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Evaluation of Acute Immunotoxicity of Aerosolized Aflatoxin B₁ in Female C57BL/6N Mice

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There is evidence for immunotoxicity of aflatoxin B₁ (AFB₁) in chronic animal feeding studies; however, little information is available as to the effects of inhalation exposure. This study evaluated the acute effects of aerosolized AFB₁ on systemic immune function of female C57BL/6N mice following a single aerosol exposure. Mice were exposed in nose-only inhalation tubes to 0, 2.86, 6.59 and 10 μg AFB₁ aerosol/L air for 90 minutes. A negative control group of untreated mice and a positive control group of cyclophosphamide-treated mice were included to account for day to day variation. Three days following exposure, mice were sacrificed and body, liver, lung, thymus and spleen weights, and complete blood counts and white blood cell differentials were measured. Splenocytes were isolated for flow cytometric analysis of CD4⁺ and CD8⁺ lymphocytes, CD19⁺ B-cells and natural killer cells (NK 1.1⁺). The effect of AFB₁ on humoral immunity was assessed by measuring serum anti-keyhole limpet hemocyanin (KLH) IgM levels. Of the tissues examined, only the thymus weight of AFB₁ exposed mice decreased significantly compared to naïve mice; however, the decrease was not dose related and was also observed in the 0 AFB₁ aerosol control group. A decrease in the mean white blood cell count of treated vs. naïve mice was observed at all dose levels but was clearly not dose related and was statistically significant only in the 0 and 2.86 $\mu\text{g}/\text{L}$ groups. Red blood cell and platelet counts and white blood cell differentials were not significantly affected by AFB₁. The number of CD4⁺ (helper T-cells), CD8⁺ (cytotoxic T-cells) and CD19⁺ (B-cells) decreased in spleens of AFB₁ aerosol exposed mice compared to naïve mice; however, the decrease was not dose-related and was also observed in the 0 AFB₁ exposure group. Dose-related changes in the CD4⁺/CD8⁺ T-lymphocyte ratios were not observed. The IgM response to KLH was not significantly different in AFB₁ compared to naïve mice, suggesting that AFB₁ did not effect antigen-specific antibody production. Based on the results of this study, a single AFB₁ inhalation exposure up to 10 $\mu\text{g}/\text{L}$ for 90 minutes (CxT = 900 $\mu\text{g}\cdot\text{min}/\text{L}$) did not significantly alter the immune parameters measured in this study. The aerosol vehicle (ethanol) and/or stress could have masked subtle AFB₁-dependent changes in thymus and spleen weights, and in splenic lymphocyte subpopulations. However, for other immunological parameters, such as the IgM response

to KLH, there was clearly no significant effect of AFB₁ aerosol exposure.

Keywords aflatoxin, C57BL/6N mice, AFB₁, mycotoxin, immunotoxicity

INTRODUCTION

Aflatoxin B₁ (AFB₁), a metabolite of the fungus *Aspergillus flavus*, is a potent hepatocarcinogen in experimental animals and humans (Groopman, 1988). Aflatoxin (AF) contaminated food has been implicated as a major cause of liver cancer in many parts of Africa and Asia (National Toxicology Program, 2002). Dietary AF has been implicated as a factor in outbreaks of infectious disease in domesticated animals (Pier et al., 1980; Pier, 1981; Pier and McLoughlin, 1985; Panangala et al., 1986; Padmanaba, 1989). In addition, there is experimental evidence indicating that AF reduces resistance to bacterial, viral, fungal, and parasitic infections in swine, rabbits, and chickens (Michael et al., 1973; Richard et al., 1973; Cysewski et al., 1975; Miller et al., 1978; Ghoneimy et al., 2000).

A number of studies have documented the immunotoxic potential of dietary AF in several species including laboratory and domestic animals (reviewed in Sharma, 1993; Bondy and Pestka, 2000). Studies in domesticated animals have shown that AF ingestion causes thymus and bursal hypertrophy in chickens (Thaxton et al., 1974), immunosuppression in turkeys (Pier and Heddleston, 1970; Pier et al., 1972; Richard et al., 1973), impaired bovine lymphocyte mitogenesis (Paul et al., 1977), suppressed antibody response to *Salmonella* in chickens (Giambrone et al., 1978), suppressed cell-mediated immunity in chickens (Ghosh et al., 1990), and delayed-type hypersensitivity in chickens (Kadian et al., 1988). In pigs, AF decreased mitogen-induced lymphocyte proliferation, complement titers, macrophage activation, and the delayed hypersensitivity response (Miller et al., 1978; Silvotti et al., 1997; Mocchegiani et al., 1998). Oral doses of AF decreased the number of lymphoid cells and reduced peritoneal macrophage phagocytosis in weanling rats (Raisuddin et al., 1990), reduced lymphocyte

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blastogenesis and antibody response in mice (Reddy et al., 1987), impaired alveolar macrophage phagocytosis in rabbits (Richard and Thurston, 1975), and reduced complement activity in guinea pigs (Thurston et al., 1972, 1974). Low dose AF reduced the humoral immune response induced by *Mycoplasma agalactiae* (Marin et al., 2002).

AF ingestion does not appear to alter immunoregulatory cytokine production (Dugyala and Sharma, 1996; Watzl et al., 1999; Marin et al., 2002) or the humoral response to sheep red blood cells (van Heugten et al., 1994) and *Erysipelothrix rhusiopathiae* (Panangala et al., 1986). Weanling pigs fed low doses of AF for 4 weeks, displayed no alterations in the relative numbers of blood cells, globulin, albumins, serum protein concentration, or phytohemagglutinin (PHA)-induced expression of IL-2 or IL-4 using whole blood, indicating that there was no effect of AF on the cell-mediated immune response (Marin et al., 2002).

Five studies with aerosol exposure to AF have been reported (Louria et al., 1974; Northrup et al., 1975; Richard et al., 1982; Zarba et al., 1992); however, only one study examined the immunotoxicity of AF (Jakab et al., 1994). Jakab et al. (1994) observed suppression of alveolar macrophage Fc_c-receptor mediated phagocytosis following aerosol inhalation of AFB₁ in rats. Suppression of systemic immune function, as determined by splenic antibody response to sheep red blood cells and suppression of peritoneal macrophage phagocytosis, was observed with intratracheal instillation of AFB₁ at 10-fold higher doses compared to inhalation.

Inhalation of AFB₁ from grain dust may be a risk factor for a number of human diseases (Zarba et al., 1992). AFB₁ levels of 12 to 700 ppb were measured in airborne dust during handling and processing of peanut and corn grain (Burg et al., 1981; Sorenson et al., 1981, 1984). In addition, there are numerous studies identifying increased incidences of total and respiratory cancer in aflatoxin-exposed workers (Hayes et al., 1984 and NTP, 2002). Since immune surveillance is an important protective mechanism against the development of cancer cells, suppression of the immune response could be at least partially responsible for increased risk of carcinogenicity. In this study we investigated the effects of AFB₁ aerosol on immune parameters in female C57BL6 mice at three dose levels. Analyses included characterization of cells and organs of the immune system, hematology, spleen cell immunophenotyping, and T-cell dependent IgM response to KLH.

MATERIALS AND METHODS

Animals

Female C57BL/6N mice, approximately 7–9 weeks old on the day of dosing, were obtained from Charles Rivers (Wilmington, MA) and housed in a controlled environment with a 12-h light/dark cycle. Food and water were provided *ad libitum*. The study adhered to the Guide for the Care and Use of Laboratory

Animals prepared by the Institute of Laboratory Animal Resources, National Research Council (National Academy Press, 1996).

Test Chemical

Aflatoxin B₁ (FW 312.3) from *Aspergillus flavus* (99% purity by TLC) was purchased from Sigma Chemical Co. (St. Louis, MO). The identity of the stock AFB₁ was verified by LC/MS. A 10 μ L aliquot of a 1 μ g/mL solution of AFB₁ in methanol was injected on a 150 \times 3.2 mm Allure C-18 column with a MicroMass Quattro MS/MS detector. The LC column was eluted for 16 min at 0.4 mL/min using with a linear gradient from 20% to 80% methanol containing 2 mM formic acid and 2 mM ammonium formate followed by a 0.1 min ramp back to 20% methanol containing 2 mM formic acid and 2 mM ammonium formate and holding for 4 min. The eluent was analyzed by MS in the ESP+ mode by scanning from mass/charge ratio (m/z) 10 to m/z 500 in 1.5 sec. LC/MS of the stock AFB₁ gave a single chromatographic peak for m/z 313.2 at 17.8 min. MS analysis of this peak gave a major ion at 313 (MH⁺) and 335 (M+Na)⁺. Nebulizer solutions of AFB₁ (0.05, 0.20 or 0.60 mg AFB₁/mL ethanol) were prepared on the day of use.

Other Chemicals

Cyclophosphamide monohydrate, ethanol (200 proof, spectrophotometric grade, >99% purity), bovine serum albumin (BSA), paraformaldehyde, sodium azide, red blood cell lysing buffer, and DNase and RNase free water were obtained from Sigma-Aldrich (St. Louis, MO). Modified Eagle medium (MEM) with 100 mM sodium pyruvate and RPMI medium 1640 with Glutamax I were obtained from Invitrogen (Carlsbad, CA). 10X Calcium- and magnesium-free phosphate-buffered saline, pH 6.7–6.9 (10X CMF PBS) was obtained from In Vitro Life Technologies (Carlsbad, CA). Penicillin-streptomycin (10,000 units penicillin, 10 mg/mL streptomycin) was obtained from Amresco (Solon, OH). Key-hole limpet hemocyanin (KLH), lyophilized powder, was obtained from Pierce (Rockford, IL) and was regenerated in sterile distilled water (Sigma). Horseradish peroxidase (HRP)-conjugated rat anti-mouse IgM monoclonal antibody was obtained from BD Biosciences Pharmingen (San Diego, CA). Mouse IgM was obtained from Caltag (Burlingame, CA). The monoclonal antibodies used for spleen cell phenotyping were rat anti-mouse CD3-R-phycoerythrin (R-PE), rat anti-mouse CD4-fluorescein isothiocyanate (FITC), rat anti-mouse CD8a-FITC, rat anti-mouse CD19-FITC (Caltag), and mouse anti-mouse (NK1.1-FITC) (BD Biosciences Pharmingen). The matched isotype control antibody conjugates used to determine background staining were anti-mouse IgG_{2a} isotype control-R-PE and anti-mouse IgG_{2a} isotype control-FITC (Caltag), and anti-mouse IgG_{2a} isotype control-FITC (BD Biosciences Pharmingen).

TABLE 1
Experimental design

Treatment day	Exposure group ¹	Number of mice		
		Aerosol exposed	Untreated (Control)	Cyclophosphamide treated (Positive control)
1	0 ug AFB ₁ /L	21	21	21
2	2.86 μg AFB ₁ /L	21	21	21
3	6.59 μg AFB ₁ /L	21	21	21
4	10.0 μg AFB ₁ /L	21	21	21

¹Values indicate aerosol exposure concentration in μg AFB₁/L air. Each aerosol exposure was for 90 min.

Experimental Design

There were four aerosol exposure groups in this study; 0, 2.86, 6.59, and 10.0 μg AFB₁/L air (Table 1). Each aerosol treatment group contained 21 mice that were exposed once to the aerosol for 90 min. Each aerosol treatment group was exposed on a different day. To account for day-to-day variation, an additional group of 21 naïve mice (Untreated Control) was processed and analyzed simultaneously with samples from the AFB₁ aerosol exposed animals on each day of exposure. On each day of aerosol exposure an additional group of 21 naïve animals was treated with cyclophosphamide, a known immunosuppressant, intraperitoneally (IP) at 225 mg/kg body weight (BW); these animals served as positive controls for the immunological analyses.

Of the 21 mice in each group, 7 were used for analysis of the IgM response to KLH and the other 14 mice for the other analyses. For analyses of splenocytes, the spleens from 14 mice were harvested and processed in sets of two (n = 7).

AFB₁ Aerosol Exposure

Female C57BL/6N mice were divided randomly by weight into three groups: aerosol-treated, untreated, and cyclophosphamide-treated mice. Mice were acclimated to exposure tubes for 0.5 and 1 h on the two days prior to aerosol exposure.

Mice were exposed once for 90 min to an atmosphere of 0, 2.86, 6.59, or 10.0 μg AFB₁ aerosol/L air. The exposure atmosphere also contained ethanol vapor due to solubilization of the AFB₁ in ethanol for nebulization. The AFB₁ aerosol exposure was generated by nebulization of solutions of 0, 0.05, 0.20, or 0.60 mg AFB₁/mL ethanol using a three jet Collison nebulizer and was delivered to rodents via a nose-only inhalation system (CH Technologies, Westwood, NJ). Total air-flow through the system was regulated to give approximately 10 L/min through the chamber. Homogeneity of an aerosol in the exposure chamber was verified with 0.7–2.9 μm polystyrene latex beads (PSL) at aerosol concentrations of 50,000 to 500,000 particles/L. PSL concentration values at all levels of the exposure chamber were within 10% of the mean. Since the AFB₁ aerosol has a smaller

particle size than the standard PSL particles; it can be safely assumed to distribute in a similar homogeneous manner throughout the system.

The aerosol atmosphere was sampled through two serial 0.2 μm filters. The aerosol particle size was estimated as the mass median aerodynamic diameter (MMAD) using a PIXE cascade impactor, Model I-1, PIXE International Corp. (Tallahassee, FL). Filters and slides from the cascade impactor were extracted with 200-proof ethanol and analyzed by UV spectroscopy at 260 nm. A standard curve was prepared using a standard solution of AFB₁ in 200-proof ethanol. The aerosol concentration in the exposure chamber was calculated from the amount of AFB₁ collected on filters as:

$$\text{Aerosol Concentration of AFB}_1 = \frac{[\text{AFB}_1 \text{ concentration in filter extract} \times \text{Volume of extract (mL)}]}{[\text{sampling rate (L/min)} \times \text{sampling time (min)}]}$$

Following exposure, the animals were placed in cages (2–4 per cage) and provided food and water ad libitum until sacrifice, at 3–5 days following exposure. All animals were weighed at randomization (3 or 4 days prior to exposure) and at sacrifice (3 days after aerosol exposure). Clinical observations were performed twice daily from the quarantine period through the day of termination.

IgM Response to KLH

Animals (7 per treatment group) were injected IP with KLH (0.2 mL of 0.5 mg KLH/mL sterile phosphate-buffered saline, pH 7.2) 1 day following AFB₁ aerosol or cyclophosphamide dosing. Untreated (control) animals were injected the same day. Five days following exposure (4 days after KLH injection), a blood sample was collected by retro-orbital bleed (or heart puncture if insufficient blood was obtained) under halothane anesthesia into a serum collection tube. Sera was stored at ≤−70°C and analyzed within 4 days of collection for the concentration of anti-KLH IgM.

Body Weight, Hematology, and Organ Weights

Three days following aerosol exposure, 14 aerosol-treated, 14 cyclophosphamide-treated, and 14 untreated mice were weighed, and a blood sample was collected by retro-orbital bleed under CO₂ anesthesia into a potassium-EDTA tube for hematology. Blood from individual animals was analyzed for white blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count (PLT), mean platelet volume (MPV), and white blood cell differentials.

The mice were euthanized while under anesthesia by cervical dislocation. The body of the rodent was washed with 95%

ethanol and the spleen was removed aseptically, placed in a sterile container, and weighed. The liver, thymus, and lung were removed and weighed individually.

Isolation of Splenocytes

Spleens were transferred to dishes (two per dish) containing 5–10 mL of growth medium (RPMI 1640 containing 10% FBS, 10 $\mu\text{g}/\text{mL}$ gentamycin). Each spleen was macerated between the frosted ends of two sterile glass slides. Splenocytes were collected and transferred to sterile conical tubes. Samples were centrifuged for 5 min at $350 \times g$ at room temperature to pellet the cells. The supernatant was removed and red blood cells were lysed using Red Blood Cell Lysing Buffer. Splenocytes were pelleted, washed with growth medium and resuspended in 2 mL of fresh growth medium. Total splenocyte counts and viability were determined using a hemocytometer. A portion of each cell solution was diluted to 1.0×10^7 cells/mL and used for flow cytometric analysis. The average cell viability was $>94\%$ for all groups of animals (range of 87.3–99.8%) except the cyclophosphamide treated mice which showed a slightly lower mean viability of 91.4% (range of 80.0–98.9%) These results indicate that the isolated splenocytes retained viability and were suitable for subsequent analyses.

Immunophenotyping

Immunostaining for CD3, CD4, CD8, CD19 and NK 1.1 antigenic markers was performed on the day of splenocyte isolation. Briefly, 20 μL aliquots of the appropriate antibody solutions were added to the wells of a 96-well microplate. The volume of each well was brought to 100 μL using PBS/BSA/azide wash buffer (0.2% bovine serum albumin and 0.2% sodium azide in 1X-CMF PBS). An aliquot (100 μL) of a solution containing 1.0×10^7 splenocytes/mL was added to each assay well for a final well volume of 200 μL . The cells were incubated for 1 hr at 37°C in the dark. The plate was centrifuged at $210 \times g$ for 3 min and washed twice with PBS/BSA/azide buffer. The cell suspension in PBS/BSA/azide was added to an equal volume of 2% paraformaldehyde and stored at $2\text{--}8^\circ\text{C}$ protected from light until analyzed. Analysis of splenic lymphocyte populations was

performed on a FACScan (Becton Dickinson, San Jose, CA) using Cell Quest software (Becton Dickinson, San Jose, CA). Splenocytes were gated by means of forward and side scatter, and approximately 10,000 gated cells were analyzed.

Statistical Analysis

Comparisons between treated and untreated mice were made using the Student's 2-tailed *t*-test with a *p* value of ≤ 0.05 being considered significant.

RESULTS

AFB₁ Aerosol Exposures

The aerosol concentrations for the 90 min exposures were 0, 2.86, 6.59 and 10.0 μg AFB₁/L air (Table 2) equivalent to C \times T exposures of 0, 257, 593, and 900 $\mu\text{g}\cdot\text{min}/\text{L}$ air. The amount of AFB₁ inhaled in the four exposures was estimated to be 0, 5.1, 11.9 and 18.0 μg assuming a mouse minute volume of 20 mL/min. Regression analysis of impactor data indicated that the aerosol had a MMAD range of 0.04–0.2 μm over the three AFB₁ exposures. This particle size ensures significant alveolar deposition of the AFB₁ aerosol. The estimated exposure concentration of the vehicle, ethanol, was similar for all aerosol exposures and ranged from 50.4–67.8 mg/L air.

Body Weights

There was no effect of AFB₁ exposure on body weight (data not shown). The cyclophosphamide-treated mice showed a slight decrease in average weight gain compared to untreated mice; however, this was statistically significant on only one of the four exposure days.

Organ Weights

A summary of the effect of treatment on organ weights and organ/body weight ratios are shown in Tables 3 and 4, respectively. Although some apparent statistical differences in liver weight and liver/body weight ratio were observed in AFB₁ treated versus untreated mice, these were within 10% and were not dose related. Although lung weight and lung/body weight ratio were significantly increased (18%) compared to untreated mice in the

TABLE 2
Exposure concentration of AFB₁ aerosol

Exposure group	Estimated ethanol exposure concentration (mg/L) ¹	Exposure duration (min)	AFB ₁ C \times T ($\mu\text{g} \times \text{min}/\text{L}$ air)	Estimated amount of AFB ₁ Inhaled (μg) ²
0 μg AFB ₁ /L	67.8	90	0	0
2.86 μg AFB ₁ /L	61.4	90	257	5.1
6.59 μg AFB ₁ /L	50.4	90	593	11.9
10.1 μg AFB ₁ /L	58.2	90	900	18.0

¹Calculated based on the amount of ethanol used/volume of air passed through chamber.

²Calculated based on exposure concentration ($\mu\text{g}/\text{L}$) \times estimated minute volume (0.02 L/min) \times exposure duration (90 min).

TABLE 3
Summary of organ weights

Exposure group	Liver weight (g) ¹			Lung weight (g) ¹		
	Untreated on same day	Aerosol exposed	Cyclophosphamide treated on same day	Untreated on same day	Aerosol exposed	Cyclophosphamide treated on same day
0 μg AFB ₁ /L	1.06 \pm 0.08	0.99 \pm 0.10* (0.93) ²	1.02 \pm 0.10 (0.97)	0.152 \pm 0.020	0.148 \pm 0.017 (0.97)	0.177 \pm 0.024* (1.17)
2.86 μg AFB ₁ /L	1.10 \pm 0.10	1.19 \pm 0.08* (1.09)	1.14 \pm 0.11 (1.04)	0.168 \pm 0.041	0.179 \pm 0.028 (1.06)	0.199 \pm 0.036* (1.18)
6.59 μg AFB ₁ /L	1.06 \pm 0.13	1.08 \pm 0.06 (1.02)	1.19 \pm 0.12* (1.12)	0.155 \pm 0.015	0.184 \pm 0.042* (1.18)	0.198 \pm 0.038* (1.27)
10.1 μg AFB ₁ /L	1.08 \pm 0.09	1.14 \pm 0.10 (1.05)	1.17 \pm 0.10* (1.08)	0.154 \pm 0.014	0.154 \pm 0.012 (1.00)	0.211 \pm 0.053* (1.37)
	Thymus weight (g) ¹			Spleen weight (g) ¹		
0 μg AFB ₁ /L	0.078 \pm 0.026	0.055 \pm 0.017* (0.70)	0.025 \pm 0.005* (0.32)	0.072 \pm 0.017	0.057 \pm 0.010* (0.78)	0.032 \pm 0.006* (0.44)
2.86 μg AFB ₁ /L	0.055 \pm 0.016	0.043 \pm 0.009* (0.77)	0.026 \pm 0.005* (0.46)	0.061 \pm 0.010	0.054 \pm 0.006 (0.89)	0.032 \pm 0.005* (0.53)
6.59 μg AFB ₁ /L	0.069 \pm 0.017	0.048 \pm 0.011* (0.69)	0.028 \pm 0.005* (0.41)	0.071 \pm 0.009	0.062 \pm 0.031 (0.87)	0.035 \pm 0.007* (0.49)
10.1 μg AFB ₁ /L	0.057 \pm 0.013	0.047 \pm 0.008* (0.82)	0.024 \pm 0.005* (0.42)	0.068 \pm 0.022	0.056 \pm 0.006 (0.83)	0.030 \pm 0.004* (0.45)

¹Values are mean \pm standard deviation of 14 individual mice.

²Values in parentheses are the ratios of the organ weight of the aerosol or cyclophosphamide treated mice to the organ weight of the untreated mice from the same exposure day.

*Significantly different from untreated mice analyzed on the same day at $p \leq 0.05$ using students 2-tailed t-test.

mid-dose group (6.59 $\mu\text{g}/\text{L}$), this increase was not dose related; the high-dose AFB₁-treated mice had the same mean lung weight as the untreated mice. Thymus weights in aerosol exposed mice decreased significantly (20–30%) compared to untreated mice, however, the decrease was similar at all AFB₁ exposure levels (including the 0 AFB₁ level) indicating that this effect was not due to AFB₁, but most likely due to either ethanol exposure (Budec et al., 1992, Budec, 1996) and/or a stress response caused by restraint of animals in nose-only exposure tubes. A similar decrease in spleen weight was observed for aerosol exposed mice, but was only statistically significant at the 0 AFB₁ exposure level, indicating that the effect was not due to AFB₁.

Cyclophosphamide affected the mean liver weight slightly, and increased the mean lung weight 17–37%. Cyclophosphamide consistently decreased thymus (54–68% decrease) and spleen (47–56%) weights.

Hematology

Although statistically different only at the 0 and 2.86 $\mu\text{g}/\text{L}$ level, the WBC count was lower than that of naïve mice at all AFB₁ aerosol concentrations (Table 5). This decrease in WBC is not due to AFB₁ since the same decrease is seen in the 0 AFB₁

(vehicle) exposure. There was no effect of aerosol exposures on blood RBC count, HCT, HGB, MCH, MCHC, MCV, or RDW (data not shown). Significant changes in platelet count and mean platelet volume related to aerosol exposures were not observed. There was no consistent AFB₁ aerosol exposure related change in percent lymphocytes, monocytes, neutrophils, eosinophils, or basophils (data not shown).

Cyclophosphamide decreased the WBC count by 81–90% (Table 5) and caused significant decreases in HCT (11–15%), HGB (10–18%), and RDW on all exposure days and a decrease in platelet count on three of the four exposure days. The percentage of neutrophils and eosinophils was significantly increased by cyclophosphamide.

Spleen Cell Immunophenotype

Flow cytometry was used to evaluate lymphocyte subpopulations in the spleen. Total T-cells (CD3⁺), T-helper cells (CD4⁺), cytotoxic T-cells (CD8⁺), B-cells (CD19⁺), and natural killer (NK) cells (CD3⁻/NK1.1⁺) are expressed as total cells per spleen (Figure 1). A statistically significant decrease in total T- and B-lymphocytes per spleen was observed at 0 μg AFB₁ /L air as well as at 6.59 and 10.0 μg AFB₁ /L air indicating that this

TABLE 4
Summary of organ/body weight ratios

Exposure group	Liver weight (g) ¹			Lung weight (g) ¹		
	Untreated on same day	Aerosol exposed	Cyclophosphamide treated on same day	Untreated on same day	Aerosol exposed	Cyclophosphamide treated on same day
0 $\mu\text{g AFB}_1/\text{L}$	0.054 \pm 0.004	0.052 \pm 0.003 (0.95) ^b	0.054 \pm 0.004 (0.99)	0.0078 \pm 0.0011	0.0078 \pm 0.0012 (1.00)	0.0094 \pm 0.0012* (1.20)
2.86 $\mu\text{g AFB}_1/\text{L}$	0.053 \pm 0.004	0.057 \pm 0.004* (1.08)	0.056 \pm 0.005 (1.07)	0.008 ^a \pm 0.002 ^a	0.0086 \pm 0.0013 (1.06)	0.0098 \pm 0.001 8* (1.21)
6.59 $\mu\text{g AFB}_1/\text{L}$	0.053 \pm 0.007	0.054 \pm 0.003 (1.03)	0.060 \pm 0.005* (1.13)	0.0078 \pm 0.0008	0.0093 \pm 0.0022* (1.20)	0.0099 \pm 0.0018* (1.28)
10.1 $\mu\text{g AFB}_1/\text{L}$	0.053 \pm 0.004	0.056 \pm 0.005* (1.07)	0.059 \pm 0.004* (1.12)	0.0075 \pm 0.0006	0.0077 \pm 0.0006 (1.02)	0.0 106 \pm 0.0026* (1.41)
	Thymus weight (g) ¹			Spleen weight (g) ¹		
0 $\mu\text{g AFB}_1/\text{L}$	0.0040 \pm 0.0013	0.0028 \pm 0.0008* (0.71)	0.0013 \pm 0.0003* (0.33)	0.0037 \pm 0.0008	0.0030 \pm 0.0005* (0.80)	0.0017 \pm 0.0003* (0.46)
2.86 $\mu\text{g AFB}_1/\text{L}$	0.003 ^a \pm 0.001 ^a	0.0020 \pm 0.0004* (0.77)	0.0013 \pm 0.0003* (0.48)	0.0029 \pm 0.0005	0.0026 \pm 0.0003* (0.88)	0.0016 \pm 0.0003* (0.55)
6.59 $\mu\text{g AFB}_1/\text{L}$	0.0035 \pm 0.0009	0.0024 \pm 0.0006* (0.70)	0.0014 \pm 0.0003* (0.41)	0.0035 \pm 0.0005	0.0031 \pm 0.0017 (0.88)	0.0017 \pm 0.0003* (0.49)
10.1 $\mu\text{g AFB}_1/\text{L}$	0.0028 \pm 0.0006	0.0023 \pm 0.0004* (0.83)	0.0012 \pm 0.0002* (0.43)	0.0033 \pm 0.00 10	0.0028 \pm 0.0003 (0.84)	0.001 5 \pm 0.0002* (0.46)

^aValues are mean \pm standard deviation of 14 individual mice.

^bValues in parentheses are the ratios of the liver/body weight ratio of the aerosol or cyclophosphamide treated mice to the liver/body weight ratio of the untreated mice from the same exposure day.

*Significantly different from untreated mice analyzed on the same day at $p \leq 0.05$ using students 2-tailed t-test.

effect was not due to AFB₁ exposure. Levels of NK cells were not significantly affected by AFB₁ exposure. Cyclophosphamide caused a decrease in all subpopulations of lymphocytes.

The CD4⁺/CD8⁺ ratio was statistically decreased in the 6.59 $\mu\text{g AFB}_1/\text{L}$ group and increased in the 10.0 $\mu\text{g AFB}_1/\text{L}$ group compared to untreated mice from the same day (Table 6). These changes in the CD4⁺/CD8⁺ ratio were within the day-to-day variation observed with untreated mice, suggesting that this finding was not related to AFB₁ aerosol exposure.

IgM Response to Keyhole Limpet Hemocyanin (KLH)

A significant IgM response to KLH was detected in all groups of mice. There was no significant immunosuppressive effect of the aerosol vehicle (0 AFB₁) or any of the AFB₁ aerosol concentrations tested on the anti-KLH IgM response of mice (Table 7). Although not always statistically significant due to variability in the individual mouse response, cyclophosphamide caused a decrease of the mean anti-KLH IgM response on all exposure days.

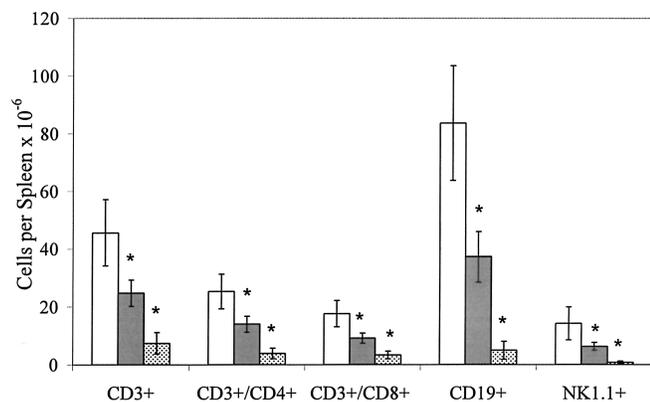
DISCUSSION AND CONCLUSIONS

Exposure of female C57BL/6N mice to aerosolized AFB₁ at concentrations of 2.9 to 10 $\mu\text{g/L}$ for 90 min had no significant

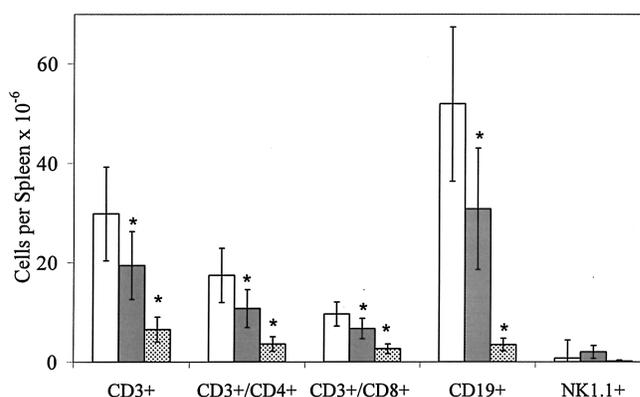
effect on the immune parameters analyzed in this study. AFB₁ did not affect measures of acute toxicity such as organ and body weight, including organs specifically involved in the immune system, such as thymus and spleen. Hematology and white blood cell differentials, a measure of the circulating levels of immune cells also were unaffected by AFB₁. Splenic T-cell (CD4⁺ and CD8⁺), B-cell (CD19⁺) and natural killer cell (CD3⁻/NK1.1⁺) populations were unaffected by AFB₁ aerosol exposure as determined by flow cytometric analysis. AFB₁ did not affect the T-cell-dependent antibody response of mice as shown by a lack of inhibition of the IgM response to KLH. Based on comparison to the aerosol control (0 AFB₁), the lymphocyte subpopulation profile (white blood cell differential) was not altered by AFB₁ exposure. These results indicate that a single, acute exposure to AFB₁ aerosol at concentrations up to 10 $\mu\text{g/L}$ for 90 min is unlikely to cause appreciable systemic immunotoxicity in the short term. Higher concentrations of AFB₁ and/or multiple exposures may be needed to observe the immunotoxic effects reported in previous feeding studies.

In this study, some measures of immune parameters were inhibited by aerosol exposure in the presence or absence of AFB₁ indicating that the immunosuppression was either due to the vehicle (ethanol vapor; 0 AFB₁), the stress response caused by

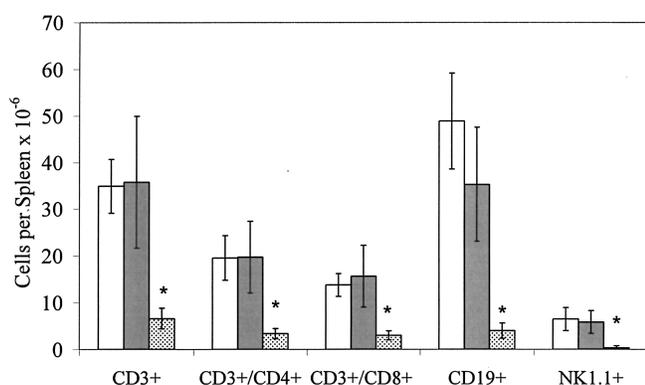
A. Ethanol x 90 min



C. 6.59 µg/L AFB₁ x 90 min



B. 2.86 µg AFB₁/L x 90 min



D. 10.0 µg/L AFB₁ x 90 min

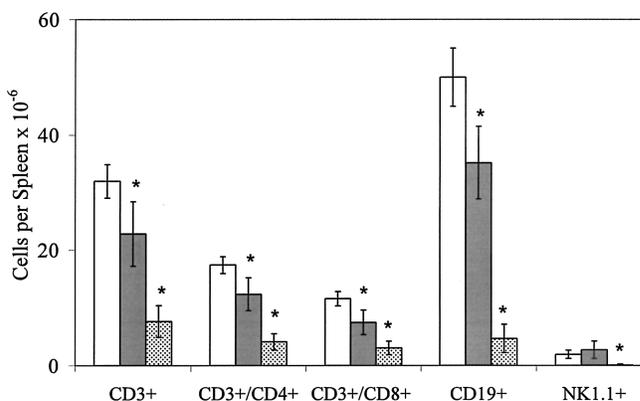


FIG. 1. Splenocytes were isolated from mouse spleen three days following exposure to aerosol containing 0 µg AFB₁/L (aerosolized vehicle, ethanol; A), 2.86 µg AFB₁/L (B), 6.59 µg AFB₁/L (C) or 10.0 µg AFB₁/L (D). The quantities of different lymphocyte subpopulations for aerosol-exposed animals is shown by the solid bar. Lymphocyte subpopulations of splenocytes isolated from untreated mice and cyclophosphamide-treated mice on the same day are illustrated by the open and dotted bars, respectively. Antigenic markers were used to identify and quantitate T-cells (CD3⁺), helper T-cells (CD3⁺/CD4⁺), cytotoxic T-cells (CD3⁺/CD8⁺), B cells (CD19⁺) and natural killer cells (NK1.1⁺) from the isolated splenocytes by fluorescent-activated cell sorting (FACS) as described in the Methods. An asterisk indicates that the value is significantly different from the untreated mice (open bar) using a Student's 2-tailed t-test at p ≤ 0.05.

restraint of mice in nose-only exposure tubes, or a combination of the two. High levels of ethanol consumption are known to affect the immune system (Szabo, 1999). Previous studies have reported that an oral dose of ethanol (4 to 7 g/kg) to B₆C₃F₁

mice slightly decreased the number of nucleated cells in the spleen but did not effect the percentage of CD4⁺ and CD8⁺ cells (Pruett et al., 2003). These results are similar to those observed in this study using ethanol vapor. Assuming a minute

TABLE 5
White blood cell count

Exposure group	White Blood Cell Count (× 10 ³ /µL)		
	Untreated on same day	Aerosol exposed	Cyclophosphamide treated on same day
0 µg AFB ₁ /L	5.97 ± 1.50	3.48 ± 0.78*	0.69 ± 0.25*
2.86 µg AFB ₁ /L	6.59 ± 1.37	4.43 ± 1.48*	1.23 ± 0.57*
6.59 µg AFB ₁ /L	6.31 ± 1.76	5.90 ± 1.69	1.17 ± 0.59*
10.0 µg AFB ₁ /L	7.41 ± 2.06	6.25 ± 1.90	0.77 ± 0.37*

Values are the mean ± standard deviation.

*Significantly different from untreated mice analyzed on the same day at p ≤ 0.05 using students 2-tailed t-test.

TABLE 6
Effect of AFB₁ on CD4⁺/CD8⁺ ratio of mouse splenocytes

Exposure group	CD4 ⁺ /CD8 ⁺ Ratio ¹		
	Untreated on same day	Aerosol exposed	Cyclophosphamide treated on same day
0 µg AFB ₁ /L	1.60 ± 0.08	1.53 ± 0.11	1.20 ± 0.14*
2.86 µg AFB ₁ /L	1.42 ± 0.24	1.28 ± 0.11	1.14 ± 0.08*
6.59 µg AFB ₁ /L	1.79 ± 0.17	1.59 ± 0.15*	1.38 ± 0.17*
10.0 µg AFB ₁ /L	1.51 ± 0.11	1.67 ± 0.15*	1.36 ± 0.09*

Values are mean ± standard deviation.

*Significantly different from untreated mice analyzed on the same day at p ≤ 0.05 using students 2-tailed t-test.

TABLE 7
IgM response to keyhole limpet hemocyanin (KLH)

Exposure group	Serum Anti-KLH IgM (ng/mL) ^a		
	Untreated on same day	Aerosol exposed	Cyclophosphamide treated on same day
0 μ AFB ₁ /L	846 \pm 563	761 \pm 347 (90.0%) ^b	366 \pm 138* (43.2%)
2.86 μ AFB ₁ /L	1960 \pm 2010	1908 \pm 672 (97.3%)	1155 \pm 1525 ^c (58.9%)
6.59 μ AFB ₁ /L	1016 \pm 606	1281 \pm 891 (126.1%)	431 \pm 453 (42.4%)
10.0 μ AFB ₁ /L	1325 \pm 225	1299 \pm 382 (98.1%)	274 \pm 111* (20.7%)

^aValues are mean \pm standard deviation of seven mice.

^bValues in parenthesis indicate the percent of the untreated value on the same day.

^cThe large variation was due to variability in individual values, which were 211, 284, 285, 346, 421, 2429, and 4109 ng/mL.

*Significantly different from untreated mice analyzed on the same day at $p \leq 0.05$ using students 2-tailed t-test.

volume of 20 mL/min, mouse body weight of 0.02 kg, ethanol vapor concentration of 60 mg/L, and 50–100% absorption of the inhaled dose, the maximum ethanol dose to mice in this study was 2.7–5.4 g/kg BW which is within the range used by Pruett et al. (2003).

Although mice were acclimated to exposure tubes prior to exposures, the possibility of some degree of stress cannot be ruled out. Restraint has been shown to transiently increase serum corticosterone levels, indicative of a stress response. This stress response decreased expression of MHC Class II receptors on splenocytes and NK cell activity within 12 h of the start of restraint (Pruett et al., 1999, 2003). In addition, the IgG response to KLH at 2 weeks after challenge was inhibited by stress (Pruett et al., 1999, 2003). In contrast, we did not observe an inhibition of the IgM response to KLH measured in our study.

The estimated inhaled dose of AFB₁ in this study ranged from 5–18 μ g per mouse over a 90 min exposure (Table 2). Assuming mice have the same percent alveolar deposition as rats (15%; Schlesinger, 1985), and a mouse body weight of 20 g, the highest dose level used in this study equates to an alveolar dose of 135 μ g AFB₁/kg. Jakab et al. (1994) reported a local immunosuppressive effect of AFB₁ on alveolar macrophage phagocytosis in male Fischer 344 rats following inhalation exposure to 33.6 μ g AFB₁/kg BW, however other immune functions using this route of exposure were not examined. At an approximately four-fold higher dose (135 μ g/kg BW), we observed no evidence of acute systemic AFB₁ immunotoxicity following inhalation exposure. It appears that for systemic immunosuppressive effects to occur a much higher dose, such as that delivered by intratracheal instillation (Jakab et al., 1994), or orally (Bondy and Pestka, 2000), would be required. The low solubility of AFB₁ in biologically

compatible solvents limits achieving higher inhalation doses by nebulization of a solution.

Local immunotoxic effects of AFB₁ on lymphocyte populations in the lung were not measured in this study. Since AFB₁ has been shown to suppress alveolar macrophage function (Jakab et al., 1994), it is quite possible that there may also be effects on alveolar lymphocytes as well. Further investigation of this area is warranted, as local suppression of lung alveolar lymphocytes would be expected to increase host susceptibility to respiratory infections.

Hatori et al. (1991) orally dosed male C57Bl/6 mice with AFB₁ once daily for 4 weeks at doses up to 750 mg/kg BW. Consistent with our findings in female C57Bl/6N mice, Hatori et al. (1991) observed no effect of treatment on helper T-cells (L3T4), suppressor and cytotoxic T-cells (Lyt 2), and the L3T4/Lyt 2 ratio at the 150 μ g AFB₁/kg dose level. AFB₁ had no effect on PHA-stimulated proliferation, mixed lymphocyte culture response, or NK cell activity at doses up to 750 μ g/kg BW. A slight (approximately 17%) decrease in plasma anti-sheep red blood cell IgM concentration was observed at 150 and 750 μ g/kg BW (Hatori et al., 1991) despite 14 days of treatment compared to our study that was a single inhalation dose.

In contrast to our findings, Hatori et al. (1991) reported a dose-related decrease in leukocyte count, a significant (~20%) decrease in helper T-cells (L3T4) at the highest dose (750 μ g/kg), and an inhibition of Con-A, LPS, and pokeweed mitogen-induced proliferation of spleen lymphocytes (150 or 750 μ g AFB₁/kg). However, these mice were exposed orally, daily for 4 weeks at higher doses (up to 750 μ g/kg BW) than that achieved in our single inhalation dose study (estimate of 135 μ g/kg BW). Hatori et al. (1991) reported that the oral dose of AFB₁ necessary to elicit immunosuppressive effects on the blastogenic response, IL-2 production, and primary antibody production of splenic cells in male C57Bl/6 mice was higher than required in previous studies using male BALB/c and male CD-1 mice.

Consistent with our study, Dugyala and Sharma (1996) reported no effect of AFB₁ on liver, kidney, spleen and thymus weights, and red blood cell and white blood cell concentrations at oral doses up to 700 μ g AFB₁/kg BW administered every other day for 2 weeks to male CD-1 mice. This study also observed no significant effect of AFB₁ treatment on Con-A-induced production of IL-2, IFN γ , and IL-3 by splenic lymphocytes. A slight and non-dose-dependent AFB₁ inhibition of LPS-induced macrophage production of IL-1, TNF, and IL-6 was observed. Dugyala et al. (1994) reported a significant decrease in proliferation and differentiation of granulocyte-macrophage progenitor cells in bone marrow cultures from male CD-1 mice treated with 145 to 700 μ g AFB₁/kg BW every other day for 2 weeks. A similar inhibition of granulocyte-macrophage progenitor cell proliferation was observed *in vitro* at approximately 10 μ M AFB₁.

Raisuddin et al. (1994) reported decreased thymus and spleen weights and cellularity, white blood cell and red blood cell counts, phagocytic function, mitogenesis of T- and B-cells, and delayed-type hypersensitivity responses in male Wistar rats at a

dose of 300 μg AFB₁/kg BW orally for two weeks every other day. Differences from the findings of our inhalation study may be due to the higher dose, the route of exposure, and/or the difference in species (rat vs. mice).

In summary, our results indicate that a single inhalation exposure to aerosolized AFB₁ at an exposure up to 900 $\mu\text{g}\cdot\text{min}/\text{L}$ (CxT) does not result in a significant suppression of the systemic immune system. However, it should be kept in mind that subtle effects of AFB₁ on some immune parameters may be masked by the effects of the aerosol vehicle (ethanol) and/or stress on some components of the immune system.

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