Aflatoxin B1 albumin adduct levels and cellular immune status in Ghanaians

Yi Jiang[1], Pauline E. Jolly[1], William O. Ellis[2], Jia-Sheng Wang[3], Timothy D. Phillips[4] and Jonathan H. Williams[5]

[1]Department of Epidemiology and International Health, School of Public Health, University of Alabama at Birmingham, Birmingham, AL 35294, USA
[2]Department of Biochemistry, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana
[3]Department of Environmental Toxicology, Texas Tech, Lubbock, TX, USA
[4]College of Veterinary Medicine, Faculty of Toxicology, Texas A&M, College Station, TX, USA
[5]Peanut Collaborative Research Support Program, College of Agricultural and Environmental Sciences, University of Georgia, Griffin, GA, USA

Abstract

Although aflatoxins (AFs) have been shown to be immune-suppressive agents in animals, the potential role of AFs in modifying the distribution and function of leukocyte subsets in humans has never been assessed. We examined the cellular immune status of 64 Ghanaians in relation to levels of aflatoxin B1 (AFB1)–albumin adducts in plasma. The percentages of leukocyte immunophenotypes in peripheral blood, CD4+ T cell proliferative response, CD4+ T_h and CD8+ T cell cytokine profiles and monocyte phagocytic activity were measured using flow cytometry. NK cell cytotoxic function was determined by perforin and tumor necrosis factor-α expression in CD3−CD56+ NK cells. AFB1–albumin adducts levels ranged from 0.3325 to 2.2703 (mean = 0.9972 ± 0.40, median = 0.9068) pmol mg^{-1} albumin. Study participants with high AFB1 levels had significantly lower percentages of CD3+ and CD19+ cells that showed the CD69+ activation marker (CD3+CD69+ and CD19+CD69+) than participants with low AFB1 levels (P = 0.002 for both). Also, the percentages of CD8+ T cells that contained perforin or both perforin and granzyme A were significantly lower in participants with high AFB1 levels compared with those with low AFB1 (P = 0.012 for both). Low levels of CD3+CD69+ (r = −0.32, P = 0.016) and CD19+CD69+ (r = −0.334, P = 0.010) cells were significantly associated with high AFB1 levels using correlation analysis. By multivariate analysis, there were strong negative correlations between the percentages of these cells (CD3+CD69+: b = −0.574, P = 0.001, and CD19+CD69+: b = −0.330, P = 0.032) and AFB1 levels. These alterations in immunological parameters in participants with high AFB1 levels could result in impairments in cellular immunity that could decrease host resistance to infections.

Introduction

Aflatoxins (AFs) are potent carcinogens that are produced as secondary metabolites of strains of the fungi *Aspergillus parasiticus* and *Aspergillus flavus* that grow on important food crops such as groundnuts, maize and other oilseeds (1). Many studies have linked AF contamination of foods with acute illness resulting in death (2) and with liver cancer (3–5). Low levels of AF ingestion in contaminated foods have been shown to cause suppression of the immune system and to increase susceptibility to diseases in several animal species (6–12). Although the major AFs, B1, B2, G1 and G2, occur together in various foods in different proportions (1), aflatoxin B1 (AFB1) is usually the predominant and most toxic form. A major metabolic product of AFB1 is AFM1 that is usually excreted in milk and urine of dairy cattle and other mammals (1). The immunosuppressive properties of AFB1, particularly on cell-mediated immunity, have been demonstrated in various animal models (6–8, 13–14). In these animal studies AFB1 has been shown to reduce T-lymphocyte function and number, suppress lymphoblastogenesis and impair delayed cutaneous hypersensitivity (11, 15–17) and graft-versus-host reaction (18–19). AF has also been shown to suppress phagocytic activity and reduce complement activity (11).

Significant decreases in splenic CD4 (T_h) cell numbers and IL-2 production have been observed in mice treated with AFB1 (20). Several studies show that AF might impair the function of macrophages in different animal species (21–23). Phagocytoses of alveolar macrophages and tumor necrosis factor (TNF)-α release were suppressed in rats and mice exposed to AF through the respiratory tract (24). AF exposure has also been shown to inhibit phygocytic cell function in normal human peripheral blood monocytes *in vitro* (25). AFB1 at concentrations ≥100 pg ml^{-1} was cytotoxic to the monocytes, and concentrations of 0.5–1 pg ml^{-1} inhibited monocyte phagocytic activity and intracellular killing of *Candida albicans*. Natural killer (NK) cell-mediated cytolysis of YAC-1 target cells in BALB/c mice has also been shown to be suppressed by AFB1 (26). More recently, a study conducted in weanling piglets exposed to low doses of AF showed that AF alters many aspects of humoral and cellular immunity in piglets and resulted in a reduction in primary and secondary immune responses and in antibody response in immunization with *Mycobacterium agalactiae* (27). The effect of AF on humoral immunity is not as evident as the effects on cell-mediated immunity, is less consistent in different species and seems to require higher doses of AF (20).

Although much data on the immune effects of AF are available from animal studies, there is a paucity of data on the effect of chronic consumption of AF-contaminated food on the human immune system. A recent study conducted in Gambian children reported that secretory IgA in saliva may be reduced by dietary levels of AF (28). The potential role of AFB1 in modifying the peripheral distribution of lymphocyte subsets and their function in humans has never been assessed. This information is urgently needed, especially as immune suppression is a significant global health problem and immune-suppressive/modulating diseases such as HIV/AIDS, malaria and other parasitic infections are rampant in developing tropical countries such as Ghana. In these countries, millions of people are chronically exposed to AF in their diet. We examined the distribution and function of lymphocyte subsets in Ghanaians in relation to AFB1–albumin adduct levels in their plasma and present the first comprehensive report of the effect of AF on the cellular immune system in humans.

Methods

Participant recruitment, data and sample collection

A cross-sectional study was conducted in four villages in the Ejura Sekyedumase District in the Kumasi Region of Ghana. Villagers who were interested in participating gave their informed consent and were asked to complete a survey on sociodemographic characteristics. A 20-ml blood sample was collected from 64 participants in EDTA vacutainer tubes. Plasma was separated and PBMCs were prepared using Ficoll–Hypaque density gradients as previously done (29) and cell viability was checked. PBMCs were stored frozen in liquid nitrogen and shipped to University of Alabama at Birmingham for analysis. The protocol for the study was approved by the Institutional Review Board of the University of Alabama at Birmingham and the Medical

School Ethics Committee of the Kwame Nkrumah University of Science and Technology.

Determination of AFB1–albumin adduct levels in plasma by radioimmunoassay

AFB1–albumin adduct levels in plasma of study participants were determined by radioimmunoassay (RIA) as published previously (30–31). The assay measures AF that is covalently bound to albumin in peripheral blood and reflects AF exposure in the previous 2–3 months. Briefly, human plasma samples were concentrated by high-speed centrifugal filtration. The concentrated protein was re-suspended in PBS and the amount of human plasma albumin and total protein was determined. The total protein was then digested with Pronase (Calbiochem, La Jolla, CA, USA) after which the sample was mixed with ice-cold acetone and kept at 4°C for 1 h. The suspension was then centrifuged and the resulting supernatant containing the bound AF was decanted and dried *in vacuo* using a Savant Speed-Vac Concentrator. The RIA procedure (30–31) was used to quantify AF–albumin adducts in duplicate human plasma protein digests, each containing 2 mg protein. Non-specific inhibition in the assay was determined by processing pooled normal human plasma standards obtained from Sigma (St Louis, MO, USA) and the average values of the background were subtracted from those of test samples in calculating AFB1–albumin adduct levels. The standard curve for the RIA was determined using a nonlinear regression method (32) and values were expressed as the amount of AFB1 per milligram of albumin (30).

Determination of percentages of leukocyte immunophenotypes using flow cytometry

We determined the percentages of certain leukocyte immunophenotypes in PBMCs from the study participants. The percentages of T cells (CD3+), subsets of T cells (CD4+ and CD8+), B cells (CD19+), NK cells (CD3−CD56+) and macrophages (CD14+) were measured by flow cytometry. CD8+ T cell subset classification has been proven to be useful in monitoring the immune system in several clinical situations (33). Therefore, we classified CD8+ T cell subsets into naive (CD8+CD45RA+CD27+), memory (CD8+CD45RA−CD27+) and effector (CD8+CD45RA+CD27−) CD8 T cells by flow cytometry. Subtypes of NK cells CD3−CD56^{bright}CD16^{dim} and CD3−CD56^{dim}CD16^{bright} were determined. The percentages of activated marker CD69 on CD3+ T cells and CD19+ B cells were also measured.

PBMCs were incubated with combinations of FITC-, PE-, PerCP-labeled mAbs against CD3, CD4, CD8, CD14, CD16, CD19, CD56, CD45RA, CD27 and CD69 (BD PharMingen, San Diego, CA, USA) for 30 min at 4°C. Isotype-matched irrelevant FITC-, PE- and PerCP-labeled mAbs (BD PharMingen) were used as controls in the experiments. After washing the cells three times in PBS, cell fluorescence for each phenotype was analyzed using Becton Dickinson (San Diego, CA, USA) FACS and CELLQuest software.

Lymphoproliferative response of CD4+ T cells

We measured CD4+ T cell proliferative response using 5-bromo-2-deoxyuridine (BrdU, Sigma) incorporation assay

by flow cytometry (34). PBMCs (1×10^6) were placed in 12×75-mm tissue culture tubes containing 2 ml of medium with or without PHA (Sigma). The cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere for 3 days. BrdU was added to the cells at a final concentration of 40 μM 16 h before the end of the 3-day incubation period. After incubation, cells were collected and stained with an anti-BrdU mAb conjugated to FITC (Becton Dickinson) together with a PE-coupled anti-CD4 mAb (Becton Dickinson) in the presence of deoxyribonuclease (Sigma). Thereafter, at least 10 000 cells were analyzed by flow cytometry.

Cytokine production by CD8+, CD4+ and CD3−CD56+ cells

The cytokine profile of participants was analyzed using intra-cytoplasmic staining of cytokine in PBMC samples. This technique enables phenotypic characterization of cytokine-producing cells and, therefore, allows a more accurate and detailed comparison of the cytokine profile of blood lymphocytes at a single cell level.

CD4+ T_h cytokine profile (IL-4, IFN-γ,) was examined after stimulation *ex vivo* with phorbol myristate acetate (PMA) and ionomycin. The proportions of CD4+ cells that stained positive for IFN-γ and IL-4 were determined by a combination of cell-surface and intracellular mAb staining and analysis by flow cytometry. CD8+ T cell cytokine production (IFN-γ, IL-4, perforin and granzyme A) was measured by intracellular cytokine staining and multiparameter flow cytometry. Also, we examined cytotoxic function of NK cells by detecting perforin and TNF-α expression in phenotypically defined NK cells (CD3−CD56+).

For intracellular cytokine staining, PBMCs (1×10^6) were placed in 12×75-mm tissue culture tubes containing 2 ml of medium containing 0.5 μg each of CD28 and CD49d mAbs with or without PMA (Sigma). These cultures were incubated at 5° slants at 37°C in a humidified 5% CO_2 atmosphere for 6 h. In the last 5 h, 10 μg ml^{-1} of the secretion inhibitor, Brefeldin A (Sigma), was added. After incubation, the cells were collected in PBS and washed once with cold PBS containing 1% BSA. Cells were then re-suspended in 100 μl of staining buffer (PBS supplemented with 0.1% sodium azide and 1% fetal bovine serum (FBS) pH 7.4; BD PharMingen) and the phenotypic mAb (CD3, CD4, CD8 and CD56) and incubated at 4°C for 30 min. After staining, the cells were washed with PBS and re-suspended in 1 ml of fix/perm buffer (BD PharMingen). The cells were then fixed for 30 min at 4°C, washed, re-suspended in perm-staining buffer and incubated with cytokine antibodies (anti-IFN-γ, anti-IL-4, anti-perforin, anti-granzyme A and anti-TNF-α; BD PharMingen) for 30 min at 4°C. The cells were then washed with perm-staining buffer and re-suspended in fixative buffer (BD PharMingen) for flow cytometric analysis on a Becton Dickinson FACS using CELLQuest software.

Determination of monocyte phagocytic function

We examined phagocytosis of peripheral blood monocytes by flow cytometry using the method of Steinkamp *et al.* (35) with some modifications. The concentration of cell suspension was adjusted to 4×10^6 cells ml^{-1} in medium without serum. Fluorescent latex particles (2.5% latex solids, 2.0 μm in diameter;

Polyscience, Warrington, PA, USA) were diluted 10 times with medium and 4 μl of the suspension of these particles added to 1 ml of cell suspension. The mixture was allowed to react at 37°C for 90 min with gentle shaking in a water bath. The cells were then washed three times with cold PBS (pH 7.4) to terminate the reaction and stained with PE-labeled anti-CD14 mAb. The final pellet was re-suspended with PBS and applied to flow cytometry to analyze the phagocytosed fluorescent particles. The phagocytic rate, which represents the percentage of cells with ingested particles relative to the total number of CD14+ cells, was determined.

Statistical analysis

Data were entered and analyzed using Windows SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). Statistical analysis was performed using the Mann–Whitney *U*-test. Correlation analyses were performed between the different immune variables and AFB1 levels. Multiple linear regression analysis was employed to assess the relationship of AFB1 levels in plasma with age, gender, percentage of T and B cells and other immune variables. Dummy variables were used to assess the effect of categorical variables or continuous variables where the relationship was not linear. A probability value of $P < 0.05$ was considered statistically significant.

Results

Age, gender and AFB1–albumin adduct levels (in picomoles per milligram of albumin) of study participants

Study participants ranged in age from 19 to 86 years. Of the 64 participants, 34 were males and 30 were females. AFB1–albumin adducts levels for the 64 study participants ranged from 0.3325 to 2.2703 pmol mg^{-1} albumin with a mean of 0.9972 ± 0.40 and median of 0.9068 pmol mg^{-1} albumin (Fig. 1). Majority (98%) of the participants had levels of AFB1 >0.4 pmol mg^{-1} albumin in their blood.

Study participants were divided into two groups based on the median AFB1 level of 0.9068 pmol mg^{-1} albumin. Those individuals who had AF levels of 0.9068 pmol mg^{-1} albumin and above were in the high-AF group and those with AF levels <0.9068 pmol mg^{-1} albumin were in the low-AF group. The
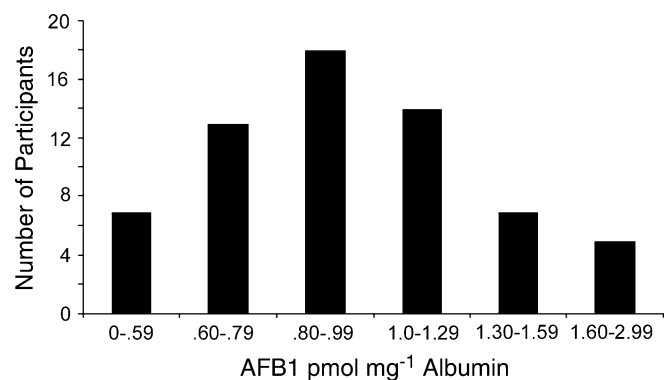

Fig. 1. Plasma AFB1–albumin adduct levels of study participants. AFB1 adduct levels ranged from 0.3325 to 2.2703 pmol mg^{-1} albumin with a mean of 0.9972 ± 0.40 and median of 0.9068 pmol mg^{-1} albumin.

participants in the two groups did not differ significantly in age and gender. The high-AFB1 group consisted of 18 males (mean age ± SD = 44.83 ± 19.80 years) and 14 females (mean age ± SD = 42.29 ± 19.48 years). There were 16 males (mean age ± SD = 47.13 ± 16.38 years) and 16 females (mean age ± SD = 38.81 ± 16.12 years) in the low-AFB1 group.

Percentages of leukocyte phenotypes in relation to AFB1 levels

When the mean percentages of the various leukocyte subsets of the study group were examined according to high and low AFB1 levels, there were no differences in the percentages of CD3, CD4, CD8, CD14, CD19 and CD3−CD56+ cells between the two groups (Fig. 2). Although the mean percentages of T (CD3+) and B (CD19+) cells were not significantly different between participants with high and low AFB1 levels, the mean percentages of CD3 and CD19 cells showing the CD69 activation marker (CD3+CD69+ and CD19+CD69+) were significantly lower (2.164 ± 1.24 and 6.92 ± 4.15, respectively) in participants with high AFB1 than in those with low AFB1 (3.905 ± 2.47 and 11.945 ± 7.07, respectively; P = 0.002 for both) (Fig. 3).

NK cells comprise ~15% of all circulating lymphocytes and are defined phenotypically by their expression of CD56 and lack of expression of CD3. In our participants, the percentage of CD3−CD56+ NK cells was lower (mean ± SD = 4.041 ± 3.023, median = 3.31, range 1.10–17.41) compared with the normal range. The high-AFB1 group had a slightly higher percentage of NK cells compared with the low-AFB1 group (Table 1). Interestingly, the high-AFB1 group had a lower percentage of CD3−CD56^{bright}CD16^{dim} cells than the low-AFB1 group. However, the difference was not significant (Table 1).

Cytokine expression by leukocyte phenotypes in relation to AFB1 levels

The mean percentages of CD4+ T cells expressing IFN-γ and IL-4 were measured after PMA stimulation as double-positive cells by flow cytometry for the two groups. There was no
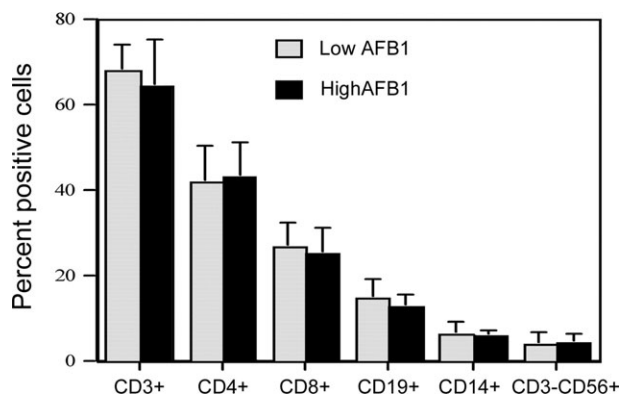

Fig. 2. Percentages of leukocyte immunophenotypes in relation to AFB1 levels. The mean percentages of various leukocyte subsets (CD3+, CD4+, CD8+, CD14+, CD19+ and CD3−CD56+ cells) were determined and examined according to AFB1 levels of the study participants. There were no significant differences in leukocyte subsets between the two groups.

difference in the mean percentages of IFN-γ- and IL-4-expressing CD4+ T cells between the two groups (Table 1). The lymphoproliferative response assay for CD4 T cells was also performed. No difference was observed between the two groups.

For CD8+ T cells, there was no significant difference in the mean percentages of IFN-γ- and IL-4-expressing cells between the high-AFB1 and low-AFB1 groups (Table 1). However, the percentages of CD8+ T cells containing perforin and CD8+ cells containing both perforin and granzyme A were significantly lower in participants with high AFB1 compared with those with low AFB1 (Table 1, Fig. 4). There was no difference in percentages of cytotoxic effector (CD8+CD45RA+CD27−), naive (CD8+CD45RA+CD27+) or memory (CD8+CD45RA−CD27+) CD8+ T cells in the two groups (Table 1).

When perforin- and TNF-α-expressing CD3−CD56+ NK cells were measured, we found no difference in the percentages of TNF-α-expressing NK cells between the two groups (Table 1). Also, there was no difference in the percentages of CD14+ cell phagocytic rate between the groups (Table 1).

Correlation and multivariate analyses

By correlation analysis, factors that were significantly associated with higher AFB1 level included low CD3+CD69+ cells (r = −0.321, P = 0.016) and CD19+CD69+ cells (r = −0.334, P = 0.010). That is, study participants with high levels of AFB1 had significantly lower percentages of CD3+CD69+ and CD19+CD69+.

Multiple linear regression analysis was employed to assess the relationship between AF levels and age, percentage of T and B cells and other variables. After adjusting for age and other immune parameters, the percentage of CD3+CD69+ and CD19+CD69+ cells showed strong negative associations with AFB1 levels (b = −0.574, P = 0.001 and b = −0.330, P = 0.032, respectively).

Discussion

While there are much available data on AF levels in staple foods, there are no data on dietary intake of AF by Ghanaians. Based on AFB1–albumin adducts in our participants and previous work conducted by J.-S.W. and collaborators on AF high-risk populations in China, we estimated that our study participants consumed at least 10 μg of AFB1 in their foods daily.

In this study, 100% of the participants had detectable AFB1–albumin adducts. In Ghana, basic staples such as groundnuts, maize and other grains are contaminated with levels of AF that far exceed the 30 p.p.b. considered tolerable in foods for human consumption. Levels of AF up to 22 168 p.p.b. have been found in 71% of damaged groundnut kernels in market samples (36). While there are much available data on AF levels in staples such as peanuts and maize marketed and consumed in Ghana (36–37), there are no data on dietary intake of AF by Ghanaians. Based on AFB1–albumin adducts in our participants and previous work conducted by Wang *et al.* (38) on AF in high-risk populations in China, we estimated that
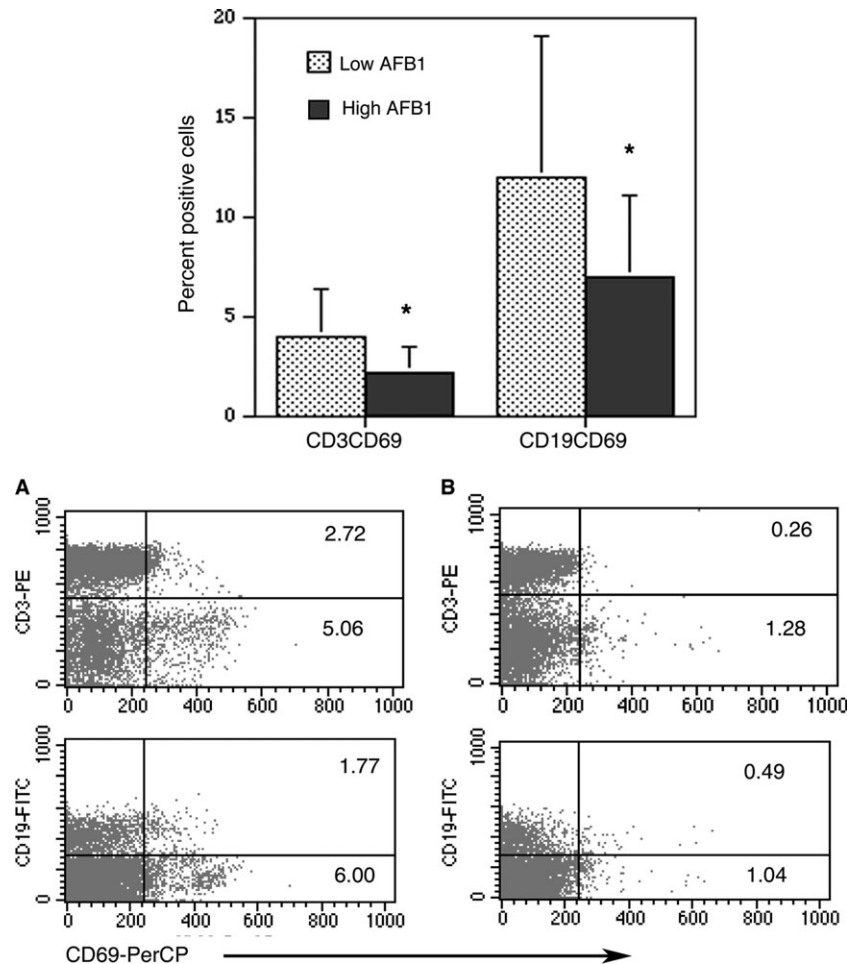
Fig. 3. Percent of activated T cells (CD3+CD69+) and activated B cells (CD19+CD69+) in relation to AFB1 levels. The mean percentages of CD3+CD69+ and CD19+CD69+ cells in the group of participants with high AFB1 were significantly lower (2.164 ± 1.24 and 6.92 ± 4.15, respectively) than in the low-AFB1 group (3.905 ± 2.47 and 11.945 ± 7.07, respectively). In the examples shown in the bottom panel, the percentages of CD3+CD69+ and CD19+CD69+ cells were 0.26 and 0.49%, respectively, for participant B who had high AFB1 and 2.72 and 1.77%, respectively, for participant A who had low AFB1.

that our study participants consumed at least 10 µg of AFB1 in their foods daily.

When the percentages of the different leukocyte subsets were estimated according to high and low AFB1 levels, we found statistically significant differences between the groups in the percentages of CD3 and CD19 cells that express the CD69 activation marker and CD8+ T cells that express perforin and both perforin and granzyme A. The percentages of CD3+CD69+ and CD19+CD69+ in participants with high AFB1 were significantly lower than in participants with low AFB1. The activation marker CD69 (previously known as activation inducer molecule) is a 60-kDa glycosylated co-stimulatory molecule for T-lymphocyte proliferation which is expressed early after triggering of CD2 or CD3/TCR complex. T-lymphocyte activation via CD69+ will progress to proliferation, thus amplifying immune responses (39). In our study, the decreased number of activated T and B lymphocytes in participants with high AFB1 may indicate that the toxic effects of AF on lymphocytes may decrease their expression of the CD69 molecule, which is essential for a normal immune response against an infectious agent or a vaccine. The CD69 down-regulation on T lymphocytes at the cell level could

partially contribute to the inability of these cells to mount immune responses.

The non-significant differences in the frequency of CD4+ IFN-γ- and IL-4-expressing cells observed in our study are similar to those found by Marin *et al.* (27) when they measured expression of mRNA regulatory cytokines in the blood of weanling piglets fed diets contaminated with AF for 30 days. The latter authors found no effect of AF on regulatory cytokines produced by either the T_h1 or T_h2 lymphocyte subsets (27). Similar results were also obtained in mice that received oral gavage with 30–700 µg AF kg^{-1} body weight (40) or in rats that received a weekly dose of 0.1 or 1 mg of AF by intra-gastric inoculation (41). Thus, the lack of effect of AF on regulatory cytokines produced by either the T_h1 or the T_h2 lymphocyte subsets in our results in humans is in agreement with results obtained in mice, rats and pigs that received AF (27, 40–41).

We observed no significant difference in proliferation of CD4+ T cells between the two groups. Previous experiments in pigs showed that AF decreased lymphocyte proliferation (42–43). Raisuddin *et al.* (44) found a dose-dependent suppression of the proliferative response of T and B cells in

Table 1. Proportion of leukocyte phenotypes and cytokine expression in study participants with high and low AFB1 levels

Leukocyte subset	Low AFB1 levels	High AFB1 levels
IFN-γ-expressing CD4+	6.36 ± 5.93	8.45 ± 8.72
IL-4-expressing CD4+	1.57 ± 1.32	1.71 ± 1.29
BrdU CD4%	13.25 ± 6.02	12.05 ± 5.00
IFN-γ-expressing CD8+	10.52 ± 7.41	14.25 ± 11.17
IL-4-expressing CD8+	1.25 ± 1.33	0.98 ± 0.72
Perforin-expressing CD8+	40.33 ± 18.55	29.40 ± 13.75*
Granzyme A-expressing CD8+	65.83 ± 12.37	61.55 ± 17.17
Perforin- and granzyme A-expressing CD8+	36.26 ± 15.04	26.91 ± 12.96*
CD8+CD45RA+CD27− (cytotoxic)	19.06 ± 8.77	18.20 ± 9.93
CD8+CD45RA+CD27+ (naive)	39.45 ± 11.52	40.10 ± 10.83
CD8+CD45RA−CD27+ (memory)	29.78 ± 10.41	31.50 ± 12.66
CD3−CD56+	3.90 ± 3.36	4.24 ± 2.67
Perforin-expressing CD3−CD56+	71.04 ± 15.83	67.97 ± 14.26
TNF-α-expressing CD3−CD56+	15.77 ± 7.97	15.46 ± 10.58
CD3−CD56^{bright}CD16^{dim}	27.12 ± 20.88	20.76 ± 19.26
CD14 phagocytic rate	11.85 ± 9.45	10.44 ± 9.25

*Significant at $P \leqslant 0.05$.

weanling rats fed with AFB1 for 4 weeks. However, a study using human cells and the MTT assay found that AFB1 at concentrations up to 10 µg ml^{-1} had no effect on lymphocyte proliferation (45). These results suggest that AFB1 could induce different T cell responses, depending on the type and amount of exposure to the toxin, the species involved and the method of exposure. Also, it may be possible that the changes observed in proliferation *in vitro* in relation to AFB1 levels could possibly be induced by acute aflatoxicosis. Our study participants are chronically exposed to subacute amounts of AF.

Effector-type CD8+ T cells can kill target cells by releasing a pore-forming protein (perforin) and serine proteases (granzymes) into the vicinity of target cell membranes. In response to antigen, this population of effector-type CD8+ cells with high levels of granzyme, perforin and Fas ligand expression that exert cytolytic activity contributes to the inhibition of pathogen spread through immediate lysis of infected cells. In our study, participants with high AFB1 levels had significantly lower levels of perforin-expressing and perforin- and granzyme A-expressing CD8+ T cells. This suggests that the CD8+ T cell function in individuals with high AFB1 level is impaired. This will definitely affect cellular immune function against infectious diseases.

NK cells are one component of the innate immune system and have the ability to both lyse target cells and provide an early source of immunoregulatory cytokines (46). NK cells can be divided into subsets based on their cell-surface density of CD56. The majority of human NK cells has low-density expression of CD56 (CD56^{dim}) and express high levels of F$_c$γR III (CD16), whereas a smaller percentage of NK cells are CD56^{bright}CD16−. A study conducted by Cooper *et al.* (47) suggests that the major function of the CD56^{bright} NK cells during the innate immune response *in vivo* might be to provide macrophages and other antigen-presenting cells with early IFN-γ and other cytokines, promoting a positive cytokine feedback
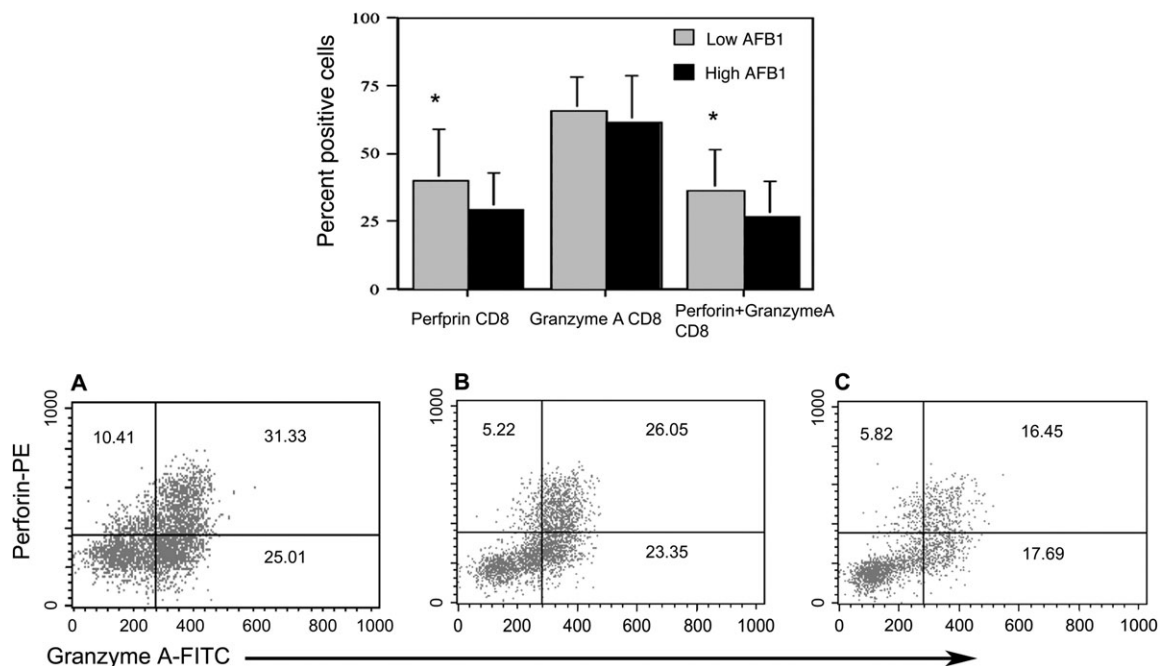
Fig. 4. Flow cytometric analysis of intracellular perforin and granzyme A expression in CD8+ T cells from participants with low and high AFB1 levels. The percentages of CD8+ T cells that contained perforin and CD8+ cells that contained both perforin and granzyme A were significantly lower in participants with high AFB1 levels (29.40 ± 13.75 and 26.91 ± 12.96, respectively) compared with participants with low AFB1 levels (40.33 ± 18.55 and 36.26 ± 15.04, respectively). In the examples shown in the bottom panel, the percentages of CD8+ T cells that contained perforin or both perforin and granzyme A were 22.27 and 16.45%, respectively, for participant C who had high AFB1. The percentages were higher for participant A (41.74 and 31.33%, respectively) and for participant B (31.27 and 26.05%, respectively) who had low AFB1 levels.

loop and efficient control of infection. Functional CD56+ NK cells can be generated *in vitro* from CD34+ bone marrow progenitors and they need IL-2 for full expression (48). The high-affinity form of IL-2R is expressed on the CD56^{bright} NK subset and the latter is highly sensitive to IL-2 (44). In our study, we found a lower percentage of CD56^{bright} NK cells in those with high AFB1. This suggests that AF may cause a decline in the availability of IL-2 and CD56^{bright} NK cells that express the high-affinity IL-2R.

Exposure of human peripheral blood monocytes to different concentrations of AFB1 *in vitro* showed that AFB1 significantly impaired the phagocytic and microbicidal activity of the monocytes (24). Previous studies conducted in mice on the effect of AF on alveolar macrophages after aerosol inhalation or intra-tracheal instillation of AF by rats and mice (25) or on peritoneal macrophages isolated from mice fed AF in their diet (23) also showed that AF suppressed macrophage phago-cytic activity. However, a study that investigated the effect of AF fed in the diet to weanling pigs found that AF did not alter the relative number of monocytes in peripheral blood similar to our study in humans (27). We also found no significant difference in phagocytosis by monocytes from our study participants in relation to AFB1 levels. Further studies need to be conducted to determine the effects of dietary AF exposure on human monocytes/macrophages.

In summary, we describe the differential subset distributions and functional alterations of specific lymphocyte subsets between study participants with high and low levels of AFB1. The major changes in the constitution of lymphocyte subsets are a decrease in activated T cells and B cells and significantly lower levels of perforin- and granzyme A-expressing CD8+ cytotoxic T cells in those with high AFB1 compared with those with low AFB1. These alterations in immunological parameters in participants with high AFB1 levels could result in impairments in cellular immunity in these individuals that could decrease their resistance to infections. Immune suppression by a toxicant can result from various mechanisms such as decreased protein and/or DNA synthesis, changes or loss in enzymatic activity and changes in metabolism or cell cycles, which may result in apoptosis or necrosis (49). Our findings should be considered exploratory, given the cross-sectional design of this study and because there may be other variables not considered such as other mycotoxins that may affect immune cell distribution and function. Other mycotoxins, such as fumonsins, ochratoxins, gliotoxin, patulin, zearaleone and trichothecenes including deoxynivalenol, have been reported to modulate the immune system (45, 50–52). The co-occurrence of fumonsins and AFs has been shown in maize from Ghana (53) and co-occurrence of fumonsins, trichothecenes and AF demonstrated in maize from China (54). Further clarification of the immune effects of mycotoxins and of other possible immune correlates of AF exposure might be afforded by additional investigation.

Abbreviations

AF	aflatoxins
AFB1	aflatoxin B1
BrdU	5-bromo-2-deoxyuridine
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
MTT	(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; Thiazolyl blue)
PBS	Phosphate buffered saline
PE	polyethylene
PerCP	peridinin chlorophyll a protein
PMA	phorbol myristate acetate
RIA	radioimmunoassay
TNF	tumor necrosis factor

References

1 Gourama, H. and Bullerman, L. B. 1995. *Aspergillus flavus* and *Aspergillus parasiticus*, aflatoxigenic fungi of concern in foods and feed—a review. *J. Food Prot.* 58:1395.

2 Center for Disease Control and Prevention (CDC). 2004. Outbreak of aflatoxin poisoning—Eastern and Central Provinces, Kenya, January–June 2004. *Morb. Mortal. Wkly. Rep.* 53:790.

3 Bosch, F. X. and Munoz, N. 1988. Prospects for epidemiological studies on hepatocellular cancer as model for assessing viral chemical interactions. In Bartsch, H., Hemminki, K. and O'Neill, I. K., eds, *Methods for Detecting DNA Damaging Agents in Humans: Applications in Cancer Epidemiology and Prevention*. IARC Scientific Publication No. 89, p. 427. International Agency for Research on Cancer, Lyon, France.

4 IARC. 1996. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. *IARC Monographs on the Evaluation of Carcinogenic Risks to Human*, vol. 56. International Agency for Research on Cancer, Lyon, France.

5 Ozturk, M. 1991. p53 mutation in hepatocellular carcinoma after aflatoxin exposure. *Lancet* 338:1356.

6 Ali, M. V., Mohiuddin, S. M. and Reddy, M. V. 1994. Effect of dietary aflatoxin on cell mediated immunity and serum proteins in broiler chicken. *Indian Vet. J.* 71:760.

7 Neiger, R. D., Johnson, T. J., Hurley, D. J., Higgins, K. F., Rottinghaus, G. E. and Stahr, H. 1994. The short-term effect of low concentrations of dietary aflatoxin and R-2 toxin on mallard ducklings. *Avian Dis.* 38:738.

8 Pestka, J. J. and Bondy, G. S. 1994. Mycotoxin-induced immunomodulation. In Dean, J. H., Luster, M. I., Munson, A. E. and Kimber, I., eds, *Immunotoxicology and Immunopharmacology*, p. 163. Raven Press, New York.

9 Azzam, A. H. and Gabal, M. A. 1998. Aflatoxin and immunity in layer hens. *Avian Pathol.* 27:570.

10 Gabal, M. A. and Azzam, A. H. 1998. Interaction of aflatoxin in the feed and immunization against selected infectious diseases in poultry. II. Effect on one-day-old layer chicks simultaneously vaccinated against Newcastle disease, infectious bronchitis and infectious bursal disease. *Avian Pathol.* 27:290.

11 Pier, A. C. 1986. Immunologic changes associated with mycotoxi-coses. 13. Immunomodulation in aflotoxicosis. In Richard, J. L. and Thurston, J. R., eds, *Diagnosis of Mycotoxicosis*, p. 143. Martinus Nijhoff, Boston.

12 Venturini, M. C., Quiroga, M. A., Risso, M. A. *et al.* 1996. Mycotoxin T-2 and aflatoxin B1 as immunosuppressants in mice chronically infected with *Toxoplasma gondii*. *J. Comp. Pathol.* 115:229.

13 Silvotti, L., Petterino, C., Bonomi, A. and Cabassi, E. 1997. Immunotoxicological effects on piglets of feeding sows diets containing aflatoxins. *Vet. Rec.* 141:469.

14 Sharma, R. P. 1993. Immunotoxicity of mycotoxins. *J. Dairy Sci.* 76:892.

15 Reddy, R. V., Taylor, M. J. and Sharma, R. P. 1987. Studies of immune function of CD-1 mice exposed to aflatoxin B1. *Toxicology* 43:123.

16 Pier, A. C., Fichter, R. E. and Cysewshi, S. J. 1977. Effects of aflatoxin on the cellular immune system. *Ann. Nutr. Aliment.* 31:781.

17 Pier, A. C., Belden, E. L., Ellis, J. A., Nelson, E. W. and Maki, L. R. 1989. Effects of cyclopiazonic acid and aflatoxin singly and in combination on selected clinical, pathological and immunological responses of guinea pigs. *Mycopathologia* 105:135.

18 Dietert, R. R., Qureshi, M. A., Nanna, U. C. and Bloom, S. E. 1985. Embryonic exposure to aflatoxin-B1: mutagenicity and influence on development and immunity. *Environ. Mutagen.* 7:715.

19 Giambrone, J. J., Ewert, D. L., Wyatt, R. D. and Edison, C. S. 1978. Effect of aflatoxin on the humoral and cell mediated immune systems of the chicken. *Am. J. Vet. Res.* 39:305.

20 Hatori, Y., Sharma, R. P. and Warren, R. P. 1991. Resistance of C57B1/6 mice to immunosuppressive effects of aflatoxin B1 and relationship with neuroendocrine mechanisms. *Immunopharmacology* 22:127.

21 Richard, J. L. and Thurston, J. R. 1975. Effect of aflatoxin on phagocytosis of *Aspergillus fumigatus* spores by rabbit alveolar macrophages. *Appl. Microbiol.* 30:44.

22 Neldon-Ortiz, D. L. 1991. Direct and microsomal activated aflatoxin B1 exposure and its effects on turkey peritoneal macrophage functions *in vitro. Toxicol. Appl. Pharmacol.* 109:432.

23 Moon, E. Y., Rhee, D. K. and Pyo, S. 1999. Inhibition of various functions in murine peritoneal macrophages by aflatoxin B1 exposure *in vivo. Int. J. Immunopharmacol.* 21:47.

24 Cusumano, V., Rossano, F., Merendino, R. A. *et al.* 1996. Immunobiological activities of mould products: functional impairment of human monocytes exposed to aflatoxin B1. *Res. Microbiol.* 147:385.

25 Jakab, G. J., Hmieleski, R. R., Zarba, A., Hemenway, D. R. and Groopman, J. D. 1994. Respiratory aflatoxicosis: suppression of pulmonary and systemic host defenses in rats and mice. *Toxicol. Appl. Pharmacol.* 125:198.

26 Reddy, R. V. and Sharma, R. P. 1989. Effects of aflatoxin B1 on murine lymphocytic functions. *Toxicology* 54:31.

27 Marin, D. E., Taranu, I., Bunaciu, R. P. *et al.* 2002. Changes in performance, blood parameters, humoral and cellular immune responses in weanling piglets exposed to low doses of aflatoxin. *J. Anim. Sci.* 80:1250.

28 Turner, P. C., Moore, S. E., Hall, A. J., Prentice, A. M. and Wild, C. P. 2003. Modification of immune function through exposure to dietary aflatoxin in Gambian children. *Environ. Health Perspect.* 111:217.

29 Jolly, P. E. 1997. Replicative characteristics of primary isolates of the human immunodeficiency virus type 1 in peripheral blood mononuclear cells, primary macrophages and CD4+ transformed T-cell lines. *Cell. Mol. Biol.* 43:1057.

30 Wang, J. S., Qian, G. S., Zarba, A. *et al.* 1996. 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. *Cancer Epidemiol. Biomarkers Prev.* 5:253.

31 Wang, J. S., Abubaker, S., He, X., Sun, G., Strickland, P. T. and Groopman, J. D. 2001. Development of aflatoxin–lysine adduct monoclonal antibody for human exposure studies. *Appl. Environ. Microbiol.* 66:2712.

32 Gange, S. J., Munoz, A., Wang, J.-S. and Groopman, J. D. 1996. Variability of molecular biomarker measurements from nonlinear calibration curves. *Cancer Epidemiol. Biomarker Prev.* 5:57.

33 Hamann, D., Baars, P. A., Martin, H. G. *et al.* 1997. Phenotypic and functional separation of memory and effector human CD8+ T cells. *J. Exp. Med.* 186:1407.

34 Bela, A. M. and Vernon, C. M. 1997. Simultaneous detection of DNA synthesis and cytokine production in staphylococcal enterotoxin B activated CD4+ T lymphocytes by flow cytometry. *J. Immunol. Methods* 208:49.

35 Steinkamp, J. A., Wilson, J. S., Saunders, G. C. and Stewart, C. C. 1982. Phagocytosis: flow cytometric quantitation with fluorescent microspheres. *Science* 215:64.

36 Awuah, R. T. and Kpodo, K. A. 1996. High incidence of *Aspergillus flavus* and alfatoxins in stored groundnuts in Ghana and the use of microbial assay to assess the inhibitory effects of plant extracts on aflatoxin synthesis. *Mycopathologia* 134:109.

37 Mintah, S. and Hunter, R. B. 1978. The incidence of aflatoxin found in groundnuts (*Arachis hypogea L.*) purchased from markets in and around Accra, Ghana. *Plant Sci.* 5:13.

38 Wang, J. S., Huang, T., Su, J. *et al.* 2001. Hepatocellular carcinoma and aflatoxin exposure in Zhuqing Village, Fusui County, People's Republic of China. *Cancer Epidemiol. Biomarkers Prev.* 10:143.

39 Rutella, S., Rumi, C., Lucia, M. B. *et al.* 1999. Induction of CD69 antigen on normal CD4+ and CD8+ lymphocyte subsets and its relationship with the phenotype of responding T-cells. *Cytometry* 38:95.

40 Dugyala, R. R. and Sharma, R. P. 1996. The effect of aflatoxin on cytokine mRNA and corresponding protein levels in peritoneal macrophages and splenic lymphocytes. *Int. J. Immunopharmacol.* 18:599.

41 Watzl, B., Neudecker, C., Hansch, G. M., Rechkemmer, G. and Pool-Zobel, B. L. 1999. Short-term moderate aflatoxin exposure has only minor effects on the gut-associated lymphoid tissue of Brown Norway rats. *Toxicology* 138:93.

42 Van Heugten, E., Spears, J. W., Coffey, M. T., Kegley, E. B. and Qureshi, M. A. 1994. The effect of methionine and aflatoxin on immune function in weanling pigs. *J. Anim. Sci.* 72:658.

43 Harvey, R. B., Edrington, T. S., Kubena, L. F., Elissalde, M. H. and Rottinghaus, G. E. 1995. Influence of aflatoxin and fumonisin B1-containing culture material on growing barrows. *Am. J. Vet. Res.* 56:1668.

44 Raisuddin, S., Singh, K. P., Zaidi, S. I., Paul, B. N. and Ray, P. K. 1993. Immunosuppressive effects of aflatoxin in growing rats. *Mycopathologia* 124:189.

45 Meky, F. A., Hardie, L. J., Evans, S. W. and Wild, C. P. 2001. Deoxynivalenol-induced immunomodulation of human lymphocyte proliferation and cytokine production. *Food Chem. Toxicol.* 39:827.

46 Robertson, M. J. and Ritz, J. 1990. Biology and clinical relevance of human natural killer cells. *Blood* 76:2421.

47 Cooper, M. A., Fehniger, T. A., Turner, S. C. *et al.* 2001. Human natural killer cells: a unique innate immunoregulatory role for the CD56bright subset. *Blood* 97:3146.

48 Silva, M. R. G., Kessler, S. and Ascensao, J. L. 1993. Hematopoietic origin of human NK cells: generation from immature progenitors. *Pathobiology* 61:247.

49 Hinton, D. M., Myers, M. J., Raybourne, R. A. *et al.* 2003. Immunotoxicity of aflatoxin B1 in rates: effects on lymphocytes and the inflammatory response in a chronic intermittent dosing study. *Toxicol. Sci.* 73:362.

50 Bondy, G. S. and Pestka, J. J. 2000. Immunomodulation by fungal toxins. *J Toxicol. Environ. Health B Crit. Rev.* 3:109.

51 Lea, T., Seien, K. and Stormer, F. C. 1989. Mechanism of ochratoxin A-induced immuno-suppression. *Mycopathologia* 107:153.

52 Pestka, J. J., Zhou, H. R., Moon, Y. and Chung, Y. J. 2004. Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox. *Toxicol. Lett.* 153:61.

53 Kpodo, K., Thrane, U. and Hald, B. 2000. *Fusaria* and fumonisins in maize from Ghana and their co-occurrence with aflatoxins. *Int. J. Food Microbiol.* 61:147.

54 Chu, F. S. and Li, G. Y. 1994. Simultaneous occurrence of fumonisin B_1 and other mycotoxins in moldy corn collected from the People's Republic of China in regions with high incidences of esophageal cancer. *Appl. Environ. Microbiol.* 60:847.