Temporal Patterns of Aflatoxin-Albumin Adducts in Hepatitis B Surface Antigen-positive and Antigen-negative Residents of Daxin, Qidong County, People's Republic of China

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Abstract

Molecular epidemiological studies of populations at high risk for liver cancer have shown that hepatitis B virus (HBV) and aflatoxin B1 (AFB1) exposure are two major risk factors for this disease. These etiological agents, combined with nutritional deficiencies, are important for the initiation and promotion of liver cancer in various parts of the world. In Qidong, People's Republic of China, liver cancer accounts for 10% of all adult deaths, and both HBV and AFB1 exposures are common. To study temporal and possible chemical-viral interactions in people, serum samples were collected during a longitudinal study designed to measure aflatoxin molecular biomarkers in residents of Daxin Township, Qidong City, People's Republic of China. In this study, the temporal modulation of aflatoxin adduct formation with albumin over multiple lifetimes of serum albumin was examined in both HBV-positive and HBV-negative people in two periods: September–December 1993 (wave 1) and June–September 1994 (wave 2). During the 12-week monitoring period of wave 1, 120 individuals (balanced by gender and HBV status) provided a total of 792 blood samples. AFB1-albumin adducts were detected in all but one of the serum samples. The range of binding detected by RIA in the Daxin population was 0.17–4.39 pmol AFB1/mg albumin with an overall mean ± SD of 1.51 ± 0.21 pmol AFB1/mg albumin. The mean ± SD for weeks 0, 2, 4, 6, 8, 10, and 12 of wave 1 were 1.21 ± 0.41, 1.28 ± 0.70, 1.36 ± 0.52, 1.71 ± 0.44, 1.18 ± 0.60, 2.00 ± 0.59, and 1.68 ± 0.34 pmol AFB1/mg albumin, respectively. During wave 2, 103 individuals from wave 1 provided a total of 396 blood samples collected monthly over wave 2, with mean ± SD aflatoxin-albumin adduct levels of 1.19 ± 0.37, 0.85 ± 0.45, 0.89 ± 0.28, and 0.61 ± 0.15 pmol AFB1/mg albumin. Using linear regression models, the mean aflatoxin-albumin adduct levels increased (P < 0.05) during the 12 weeks of wave 1 and decreased (P < 0.05) over the 4 months of wave 2. Neither HBV surface antigen status nor gender modified either the baseline mean or the temporal trend. High-performance liquid chromatography confirmation was done on a subset of serum samples, and the results show an excellent association between the immunoassay data and high-performance liquid chromatography. Taken together, these data demonstrate that AFB1-albumin is a sensitive and specific biomarker for assessing exposure to this carcinogen in the population in Qidong.

Introduction

AFB1 is a naturally occurring mycotoxin that has been demonstrated to be one of the most potent liver carcinogens examined in experimental animals, including nonhuman primates (1, 2). Data from human epidemiological studies have demonstrated that exposure to AFB1 is one of the major risk factors in the multifactorial etiology of HCC (3–6). The degree that AFB1 contributes to HCC has been shown to be influenced by a number of health factors, such as HBV and/or hepatitis C virus infection, nutritional status, metabolic polymorphism, and age, as well as the extent of AFB1 exposure. Nonetheless, AFB1 has now been classified as a known (group 1) human carcinogen by the IARC (7).

The development and application of molecular biomarkers for AFB1 in human HCC etiological studies is based upon knowledge of AFB1 metabolism, critical macromolecular adduct formation, and possible target sites in vivo. A comprehensive review of the literature on the toxicology of aflatoxins in experimental models and humans has been published recently (1). Many studies have confirmed that the toxic and carcinogenic effects of the aflatoxins are manifested only after metabolism by members of the endogenous cytochrome P-450 enzyme superfamily (8, 9). These enzymes catalyze oxidative metabolism, resulting in the formation of various hydroxylated derivatives, as well as two highly reactive 8,9-epoxide metabolites, which can covalently interact with cellular DNA and proteins (10, 11). The two major epoxide-derived macromolecular adducts identified to date are the AFB1-N7-guanine adduct in DNA and the AFB1-lysine adduct in albumin. The aflatoxin-
Longitudinal Patterns of Aflatoxin-Albumin Adducts in Humans

Longitudinal patterns of aflatoxin-albumin adducts in humans (12-17) have been reported, and these adducts have been used as biomarkers of aflatoxin exposure (18). The sensitivity and specificity of this assay permits using <100 μL of blood to quantitate exposure in many populations. In addition, the albumin biomarker correlates well with other aflatoxin biomarkers, such as urinary aflatoxin metabolites and serum aflatoxin-lysine adducts (19). The measurement of aflatoxin-albumin adducts has been shown to be a useful tool for epidemiological studies (20, 21).

Liver cancer is a major public health problem in China and is the third leading cause of cancer mortality in the country, resulting in more than 200,000 deaths annually. In recent studies of a cohort in Shanghai, both HBV and aflatoxin have been found to be closely linked to liver cancer development (5, 19). Qidong City, located just north of Shanghai on the southeast coast of Jiangsu Province, is one of the several regions with the highest HCC incidence and mortality in China (20, 21). In Qidong County, liver cancer accounts for 10% of all adult deaths, and both HBV infection and aflatoxin exposure are common in this region. Uniformly throughout Jiangsu Province, almost 10% of the people are HBV surface antigen positive. However, a >10 fold gradient of liver cancer rates across the province suggests that HBV is not the only risk determinant in developing the disease. Current studies that have examined the pattern of mutations in the p53 tumor suppressor gene of liver tumors in Qidong find a consistency of mutations with exposure to aflatoxins (2).

The long-term goal of our research is to design strategies for reducing the incidence of this largely fatal disease. The application of molecular biomarkers of aflatoxin, to identify individuals at high risk has proven to be valuable for epidemiological studies (22, 23); however, our knowledge base about temporal patterns of formation and persistence of aflatoxin-macromolecular adducts in human samples is still limited. Therefore, we conducted a longitudinal study to determine the formation and persistence of serum albumin adducts with respect to HBV infection status in carriers and noncarriers and gender.

Materials and Methods

**Chemicals**

[^1]H]AFB1 (28 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA) and was purified by using a Waters Sep-Pak C18 cartridge (Millipore Corp., Milford, MA) and then stored in 100% ethanol at -20°C. The radiolabeled aflatoxin was >98% pure when assessed by HPLC. [^3]H]toluene was purchased from DuPont NEN Research Products (Boston, MA). Unlabeled AFB1, albumin determination reagent (BCP), human albumin standards, normal human serum, BSA (fraction V), and horse serum were obtained from Sigma Chemical Co. (St. Louis, MO). Protein assay dye reagent concentrate and protein standards were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Promase (70,000 proteolytic units/g dry weight) was obtained from Calbiochem (La Jolla, CA). AFB1-lysine adduct standard was synthesized by the reaction of 8,9-dihydro-8,9-dibromo-AFB1 with N-acetyl-L-lysine (Aldrich Chemical Co., Milwaukee, WI) and purified by HPLC as previously described (24, 25). All other chemicals were of the highest purity that was commercially available.

**Study Population**

**Study Design for Wave 1 (September–December 1993).** Serum was repeatedly collected from 120 individuals enrolled in a longitudinal study. In July 1993, 10-mL blood samples, obtained from 600 volunteers in an initial screening physical examination, were used to assess HBsAg status using the AUSRIA II kit (Abbott Laboratories, North Chicago, IL). All residents of villages 4 and 5 in Daxin Township, Qidong City, Jiangsu Province, People’s Republic of China, were eligible for participation in the study if they were older than 18 years, did not have a medical history precluding repeated venipuncture, had no a-fetoprotein detected in the ELISA test, were not currently pregnant or lactating, and did not have any history of cancer. Individuals meeting the eligibility criteria were interviewed in Chinese and informed of the study design by staff from the Shanghai Cancer Institute and the Qidong Liver Cancer Institute. Study subjects agreeing to participate were then administered an informed consent form, in both Chinese and English, that had been approved by the Institutional Review Board for Human Research at the Johns Hopkins School of Public Health and the Qidong Liver Cancer Institute. Consent ing individuals were stratified by both gender and HBsAg positivity status, and 30 individuals from each of the four strata were selected as they became eligible. Of these 120 adults, 116 returned to the clinic for their baseline serum collection and physical examination in September 1993. Table I delineates the baseline characteristics of the study sample by HBV status and gender strata.

![Table I: Mean (SD) Wave 1 baseline measurements of liver function and physical examination by gender and hepatitis B surface antigen status](https://example.com/table1.png)

<table>
<thead>
<tr>
<th>HBsAg Negative</th>
<th>HBsAg Positive</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>No. enrolled</td>
<td>32</td>
<td>29</td>
</tr>
<tr>
<td>No. with baseline measurements</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>Liver function markers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST/SGOT, IU/L</td>
<td>24.7 (13.5)</td>
<td>20.0 (15.1)</td>
</tr>
<tr>
<td>ALT/SGPT, IU/L</td>
<td>9.0 (5.4)</td>
<td>7.9 (3.8)</td>
</tr>
<tr>
<td>SUN, mg/dL</td>
<td>18.0 (3.6)</td>
<td>15.6 (4.2)</td>
</tr>
<tr>
<td>Bilirubin, mg/dL</td>
<td>0.7 (0.07)</td>
<td>0.73 (0.07)</td>
</tr>
<tr>
<td>Serum creatinine, mg/dL</td>
<td>1.48 (0.21)</td>
<td>1.29 (0.33)</td>
</tr>
<tr>
<td>Physical attributes</td>
<td></td>
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</tr>
<tr>
<td>Mean systolic BP, mmHg</td>
<td>125 (11)</td>
<td>119 (12)</td>
</tr>
<tr>
<td>Mean diastolic BP, mmHg</td>
<td>81 (8)</td>
<td>78 (8)</td>
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<tr>
<td>Body mass index, kg/m²</td>
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<td>23 (3)</td>
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<tr>
<td>Age, years</td>
<td>48.9 (14.1)</td>
<td>44.1 (11.7)</td>
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<tr>
<td>% smoked cigarettes</td>
<td>46.7%</td>
<td>0.0%</td>
</tr>
</tbody>
</table>

*BP, blood pressure.*

Legend: AFB1, aflatoxin B1; ALT, alanine transferase; AST, aspartate transaminase; bilirubin, serum bilirubin; BSA, bovine serum albumin; BCP, albumin determination reagent; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; SCT, serum creatinine; SGOT, serum glutamic-oxaloacetic transaminase; SGPT, serum glutamic-pyruvic transaminase; T, testicular; TCGA, tumor suppressor gene.

Table I: Mean (SD) wave 1 baseline measurements of liver function and physical examination by gender and hepatitis B surface antigen status

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This content is a summary of the study on aflatoxin-albumin adducts in humans, focusing on the methodology and results. The study aimed to design strategies for reducing the incidence of liver cancer, particularly in regions with high aflatoxin and HBV exposure. The data collected included various biochemical and physical measurements to assess the health status of the study participants. The table outlines the baseline characteristics of the study sample by HBV status and gender strata, highlighting the differences in liver function and physical examination results.

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Study Design for Wave 2 (June-September 1994). 103 individuals from wave 1 [51 males (28 HBV-, 23 HBV+) and 52 females (27 HBV-, 25 HBV+)] were available and willing to continue the monitoring study.

Data Collection. At the baseline visit of wave 1, participants were administered a questionnaire at a health clinic to elicit information related to health status, medication usage, and smoking and drinking patterns. Five-mL blood samples were drawn from the participants at the baseline visit, and every 2 weeks when the participant returned to the health clinic over a 3-month period between mid-September and early December 1993. Thus, a maximum of seven individual blood samples were collected from each participant. For wave 2, blood was drawn from participants at the initial visit and every 4 weeks over a 4-month period between June and September 1994.

All bloods were drawn into red-top Vacutainer tubes (Becton Dickinson BB-6435) that did not contain any anticoagulants. These samples were allowed to clot, and then serum was isolated after centrifugation. Serum samples were then divided into three separate tubes and stored frozen at -20°C until shipment to Baltimore. Every 4 weeks, samples were shipped on dry ice to Baltimore, where they were stored at -80°C until analysis.

Measurement of Aflatoxin Serum-Albumin Adduct Levels

The human serum samples were first concentrated by high-speed centrifugal filtration using Microcon-50 microconcentrators that had a 50,000-MW filter cutoff (Amicon, Inc., Beverly, MA). Briefly, 150 μL of human serum were loaded into the top reservoir of a Microcon-50 that had been prefilled with 100 μL of water, then 250 μL of PBS (pH 7.0) was added to dilute the serum. The samples were centrifuged in a Labnet Z 230 MR microcentrifuge (Woodbridge, NJ) for 20 min at 13,000 rpm (13,700 × g) at room temperature. After the centrifugation, 200 μL of PBS was added to the top reservoir, and the samples were centrifuged at 13,000 rpm for 10–15 min until the total volume in the top reservoir was <50 μL. The top reservoir was then disassembled, placed upside down in a new vial, and centrifuged for 5 min at 5,000 rpm to transfer the concentrated protein into the vial. Finally, the concentrated protein was resuspended in 100–150 μL of PBS, and the volume of each sample was measured and recorded. The amount of human serum albumin was determined in each sample by a brom cresol purple dye binding method (26), which measures a stable blue-purple color complex between the dye and albumin with an absorption maximum at 560 nm. Standard curves for this assay were constructed using human albumin. In addition, the amount of total protein was determined by the procedure of Bradford (27) using a Bio-Rad protein assay kit calibrated with serum albumin levels in individual samples, we also report an analysis of the aflatoxin levels/ml serum, obtained by multiplying the albumin-normalized aflatoxin data by the amount of albumin/ml serum.

HPLC-Fluorescence Confirmation of AFB1-Lysine Adduct

Human serum albumin was concentrated and digested using Pronase as described above. After digestion, the aflatoxin adducts were further purified using an aflatoxin monoclonal antibody (2B11) affinity column as previously described (28, 31). Chromatographic separation of the aflatoxin adducts was achieved using an HPLC system consisting of two Beckman Model 110 A pumps controlled by a Beckman Model 420 programmer, a Rheodyne 7125 injector (Rhodeyne, Inc., Cotati, CA), a Hewlett-Packard 1040M Diode-Array UV detector, a Hewlett-Packard 1046A programmable fluorescence detector (excitation, 405 nm; emission, 470 nm), and a Partisil 5 ODS-3 WCS analytical column (4.6 × 250 mm; Whatman, Inc., Clifton, NJ) equipped with a pre- and a guard-column. The column temperature was maintained at 50°C. Solvent A was 20 mm ammonium phosphate (pH 7.3), and solvent B was 100% methanol. The purity of the ammonium phosphate used in the HPLC buffers proved to be very important. Several lots of ammonium phosphate were found to contain fluorescent materials that chromatographed near the aflatoxin-lysine adduct. Thus, lots of ammonium phosphate were prescreened to select only those batches containing no interfering contaminants. Isocratic elution with solvent A for 2 min preceded a linear methanol gradient elution (0–60% B) for 20 min and then isocratic elution with 60% methanol:20 mm ammonium phosphate for an additional 13 min. The flow rate was 1 ml/min. Authentic aflatoxin-lysine adduct standard was synthesized as described previously (24, 25). This adduct was characterized by UV absorption, mass spectrometry, and NMR and then used to construct standard curves to quantitate the levels in the human samples.

Data Analysis

Linear regression methods for correlated data were used to describe the temporal trend of AFB1, in units of pmol/mg albumin and pmol/ml serum over the 12 weeks of wave 1 and over the 4 months of wave 2 (32). Graphical analysis indicated the data were symmetrically distributed with an approximately...
constant variance over time. Separate regression models for albumin-normalized and serum-normalized aflatoxin data and serum albumin data were constructed for each wave by including parameters for an intercept (i.e., baseline mean) and slope (mean change per 2-week cycle). Correlation between measurements made on the same individual was assessed by including parameters that represent the within-individual correlation. This corresponds to a compound symmetry correlation structure, in which the correlation is assumed to be independent of the time lag between measurements. Alternative correlation structures, including an autoregressive structure, were also investigated, but the results were not qualitatively different when using the compound symmetry structure. Using the wave 1 data, model parameters were estimated for the entire cohort (i.e., overall for all 120 individuals) and for each of the four HBV status and gender combinations (i.e., four intercept, slope, and correlation parameters). Using the wave 2 data, model parameters were estimated only for the entire cohort. Differences among HBV and gender group baseline means, 2-week slopes, and within-individual correlations were assessed using standard likelihood methods. All computations were done using PROC MIXED in SAS 6.09 (SAS Institute, 1994).

Results
Modification of the Method for the Measurement of Aflatoxin-Albumin Adducts in Human Samples. A number of refinements to the analysis of aflatoxin-albumin adducts in human samples have been made since the earliest reports in the literature (16, 17, 28, 31, 33). A significant modification to our previously described methods (28) is the use of centrifugal microfiltration units. These devices contain molecular weight cutoff membranes that greatly facilitate the isolation and purification of albumin from human serum samples. Because the only major blood protein bound by aflatoxin is albumin, it is a goal to rapidly isolate albumin from other proteins and low-molecular weight substances found in serum. Past reports have used precipitation and other purification methods (17, 28), and often these isolation techniques introduce high salt levels in samples that then have to be removed before enzyme digestion. In addition, other methods involve a series of transfers that can lead to sample loss. One of the objectives in this study was to devise a method that would lead to high albumin recovery in a short period of time and minimize sample transfer. We found that a number of commercially available centrifugal filtration devices could be used that not only permitted rapid isolation of albumin, but also provided a unit that permitted the use of proteolytic enzymes necessary to digest modified albumin in the same unit used to isolate the albumin fraction. This eliminated a number of transfer steps, increased albumin yield, and increased the number of samples that could be processed per day.

Initial experiments were done to determine the appropriate molecular weight cutoff membrane to be used in the analysis. In this pilot study, 10,000-, 30,000-, and 50,000-MW cutoff filter units from a number of manufacturers were examined using BSA as a test material. The BSA, at levels ranging from 1 to 20 mg, was applied to the filter unit as described in “Materials and Methods.” Levels of albumin were determined before and after centrifugation and recovery yields determined. All of the molecular weight cutoff filters were capable of yielding between 95 and 100% of the applied serum albumin. The 50,000-MW filters were selected for use in the following investigation because the lower-molecular weight filter devices, although working well, required much longer centrifugation times to filter the samples. This test was also used for quality assurance when different lots of filter units were used. This proved to be important because we unfortunately found that the efficiency of the filter units in recovering albumin from different manufacturers varied from lot to lot. Thus, these high-speed centrifugal filtration units permitted the concentration and washing of samples to be completed in <1 h, providing a sample that could be immediately used for Pronase digestion.

A final series of quality control tests was done with the microfiltration units using [3H]toluene spiked in BSA and [3H]AFB1 spiked into control human serum samples. In all instances, >99.5% of the spiked radiolabeled tracers were removed from the albumin samples after three separate wash steps as described in “Materials and Methods.” One critical observation in these studies was that the filter units should always be prewetted with water before loading the test sample. Without prewetting, nonspecific losses of albumin were increased. Thus, after optimization, 4–6 mg of albumin were easily purified from 100–150 μL of human serum, which was sufficient for duplicate or triplicate analysis by RIA.

Detection of AFB1-Albumin Adducts by RIA in Residents of Daxin, Qidong, People’s Republic of China. The 120 people enrolled at the start of the study provided 792 serum samples during wave 1: 90 people completed all 7 follow-up visits, 20 people provided 6 measurements each, 5 provided 5 measurements each, 1 provided 4 measurements, and 4 provided 3 measurements each. For wave 2, totals of 1, 14, and 88 people provided 2, 3, and 4 monthly blood samples, respectively, resulting in a total of 396 samples for analysis. The lack of complete longitudinal follow-up was not due to drop-out of individuals from the study or any adverse medical response to blood sampling; incomplete observations occurred uniformly throughout the course of the study and were most commonly due to the subject being out of the village on the day of collection.

Several of the key results are depicted in Fig. 1, which displays the albumin-standardized aflatoxin (Fig. 1A), serum-standardized albumin (Fig. 1B), and serum-standardized aflatoxin (Fig. 1C) data superimposed with box plots indicating the range and the 25th, 50th, and 75th percentiles of the data at each time period. AFB1-albumin adducts could be detected in all but one of the serum samples tested. To determine the final specific activity of the albumin samples, the mean level (0.16 pmol AFB1/mg albumin) of the control negative human serum samples used in the RIA was subtracted from each result. Thus, the range of binding in the Daxin population was between 0.14 and 4.39 pmol AFB1/mg albumin with an overall mean ± SD of 1.53 ± 0.59 pmol AFB1/mg albumin in wave 1 and 0.89 ± 0.39 pmol AFB1/mg albumin in wave 2. The mean ± SD for weeks 0, 2, 4, 6, 8, 10, and 12 of wave 1 were 1.21 ± 0.41, 1.58 ± 0.70, 1.36 ± 0.52, 1.71 ± 0.44, 1.18 ± 0.60, 2.00 ± 0.59, and 1.68 ± 0.34 pmol AFB1/mg albumin, respectively; the mean for weeks 0, 4, 8, and 12 of wave 2 were 1.19 ± 0.37, 0.85 ± 0.45, 0.89 ± 0.28, and 0.61 ± 0.15 pmol AFB1/mg albumin, respectively. The >25-fold range of the serum albumin-normalized aflatoxin in Fig. 1A within an exposed population is consistent with previous studies (14, 16, 17). As indicated in the previous section, there was wide variability in the serum albumin levels; the mean for wave 1 (wave 2) was 41.9 ± 5.7 (59.4 ± 8.8) mg albumin/ml serum, with a minimum of 21.2 (31.1) mg/ml and maximum of 61.9 (84.1) mg/ml. The serum albumin-normalized aflatoxin levels had a mean of 48.5 ± 17.2, 65.3 ± 28.5, 54.5 ± 18.3, 67.2 ± 17.0, 47.1 ± 24.1, 85.9 ± 24.9, and 80.1 ± 17.8.
pmol aflatoxin/ml serum at the seven time periods of wave 1, respectively, and were 67.1 ± 21.7, 52.2 ± 28.1, 53.6 ± 16.5, and 35.3 ± 9.0 pmol aflatoxin/ml serum at the four time periods, respectively, of wave 2.

The results from the regression models for the data in Fig. 1 are shown in Table 2. Each of the baseline (intercept) parameters was significantly different from 0 ($P < 0.01$). A significantly increasing trend ($P < 0.001$) in AFB$_1$ adducts and albumin was observed over the 12-week period of wave 1. The overall biweekly aflatoxin-albumin adducts were increasing in wave 1 by 0.074 pmol AFB$_1$/mg albumin and 4.62 pmol AFB$_1$/ml serum, and the mean albumin was increasing by 0.86 mg albumin/ml serum per 2-week period. In wave 2, there was a significantly decreasing trend ($P < 0.001$) in AFB$_1$ adducts but no change ($P = 0.96$) in albumin. The aflatoxin decreased at a rate of 0.086 pmol AFB$_1$/mg albumin and 4.74 pmol AFB$_1$/ml serum per 2-week period. The estimated regression lines are plotted in Fig. 1.

Table 2 also displays the results for wave 1 analyzed for each of the four groups defined by HBsAg status and gender. There were no significant differences among the four groups with respect to the aflatoxin measurements. HBV-negative females had albumin levels 2.6 mg albumin/ml serum lower than HBV-negative males ($P = 0.012$) and 2.7 mg albumin/ml serum lower than HBV-positive males ($P = 0.013$) at baseline. The mean slope of HBV-negative females was
also higher by 0.79 mg albumin/ml serum per 2-week period relative to HBV-negative males \( (P = 0.0009) \) and HBV-positive males \( (P = 0.0026) \).

The within-individual correlation estimates for each HBV/gender group are also shown in Table 2. None of the correlation measurements were significantly different from 0 except the overall correlation among albumin levels, with a small positive intraclass correlation of 0.138 \( (P < 0.001) \), and there was no difference in the wave 1 correlations by HBV or gender category \( (P > 0.15) \).

**Confirmation of HPLC-Fluorescence with Synthesized AFB1-Lysine Adduct.** The RIA data described above detect aflatoxin adducts derived from the enzymatic hydrolysis of the modified protein. The enzymatically-produced hydrolysate is a complex mixture of the aflatoxin-lysine adduct and a number of other aflatoxin-polypeptide adducts. Historically, the single aflatoxin-lysine adduct released from the albumin represents only 2–10% of the fraction of total aflatoxin content in the albumin (33). Fortunately, the released aflatoxin-lysine monoadduct has unique absorbance and fluorescence properties (24, 25) that permit sensitive and specific measurement. This analysis is most sensitive after immunoaffinity column clean-up and HPLC or ELISA specific assays (24, 25, 33). Because the HPLC method is a completely different analytical technique than RIA, this method was used to confirm the detection of the aflatoxin-lysine adduct in these human samples. The HPLC conditions were first optimized by testing several buffers and solvents. In particular, it was found that phosphate buffers often contain interfering fluorescent materials that chromatograph close to the aflatoxin-lysine adduct. Although these contaminants can be removed by immunoaffinity chromatography as a preparative step, we sought to avoid such contaminants affecting any of our mobile phases or standards. The standard curve for HPLC-fluorescence detection of the synthesized AFB1-lysine adduct is shown in Fig. 2. The limit of detection of this method was 10 fmol AFB1-lysine. A representative chromatogram of a AFB1-lysine adduct standard is shown in Fig. 3A with a retention time of 24.5 min. These conditions were then employed using 17 randomly selected serum samples previously analyzed by RIA to confirm the presence of the AFB1-lysine adduct. In this study, 12 mg of serum albumin were digested for HPLC analysis compared to 2 mg used for RIA. This higher amount was chosen because of the relatively low yield of the mono aflatoxin-lysine adduct from proteins. Representative chromatograms of lower and higher levels of AFB1-lysine adduct detected from serum samples are shown in Fig. 3, B and C, respectively. The correlation of the results from RIA and HPLC-fluorescence method done on the 17 randomly selected human serum samples is shown in Fig. 4. A very good association \( (r^2 = 0.949; P < 0.001) \) is found between the two methods. The range of the AFB1-lysine adduct released from the albumin was from 4 to 121.1 fmol AFB1/mg albumin (or 1.82 to 55.10 pg AFB1-lysine/mg albumin), and this represented 2.2 ± 0.9% (mean ± SD) of the amount of aflatoxin detected by the RIA. Thus, the HPLC technique can be used to confirm the presence of the aflatoxin-lysine adduct in this population. Unfortunately, the average time for a single HPLC analysis was about 1 h; hence, the immunoassay method is preferable for large population-based studies.

\[ \text{Table 2: Regression model estimates (SE) by participant HBV antigen status and gender for wave 1 (September-October 1993) and wave 2 (June-September 1994)} \]

<table>
<thead>
<tr>
<th>Wave 1</th>
<th>Wave 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg negative</td>
<td>HBsAg positive</td>
</tr>
<tr>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Number of individuals</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>32</td>
<td>29</td>
</tr>
<tr>
<td>Number of contributed measurements</td>
<td></td>
</tr>
<tr>
<td>212</td>
<td>198</td>
</tr>
<tr>
<td>Aflatoxin, pmol/mg albumin</td>
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</tr>
<tr>
<td>Baseline</td>
<td>1.181 (0.062)</td>
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<tr>
<td>Slope/14 days</td>
<td>0.097 (0.017)</td>
</tr>
<tr>
<td>Within-individual correlation</td>
<td>-0.005</td>
</tr>
</tbody>
</table>

| Albumin, mg/mL serum | | | | | | |
| Baseline | 40.18 (0.78) | 37.60 (0.66) | 40.35 (0.88) | 38.97 (0.64) | 39.26 (0.37) | 58.74 (0.73) |
| Slope/14 days | 0.60 (0.17) | 1.39 (0.16) | 0.60 (0.21) | 0.89 (0.17) | 0.86 (0.09) | 0.20 (0.19) |
| Within-individual correlation | 0.141 | 0.136 | 0.082 | 0.061 | 0.138 | 0.021 |

| Aflatoxin, pmol/mL serum | | | | | | |
| Baseline | 46.12 (2.50) | 53.18 (3.28) | 51.40 (3.18) | 50.48 (3.12) | 50.22 (1.51) | 66.17 (1.68) |
| Change/14 days | 5.12 (0.72) | 4.48 (0.90) | 4.11 (0.91) | 4.69 (0.90) | 4.62 (0.43) | -4.74 (0.47) |
| Within-individual correlation | -0.036 | 0.010 | -0.038 | -0.049 | -0.020 | -0.009 |

Fig. 2. Standard curve of synthesized and purified AFB1-lysine adduct in HPLC-fluorescence method. The stock solution of adduct was quantitated by measuring UV absorbance at 400 nm and diluted in 100 μl of 20 mm ammonium phosphate before injection into the HPLC. The conditions of HPLC-fluorescence analysis and integration are described in detail in “Materials and Methods.” Each ○ represents the average of three independent measurements.
The application of molecular biomarkers of carcinogens in human studies has been an important advance in improving the quality of environmental epidemiological data, especially in identifying individuals at high risk for disease and in establishing the linkage between carcinogen exposure(s) and the cancer(s) they initiate. The development of aflatoxin-specific biomarkers has been very valuable for associating exposure to aflatoxins with improved sensitivity in the blood of people. The time, we have sequentially assessed aflatoxin-albumin biomarkers has been very valuable for associating exposure to aflatoxins and human liver cancer (5).

All of these studies confirm the validity of using this aflatoxin biomarker in exposed populations, and these data have been correlated with the prevalence of specific mutations in the p53 gene of liver tumors in these different geographic areas (4).

Despite the many different advances in aflatoxin-albumin adduct detection methodologies, some questions still remain about the comparison of measured values of aflatoxin-albumin adducts using different analytic methods. There are three major analytical techniques currently available for measuring aflatoxin-albumin adducts in human blood. They are two immunoassays, RIA and ELISA, which use either monoclonal or polyclonal antibodies recognizing aflatoxins and a HPLC-fluorescence method (28, 31, 33). All of these methods involve a large number of experimental steps, resulting in significant variations of recovery and yield among the various laboratories. Losses of aflatoxin-albumin adducts have been reported to range from 35 to 95%, and such variation makes quantitative comparisons among the published data difficult (16, 17, 28, 31, 33).

The concentration and purification of albumin from human serum is the first step for all these published methods, and this step is a major source of losses of the protein adducts. In the study reported here, we have used microfiltration units to concentrate albumin and to wash the proteins free of aflatoxin by this method modification should improve the accuracy of the adduct analyses.

Wild et al. (33) proposed that an HPLC-fluorescence detection method be used to confirm immunoassay data and also be used as a routine quality control method. Results in this report confirm an excellent agreement between the immunoassay and HPLC method. Because only the HPLC method can use...

Discussion

The application of molecular biomarkers of carcinogens in human studies has been an important advance in improving the quality of environmental epidemiological data, especially in identifying individuals at high risk for disease and in establishing the linkage between carcinogen exposure(s) and the cancer(s) they initiate. The development of aflatoxin-specific biomarkers has been very valuable for associating exposure to aflatoxins and human liver cancer (5). The study reported in this paper extends the methods used to measure aflatoxin-albumin adducts with improved sensitivity in the blood of residents at high risk for liver cancer. Furthermore, for the first time, we have sequentially assessed aflatoxin-albumin biomarkers over at least two lifetimes of the protein in people. The results reported here show that exposure to aflatoxin is very prevalent in the population of Qidong, and almost 100% of serum samples tested in this study contained detectable aflatoxin-albumin adducts. These data are consistent with previous reports (17, 28) in residents of Fushui County, Guangxi Autonomous Region, and of Chongming County, Shanghai, People’s Republic of China. These regions in China also have elevated rates of HCC. A high percentage of positive serum samples for aflatoxin-albumin adducts has also been reported by other investigators studying residents of The Gambia and other regions in Africa that are known to have high incidence of liver cancer (14–16). Furthermore, in recently completed studies in Mexico, levels of aflatoxin-albumin adducts were found to be high (34).

Fig. 3. Chromatograms of HPLC-fluorescence detection for AFB1-lysine adduct. A, 0.22 pmol (100 pg) of synthesized and purified AFB1-lysine adduct was passed through aflatoxin monoclonal antibody affinity column and analyzed. B, lower level of AFB1-lysine adduct detected in human serum sample from residents of Daxin, Qidong. C, higher level of AFB1-lysine adduct detected in human serum sample from residents of Daxin, Qidong.

Fig. 4. Comparison of the specific activity of aflatoxin-albumin adducts measured by RIA and AFB1-lysine adducts measured by HPLC-fluorescence. Details are described in "Material and Methods."
an internal standard, this procedure is very valuable to independently assess recovery and yield of aflatoxin adducts. This is much more difficult to accomplish in immunoassays because the addition of an internal standard often involves using a compound for which antibody may not have an identical affinity as compared to the adduct. The significant disadvantage of the HPLC-fluorescence methods is that it requires more albumin because the proteases incompletely digest the modified albumin. This leads to the relatively low yield of the HPLC-fluorescence method for the AFB1-lysine adduct, generally in the 2–10% range. The AFB1-lysine adducts in this study, ranging from 1.9 to 55.4 pg AFB1-lysine/mg albumin, are very similar to other published values for exposed populations (14, 16, 28, 31), indicating that the exposure in this population is similar to that reported in other studies in China and West Africa. In summary, although the use of the HPLC-fluorescence method might be limited for large-scale human investigations, this tool is an important confirmation technique for immunoassays, and it should be used as a quality control procedure in future human studies concerning measurement of AFB1-albumin adducts.

Aflatoxin-albumin binding is a longer-term biomarker of aflatoxin exposure than any of the urinary aflatoxin markers. Several studies have investigated the rate of formation and turnover of aflatoxin-albumin biomarkers in rats chronically dosed with the carcinogen. In one of the earlier reported studies, the half-life for loss of AFB1-albumin adducts after multiple dosing was found to be 55 h, which is similar to the determined half-life of albumin measured in these animals (35). Recently, these studies have been extended to rats multiply dosed with aflatoxin in the presence or absence of the chemopreventive agent oltipraz. These data confirmed that the rate of turnover of the aflatoxin-albumin adducts was similar to the normal turnover of the blood protein (36). Unfortunately, much of our knowledge of albumin turnover in humans stems from a limited number of experiments done using radiolabeled albumin injected into people and the time course of removal being monitored (37). These data showed that the half-life of albumin in normal, healthy people is between 14 and 20 days. This indicates that AFB1-albumin adducts may be stable and serve as a molecular biomarker integrating AFB1 exposure over a period of weeks or months (38). However, in a limited series of studies that have been done in people with serious liver diseases, such as cirrhosis and hepatitis infections, the turnover of albumin is much more variable (39). Because many of the populations at risk for liver cancer have these types of chronic disease states, the simple assumption of albumin half-life being about 3 weeks may be incorrect. Perhaps the apparent lack of tracking observed in our study relates to our limited understanding of the nutritional, metabolic, and health-related components of albumin turnover and aflatoxin metabolism in people. This observation needs to be extensively followed up in future investigations with studies that have more frequent sampling intervals for albumin adduct determinations.

Direct literature evidence about the stability and half-life of AFB1-albumin adducts in humans has been lacking because most published studies have not continuously measured serum samples from the same population over weeks or months. In this study, AFB1-albumin adducts were longitudinally monitored over a period of 3 months (at least two lifetimes of human serum albumin) in a large number of human subjects. We found that the AFB1-albumin adducts were prevalent and persistent and that an 8-fold range of adduct formation existed among individuals within a cycle. Recently, it has been reported that genetic variations in metabolic enzymes responsible for the detoxication of aflatoxin-epoxides play a role in both the risk of developing liver cancer and in the enhanced formation of albumin adducts (6). Although we do not have similar genotyping data at this time for the population examined in this study, the cycle-to-cycle variation in albumin adduct formation seen in this report could readily be explained on the basis of changes in dietary exposure from week to week. Indeed, preliminary analysis of corn and peanut consumption, major sources of aflatoxin exposure, in this population shows wide fluctuations in daily-to-day consumption of these staple foods. Future studies will need to be done to sort out the relative contributions of exposure and host-susceptibility factors in the formation of aflatoxin-albumin adducts in people.

Chronic infection with HBV and ingestion of AFB1-contaminated foods have been found to be two major risk factors for the development of liver cancer in China (3, 5, 19). A simple hypothesis that has now been examined in a number of populations is that HBV enhances aflatoxin metabolism to genotoxic derivatives. In this study, there was no significant difference for albumin adduct formation observed for HBV carriers and noncarriers. These data are similar to previous results in adult populations in West Africa (40, 41) and in a recent case-control study of adults in Taiwan (42). Allen et al. (15) had found a significantly higher level of AFB1-albumin adduct in HBsAg-positive children living in The Gambia. However, a follow-up study in children in The Gambia did not find a statistically significant difference between HBV-positive and HBV-negative individuals, but there was a trend toward more adduct formation in the HBV-positive children (12). Thus, it may turn out that although HBV is not a metabolic modifier of aflatoxin in adults, it may affect the metabolism of this carcinogen in children at a time when hepatocytes are maximally dividing. Finally, in agreement with previous findings, gender was not a significant modifier of aflatoxin metabolism in this population (31, 43), although it does play a role for albumin levels. Taken together, these data demonstrate that AFB1-albumin is a sensitive and specific biomarker for assessing exposure to aflatoxin in the population in Qidong. Furthermore, this biomarker should be a useful end point for assessing the efficacy of interventions designed to lower genotoxic damage by aflatoxin in people.

References

Temporal patterns of aflatoxin-albumin adducts in hepatitis B surface antigen-positive and antigen-negative residents of Daxin, Qidong County, People's Republic of China.

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