

Modification of Immune Function through Exposure to Dietary Aflatoxin in Gambian Children

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Aflatoxins are immunotoxins that frequently contaminate staple foods in The Gambia and other parts of sub-Saharan Africa, resulting in high exposure throughout life. Impaired infant immune system development may be a key predictor of mortality from infectious disease. In this study we aimed to determine the effect of dietary aflatoxin exposure on a number of immune parameters in Gambian children. A cohort of 472 Gambian children 6–9 years of age was recruited. Serum aflatoxin-albumin (AF-alb) adducts were analyzed to provide a measure of exposure. Immune parameters included secretory IgA (sIgA) in saliva, cell-mediated immunity (CMI), determined using the CMI multitest where test antigens are applied to the skin, and antibody responses to both rabies and pneumococcal polysaccharide vaccines. Birth weight, current anthropometry, and micronutrient status were also recorded. AF-alb adducts were detected in 93% of the children (geometric mean level 22.3 pg/mg; range 5–456 pg/mg). AF-alb level was strongly influenced by month of sampling. In a multivariable analysis, sIgA was markedly lower in children with detectable AF-alb compared with those with nondetectable levels [50.4 µg/mg protein (95% confidence interval [CI] 48.0–52.8) and 70.2 µg/mg protein (95% CI 61.1–79.2), respectively; $p < 0.0001$]. Antibody response to one of four pneumococcal serotypes, but not rabies vaccine, was weakly associated with higher levels of AF-alb. There was no association between CMI responses to test antigens and AF-alb. These data confirm that children in rural Gambia are frequently exposed to high levels of aflatoxin. The study provides evidence that sIgA in saliva may be reduced because of dietary levels of aflatoxin exposure. Given the high burden of infection-related mortality in West Africa, further investigation of the immune effects of aflatoxin exposure in children is merited. **Key words:** aflatoxin, children, diet, Gambia, immunity. *Environ Health Perspect* 111:217–220 (2003). [Online 30 October 2002]

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In rural Gambia season can strongly influence the nutritional status of both children and adults (Cole 1993). During the annual wet season from July to November (the hungry season), weight loss occurs in pregnant and lactating women, and there is a reduction in birth weight of 200–300 g compared with that in other seasons; this difference can be reduced by maternal dietary supplementation (Ceesay et al. 1997). Season of birth has also predicted infection-related mortality in adults (Moore et al. 1997), suggesting the importance of intrauterine and early childhood environment on health and disease in later life (Moore et al. 1999).

In addition to frequent exposure to infectious pathogens, Gambian populations have some of the highest recorded levels of chronic exposure to a family of fungal metabolites known as aflatoxins. Aflatoxin contamination is associated with the storage of groundnuts and maize, dietary staples, colonized by *Aspergillus flavus* and *A. parasiticus* (Hall and Wild 1994). Aflatoxins are human carcinogens (IARC 1993), but also have an immunosuppressive effect in many species (Bondy and Pestka 2000). A major effect has been suppression of cell-mediated immunity (CMI), most notably an impairment of delayed-type

hypersensitivity, which has been a consistent observation at low-dose levels in different species (Pier et al. 1977; Pier and McLoughlin 1985). Aflatoxin also increases susceptibility to bacterial and parasitic infections and adversely affects acquired immunity, as evidenced following experimental challenge with infectious agents after vaccination (Denning 1987).

Despite data from animal studies, evidence of the immunosuppressive action of aflatoxins in humans is limited to *in vitro* studies. Extremely low doses (0.05–0.1 pg/mL) of aflatoxin B₁ (AFB₁) decreased phagocytosis and the microbiocidal activity (against *Candida albicans*) of human monocytes *in vitro* (Cusumano et al. 1996) and decreased the secretion of interleukin-1, interleukin-6, and tumor necrosis factor- α (Rossano et al. 1999). These studies are striking when considered in the light of levels of free AFB₁ reported in sera in West Africa, which range up to 3 ng/mL (Denning et al. 1988).

Aflatoxin exposure can be assessed accurately by measuring aflatoxin-albumin (AF-alb) adducts in peripheral blood (Chapot and Wild 1991; Montesano et al. 1997). Using this biomarker, we demonstrated previously that rural Gambian populations are exposed

to aflatoxin throughout life, including *in utero* (Allen et al., 1992; Groopman et al. 1992; Turner et al. 2000; Wild et al. 1991, 1992, 1993, 2000). In this study we examined whether aflatoxin exposure was associated with several immune parameters in a cross-sectional study of 478 Gambian children. We reported previously the effects of season of birth, birth size, and maternal supplementation on immune function in the same children (Moore et al. 2001). A preliminary report on the aflatoxin data in the latter manuscript used incomplete statistical analysis and consequently did not explore in detail the associations between aflatoxin exposure and various immune parameters.

Materials and Methods

Recruitment. This study involved children born during a 5-year maternal dietary supplementation trial in 28 villages in the West Kiang region of The Gambia, commencing in 1989 (Ceesay et al. 1997). The children recruited into the current study were all those born during the first 2 years of the supplementation study, still living in West Kiang, and willing to participate. Scientific approval for the study was granted by the Medical Research Council (MRC), Gambia Scientific Coordinating Committee. Ethical permission was granted by the joint Gambian Government and MRC Ethical Committee. During the original study, 2,047 live infants were born, and the present study recruited 472 of these children 6–9 years of age (251 male, 221 female). The majority ethnic group was Mandinka, with a few Fula. Weight for age Z-score (WAZ), height for age

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Z-score (HAZ), and weight for height Z-score (WHZ) were calculated according to the median value of the international reference population recommended by the National Center for Health Statistics/World Health Organization (WHO; 1986) using EpiInfo 2000 (U.S. Department of Health and Human Services, Washington, DC). All measurements and blood samples were obtained between May 1998 and February 1999.

Study protocol. On day 0, a fasted venous blood sample was collected for *a*) plasma micronutrient status (zinc, vitamin C, vitamin A, and related retinoids); *b*) prevaccination antibody titers; and *c*) serum AF-alb analysis. At this time the CMI multitest was applied (see "Immune Measurements"). Forty-eight hours later the CMI test was assessed, at which time both rabies and pneumococcal vaccines were given. On day 16, a finger-prick blood sample was obtained to assess response to the first dose of rabies vaccine. On day 30, a second finger-prick blood sample was obtained to assess the response to pneumococcal vaccination, and the second dose of rabies vaccine was given. On day 60, a third blood sample was taken to assess response to the second dose of rabies vaccine. The vaccines used in the current study were chosen to represent a primarily T-cell-dependent response (rabies) and a primarily T-cell-independent response (pneumococcal). The protective effect of the pneumococcal vaccine is related to the development of antipneumococcal capsular polysaccharide antibodies that enhance phagocytosis of the bacteria. Although pneumococcal polysaccharides directly trigger the activation of B cells, T cells and other genetic factors influence the immunoglobulin class and the magnitude of the antibody response, so the response cannot be considered entirely T-cell-independent. The production of antibody to human diploid-cell rabies vaccine, however, is primarily T-cell-dependent. The use of the rabies vaccine additionally allowed assessment of antibody production against a naïve vaccine antigen.

Prevaccine blood sample analysis. Blood samples were available for 466 of 472 children. Serum was separated from the blood sample obtained from each child before the vaccination regimes began (day 0). The levels of AF-alb adduct were determined by albumin extraction, digestion, and enzyme-linked immunosorbent assay (ELISA), as previously described (Chapot and Wild 1991). The detection limit was 5 pg AF-lysine equivalents/mg albumin. Three positive and one negative control sample were analyzed with each batch of samples. Samples were measured in quadruplicate on at least two occasions on separate days; coefficients of variation were less than 25%. Micronutrient

analysis has been detailed elsewhere (Moore et al. 2001).

Immune measurements. Secretory IgA (sIgA) in saliva was measured by modification of an ELISA used to determine breast milk antimicrobial factors (Prentice et al. 1984, 1991). The sIgA level was expressed as micrograms per milligram total salivary protein. CMI was tested using the Merieux CMI multitest kit (Marcel Merieux, Lyon, France) according to the manufacturer's instructions. The CMI recall test antigens are tetanus, diphtheria, streptococcus, tuberculin, candida, trichophyton, proteus, and glycerin as a control. The antigens were applied to the skin of the forearm. The response was determined 48 hr after application, with an induration of > 2 mm considered positive for each antigen. Anergy is defined as a failure to respond to any of the antigens.

Antibody responses to pneumococcal serotypes 1, 5, 14, and 23 antigens were determined after administration of 23-valent pneumococcal capsular polysaccharide vaccine (Pneumovax 23; Merck and Co., Inc, West Point, PA, USA) and after each of two doses of rabies vaccine (Rabies Vaccine BP; Pasteur-Merieux Connaught, Lyon, France). Antibody titers against pneumococcal vaccine were measured at the Department of Immunology, Institute of Child Health (London, UK). Antibody titers were tested against three capsular polysaccharide components of the vaccine that are usually immunogenic (serotypes 1, 5, and 14) and one component that is less immunogenic (serotype 23). Antirabies antibody titers were determined at the Central Veterinary Laboratories (Surrey, UK), using the rapid-focus fluorescence inhibition test of the WHO (Smith et al. 1973).

Statistical analysis. The AF-alb level and rabies and pneumococcal vaccine outcomes were not normally distributed and were consequently natural log transformed before statistical analysis. For clarity of presentation the log-transformed levels and 95% confidence intervals (CIs) were back transformed, and data throughout are presented as geometric means with 95% CIs. AF-alb adduct level was divided into six groups. Group 1 samples were below the detection limit; the remaining samples were divided into quintiles of increasing adduct level (groups 2–6). For sIgA the AF-alb level was additionally considered as a dichotomous variable, with all samples in group 1 below the detection limit. The effect of each independent variable (AF-alb, plasma micronutrient, anthropometry, age) on each dependent variable (immune outcome) was determined by regression analysis in STATA 7.0 (Stata Corp., College Station, TX, USA). The contribution of each variable was then examined in a multivariable model that

always included month of blood sample collection and sex.

Results

Aflatoxin exposure. The sera of 466 of 472 children 6–9 years of age were tested for the level of AF-alb adducts; 93% ($n = 434$) of the samples were positive (geometric mean adduct level 22.3 pg/mg, 95% CI 20.3–24.5; range 5–456 pg/mg). AF-alb level was not significantly related to the age or sex of the children. However, the level of AF-alb was significantly ($p = 0.0001$) related to month of blood sample collection, although sample collection covered two cycles of harvest and storage, thus reflecting both annual and seasonal variation.

Micronutrient status. The levels of micronutrients in the prevaccination bleeds are detailed elsewhere (Moore et al. 2001). In brief, there were seasonal variations in a number of micronutrients—for example, vitamin C, α - and β -carotene, and lycopene—reflecting periods in January–May when citrus fruits and mangos were available, and September–October when more green leafy vegetables are included in the diet. However, of all the micronutrients measured, the only association with AF-alb was a negative correlation with vitamin C ($p = 0.01$).

Anthropometry. Anthropometric results are presented as Z-scores according to WHO criteria (WHO 1986), where a score < -2 is recognized as a state of malnutrition, and a score < -3 as severe malnutrition. In this study, 11.5% of the children were stunted (HAZ-score < -2), 17.5% were underweight (WAZ-score < -2), and 14.9% were classified as wasting (WHZ-score < -2). AF-alb level was weakly associated ($p = 0.034$) with a lower WHZ score and subsequently fitted to a regression model. AF-alb was grouped into nondetectable and quintiles of detectable adducts. In this model, AF-alb level ($p = 0.028$), month of sampling ($p = 0.003$), sex ($p = 0.044$), and birth weight ($p = 0.024$) were all associated with WHZ. When adjusted for all other factors contributing to the model, there was a decrease in WHZ score up to 21 pg/mg,

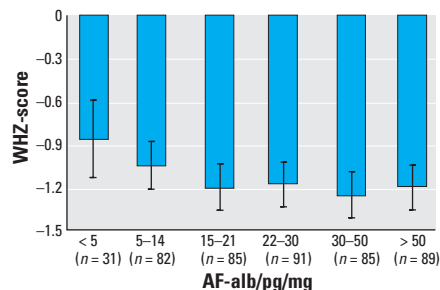


Figure 1. WHZ compared with AF-alb level. Data are presented as adjusted mean with 95% CI.

after which the WHZ-score reached a plateau (Figure 1). AF-alb level was not associated with either HAZ or WAZ scores.

Immune outcomes. Salivary secretory IgA. The mean sIgA was 51.8 $\mu\text{g}/\text{mg}$ protein (range 10.0–343 $\mu\text{g}/\text{mg}$). Aflatoxin exposure was strongly associated ($p = 0.006$) with reduced sIgA levels and subsequently fitted to a regression model. AF-alb was used as a dichotomous variable: those with nondetectable levels of adduct ($n = 32$) and those with detectable adduct levels ($n = 432$). In this model, AF-alb ($p < 0.0001$), sex ($p = 0.041$), age ($p = 0.040$), month of birth ($p = 0.016$), and mid-upper-arm circumference ($p = 0.002$) were all associated with the sIgA level. When adjusted for all other factors contributing to the model, the mean adjusted sIgA level was lower in children with detectable AF-alb adducts (50.4 $\mu\text{g}/\text{mg}$; 95% CI, 48.0–52.8) compared with those without detectable adducts (70.2 $\mu\text{g}/\text{mg}$; 95% CI, 61.1–79.2) (Figure 2). No dose–response effect was observed when examined as quintiles of exposure.

CMI response. All but three children were successfully tested for CMI using the skin test. The responsiveness to the individual antigens, however, was low, ranging from 6.0 to 21.3%, with 50% of children being anergic (unresponsive to any test antigens). None of the children reacted to the glycerin control. The geometric mean AF-alb adduct levels in anergic children (23.8 pg/mg ; 95% CI, 21.1–26.8) and nonanergic children (24.3 pg/mg ; 95% CI, 21.5–27.4) were not statistically different. In addition, no significant association between level of AF-alb and responsiveness to the individual antigens or to the total number of responses to test antigens was observed.

Vaccination response. The rabies antibody titers and three of the four pneumococcal antibody titers were not associated with AF-alb level. In a multivariable regression model for pneumococcal serotype 23, antibody titer was associated with AF-alb, but only with marginal statistical significance ($p = 0.05$). WHZ-

($p < 0.0001$) and weight ($p < 0.0001$) were also positively associated with antibody titer. However, there was no strong trend in adjusted geometric mean antibody titer with increasing AF-alb adduct level (Figure 3).

Discussion

In The Gambia, season of birth has been associated with altered morbidity and mortality in adulthood, which are frequently infection related (Moore et al. 1999). Aflatoxins are also prevalent in this population, and there are seasonal variations in the level of food contamination and therefore exposure (Turner et al. 2000; Wild et al. 2000). Because aflatoxins are potent immunosuppressors in animals (Bondy and Pestka 2000; Richard 1998), we examined whether aflatoxin exposure was associated with a spectrum of immune tests reflecting T-cell, B-cell, and mucosal secretion as measured by the CMI test, vaccine responses, and the level of sIgA in saliva. The present cross-sectional study involved a cohort of children 6–9 years of age that was part of a larger study investigating fetal nutrition by maternal nutritional supplementation on long-term immune outcome. In addition to aflatoxin, the levels of many of the nutritional factors were seasonal, so month of sampling was an important parameter in the multivariable models. The supplementation status of mothers during pregnancy was not significantly associated with the measured immune parameters in this study.

AF-alb in peripheral blood reflects consumption of toxin over the preceding 2–3 months. In this study, 93% of the children had detectable AF-alb. We previously demonstrated similar high levels of exposure in both Gambian adults and children (Allen et al. 1992; Groopman et al. 1992; Turner et al. 2000; Wild et al. 1991, 1992, 1993, 2000). However, it is worth noting that the mean level in the present study, although high, was somewhat lower than previously observed. This may reflect geographic, seasonal, and annual variations in the levels of aflatoxin in contaminated food.

This study showed a weak association between adduct level and wasting (WHZ-

scores), but not for stunting (HAZ-score) or being underweight (WAZ-score). We previously showed a very strong association between AF-alb adduct level and stunting and being underweight (Gong et al. 2002), but this was in a younger group of children (9 months to 5 years of age) from Benin and Togo, who may be more sensitive to the growth-inhibitory effects of aflatoxin. In addition, the children in Benin and Togo had some of the highest AF-alb levels observed in any of our studies in West Africa.

Aflatoxin exposure was significantly associated ($p < 0.0001$) with a decreased level of sIgA [from 70.2 $\mu\text{g}/\text{mg}$ (95% CI, 61.1–79.2) in children with no detectable AF-alb to 50.4 $\mu\text{g}/\text{mg}$ (95% CI, 48.0–52.8) in those with detectable adduct level]. In saliva, breast milk, tears, and mucus of the bronchial, genitourinary, and digestive tracts, sIgA binds to bacterial and viral surface antigens, providing an important component of the mucosal barrier. Given the high levels of aflatoxin exposure and frequency of infectious insult in African populations, this observation suggests that toxin exposure may influence susceptibility to infectious disease. The mechanism behind this observation is uncertain, although aflatoxin can disrupt protein synthesis through binding to DNA, RNA, and proteins (Bondy and Pestka 2000).

In this study using the CMI test, 50% of the children were classified as anergic. There are no previous data from The Gambia, but in nearby Guinea Bissau, between 17 and 31% of children 3–13 years of age were anergic using the same test, with higher prevalence in the rainy season (Shaheen et al. 1996). Despite the high prevalence of anergy, there was no association between the CMI response and the aflatoxin biomarker level. This appears to contradict the strong effects of aflatoxin on CMI in animals. Previous studies—e.g., the one in Guinea-Bissau (Shaheen et al. 1996)—have shown the CMI test can detect significant differences between exposures variables and predict outcome measures, so this test should have been adequate to detect modest differences between exposure groups in this population. One possibility is that even these high aflatoxin exposures are insufficient to cause this effect in humans. Alternatively, the timing of the aflatoxin exposure assessment may not have been relevant to the effects on immune status, because it is unknown whether recent or past exposure is important in determining immune modulation. In the cross-sectional design employed, only a single measure of AF-alb was made (at the time of the CMI test). The AF-alb marker integrates aflatoxin exposure over the previous 2–3 months; ideally, exposure needs to be considered in the context of the dynamic of the immune system for each of the parameters

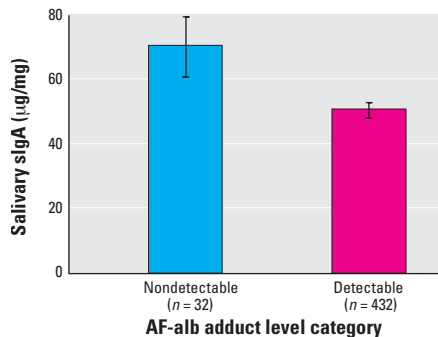


Figure 2. sIgA levels in saliva in children with and without detectable aflatoxin exposure. Data are presented as adjusted mean with 95% CI.

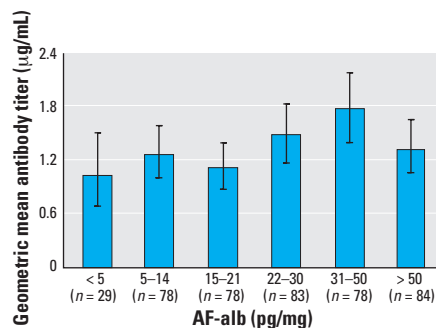


Figure 3. Pneumococcal vaccine, type 23, antibody titer response compared with AF-alb level. Data are presented as adjusted mean with 95% CI.

under consideration. A longitudinal study may be more informative in this regard. In addition, in developed countries only about 2% of the population are anergic (Beier-Holgersen and Brandstrup 1999), and therefore strong confounding factors in the Gambian population may be masking any more-modest effects of aflatoxin exposure. It is also possible that the low levels of response to the CMI testing in our study could indicate malfunction of the test kits; however, the cold chain was meticulously preserved, and the kits were applied by a single investigator according to the manufacturer's instructions.

A weak association between AF-alb adduct level was observed with pneumococcal serotype 23 ($p = 0.05$) vaccination responses but, contrary to expectations, there was a tendency to higher antibody titers with increasing levels of AF-alb. No associations were observed for the other test vaccines. The effects of aflatoxin on the immune system are complex. Aflatoxin exposure has occasionally been shown to increase antibody production (Richard et al. 1998), but generally reductions are observed (Azzam and Gabal 1997; Dimitri and Gabal 1996; Fernandez et al. 2000; Gabal and Dimitri 1998). At present it is not possible to say whether these associations we report are chance findings or represent an unexpected effect of aflatoxin exposure.

In conclusion, populations in West Africa are frequently exposed to high levels of aflatoxin, and exposures in childhood may have a critical influence on disease outcomes in later life (Turner et al. 2000). Turner et al. (2000) estimated that 30% of Gambian children are exposed to food contaminated with more than 100 ppb aflatoxins. Previous studies in poultry indicate that immune competence is compromised when feeds contain similar levels of contamination (reviewed by Coulombe 1994). Despite the inherent limitations in cross-species comparisons, the data nevertheless indicate that children are naturally exposed to aflatoxin through the diet at levels that compromise the immune system in other species. This study specifically observed a highly significant association between aflatoxin exposure and reduced salivary sIgA. The possible impact of this effect on health outcomes in this population merits consideration, particularly within the context

of intervention studies to reduce aflatoxin exposure.

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