Aflatoxin, Liver Enzymes, and Hepatitis B Virus Infection in Gambian Children

Christopher P. Wild, Marijke Fortuin, Francesco Donato, Hilton C. Whittle, Andrew J. Hall, C. Roland Wolf, and Ruggero Montesano

International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France (C. P. W., R. M.); The Gambia Hepatitis Intervention Study, International Agency for Research on Cancer, P.O. Box 273, Banjul, The Gambia, West Africa (M. F.); Institute of Hygiene, University of Brescia, Via Valsabbina 19, 25124 Brescia, Italy (F. D.); Medical Research Council Laboratories, Fajara, P.O. Box 273, Banjul, The Gambia, West Africa (H. C. W.); Communicable Disease Epidemiology Unit, London School of Hygiene and Tropical Medicine, 7 Keppel Street, London WC1 7HT, United Kingdom (A. J. H.); and Imperial Cancer Research Fund, Molecular Pharmacology Unit, Biomedical Research Centre, University of Dundee, United Kingdom (C. R. W.)

Abstract

The relative contribution of, and possible mechanism of interaction between, aflatoxin and hepatitis B virus (HBV) in the development of primary hepatocellular carcinoma can be better investigated now that markers of individual exposure to both factors are available. In this study, blood samples were collected over a 1-month period from 117 children aged 3 to 4 years, resident in Kuntair or Kerr Cherno in the Upper Niumi District of The Gambia. Samples were analyzed for aflatoxin-albumin (AF-alb) adducts, markers of HBV infection, liver enzymes [serum alanine aminotransferase (ALT)] as markers of liver damage, and glutathione S-transferase M1 (GSTM1) genotypes. All but two children showed detectable serum AF-alb with levels ranging from 2.2 to 250.4 pg aflatoxin B1-lysine equivalent/mg albumin. There was a significant positive correlation between AF-alb and ALT (r = 0.4; P < 0.001). HBV carriers showed moderately higher levels of AF-alb than noncarriers but the difference was not statistically significant and the association between AF-alb and ALT was unchanged when the HBV carriers were excluded from the analysis, suggesting that factors other than HBV infection contributed to the association. The null glutathione S-transferase M1 genotype was infrequent (17.7%) in this population and was not associated with any difference in AF-alb adduct levels compared to glutathione S-transferase M1-positive individuals. However, the percentage of individuals with the null genotype varied significantly between ethnic groups with 32.1% in Fula, 8.8% in Mandinka, and 13.3% in Wolof. The association between AF-alb and ALT could be a result of the hepatotoxicity of aflatoxin, but the data are also consistent with the hypothesis that liver damage resulting from HBV and/or other factors can alter aflatoxin metabolism resulting in an increased binding to cellular macromolecules including DNA. This hypothesis merits further investigation in appropriate animal models and future field studies in aflatoxin-exposed populations.

Introduction

The high incidence of hepatocellular carcinoma in parts of Africa and southeast Asia is strongly associated with chronic HBV infection (1) and another important risk factor is exposure to aflatoxins. These are secondary fungal metabolites contaminating a significant part of the human food supply in many parts of the world (2) and are potent animal hepatocarcinogens (3) with strong evidence of hepatocarcinogenicity in humans (1, 4, 5). The understanding of the degree and nature of interaction between aflatoxin exposure and HBV infection in the development of this disease is limited. This understanding could be facilitated by the development of markers of individual exposure to aflatoxin, e.g., the AF-alb in peripheral blood which provides a marker of exposure over the previous 2–3 months (6–8). This albumin adduct is formed following metabolism of aflatoxin in the liver and its level correlates with both dietary aflatoxin exposure and with another exposure marker (aflatoxin-N7-guanine adduct) excreted in the urine (9, 10). An immunoassay for AF-alb has permitted a more precise demonstration of the extent of exposure in different parts of the world (11). In The Gambia, exposure to aflatoxin is widespread at a high level at all ages, including exposure during the perinatal period (7, 12–14).

One possible mechanism of interaction between aflatoxin and HBV, among several which have been discussed (15, 16), is by way of alteration of carcinogen metabolism resulting from the viral infection. Increased activation of chemical carcinogens, including aflatoxin, has been reported in woodchucks infected with woodchuck HBV (17). The mechanism of the altered metabolism in the woodchucks is not known and could be either a nonspecific response to liver damage or due to a more specific effect of the virus, including transactivation of cellular genes (18). Recently, an increased level of AF-alb was observed in Gambian children who were HBsAg positive and this increase could reflect altered aflatoxin metabolism in the HBsAg-positive children (12). Serum transaminases (ALT and AST)

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are increased in the case of hepatocellular damage resulting from viral hepatitis, thus, it was considered appropriate to examine the association between the level of aflatoxin-albumin adduct, markers of HBV infection, and the level of these markers of liver damage.

The specific enzymes involved in human hepatic metabolism of aflatoxin are being identified and eventually their expression could be examined using noninvasive markers (see Ref. 19). The enzymes include cytochromes P450 which activate aflatoxins, to the aflatoxin 8,9-epoxide, (20-22) and the GST enzymes which conjugate aflatoxin 8,9-epoxide to glutathione (23, 24). Conjugating capacity of human hepatic GST for the aflatoxin 8,9-epoxide appears to be low (25), although an important contribution of the GSTM1 (μ) isoenzyme has been suggested in one study (23). The GSTM1 enzyme is polymorphic in humans due to a gene deletion (26) and the genotype can be easily determined (27).

The present cross-sectional study in a group of children from The Gambia, therefore, addressed two hypotheses. The first was that there is an association between serum transaminase levels (indicative of liver damage) and the level of aflatoxin-albumin adducts. The second was that there is an association between GSTM1 genotype and the level of aflatoxin-albumin adducts.

Materials and Methods

Materials. Taq polymerase was from Promega, as were nucleotide triphosphates. Oligonucleotide primers for GSTM1 genotyping were prepared by Operon. All reagents for enzyme-linked immunosorbent assay of AF-alb adducts were as previously described (28).

Study Population. Venous blood samples were used which had been obtained from 117 children aged 3 to 4 years as part of the Gambia Hepatitis Intervention Study (29) as approved by the GHIS Steering Committee and the Medical Research Council/Gambian Government Ethical Committee. All subjects were resident in Kuntain or Kerr Cherno in the Upper Niumi District on the north bank of the river Gambia in the west of the country and all samples were collected in a 1-month period between May 30 and July 2, 1991. This time period was chosen as aflatoxin exposure was expected to be high, based on previous studies (12). A second blood sample was taken between July 6, and July 20, 1992, for all but 2 of the 17 children who were HBsAg-positive at the first examination. Ethnic group was recorded from the child’s Infant Welfare Card, which is filled in by nurses at vaccination clinics. A 5-ml blood sample was obtained, of which 3 ml was collected in a heparinized tube and the WBC separated from the plasma by centrifugation, the buffy coat being washed twice in phosphate-buffered saline prior to DNA extraction. Plasma was stored at -70°C prior to shipment to Lyon on dry ice. DNA was extracted by a phenol-chloroform procedure and was transferred to Lyon in precipitated form in methanol.

GST Genotyping. The genotyping was performed as described previously (27) using three oligonucleotide primers in exons 4 and 5 region of the GSTM1 and a related gene (GSTM4) of the same multigene family. The polymerase chain reaction yields a constant 160-base pair fragment in all samples and a 232-base pair fragment seen only in the GSTM1-positive genome.

For a few individuals, the genotype and phenotype in peripheral blood lymphocytes were shown to correlate following the protocol of Liu et al. (23) with minor modifications (data not shown). Although the GSTM1 genotypes have not been characterized in Gambian subjects, it appears that the same gene deletion is present as in Caucasians (26). Aflatoxin-Albumin Adduct Determination. The presence of AF-alb was determined by enzyme-linked immunosorbent assay following albumin hydrolysis and purification of aflatoxin residues on a C18 Sep-pak (Waters) cartridge (28). The detection limit was 2 pg AF-B1-lysine equivalent/mg albumin.

Markers of HBV Infection. Sera were assayed for HBsAg by reverse passive hemagglutination (Wellcome Diagnostics) and for antibodies to hepatitis B core antigen by radioimmunoassay (Sorin). Children who were HBsAg negative and hepatitis B core antigen positive were regarded as having recovered from a past infection with HBV. Those who were HBsAg-positive at the two examinations 1 year apart were considered as chronic carriers and those who were positive at the first and negative at the second were considered as acute infections.

Liver Enzyme Function. Sera from the first blood sample were analyzed for ALT and AST aminotransferases by use of a Cobas-Mira auto-analyzer.

Statistical Methods. Since the distributions of AF-alb, ALT, and AST were skewed, a natural logarithmic transformation of these variables was performed. All statistical tests of hypotheses were performed on the transformed variables because they gave a better approximation to a normal distribution than the untransformed data. However, for greater clarity, original data or antilog of the log-transformed data (back transformations) are given in text and tables whenever possible. The P values presented throughout the manuscript are two-sided.

ALT and AST were strongly correlated (r = 0.6; P < 0.001). ALT is a more specific marker of liver damage and was therefore used for further analysis. We assessed the independent associations between sex, HBV infection status, serum ALT, GSTM1 genotype and ethnic group, and AF-alb level by analysis of variance. In a further analysis, the same associations were assessed adjusting for serum ALT levels by covariance analysis in order to examine these associations after taking into account the effect of ALT on AF-alb levels. In the case of HBV infection, since no differences were found in the data for AF-alb levels between those children never having been infected and those who had recovered from a past infection, the two groups were considered together in some analyses and HBV infection considered as a dichotomous variable (noncarrier/carrier). The four subjects with acute HBV infection and the two who were HBsAg positive at the first examination but were not retested were excluded from covariance analysis. The assumptions of the model were assessed using a test of parallelism of the regression lines and through the examination of the residual plots (30). For comparisons of proportions, χ² test was used without Yates correction when the expected value was less than 5 as the marginal frequencies of the tables were subject to random variability (31).
All the statistical analyses were performed using the BMDP statistical package for mainframe, 1990 version (32).

Results
The results of this study demonstrate a positive association between AF-alb adduct level and ALT, a marker of liver damage. This association was observed independent of HBV infection. Between the three ethnic groups, there were also differences in the levels of AF-alb and ALT and in the frequency of the GSTM1 genotype. These results are presented in detail below.

Aflatoxin Exposure. The distributions of subjects by sex, ethnic group, HBV infection status, and GSTM1 genotype are shown in Table 1. The distributions of AF-alb, ALT, and AST were all skewed (Table 2). A high intensity distribution as the cutpoints, the AF-alb means showed a linear relationship in the three ethnic groups. The Wollof and Mandinka, further analysis was performed by testing for parallelism of slopes, the result was at the limit of significance (P = 0.03). Of the four children with acute HBV infection at the time of measurement of AF-alb, three had high AF-alb adduct levels, exceeding 100 pg/mg, and all had ALT levels >35 units/liter. In contrast to the result with HBV infection, strong associations were found between ln(AF-alb) and both ALT level and ethnic group. The lack of association between ln(AF-alb) level and sex, HBV infection status, and GSTM1 genotype was maintained when ethnic group was taken into account by a two-factor analysis of variance (data not shown) and when ln(ALT) was included as covariate in the covariance analysis (Table 3). The differences among the ln(AF-alb) means in the ethnic groups, however, although reduced, remained significant when they were adjusted for ln(ALT) (Table 3).

The Fula group had higher adduct levels than the other two ethnic groups, while there was no significant difference between Wollof and Mandinka groups when Fula was excluded from the analysis. When the relationship between ln(AF-alb) and ln(ALT) was examined in the three ethnic groups separately by testing for parallelism of slopes, the result was at the limit of significance (P = 0.05), suggesting differences between ethnic groups in this relationship. This was because the slope of the regression line for Wollof (y = 0.56 + 1.02x) was different than that for Mandinka (y = 2.40 + 0.25x) and Fula (y = 2.73 + 0.37x).

Since the AF-alb level was significantly higher in Fula compared to Wollof and Mandinka, further analysis was performed among the ethnic groups. The Wollof and Mandinka ethnic groups were similar with respect to levels of AF-alb, ALT, and the proportion of GSTM1 positive and negative individuals, whereas the Fula differed from the other two groups with respect to the same variables (Table 4). Fula show the highest AF-alb and ALT mean levels and the highest proportion of GSTM1-negative subjects. The overall frequency of the null genotype in The Gambian population examined was 17.7% (Table 1) but in the Fula this was approximately 3 times more frequent than in the other 2 groups (Table 4).

Further analysis was performed, in which the 11 HBV chronic carriers were excluded in order to examine whether this condition modified the relationships seen in the study population as a whole. However, no substantial changes were observed in the associations described above: (a) ln(AF-alb) was still linearly correlated with ln(ALT) (r = 0.4; P = <0.0001); (b) and ethnic group was still associated with ln(AF-alb) (P = 0.02).

Discussion
Almost all the children in this study had detectable serum AF-alb adducts with a wide variation from 2.2 to 250.4 pg levels of AF-alb (geometric mean, 36.3) than noncarriers (geometric mean, 26.4), but the difference was not statistically significant (P = 0.3). Of the four children with acute HBV infection at the time of measurement of AF-alb, three had high AF-alb adduct levels, exceeding 100 pg/mg, and all had ALT levels >35 units/liter. In contrast to the result with HBV infection, strong associations were found between ln(AF-alb) and both ALT level and ethnic group. The lack of association between ln(AF-alb) level and sex, HBV infection status, and GSTM1 genotype was maintained when ethnic group was taken into account by a two-factor analysis of variance (data not shown) and when ln(ALT) was included as covariate in the covariance analysis (Table 3). The differences among the ln(AF-alb) means in the ethnic groups, however, although reduced, remained significant when they were adjusted for ln(ALT) (Table 3).
AFB1-lysine equivalent/mg albumin. This wide range, together with an indication of a bimodal distribution of adduct level (Fig. 1), has been already observed in children from The Gambia (11, 12). AF-alb is strongly related to dietary aflatoxin intake as shown in earlier reports from The Gambia (9) and People’s Republic of China (6), and there is evidence that it is a valid indicator of formation of hepatic aflatoxin DNA adducts in animals and humans (10, 33). No food intake data are available for the subjects in the present study. However, all samples were collected from children of the same age living in the Upper Niumi District, and the diet in this area is relatively stable and predictable, being based on groundnuts, millet, and rice. Furthermore, the samples were collected within a short time period, making it unlikely that seasonal variations (11) had an important effect on dietary aflatoxin exposure.

We observed a positive linear relationship between serum AF-alb and ALT. ALT is relatively specific for liver damage, being expressed only in low concentrations in other tissues while an alternative marker, AST, is also found in high concentrations in heart muscle, skeletal muscle, kidney, pancreas, and red cells (34). These data are consistent with the hypothesis that liver cell damage leads to an increased activation of aflatoxin in the liver, which would consequently be associated with an increased level of aflatoxin DNA adducts and increased serum AF-alb. However, an alternative explanation for our data is that the hepatotoxic effects of aflatoxin (3) are a direct cause of the increased ALT level. Some experimental data support the former hypothesis that liver damage could potentiate the mutagenic and carcinogenic effects of aflatoxin. In woodchucks infected with HBV, an increased activation of aflatoxin was observed compared to noninfected animals, although no specific isoenzymes were studied (17). In one strain of HBV transgenic mice which overexpress the HBsAg, leading to liver damage, a synergistic effect between HBV and aflatoxin was observed in the development of hepatocellular carcinoma (35). In these same mice, in collaboration with Chisari et al., we have observed an increased expression of CYP2a5, specifically associated with cells adjacent to areas of liver cell damage. The equivalent human isoenzyme, CYP2Ab, is capable of metabolizing aflatoxin (36), and preliminary observations in human cirrhotic livers showed that CYP2A6

\[ \text{AFB}_1 \text{-lysine eq. per mg albumin} \]

Fig. 1. Frequency distribution of AF-alb in the 117 children included in the study.

Fig. 2. Scatter diagram of AF-alb and ALT. The linear regression line is presented and statistical analysis was performed on the log-transformed data ($r = 0.4; p < 0.001$).

\[ \text{AF} \text{-} \text{alb} \text{ (pg per mg)} \]

\[ \text{ALT (U/L)} \]
can also be induced in hepatocytes adjacent to areas of liver damage (37). Such an increase in expression of enzymes activating aflatoxin would be expected to result in an increased formation of aflatoxin 8,9-epoxide, increased DNA and albumin adducts and a consequent increase in mutagenesis. It would also suggest that a more complete analysis of the data concerning liver damage would be valuable in complementing the data on transaminases and permitting the investigation of the possible mechanism of interaction between aflatoxin and HBV infection state and GSTM1 genotype according to ethnic group

### Table 3

<table>
<thead>
<tr>
<th>Ethnic group</th>
<th>Unadjusted</th>
<th>Adjusted</th>
<th>NS</th>
<th>NS</th>
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<tbody>
<tr>
<td>Male</td>
<td>27.7</td>
<td>26.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>26.8</td>
<td>27.5</td>
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### Table 4

<table>
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<tr>
<th>Ethnic group</th>
<th>Geometric means of AF-alb and ALT and the number and percentage of subjects by sex, HBV infection state, and GSTM1 genotype according to ethnic group</th>
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<td>Geometric means (antilog of the means of log-transformed data). NS = P &gt; 0.1 (not significant).</td>
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<td></td>
<td>F test of the analysis of variance.</td>
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<td></td>
<td>Means are adjusted for ln(ALT).</td>
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<td>F test of the covariance analysis with ln(ALT) as covariate.</td>
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Aflatoxin itself causes the liver damage and associated increase in ALT, is also possible and in the present cross-sectional study, with the contemporary measurement of the AF-alb and ALT, no definite conclusion can be drawn about the cause and effect relationship between the two factors. Liver injury due to aflatoxin exposure has been reported previously in children given daily dietary supplements of peanut meal later found to contain AFB1 (38). Daily aflatoxin intakes were estimated to be >1 µg/kg body weight and up to 20 µg/kg, although these children may have already had liver damage due to kwashiorkor prior to aflatoxin exposure via the dietary supplements. Nevertheless, based on the levels of AF-alb in The Gambia, the highest daily intakes have been estimated to exceed 1 µg/kg body weight in children (11) and thus the potential hepatotoxic effect merits further attention. The direct hepatotoxic effect of aflatoxin does not, of course, rule out the concomitant alteration of carcinogen metabolism and may in fact itself contribute to this effect. The situation is likely to be complex, however, given that in rats, chronic exposure to AFB1 results in decreased liver AFB1-DNA adducts due to induction of glutathione-S-transferase activity (39, 40).

In both this study and an earlier one in Gambian children (12), we observed a moderate increase in AF-alb level in HBV carriers compared to noncarriers. In the previous study, no data were available on ALT or other indicators of liver damage. However, while in the present study ALT levels were higher in the chronic HBV carriers compared to other children, the association between AF-alb and ALT did not change when HBV chronic carriers were excluded from the analysis. This finding suggests that it is the liver damage per se which is associated with increased AF-alb levels and one should consider that causes of liver damage other than the HBV associated chronic active hepatitis may occur in this population (e.g., nutritional status or infections with other hepatic viruses or parasites). It is noteworthy that of the four children with acute HBV infection (HBsAg positive at the first bleed and negative at the second) three had AF-alb levels >100 pg/mg and all had ALT levels >35 units/liter. This again indicates that a more complete analysis of the temporal relationship between HBV infection, ALT elevation, and AF-alb adducts would be informative. It would also be of interest to examine the relationship between AF-alb and liver damage in older age group individuals identified as chronic HBV carriers over a number of years and such studies are underway in The Gambia. The clinical relevance of the ALT levels associated with high AF-alb levels in the present study in children remains unclear, particularly given that the majority of ALT values are lower than generally considered to be significant for an adult Caucasian population (40 units/liter). Therefore, in future studies, other clinical data concerning liver damage would be valuable in complementing the data on transaminases and permitting the importance of the association with AF-alb to be better defined.

The possible mechanism of interaction between aflatoxin and HBV is unknown, but among several hypotheses,
in addition to the alteration of carcinogen metabolism discussed above, it has been suggested that chronic HBV infection could act by causing cell death, increased cell replication, and consequently a clonal expansion of mutated cells (for discussion of mechanisms see Refs. 15 and 16). Liver damage, therefore, could act both by alteration of carcinogen metabolism leading to increased DNA damage and mutation and also by subsequently promoting the clonal expansion of mutated cells by increasing cell replication associated with cell death.

Significant differences between the three ethnic groups were observed with respect to some of the examined variables. The data on ethnic group should be considered with some caution, given the possible misclassification of this variable (e.g., in mixed ethnic marriages the child will take the ethnic group of the father and interethnic marriage could be more common in some ethnic groups). Nevertheless, the association between ALT and AF-alb was much stronger in Wolof than in Mandinka and Fula (see “Results”). The individuals of the three ethnic groups were living in the same villages and sharing the same food, so that dietary exposure of the three groups to aflatoxin would be expected to be essentially the same, although the Fula may differ slightly in having a higher milk intake as they are traditionally cattle herders. It seems plausible, therefore, that the apparent difference in the strength of association between ALT and AF-alb in the different ethnic groups could represent a genetic difference in the response to liver damage or in the sensitivity to aflatoxin-induced liver damage. Previously in The Gambia, we reported a higher level of AF-alb in Wolof children compared to Fula and Mandinka (12) in apparent contrast to the present study, where the Fulas had higher adduct levels than the other two groups. In the study by Allen et al. (12), no adjustment was made for ALT level and different degrees of liver damage in the three ethnic groups in that study could conceivably be an explanation for the differing observations. However, in the present study, adjustment for ALT between ethnic groups made little difference to the AF-alb adduct levels, therefore suggesting that other factors are responsible for the difference between the two studies.

Genetic differences between the ethnic groups are evident from the data on GSTM1 genotype (Table 4). The low frequency of deletion of this gene (17.7% in the total population and only 8.8 and 13.3% in Mandinka and Wolof, respectively) is striking compared to previous studies in Europe (approximately 50% deletion) and parts of Asia (2-90%) (see 41). Liu et al. (23) proposed a significant role for human GSTM1 in aflatoxin conjugation but this was an in vitro study using high protein concentrations. The present results suggest that this enzyme does not have a major impact on aflatoxin detoxification in this population and this in agreement with our own data and others on the low level of GST conjugation of aflatoxin by human liver in vitro (24, 25).1 It is worth noting, however, that the Fula ethnic group with the highest AF-alb adduct levels do have the highest frequency of the null genotype and the association with GSTM1 genotype would be important to verify in studies with larger sample sizes.

This study provides data showing an association between AF-alb adducts and ALT. This may be a direct consequence of aflatoxin-induced hepatotoxicity, but the observation is also consistent with the hypothesis that liver damage could increase activation of carcinogen and consequently increase hepatic DNA damage, as one component in the multistep process of carcinogenesis. It is now important to examine the expression of specific cytochrome P450s and GSTs using noninvasive markers (19) in individuals with liver damage induced by various agents including chronic HBV infection.

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