundnut oil), *benechin* (rice, *maggi* cube, chili, onion, tomato paste, groundnut oil), and *jambanduro* (leaves, *maggi* cube, chili, and salt).

as a staple. The new groundnut crop had not been harvested, and the stored supplies were generally low. Details of the typical rural Gambian diet have been published previously (15), and the details from the present study will be presented fully elsewhere.[4]

The source of aflatoxin contamination in this study was clearly not linked to one food type. One hundred and eighteen of 186 samples were positive for AF, with groundnuts being an ingredient in only 35% of the contaminated meals. The highest levels of AF tended to be found in the groundnut sauces (Table 2), although other ingredients cannot be ruled out as the source of AF. Rice was rarely found to contain detectable levels of AF in 30 samples analyzed and was therefore excluded from exposure calculations. The food intakes were calculated from weight measurements made on ready-to-eat foods and on the number of individuals eating from the same bowl. Average intakes of 100 g of sauce/60 kg of body weight were calculated. Where a subject was eating from two food bowls, calculations were based on an intake of 50 g/bowl. The average water content of these sauces is 62%. Daily intakes in these subjects ranged from nondetectable to 29.6 μg/day. Average measured daily exposure was 1.4 μg/day.

AF-Albumin Adducts: Day 1 and Day 8. The AF-albumin adducts were measured prior to food collection and after the 7-day collection period. Fig. 1 shows the correlation between the two time points for each individual as measured by ELISA. There was a good correlation between these two measurements with a correlation coefficient of 0.79 ($P < 0.001$). In one subject (R) the level of adduct increased by 8-fold, but for the other individuals the level of adduct was generally altered by less than a factor of 2. The albumin adduct levels were stable over the time period despite large variations in dietary aflatoxin intake as illustrated for subjects I and J in Fig. 2.

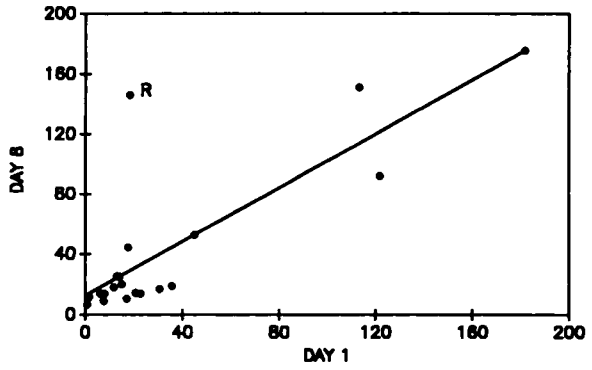

Fig. 1. Comparison of the levels of aflatoxin-albumin adducts in the same individuals on days 1 and 8 of the study. Each point represents one individual. Levels of adduct are expressed as pg AFB_1-lysine equivalent/mg albumin. The line represents linear regression through all the points. The individual *R* is clearly an outlier and is discussed in the text.

Of the 40 sera analyzed by ELISA, 34 samples were also analyzed blind in a separate laboratory by HPLC-fluorescence specifically for AFB_1-lysine (Fig. 3). There was a strong association between these two sets of data ($r = 0.97$; $P < 0.001$) with similar quantitative differences as have been found previously for the two approaches, due to differences in adduct recovery (Ref. 6, and see "Discussion"). No corrections for adduct recoveries are given here, however, because it is not known whether the recoveries from human samples are comparable to those established in rats. HPLC-fluorescence was less sensitive than ELISA in this series of experiments, where the same quantity of albumin was used for both assays (6).

Dietary Intake Correlated with AF-Albumin Adduct. The measured 7-day dietary intake of AF was compared with the AF-albumin adduct level on day 8 of the study and is presented in Fig. 4. There was a significant correlation between these two parameters (correlation coefficient = 0.55; $P < 0.05$), and this was a stronger association than between the same dietary data and the AF-albumin adduct level on day 1 of the study (data not shown). There were some individuals who were clearly outside this distribution, and this is addressed in the "Discussion."

There was no significant difference between paired HB_sAg carriers and noncarriers in the level of AF-albumin adduct expressed as a function of dietary AF intake (Fig. 4). Similarly, there was no difference between males and females in the adduct levels (Fig. 4).

Discussion

Dietary levels of total AF were measured by immunoaffinity chromatography and fluorescence detection (16); the average intake of 1.4 μg/person/day is relatively low and appears to be underestimated (see below). However, the time at which the study was conducted (October, at the end of the rainy season) has since been shown to be a period of lower AF exposure in another region of The Gambia (7). This may reflect the fact that the individuals studied were eating few groundnuts and no maize. The groundnuts that were being consumed were from the new harvest rather than stored crops and thus by defi-

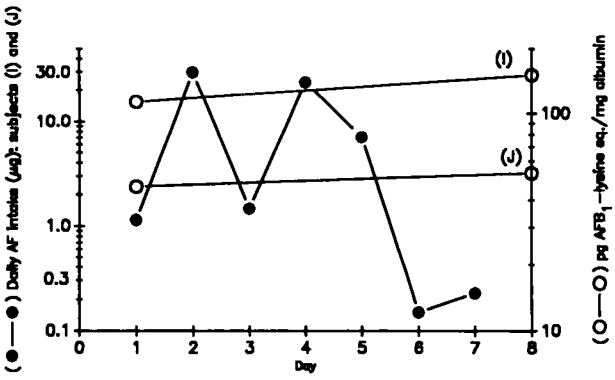

Fig. 2. The food intake of individuals I and J (see Table 1) over the 7-day period of the study is given (●) together with the aflatoxin-albumin adduct levels (O) found in these individuals. Food intake was from the same food bowl for the two subjects. The small estimated differences in quantity of food consumed (based on body weight of the individuals) is not presented in this figure, for clarity, but is included in all other data analyses.

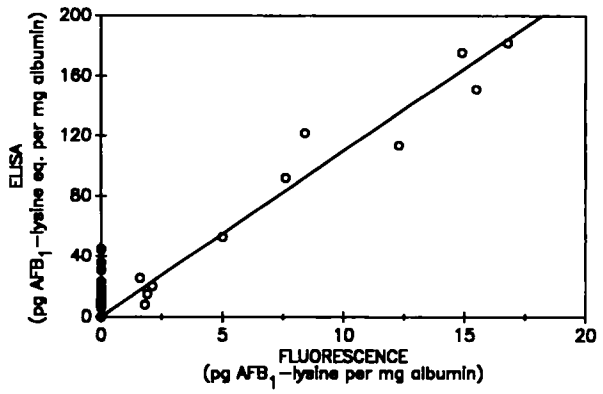

Fig. 3. Comparison of the level of aflatoxin-albumin adducts measured by ELISA and HPLC-fluorescence techniques. The *line through the points* represents the situation where the ELISA measure of "total" hydrolysate products is 11-fold higher than the specific measure of AFB_1-lysine by HPLC-fluorescence. An 11-fold difference was seen previously between the two methods (6). Comparison was made for 34 samples.

nition would be free from postharvest contamination. A population in The Gambia from whom samples were obtained in May and November showed considerably higher levels at the former time (end of dry season) when stored groundnuts would be consumed (7).

In some of the samples containing AF, the content of AFB_1, AFB_2, AFG_1, and AFG_2 was determined by HPLC with UV detection; between 25 and 50% of the aflatoxin content was AFG_1 (4). This contrasts with data from the People's Republic of China, where maize was the major source of AF contamination and AFG_1 was not detected (17). This may reflect different substrate specificities for the various *Aspergillus* strains producing AF.

The correlation between food intake of AF and albumin-bound AF (Fig. 4) is clear but is not as strong as the correlation with urinary AF nucleic acid adducts (4). Several factors probably contribute to the scatter of the points in Fig. 4. First, the imprecision of making individual dietary measures of AF intake (see also below) would affect the correlation with albumin adduct (Fig. 4) and urinary AF adducts (4). The field workers in this study were present at all meal preparations and sampled food directly from the subject's bowl, but AF intake from snacks could have contributed to the total intake. In addition, the contribution of AFG_1 to the total AF in the food was significant, but this part of the dietary AF intake would not contribute to the AF-albumin adduct level due to the apparent lack of metabolism of AFG_1 in these individuals (4). Second, the AF-albumin marker is a measure of exposure over the past 2 to 3 months, and therefore dietary intake of AF prior to the 7-day study period would contribute to the adduct level observed at the end of this study. Third, one of the reasons for using an individual marker of biologically effective dose is that individuals would be expected to show differences in the amount of carcinogen-macromolecular adduct formed for a given intake of carcinogen (18), and such interindividual variation would influence the correlation observed. This interindividual variation could be the result of variations in expression of cytochrome P450s or glutathione *S*-transferases involved in aflatoxin metabolism (19). The ELISA used to measure the AF-albumin adduct would not appear to be a significant source of error, given the strong association between the data obtained by the ELISA method and the HPLC-fluorescence approach (Fig. 3 and Ref. 6).

Another feature of the assay of AF-albumin adducts is the integration of variations in levels of dietary exposure to AF over a period of days. It is clear from Fig. 2 that the dietary intakes can vary by orders of magnitude from day to day, making meaningful estimations of individual intake virtually impossible based on dietary assessment. Studies over longer periods (months) will establish the stability of the albumin marker, given the half-life of 3–4 weeks of albumin in humans. It is clear that this approach is a more appropriate way to assess individual exposure to AF. The adduct reflects aflatoxin DNA adduct levels in the liver in rats (20), and this is at least partially because the adduct is formed in the hepatocyte, the site of albumin synthesis (21). In addition to the biological significance of the marker, it is also valuable because logistically it can easily be integrated into field studies (see Ref. 7).

As mentioned above, considerable difficulties are involved in obtaining quantitative AF dietary exposures at an individual level. In the present study we sampled cooked foods from the subjects' bowls, which eliminates many potentially confounding factors such as food selection during preparation, effects of cooking, quantities of ingredients used, etc. Despite this, the quantitative accuracy of AF food intake is difficult to ensure. This is reflected in the present study and in the companion paper (4) when quantitative comparisons are made of the dietary levels of AF and the AF-albumin adduct levels. Previous studies in experimental animals have demonstrated that 1 to 3% of an administered dose of AFB_1 is bound covalently to albumin (20, 22). Similarly, calculations made for Chinese subjects yielded a figure of 2 to 3% (5, 8). In the present study the average level of AF-albumin was 44 pg AFB_1-lysine/mg albumin, representing a 25% recovery of adduct (6). Assuming 40 mg albumin/ml plasma and 40 ml plasma/kg body weight and adjusting for adduct recovery, a 50-kg person (average weight of our study population) would have 14.08 μg AF bound

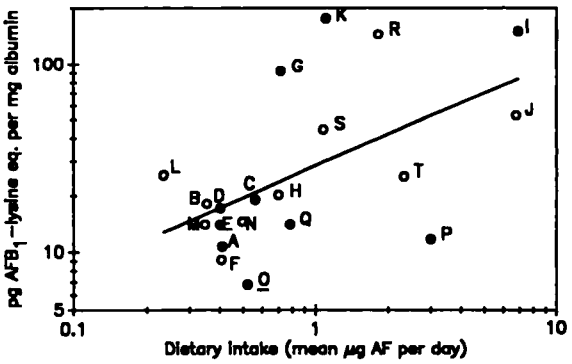

Fig. 4. Mean daily aflatoxin food intake over the 7-day period, plotted against the level of aflatoxin-albumin adduct on day 8 of the study. *Each point* represents one individual. ●, HB_sAg carriers; O, noncarriers. The *letters next to the points* represent the individuals in Table 1. Linear regression is plotted (correlation coefficient, $r = 0.55$; $P < 0.05$ on log-transformed values).

to albumin (4 × 44 × 40 × 40 × 50 pg). The half-life of albumin would give a 30-fold accumulation (22) under chronic exposure, and therefore the amount of AF due to 1 day's exposure is 469 ng. As the average daily intake of AF was measured as only 1.4 μg, this represents some 33.5% of the intake bound to albumin. This percentage would be further increased if the contribution of AFG_1 to the intake was subtracted, because AFG_1 is likely to contribute a negligible amount to the ELISA measurement (23), and no AFG_1-lysine adduct was observed, consistent with observations of the excretion of unmetabolized AFG_1 in these subjects (4). Thus, it appears that there is a discrepancy in the quantitative data for food intake of AF and albumin-bound AF.

In considering the above discrepancy, several explanations could be invoked. It is unlikely that the reason is an overestimate by ELISA of the adduct level because (*a*) the HPLC-fluorescence data indicate a similar level of adduct if adduct recoveries are taken into account (see Ref. 6 for discussion) and (*b*) similar quantitative observations were made between urinary markers and AF intake where the analyses are independent of those of the albumin adduct. While we cannot rule out that the 25% adduct recovery which we assume, based on animal studies (6), is higher in human samples, this would be unlikely to account for the large observed differences. In our opinion it is more likely that this problem is a result of the difficulty in making accurate measurements of individual food intakes.

In the present study, several factors could contribute to the inaccuracy of food intake: (*a*) false negatives in the food analysis of AF (this is unlikely, given the validation to which this assay has been subjected) (16); (*b*) food intake other than at the two fixed meals of the day [This could contribute to the AF exposure, but at the time of study food was not abundant in the village, as evidenced by the fact that subjects occasionally missed a meal. The snacks eaten would include a type of pounded rice (*dempetengo*), some fruits and vegetables, bread, raw groundnuts taken prior to the new harvest, and some cooked snack foods including fish pies and pancakes.]; (*c*) contribution of AF from nondetectable samples. Since the AF exposure was relatively low in the subjects studied, and given that the detection limit in the small sample size available for analysis was 1 ppb, samples that were nondetectable (*e.g.*, rice samples) but that contained AF could still contribute a significant percentage to the total exposure. Any explanation must account for the fact that good correlations were observed at the individual level for dietary intakes and both AF-albumin adducts (this study) and urinary AF (4), suggesting that the error is reasonably uniform with respect to all subjects.

No differences were observed in adduct levels in the HB_sAg carriers and noncarriers. In a larger study of 323 children in The Gambia, we observed a significantly higher level of AF-albumin adduct in HB_sAg-positive children,[5] but in this case no food intake measurements were made. Given the importance of understanding any interaction between HBV and AF in the etiology of hepatocellular carcinoma, it is clearly of importance to pursue this question. The availability of this marker of AF exposure allows investigations to be made in field studies that can be complemented by the available experimental animal models (24, 25).

The present study has demonstrated that the AF-albumin adduct reflects dietary exposure to AF at an individual level and that the marker is therefore a reliable tool for integration into various types of field studies (see Ref. 9). This marker should greatly facilitate the investigation of the relative roles of AF and HBV in the etiopathogenesis of hepatocellular carcinoma, and this area has received particular attention given recent observations regarding point mutations in the *p53* tumor suppressor gene that may be related to aflatoxin exposure (26, 27). The albumin adduct is also a suitable marker with which to monitor AF exposure in order to permit a complete evaluation of the efficiency of HBV vaccination programs in reducing liver cancer incidence (28, 29).

Acknowledgments

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