Exposure to aflatoxin B$_1$ (AFB$_1$), an important cofactor in the etiology of hepatocellular carcinoma in Taiwan, is influenced by dietary and other factors. The present study examined the intraindividual variability in AFB$_1$-albumin adducts, the most reliable long-term biomarker of AFB$_1$ exposure, and whether the baseline or follow-up adduct levels and the intraindividual variability in adduct levels are modified by endogenous and environmental factors. The study measured AFB$_1$-albumin adduct levels among 264 healthy male residents of three townships (H-u-Hsi, M-a-Kung, and Pai-Hsa) of Penghu Islands, Taiwan, at two different time points with a median interval of 1.68 years (range 1.00–3.17 years). There was a generalized reduction in the adduct levels, with the median values being 22.1 pmol/mg (range 5.0–355.8 pmol/mg) at time 1 and 14.3 pmol/mg (range 5.0–205.2 pmol/mg) at time 2. This intraindividual variability in adduct levels was inversely associated with the age of subjects and the time interval between the two blood draws. The variability in adduct levels was lower among subjects in H-u-Hsi and Pai-Hsa townships as compared to those in M-a-Kung. No significant association was observed for the intraindividual variability in AFB$_1$-albumin adducts with regard to the season when blood was drawn. There was also no significant association between intraindividual variability and hepatitis B surface antigen, anti-hepatitis C virus (anti-H CV), glutathione S-transferase (GST) M1, or GSTT1 status. In conclusion, we found substantial intraindividual variability in the AFB$_1$ exposure (as determined by AFB$_1$-albumin adducts) in Taiwan, which was probably more likely related to dietary or other environmental influences rather than to endogenous factors (e.g., hepatitis B/C viral infection or GST M1/T1 genetic status). Key words: aflatoxin–albumin adducts, biomarkers, intraindividual variability, liver cancer, molecular epidemiology. Environ Health Perspect 109:833–837 (2001). [Online 13 August 2001] http://ehpnet1.niehs.nih.gov/docs/2001/109p833-837ahsan/abstract.html

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related mortality in the world. In Taiwan, it is the most common cause of cancer death among men (1). Risk factors for HCC include chronic hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, cigarette smoking, and dietary exposure to aflatoxin B$_1$ (AFB$_1$). AFB$_1$ is a naturally occurring mycotoxin derived from certain species of Aspergillus fungi that contaminate various foods, particularly peanuts/groundnuts, corn/maize, and oil seeds and nuts such as cottonseed, almonds, and pistachios. AFB$_1$ is a potent liver carcinogen in animals (2,3) and has been categorized as a group I human carcinogen by the International Agency for Research on Cancer (4).

Human exposure to AFB$_1$ and its biological effects are highly variable and are influenced by several factors. AFB$_1$ production by Aspergillus fungi is influenced by humidity and temperature. Dietary exposure to AFB$_1$ is influenced by harvesting and storage conditions of foods and procedures used for cooking. In the body, AFB$_1$ is metabolized by the microsomal mixed-function enzymes, which are inherently polymorphic, to various reduced and oxidized derivatives, including an unstable reactive AFB$_1$–8, 9-epoxide, which can bind covalently to DNA and proteins. The biologically effective dose of AFB$_1$ can be measured in urine as AFB$_1$–guanine, in liver tissues as AFB$_1$–DNA, and in peripheral blood as AFB$_1$–albumin adduct, but AFB$_1$–albumin adducts have been considered the most reliable biomarker of AFB$_1$ exposure in humans (5). Because of a relatively long half-life of serum albumin, the AFB$_1$–albumin adduct reflects AFB$_1$ exposure from the past 2–3 months (5). AFB$_1$–albumin adduct has been shown to be related to HCC risk in a dose-response fashion among HBV surface antigen (H BsAg) carriers, and the biological gradient was particularly pronounced among subjects who carried null genotypes of glutathione S-transferase (GST) M1 and/or T1 metabolism genes (6).

The temporal trend in the AFB$_1$–albumin adduct level was examined during two 12-week monitoring periods within a year among 120 individuals in Qidong, China (7). The study subjects, comprising roughly equal numbers of males and females and HBsAg carriers and noncarriers, were selected by screening 600 healthy volunteers older than 18 years of age. The authors found continuously detectable levels of AFB$_1$–albumin adducts among the study participants, with an increasing trend in adduct levels during the first wave (September–December) and a somewhat decreasing trend during the second wave (June–September). These trends were not modified by gender or HBsAg status (7).

In the present study, we examined AFB$_1$–albumin adducts among a sample of healthy adults in Taiwan over a period of 1–3 years and examined whether the AFB$_1$ exposure as measured by AFB$_1$–albumin adduct levels and its temporal (or intraindividual) variability are modified by HBsAg, anti-H CV, GST M1 or GST T1 genotype, seasonal variation, and other factors. Examination of temporal variability in adduct levels is useful in evaluating whether adduct levels indicate changes in the exposure to aflatoxin in the population (e.g., monitoring effectiveness of intervention/prevention trials). Understanding the roles of host factors (i.e., HBsAg, anti-H CV, and GST genotype status) on the variability in adduct levels may help in identifying susceptible populations for targeting specific public health interventions.

**Methods**

**Study subjects** The study subjects were a sample drawn from a cancer screening project cohort that was assembled during 1990–1992. Details of the recruitment methods and characteristics of this parent cohort have been published previously (8). Briefly, 25,618 (12,024 men and 13,594 women) volunteer respondents 30–64 years of age were recruited from seven urban and rural townships in Taiwan (four townships on Taiwan Island and three townships on the Penghu Islands). These people responded to the letter of invitation sent to residents in the selected townships who were born between 1927 and 1961. In addition to the cancer screening, in-person interviews with a
structured questionnaire were conducted at the local research centers with the participants at the time of recruitment. We collected 20 mL of fasting blood (10 mL in a heparinized tube and 10 mL in a tube without anticoagulant) from each participant at the time of recruitment; buffy coat, plasma, and red blood cells were separated and stored at the central laboratory of National Taiwan University at -70°C. Subjects in this cohort who were screened positive by serological markers (i.e., high serum alanine transaminase, aspartate transaminase, or α-fetoprotein levels) or had abnormal liver ultrasound provided blood samples every 6–12 months for further clinical monitoring. The cohort members who were apparently healthy and had normal levels of serological markers and ultrasound provided a repeat sample of blood within 1–3 years as part of follow-up visits. From these healthy members of the cohort, we selected 264 apparently healthy men for the current study for the examination of intraindividual variability of AFB1–albumin adducts. These 264 individuals were selected randomly from the participating residents of three townships (H u-h si, M a-k ung, and P a-h sa) in Penghu Islets, an area with the highest HCC incidence in Taiwan, but in a manner so that half (n = 132) of them were HBsAg carriers and the other half (n = 132) were noncarriers.

**Laboratory analysis.** As mentioned above, HBsAg and HCV assays were conducted on all participants as part of the parent cancer screening project cohort study at National Taiwan University. HBsAg status was tested by enzyme immunoassay using commercial kits (Abbott Laboratories, North Chicago, IL, USA). Antibodies against H CV were also tested using an enzyme immunoassay with second-generation commercial kits (HCV; Abbott).

The biological samples of the 264 subjects selected for the current study were shipped to Regina Santella’s laboratory at Columbia University, New York, for further analyses. At Columbia, GSTM1 and GSTT1 genotypes were determined by polymerase chain reaction amplification of genomic DNA with published primers using β-globin as an internal control and gel electrophoresis as described (9,10).

To determine the AFB1–albumin adduct levels in serum samples, we used an enzyme-linked immunosorbent assay (ELISA) as described previously (6,11). An antiserum for detection of AFB1–albumin adduct was generated by immunizing New Zealand white rabbits with bovine γ-globulin (BGG) modified with AFB1 epoxide synthesized as described (12). Antiserum (#7) was used in a competitive ELISA. For the ELISA standard curve, [3H]AFB1–human serum albumin was digested with proteinase K as described below for the human samples and adducts isolated by Seppaks C18 (Waters, Milford, MA, USA) extraction. We determined adduct level from the specific activity.

Albumin was prepared from plasma essentially as described (13), and its concentration was determined with bicinechonic acid (BCA Reagent, Pierce, IL, USA). For the digestion, 2 mg albumin and 0.5 mg proteinase K were incubated 15 hr at 37°C. Adducts were isolated by the procedure as described (14), dissolved in 0.5% mL phosphate-buffered saline containing 1% fetal calf serum and 1 mmol/L phenylmethylsulphonylfluoride to inhibit residual protease activity, and assayed by competitive ELISA. This assay had 50% inhibition of antiserum binding at 10–20 fmol AFB1 adduct per well. The limit of sensitivity (15% inhibition), when assaying the equivalent of 200 μg albumin/well, was 0.01 fmol/μg. Samples with less than 15% inhibition were given a value of 0.005 fmol/μg. Samples were assayed by duplicate analysis in duplicate wells. We analyzed two control samples with each batch of sera: a pooled sample of plasma from non-smoking American subjects and a positive control of serum from a rat treated with 1.5 mg AFB1. Although samples were drawn at different time points, all samples were assayed at one time with the laboratory personnel being blinded to the time of blood drawing and other individual information.

**Statistical analysis.** Median, mean, and standard deviation (SD) of the adduct levels were calculated for all 264 samples as well as for the subgroups of samples separately for the baseline and repeat samples. The adduct levels between the two groups at a given time point were compared by using t-tests or Wilcoxon rank tests, depending on the distribution of the data. To examine the temporal variability of adduct levels among the same subjects, the mean adduct levels between two time points were compared using paired t-tests. The effect of seasonal variability on the adduct level was measured in two different ways. First, to examine the effect of seasonal variability on adduct level at any given time point, the mean and median adduct levels were examined for four different calendar periods separately for time 1 (i.e., baseline sample) and time 2 (i.e., follow-up sample). Second, to examine the effect of season on the temporal variability in adduct levels, subjects who provided blood samples during the same months in two different calendar years were compared with subjects who provided blood samples during different months in two different calendar years. Because the adduct levels were not normally distributed (as evident from the differences between the mean and median values presented in Table 1), we used log-transformed values in all multivariate analyses.

In multivariate analysis, to examine the impact of different environmental and genetic factors on adduct levels at a given time point, linear regression models were fit using the adduct level as the outcome variable separately for time 1 and time 2. To examine the effect of different factors on the temporal variability of adduct levels, a single regression model was fit using the differences in the adduct levels between two time points as the outcome variable. All regression models were rerun including a term for ELISA batch in the model to adjust for the laboratory assay variability.

**Results** AFB1–albumin adducts were measured in 264 subjects at two time points with a median duration between the sample draws of 1.68 years and a range of 1.00–3.17 years. Table 1 shows the median (and also the mean) adduct levels (picomoles of AFB1 per milligram of albumin) at two time points for all subjects as well as at different levels of other characteristics. The median adduct level for all subjects was 22.1 pmol/mg (range 5.0–355.8) at time 1 and 14.3 pmol/mg (range 5.0–205.2) at time 2. Within each time point, adduct levels were similar across different categories of HBsAg, anti-H CV, and GSTM1 and GSTT1 genotype status. The effect of season on the adduct levels appeared to be different for the two time points. At time 1, adduct levels were higher for subjects whose blood was drawn in June–November, but at time 2, adduct levels were lower for subjects whose blood was drawn in June–November. At time 2, the adduct levels were substantially lower among the youngest age group as compared to other age groups and also among subjects who had a shorter time interval between the blood draws as compared to those with a longer time intervals.

Regarding the intraindividual variability in adduct levels between two time points, the median (and also the mean) adduct levels were uniformly lower at the second time point almost for all categories of different environmental and endogenous factors. These differences in adduct levels between the two time points did not seem to vary by levels of age, HBsAg and anti-H CV status, GSTM1 and GSTT1 genotype, and time interval or seasonal variability between the blood draws. The differences in the adduct levels between two time periods, however, appeared to vary with respect to geographic area, with Ma-Kung having the highest variability.

Table 2 shows the results of multivariate analyses examining the effects of different variables on the adduct levels separately for...
time 1 and time 2 and also on the intra-individual variability between two time points. In both time periods, adduct levels seemed to be significantly positively associated with age. Scattered plots of adduct levels against age showed somewhat linear trends, especially for data in time 2 (data not shown). In time 2, but not in time 1, adduct levels were higher among subjects in H u-H si and Pai-H sa as compared to those in Ma-Kung and also higher among H BsAg carriers than among noncarriers. The adduct levels were also related to the season variability in time 2 but not in time 1.

As shown in Table 2, the intraindividual variability in adduct levels between two time points was inversely associated with the age of subjects and the time interval between the two blood draws. The variability in adduct levels was lower among subjects in H u-H si and Pai-H sa as compared to those in M a-K ung. No significant association was observed for the intraindividual variability in AFB1–albumin adducts with regard to the differences in season when blood was drawn. There was also no significant association between intraindividual variability and H BsAg, anti-H CV, or GST M 1 or GST T 1 status. These results were similar when the analysis was conducted excluding the 42 subjects whose GST genetic status could not be determined.

**Discussion**

Although H BV infection is the key etiologic element in H CC, AFB1 exposure is an important cofactor in H CC carcinogenesis. AFB1–albumin adduct is considered a reliable indicator of the biologically effective dose of AFB1 exposure. In the present study we examined how different environmental and endogenous factors influence the effect of AFB1 exposure as measured by the AFB1–albumin adduct and also whether these factors play roles in the temporal variability of adduct levels. To our knowledge, only one previously published study has examined the temporal variability in AFB1–albumin adduct level among residents of O idong County, China, by examining adduct levels at two different time periods within a 1-year period (7).

**Table 1. AFB1–albumin adduct levels at two time points by age, HBsAg status, anti-HCV status, GSTM1/T1 genotype, geographic area, and season.**

| Genotype | Townships | All subjects | Age at time 1 (quartiles) | Time interval between assays (quartiles) |
|----------|------------|--------------|--------------------------|----------------------------------------|
| GSTM1/T1 |            |              |                          |                                        |
| Null     |            | 264          | 66                       | 66                                      |
| Non-null |            | 221          | 43                       | 43                                      |
| Unknown  |            | 132          | 32                       | 32                                      |
| GSTT1    |            |              |                          |                                        |
| Null     |            | 132          | 32                       | 32                                      |
| Non-null |            | 221          | 43                       | 43                                      |
| Unknown  |            | 131          | 34                       | 34                                      |

In the present study we measured AFB1–albumin adduct levels among 264 subjects at two time points with an interval of 1–3 years among residents of three different geographic areas in Taiwan. The adduct levels observed in the current study are comparable to those observed in other studies in Asia (7), but somewhat higher than those reported in Africa (15). However, it is difficult to directly compare adduct levels across studies because different detection methods are used in different studies. The adduct levels were on average substantially lower for the second measure among all subjects, regardless of their environmental or genetic characteristics. This general reduction in adduct levels was difficult to explain. The study subjects were apparently healthy residents who participated in the cancer screening project. Although specific interventions for dietary modification were not done at the time of recruitment (i.e., at time 1), the respondents were briefed about the risk factors for H CC, including hepatitis viruses and dietary aflatoxin exposure. Therefore, one possibility is that the participants modified their dietary habits after participating in the cancer screening project. Although AFB1–albumin adduct is considered to reflect exposure for a relatively long period (up to 3 months), the time intervals of blood draws for the study subjects were sufficiently large (at least 1 year) for the adduct measures to reflect dietary exposures from non-overlapping time periods.

Both the baseline as well as the follow-up measures of adduct levels were weakly but somewhat positively correlated with age. The temporal variability in the adduct levels was, however, negatively correlated with age (i.e., the reduction in adduct level was higher among younger subjects). Despite the statistical significance of the associations with age, because the intercepts were large and the coefficients were very small, it is difficult to make any concrete inferences about the effects of age on adduct levels and its temporal variability. However, if the observed reduction in adduct levels was at least partly due to modification in their dietary behavior, then this finding, if real, would reflect a trend that younger subjects were more likely to change their diets after participating in the cancer screening project.

The temporal variability in adduct levels between two time points was inversely related to the duration of time interval between time points (i.e., the reduction in adduct levels was higher among subjects for whom the time interval between two blood draws was shorter). The reasons for and implications of this finding are not clear. The intraindividual variability was higher among subjects with a higher baseline adduct level. This finding may indicate that subjects who had a higher...
initial adduct level were more likely to have altered their dietary habits. An alternative explanation for the finding could be that, although the study subjects were apparently healthy at the time of enrollment, it is possible that subjects who were seropositive for HBsAg had subclinical impaired liver function and thus could have a defective AFB1–albumin adduct metabolism. The study by Wang et al. (7) found that HBsAg status was not correlated to the temporal variability of AFB1–albumin adduct level.

One previous study found a higher variability of polycyclic aromatic hydrocarbon–DNA adducts among subjects who carried null genotypes for the GSTM1 gene (16). The present study was the first to examine the effect of GSTT1 and GSTM1 genotype and anti-HCV status on the intraindividual variability of the AFB1–albumin adduct levels. We did not find the GSTM1 or GSTT1 genotype status or anti-HCV status to have any impact either on the baseline or on the temporal variability of the AFB1–albumin adduct levels. Wild et al. (15) recently published a study showing no association between AFB1–albumin adduct levels and GSTT1 genotype in a Gambian population, but the authors found higher adduct levels among non–HBV-infected subjects with the GSTM1 null genotype.

Several limitations of the current study should be borne in mind while interpreting the findings. First, we examined aflatoxin–albumin adducts at only two time points with an interval of 1–3 years, limiting the capability to examine the temporal trends over a shorter duration. Second, although dietary data (as sources of aflatoxin exposure) were collected at the time of recruitment (i.e., at time 1) on each subject as part of the structured interview, data on the changes of dietary and nondietary exposure at the time of second blood draw (i.e., at time 2) were not collected. The lack of sufficient dietary and nondietary data on aflatoxin exposure at time 2 limits our ability to directly correlate the observed temporal trends in adduct levels with changes to specific dietary and/or nondietary sources from the present data. However, questionnaire-based dietary information is of limited utility in determining individual exposure to aflatoxin. For example, a nested study carried out in Shanghai that found an association between aflatoxin–albumin adducts and HCC found no association with dietary aflatoxin consumption based on in-person food frequency interview combined with the survey of market foods in the study region (17). In 1988, a study of 42 residents of Guangxi Province in China established that aflatoxin adducts were a valid marker of aflatoxin exposure by comparing adduct levels to the levels of aflatoxin in portions of the food consumed (18).

A third limitation of the present study is that the study participants were recruited from three different townships where the distribution of sources of aflatoxin as well as their changes over time may not have been homogenous. Although inclusion of township variables in the multivariate model adjusted the impact of results in relation to other covariates, the interpretation of the observed relationship between township itself and temporal variability in adducts is somewhat complicated by the lack of data on the distribution and changes in sources of aflatoxin within individual townships.

In conclusion, we found that AFB1–albumin adduct levels varied over time among apparently healthy individuals residing in three townships of Taiwan. This temporal intraindividual variability in adduct levels was higher among younger subjects and subjects with an initial high adduct level. HBsAg or anti-HCV infection and GSTM1 or GSTT1 genotype status did not have any significant effects on the intraindividual variability of AFB1–albumin adduct levels. Dietary modification appears to be an important determinant of aflatoxin exposure reduction in Taiwan.

Table 2. Effect of different environmental and genetic factors on the AFB1–albumin adduct level and its intraindividual variability

| Predictors | Log (adduct) Level at time 1 | Log (adduct) Level at time 2 | Temporal variability |
|------------|------------------------------|------------------------------|---------------------|
|             | β SE p*                       | β SE p                       | β SE p              |
| Intercept  | -1.018                        | -1.104                       | -0.509              |
| Age        | 0.006 0.002 0.031             | 0.005 0.002 0.039            | 0.005 0.002 0.046   |
| Time interval |                            | -0.071 0.033 0.032           | -0.071 0.033 0.032  |
| Baseline AFB1 |                        | -0.038 0.055 0.000          | -0.038 0.055 0.000  |
| Hu-Hsi vs. Ma-Kung |                   | 0.177 0.049 0.000           | -0.199 0.050 0.000  |
| Pai-Hsia vs. Ma-Kung |                 | 0.201 0.098 0.041           | -0.191 0.099 0.054  |
| HBsAg status | 0.058 0.052 0.269           | 0.083 0.045 0.073           | -0.079 0.046 0.090  |
| Anti-HCV status | 0.008 0.086 0.922        | -0.024 0.076 0.756          | 0.019 0.076 0.803   |
| GSTM1 status | 0.015 0.025 0.536          | 0.015 0.022 0.516           | -0.013 0.022 0.565  |
| GSTT1 status | -0.026 0.025 0.315         | -0.027 0.022 0.234          | 0.025 0.022 0.281   |
| Seasonal variation | -0.067 0.051 0.192     | 0.097 0.043 0.026           | -0.003 0.043 0.947 |

*Significance for null hypothesis that β = 0.
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