Research Article

Received: 6 January 2018 Revised: 29 May 2018 Accepted article published: 17 July 2018 Published online in Wiley Online Library: 23 August 2018

(wileyonlinelibrary.com) DOI 10.1002/jsfa.9240

Immunotoxicity of aflatoxin M_1: as a potent suppressor of innate and acquired immune systems in a subacute study

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Abstract

BACKGROUND: Although, to date, there have been several *in vitro* and *in vivo* studies of immunomodulatory effects of aflatoxin M_1 (AFB_1), little is known about the effect of AFM_1 on various aspects of innate and acquired immunity. In the present study, AFM_1 was administered intraperitoneally, at doses of 25 and 50 μg kg^{-1}, body mass for 28 days and various immunological parameters were measured.

RESULTS: Several parameters related to immune function were suppressed: organ mass, cellularity of spleen, proliferation response to lipopolysaccaride and phytohemagglutinin-A, hemagglutination titer, delayed type of hypersensitivity response, spleen cell subtypes, serum hemolytic activity, serum immunoglobulin G level and cytokine production. AFM_1 did not cause changes in body mass, hematological parameters or the concentration of immunoglobulin M in blood serum.

CONCLUSIONS: Overall, the data suggested that AFM_1 suppressed innate and acquired immunity. Therefore, with respect to consumer safety, it is extremely important to further control the level of AFM_1 in milk, and this should be considered as a precedence for risk management actions.

Supporting information may be found in the online version of this article.

Keywords: immunotoxicity; innate immunity; acquired immunity; fungus; milk

ABBREVIATIONS

APC	antigen-presenting cell
CFA	complete Freund's adjuvant
CH_{50}	serum hemolytic activity
CTX	cyclophosphamide
DNA	deoxyribonucleic acid
DTH	delayed type of hypersensitivity
DTNB	5,5′-dithiobis (2-nitrobenzoic acid)
FBS	fetal bovine serum
H&E	hematoxylin & eosin
HA	hemagglutination titer
IFA	incomplete Freund's adjuvant
IFN-γ	interferon gamma
IL	interleukin
NOAEL	no-observed-adverse-effect level
PBS	phosphate buffered saline
PHA	phytohemagglutinin-A
PI	proliferation index
ROS	reactive oxygen species
SRBC	sheep red blood cells
Th	T helper

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INTRODUCTION

Mycotoxins are potent secondary metabolites of filamentous fungi that can cause economic losses and human diseases.[1,2] To date, more than 300 mycotoxins have been identified. Mycotoxins differ in their chemical structures, biosynthetic origins and biological effects. Aflatoxins are a group of extremely potent metabolites produced by the common fungi *Aspergillus flavus* and *Aspergillus parasiticus*.[3,4] Aflatoxins can cause toxicity, by being immunosuppressive, mutagenic, teratogenic and carcinogenic.[5] Aflatoxin B_1 (AFB_1) is the most potent natural hepato-carcinogen for several animal species.[6] The greatest potential for adverse effects of AFB_1 is related to its main hydroxylated metabolite, aflatoxin M_1 (AFM_1).[7,8] Approximately 0.3–6.2% of AFB_1, fed to domestic animals, is transformed to AFM_1, which is then excreted in milk. However, this rate of transmission varies among individuals and also from day-to-day and from one milking to the next.[9,10]

AFM_1 exhibits the same toxicological profile as its parent compound, AFB_1, including being a class 2B possible human carcinogen that can cause mutations of genes and anomalies to chromosomes via similar mechanisms of action as AFB_1, although with lower potency.[11,12] Because of its toxic potential and resistance to the temperatures used during autoclaving and pasteurization, some countries have set maximum limits for concentrations of AFM_1 in various foods with range of 0.01–0.5 $\mu g\ L^{-1}$.[13,14]

Aflatoxins can impair both innate and acquired immune responses, which results in decreased resistance to infections that might make individuals more susceptible to secondary infections.[15,16] Several studies have demonstrated that AFB_1 suppressed cell-mediated responses by affecting the functions of T or B lymphocytes and inhibiting their proliferation. AFB_1 also inhibited functions of natural killer cells numbers of macrophages and their functions such as phagocytic activity were suppressed.[17]

Although, to date, there have been several *in vitro* and *in vivo* studies of immunomodulatory effects of AFB_1, there have been no comprehensive study of immunotoxicity of AFM_1.[18] The results of *in vitro* studies have shown that AFM_1 inhibited the proliferation of Jurkat cells. Another study indicated that exposure to a combination of AFM_1 and AFM_2 significantly reduced the release of nitric oxide by lipopolysaccaride (LPS)-induced macrophages in a concentration-dependent manner.[18,19] Exposure of mice to 100 μg of AFM_1 kg^{-1} resulted in significantly lower gains in body mass (BM), greater numbers of total white blood cells (WBCs) in blood, and altered absolute and relative numbers of types of cells in thymus and spleen.[20] Exposure of Balb/c mice to AFM_1 negatively affected several parameters in the immune system, including total numbers of CD^{3+}, CD^{54+}, CD^{4+} and CD^{56+} cells and indices of lymphoid organs.[21] The present study aimed to investigate the toxicity of AFM_1 on different aspects of immune systems (innate and adaptive immune responses) after repeated intraperitoneal (IP) administration for 28 days.

MATERIALS AND METHODS

Animals

Male Balb/c inbred mice (6–8 weeks old; weighing 19–21 g) were purchased from the Animal Room of the School of Pharmacy, Mashhad University of Medical Sciences (Mashhad, Iran). Mice were housed in polystyrene cages to which they were acclimatized for 1 week prior to usage. Mice had free access to food and water with an ambient temperature of 20–25 °C and a relative humidity of 50%.

Doses and exposure schedules

One mg of AFM_1 (Apollo Scientific Ltd, Stockport, UK) was dissolved in 1 mL of methanol, divided into aliquots and kept refrigerated. For dosing, AFM_1 solutions were dissolved in normal saline. Animals were dosed with 25 or 50 $\mu g\ kg^{-1}$, BM. Eighty mice were randomly divided into four groups to be used for four experiments. Each of these groups was further subdivided into four subsets ($n = 5$): (i) IP injection of 25 μg AFM_1 kg^{-1}, BM, 5 days/week for 4 weeks; (ii) IP injection of 50 μg AFM_1 kg^{-1}, BM, 5 days/week for 4 weeks; (iii) IP injection of positive control of 20 mg cyclophosphamide (CTX) kg^{-1}, BM) 5 days; and (iv) IP injection of negative control of normal saline and methanol 5 days/week for 4 weeks.

Masses of body and organs

Two hours after the last dose, on day 28, mice were killed by cervical dislocation. BM and organ masses of spleen, thymus and their organ/BM ratios were determined for each mouse. Single-cell suspensions were prepared in RPMI-1640 medium to count cells. Cells were counted using a Neubauer chamber.

Histopathological examinations

Thymus and spleen were collected from each mouse and fixed in 10% formalin. Following mounting, 5-μm thick sections of these tissues were stained with hematoxylin and eosin (H&E). In addition, femurs of each mouse were collected and smears of bone marrow prepared and stained with H&E. These organs were then analyzed via light microscopy and scored based on the degree of histopathological changes.[22]

Quantification of hematological parameters

Hematology was performed on samples of blood obtained from the retro-orbital plexus of individual mice. Before mice were killed, blood was collected using heparinized capillary tubes. Blood (0.2 mL) was collected in sterile tubes that were coated with K_2-ethylenediaminetetraacetic acid as an anticoagulant. Total WBC counts, as well as differential counts of relative proportions of types of WBCs, were determined. Smears of blood were prepared for visual evaluation.

Preparation of single-cell suspension

The spleen was transferred to a small petri dish that contained 10 mL of RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin, 100 $\mu g\ mL^{-1}$ streptomycin and 2 mmol L^{-1} glutamine. The spleen was teased between two frosted slides and the tissue dispersion was centrifuged at 300 *g* at 4 °C for 10 min. Supernatants were discarded and pellets resuspended in 3 mL of red blood cell (RBC) lysing buffer that contained 0.83% NH_4Cl in 100 mol L^{-1} Tris buffer, pH 7.4, then kept at room temperature for 3 min. Cells were washed three times with medium and suspended in 1 mL of the medium containing 10% FBS. Viability of cells was determined by trypan blue exclusion.[23]

Serum antibody titer: hemagglutination (HA) titer

Four days before ending dosing, mice were immunized by IP injection of 5×10^8 sheep RBCs (SRBCs) in saline. At the termination of the experiment, after preparing sera from peripheral blood samples, an aliquot (25 μL) of two-fold diluted serum in phosphate-buffered saline was challenged with 25 μL of 1% v/v SRBCs in glass tubes. Tubes were incubated at 37 °C for 1 h and then observed for HA. Antibody titer was defined as the greatest dilution that still resulted in HA.[23]

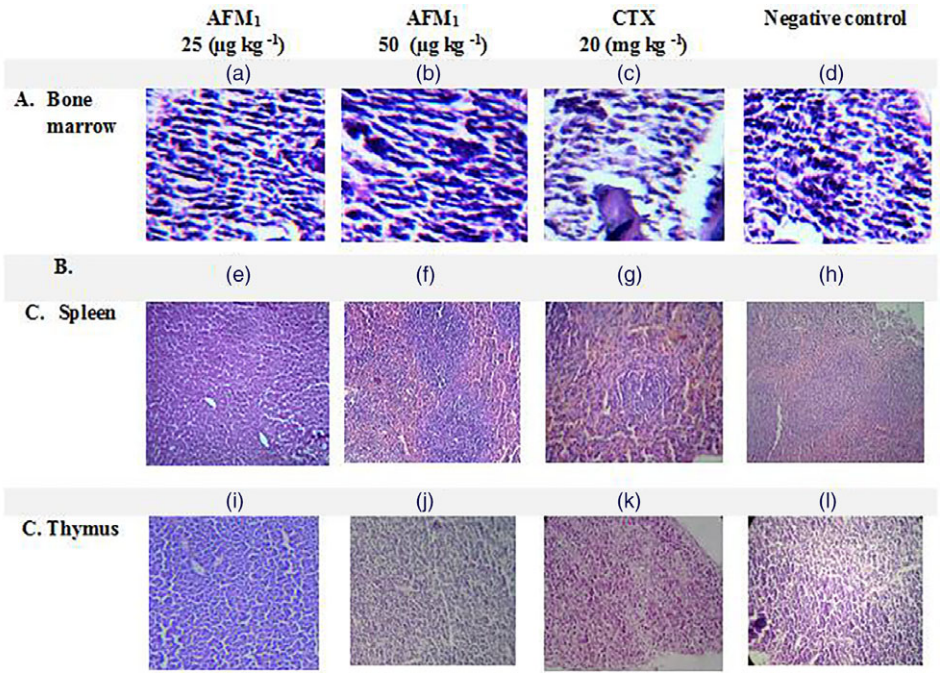

Figure 1. Effects of subacute IP exposure of AFM_1 25 and 50 ($\mu g\ kg^{-1}$), CTX 20) mg kg^{-1} (and negative control for 28 days on (A) bone marrow, (B) spleen and (C) thymus of BALB/c mice. The AFM_1 25 and 50 ($\mu g\ kg^{-1}$) treated group did not show any pathologic damages in different organs (a, b, e, f, j, k). (c, h, l) Histological damage in bone marrow, spleen and thymus in the CTX treated group.

Table 1. Effects of subacute IP exposure of mice to aflatoxin M_1 (AFM_1) for 28 days on masses of organs

	AFM_1 25 ($\mu g\ kg^{-1}$)	AFM_1 50 ($\mu g\ kg^{-1}$)	CTX20 (mg kg^{-1})	Negative control
BM (g) (before)	19.91 ± 0.34	20.01 ± 0.14	20.6 ± 0.46	20.6 ± 0.722
BM (g) (after)	20.08 ± 1.30	19.78 ± 0.54	19.81 ± 0.83	20.4 ± 0.54
Spleen mass (mg)	135 ± 11[a]	140 ± 10[a]	110 ± 13.1[a]	176 ± 9.23
Relative mass of spleen	0.67 ± 0.05[a]	0.70 ± 0.06[a]	0.55 ± 0.02[a]	0.86 ± 0.04
Thymus mass (mg)	58 ± 12[c]	52.8 ± 9[c]	40.6 ± 7.23[a]	74.6 ± 4
Relative mass of thymus	0.28 ± 0.04[b]	0.27 ± 0.03[b]	0.20 ± 0.06[a]	037 ± 0.01

Data are the mean ± SD.
[a] $P < 0.001$, [b] $P < 0.05$, [c] $P < 0.01$: significant changes compared to the control group (NS).

Lymphocyte proliferation

Proliferation of lymphocytes was determined in triplicate as described previously. Briefly, 100 μL aliquots of splenocytes, standardized to 2 million cell per millilitre, were pipetted into wells of a 96-well microtiter plate. Either no mitogen or phytohemagglutinin-A (PHA) or LPS at final concentrations of 5 and 1 $\mu g\ mL^{-1}$, respectively, was added to each well. After incubating for 48 h at 37 °C and 5% CO_2 in a humid incubator, proliferation of cells was determined using the 3-(4,5-diamethyl-2-thiazolyl) 2,5-diphenyl-2H-tetrazolium (MTT) assay. Briefly, 10% of MTT (5 mg mL^{-1}) was added to each well and incubated at 37 °C in a humid incubator under a CO_2 atmosphere for 4 h. The blue formazan precipitate was then dissolved in acidic isopropanol and its optical density was measured at 570 nm using a Stat-Fax™ (Awareness Technology, Inc., Palm City, FL, USA) enzyme-linked immunosorbent assay (ELISA) reader. The index of proliferation (PI) was calculated:[23]

$$PI = \text{absorbance of stimulated cells} / \text{absorbance of unstimulated cells} \quad (1)$$

Delayed-type hypersensitivity (DTH) response

On day 23 of dosing, mice were sensitized IP with 1×10^9 SRBC in complete Freund's adjuvant (sensitization phase). After 5 days of immunization, sensitized mice were again challenged with a booster dose of 1×10^8 SRBCs in the left hind footpad (effector phase). To serve as a negative control for non-specific swelling, the right, hind footpad was injected with the same volume of incomplete Freund's adjuvant. The increase in volume of the left footpad was measured using a pressure sensitive micrometer screw gauge (Mitutoyo, Kawasaki, Japan) 24 and 48 h after the challenge with SRBCs and the difference between left and right hind footpad volumes was calculated.[24]

Subtyping of spleen cells

Suspensions of spleen cells were prepared and evaluated for subset distribution by three-color flow cytometry. Briefly, single-cell suspensions of splenocytes in RPMI-1640 (1×10^6 cells mL^{-1}) were prepared, and numbers of spleen cell were determined by exclusion of trypan blue dye. Subtypes of cells CD^{19+}, CD^{49b}, CD^{3+}, CD^{4+} and CD^{8+} were then determined using a BD FACS Calibur™ flow

Table 2. Effects of subacute IP exposure of mice to aflatoxin M_1 (AFM_1) for 28 days on numbers of white blood cells

	$AFM_1$25 (μg kg^{-1})	$AFM_1$50 (μg kg^{-1})	CTX20 (mg kg^{-1})	Negative control
WBC (μL) 10^3	7.16 ± 0.15	6.82 ± 0.68	3.26 ± 0.21[a]	6.86 ± 0.97
LYM (μL) 10^3	4.52 ± 0.23	4.41 ± 0.16	2.44 ± 0.97[a]	4.44 ± 0.48
NEU (μL) 10^3	2.41 ± 0.48	2.29 ± 0.04	0.67 ± 0.03[a]	2.34 ± 0.08
MONO (μL) 10^3	0.2 ± 0.01	0.1 ± 0.3	0.1 ± 0.02	0.1 ± 0.01

Data are the mean ± SD.
[a] $P < 0.001$: significant changes compared to the control group (NS).

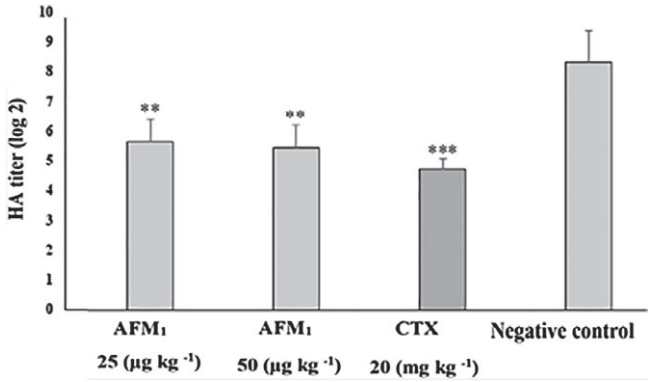

Figure 2. Effect of AFM_1 treatment on HA titer assay. Data are expressed as the mean ± SD ($n = 5$). $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* control group according to ANOVA followed by Tukey's post-hoc test. AFM_1, aflatoxin M_1; CTX, cyclophosphamide.

cytometer (Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA) with a mouse T lymphocyte subset antibody cocktail kit (with isotype control) (PE-Cy 7 CD^{3e}, PE CD^4, FITC CD^8; BD Pharmingen, San Diego, CA, USA) and FITC rat anti-CD^{19}, FITC anti-mouse CD^{49b} (with isotype control, ebioscience) in accordance with the manufacturer's instructions. The absolute number of cells in each spleen was determined by multiplying differential ratios of these subtypes by the total numbers of spleenocytes.[25]

Production of cytokines

Cells were stimulated by mitogens LPS or PHA and incubated for 48 h at 37 °C in 5% CO_2. Cell-free supernatants were collected and frozen and then maintained at −70 °C until testing. Concentrations of interferon (IFN)-γ, interleukin (IL)-4 and IL-10 in collected supernatants were measured using commercially available ELISA kits in accordance with the manufacturer's instructions.[25]

Quantification of immunoglobulin isotypes in blood serum

Concentrations of antibodies immunoglobulin (Ig)G and IgM of the compliment system in blood serum were measured using ELISA kits.[26]

Serum hemolytic assay (CH_{50})

Measures of total serum hemolytic activity (CH_{50}) were performed using rabbit anti-sheep erythrocyte IgG antibodies and sheep erythrocytes. Briefly, a 1:50 dilution of sample serum in gelatin veronal buffer (GVB) (0.1% gelatin, 5 mmol L^{-1} veronal, 145 mmol L^{-1} NaCl, 0.025% NaN_3, pH 7.3, buffer) was prepared. This then underwent a further series of serial dilutions with GVB. An aliquot (50 μL) of each diluted sample of serum was then placed into a tube. An aliquot (50 μL) of EA (antibodysensitized sheep erythrocytes, in GVB at 10^9 mL^{-1}) was added. All tubes were incubated at 37 °C for 30 min. Thereafter, 150 μL of ice-cold GVB was added to all serum-bearing tubes and cell blanks and 200 μL of H_2O was added to the 100% lysis tubes, (control tubes). Tubes were centrifuged ay 1000 × *g* for 5 min at 4 °C. Aliquots of supernatants (200 μL) from each tube were then transferred to flat-bottom 96-well plates and the absorbance (OD) of each supernatant was measured at 540 nm using a Stat-Fax™ (Awareness Technology, Inc.) ELISA reader. Following correction for background absorbance, by subtracting cell blank absorbance (value associated with spontaneous lysis) from each value, the fractional hemolysis in each well relative to that in 100% lysis wells was calculated:

$$\text{Fractional hemolytic } (y) = (\text{OD serum}/\text{OD 100\%total lysis}) \quad (2)$$

The amount (μL) of each serum causing 50% hemolysis (K) was determined by plotting (on log–log graph paper) serum volume in μL added (x) *versus* [$y/(1 - y)$]; this plot is expected to be linear. At 50% hemolysis, $y/1 - y = 1$; hence, the intercept on the x-axis from this point corresponds to 1 CH_{50} unit for that sample of serum.[27]

Assay of phagocytic activity

Phagocytic capacities of monocytes and granulocytes in each sample of mouse blood were measured by use of a Phagotest kit (Becton-Dickinson Biosciences) in accordance with the manufacturer's instructions. Each kit contained all of the necessary reagents for flow cytometric analyses of the phagocytic uptake of fluorescein-labeled opsonized *E. coli* by the cells. Ingestion of labeled particles by phagocytes was evaluated using a BD FACS Calibur™ flow cytometer. In each case, a minimum of 100 000 events was analyzed per sample. Ultimately, each presenting cell value was expressed as fluorescence intensity/phagocytic cell.[27]

Statistical analysis

All data are presented as the mean ± SD. Normality of data were verified using the Kolmogorov–Smirnov test and homogeneity of variance was checked by Levene's test. If the data failed to pass the test, a logarithmic transformation of the data was performed and then tested again to determine whether it met the assumptions of parametric statistical tests. Significant differences were assessed between each treatment and the control using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. $P < 0.05$ was considered statistically significantly different. All statistical tests were conducted using PRISM, version 6.00 (GraphPad Software Inc., San Diego, CA, USA).

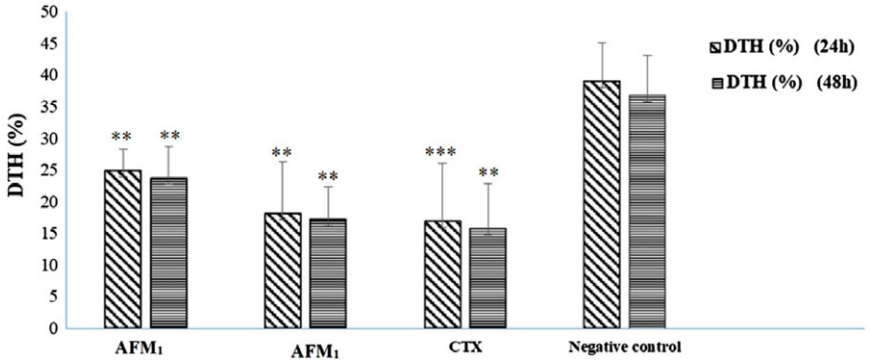

Figure 3. Effect of AFM_1 treatment on delayed-type hypersensitivity (DTH) response. Data are expressed as the mean ± SD ($n = 5$). $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* control group according to ANOVA followed by Tukey's post-hoc test. AFM_1, aflatoxin M_1; CTX, cyclophosphamide.

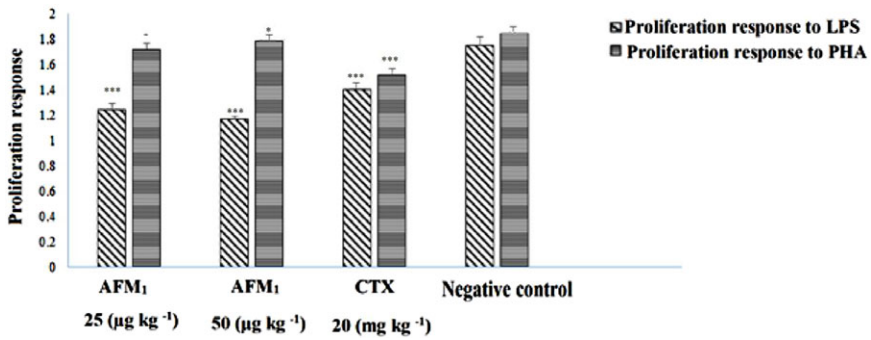

Figure 4. Effect of AFM_1 treatment on proliferative response to PHA and LPS. Data are expressed as the mean ± SD ($n = 5$). $^{*}P < 0.05$ and $^{***}P < 0.001$ *versus* control group according to ANOVA followed by Tukey's post-hoc test. AFM_1, aflatoxin M_1; CTX, cyclophosphamide.

RESULTS

Histopathology

Spleen was evaluated for white pulp atrophy (or hyperplasia), red pulp:white pulp. These changes were not observed at doses of 25 and 50 μg AFM_1 kg^{-1}. Analyses revealed that CTX induced splenic white pulp atrophy and an increase in the red:white pulp ratio. Cortex thickness, relative size of medulla, ratio of cortex to medulla and capsular changes were evaluated in thymus. The thymic tissues were also evaluated for the presence of necrosis, apoptosis and any abnormal infiltration of cells. Light microscopic examinations of the thymus samples did not reveal any significant effects from the AFM_1 treatments. A significant reduction in cortex thickness, as well as in the number of cortical lymphocytes, was observed in the CTX-treated group. Cellularity, the existence and maturation of hematopoietic cell subtypes and the erythroid:myeloid cell ratio were evaluated in each bone marrow specimen isolated. Using light microscopic examination, no significant pathologic differences were noted among the samples from the AFM_1 treatment groups. A mild reduction in cellularity was observed in the CTX-treated group (Fig. 1).

Masses of whole body and organs and numbers of spleen cells

After 4 weeks, BMs in the treatment groups were not significantly different from those of controls. However, masses of spleen and thymus of mice exposed to all doses of AFM_1 were significantly lower than those of the control group. There were significant

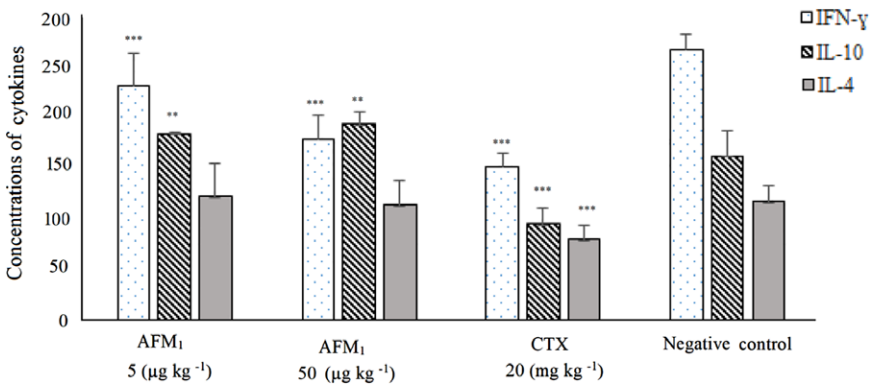

Figure 5. Effect of AFM_1 treatment on production of cytokines. Data are expressed as the mean ± SD ($n = 5$). $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* control group according to ANOVA followed by Tukey's post-hoc test. AFM_1, aflatoxin M_1; CTX, cyclophosphamide.

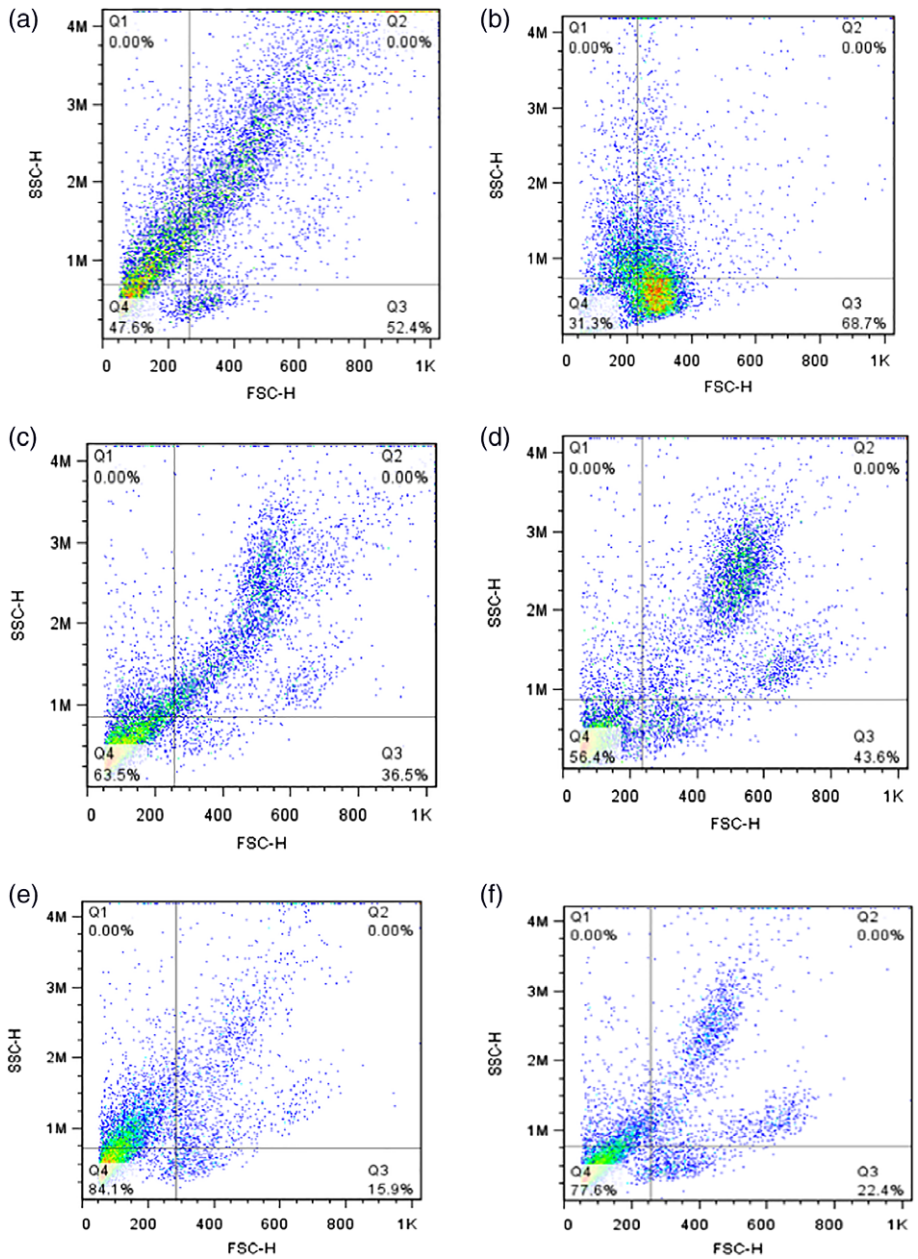

Figure 6. Dot plots from BD FACS Calibur™ flow cytometry system indicate CD^{3+} (a), CD^{+4} (c) and CD^{8+} (e) expression in BALB/c splenocytes after treatment with AFM_1 50 ($\mu g\ kg^{-1}$) and CD^{3+} (b), CD^{+4} (d) and CD^{8+}. (f) Expression in BALB/c splenocytes after treatment with negative control.

differences in the organ/BM ratios of spleen and thymus of the treated groups compared to those of control mice. There was no statistically significant difference between mice exposed to 25 or 50 μg AFM_1 kg^{-1}) (Table 1).

Hematological parameters

There were no significant differences in numbers of total WBC, lymphocytes, monocytes or neutrophils among mice treated with the two doses of AFM_1 or with unexposed controls (Table 2).

Serum antibody titer: HA titer assay

Serum anti-SRBC titer indicated a significant suppression in AFM_1 treatment groups compared to the negative control group. There was no significant difference between AFM_1 treated groups (Fig. 2).

DTH response

DTH was observed in mice exposed to either 25 or 50 μg AFM_1 kg^{-1} compared to the negative control. The DTH response was significantly ($P < 0.001$) suppressed by the positive control, CTX (Fig. 3). There were no statistically significant differences in DTH in mice exposed to the two doses of AFM_1.

Proliferative response to PHA and LPS

Exposure of mice to 25 or 50 μg AFM_1 kg^{-1} suppressed the proliferative responses of splenocytes exposed to PHA or LPS. Statistically significant suppression of proliferative responses of splenocytes was observed in cells exposed to the positive control (Fig. 4). There were no statistically significant differences between mice dosed at 25 or 50 μg AFM_1 kg^{-1}.

Table 3. Effects of subacute IP exposure of mice to 25 or 50 μg AFM_1 kg^{-1} or positive control of 20 CTX mg kg^{-1} for 28 days on mice absolute and relative numbers of types of spleenocytes

	AFM_1 25 (μg kg^{-1})	AFM_1 50 (μg kg^{-1})	CTX20 (mg kg^{-1})	Negative control
Spleen cell numbers ($\times 10^7$)	7.31 ± 0.32^a	7.04 ± 0.24^a	6.25 ± 0.13^a	8.08 ± 0.34
CD^{19+} cell (%)	23.52 ± 1.03	23.86 ± 0.83	17.44 ± 2.05^a	23.88 ± 3.24
CD^{19+} content	1.72 ± 0.16^b	1.68 ± 0.07^c	1.09 ± 0.03^a	1.93 ± 0.05
CD^{49b} cell (%)	6.15 ± 0.13	5.39 ± 0.71^c	4.96 ± 0.97^c	7.30 ± 1.02
CD^{49b} content ($\times 10^7$)	0.45 ± 0.07	0.38 ± 0.13^b	0.31 ± 0.01^c	0.59 ± 0.11
CD^{3+} cell (%)	52.31 ± 4.43^a	55.11 ± 1.83^a	57.54 ± 3.45^a	67.2 ± 5.04
CD^{3+} content	3.82 ± 0.23^a	3.88 ± 0.53^a	3.59 ± 0.07^a	5.43 ± 0.05
CD^{4+} cell (%)	36.28 ± 3.24	35.65 ± 6.21^b	35.44 ± 6.21^c	43.44 ± 3.66
CD^{4+} content	2.66 ± 0.23^c	2.51 ± 0.06^a	2.14 ± 0.09^a	3.51 ± 0.61
CD^{8+} cell (%)	15.45 ± 2.26^a	14.91 ± 1.88^a	15.84 ± 1.24^a	22.64 ± 3.11
CD^{8+} content	1.13 ± 0.05^a	1.05 ± 0.11^a	0.99 ± 0.04^a	1.83 ± 0.23

Data are the mean $\pm$ SD.
[a] $P < 0.001$, [b] $P < 0.05$, [c] $P < 0.01$: significant changes compared to the control group (NS).

Production of cytokines

Cytokines responded differently to exposure to AFM_1 (Fig. 5). Significantly lower concentrations of IFN-γ were observed in mice exposed to both doses of AFM_1 (Fig. 5). Alternatively, there was significantly more IL-10 produced in mice exposed to AFM_1. Concentrations of IL-4 were not significantly different in mice exposed to AFM_1 compared to that of controls.

Spleen cell subtyping

Exposure to AFM_1 for 4 weeks resulted in significant ($P < 0.001$) differences among relative proportions of phenotypes of lymphocytes, relative to that of the negative control (Table 3). Significantly fewer CD^{3+}, CD^{4+}, CD^{8+} and CD^{19+} were observed in spleens of mice exposed to 25 or 50 μg AFM_1 kg^{-1}. Percentages of CD^{3+} and CD^{8+} T lymphocytes were lower in spleens of mice exposed to 25 or 50 μg AFM_1 kg^{-1}. AFM_1 at 50 μg kg^{-1} significantly decreased the percentage of CD^{4+} in mice. However, the percentage of CD^{4+} exposed to 25 μg AFM_1 kg^{-1} was not significantly different from the control. Absolute and relative numbers of CD^{49b} were significantly lower in mice exposed to 50 μg AFM_1 kg^{-1}, relative to that of the control, but not compared to those exposed to 25 μg AFM_1 kg^{-1} (Fig. 6 and Table 3).

Phagocytic activity

Although phagocytosis of mice exposed to both doses of AFM_1 was significantly impaired relative to that of controls, there was no statistically significant difference between phagocytosis of mice exposed to 25 or 50 μg AFM_1 kg^{-1} (Fig. 7).

Concentrations of immunoglobulin isotypes in blood serum

Immunoglobulins responded differently to exposure to AFM_1 (Fig. 8). Concentrations of IgM did not exhibit differences among mice exposed to AFM_1 or unexposed, negative controls. Alternatively, concentrations of IgG were lower in the blood serum of mice exposed to AFM_1.

CH_{50} hemolytic assay

The ability to lyse target RBCs was significantly lower in blood samples obtained from mice exposed to AFM_1 relative to that collected from unexposed controls (Fig. 9).

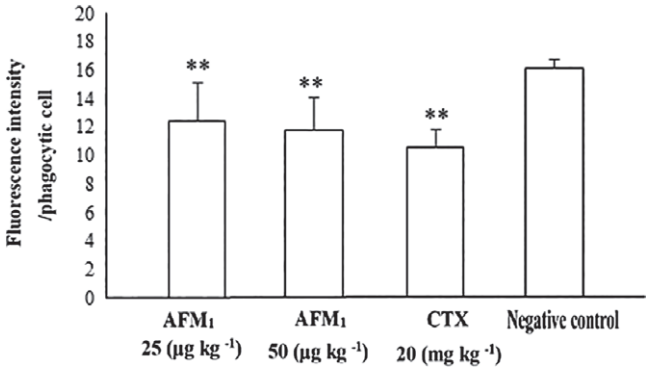

Figure 7. Effect of AFM_1 treatment on phagocytic activity. Data are expressed as the mean $\pm$ SD ($n = 5$). **$P < 0.01$ and ***$P < 0.001$ *versus* control group according to ANOVA followed by Tukey's post-hoc test. AFM_1, aflatoxin M_1; CTX, cyclophosphamide.

DISCUSSION

Several effects on the immune system have been attributed to aflatoxins. However, until now, little information has been available concerning the effects of AFM_1 on immune system cellularity and function. Despite the presence of AFM_1 in milk and milk by-products, as a result of the limited availability of AFM_1, which must be isolated from biological sources, there is little information available on acute and chronic toxicity of AFM_1 with respect to animals.[28]

Exposure of humans to AFM_1 is a result of either endogenous production of AFM_1 or the consumption of contaminated dairy products. In the present study, to more closely simulate the appropriate route of exposure and mimic crossing cellular barriers in the lung and gastrointestinal tract, at the same time as limiting systematic metabolism, IP injection was used to administer AFM_1.[29] The doses selected for use were based on the predicted no observable effect level (NOAEL), which was estimated to be 2.5 μg AFM_1 kg^{-1}. Doses that were ten- and 20-fold greater than the NOAEL were used to evaluate potency to affect immune function.[22,30]

The results obtained confirmed the adverse effects of AFM_1 on the murine immune system. Exposure to AFM_1 resulted in a lower mass of spleen, which might be because of an overall lower number of T and B cell subpopulations.[31] Furthermore,

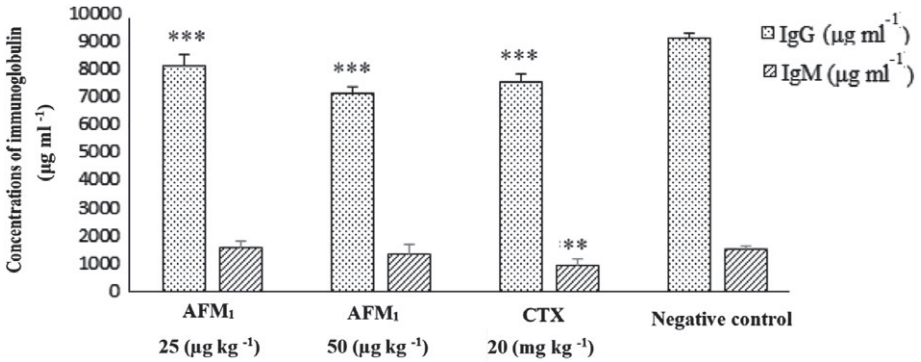

Figure 8. Effect of AFM_1 treatment on concentrations of immunoglobulin isotypes in blood serum. Data are expressed as the mean ± SD ($n = 5$). $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* control group according to ANOVA followed by Tukey's post-hoc test. AFM_1, aflatoxin M_1; CTX, cyclophosphamide.

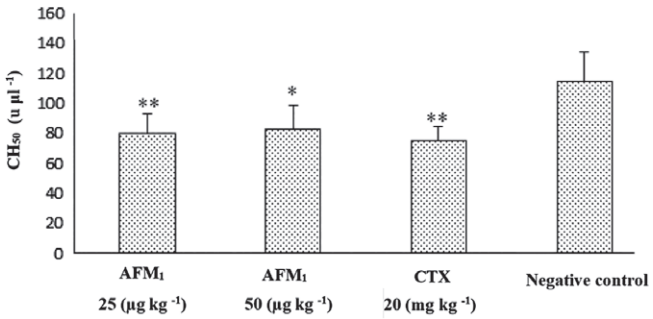

Figure 9. Effect of AFM_1 treatment on CH_{50} hemolytic assay. Data are expressed as the mean ± SD ($n = 5$). $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* control group according to ANOVA followed by Tukey's post-hoc test. AFM_1, aflatoxin M_1; CTX, cyclophosphamide.

there was no obvious damage in bone marrow with respect to a blood cell-derived tissue that results in an unchanged leukocyte count. On the other hand, no histopathologic significant changes in thymus and spleen were observed in mice exposed to AFM_1 (Fig. 1).

A decrease in the number of spleenocytes (CD^{3+}, CD^{4+}, CD^{8+}, CD^{19+} and CD^{49b} cells) might be associated with the direct toxicity of AFM_1. AFM_1 was found to be a potent producer of free oxygen radicals.[32,33] It is possible that the free oxygen radicals generated could inhibit the function of immune cells as a result of the peroxidation of lipids in membranes, which could then result in impairment reactions of lymphocytes with antigens, lymphokines or other cell subsets.[23] Therefore, the observed immunotoxicity might be related to the reactive oxygen species generated by AFM_1.[34]

AFM_1 might have caused immunotoxicity via the formation of adducts with DNA (AFM_1-N7-guanine) or blockage of DNA, which suppressed the proliferation of cells required for immune responses.[35,36]

However, there was no obvious damage in bone marrow with respect to a blood cell-derived tissue that results in an unchanged leukocyte count.

Indices of proliferation for PHA and LPS indicated the direct effects of AFM_1 on the normal function of T and B lymphocytes, which have been shown to result in impaired responses of DTH and HA.[37,38] Because CD^{4+} cells play a key role in helping B-cells produce antibodies, a decrease in anti-SRBC titers might be secondary to direct effects on T-cells.[39] Significant reductions in phagocytic activity, numbers of natural killer cells in the spleen, and CH_{50} and concentrations of IgG in blood serum indicate an impairment of innate immunity by AFM_1.[27] The complement system and IgG, which facilitate phagocytosis through opsonisation, might also result in less phagocytosis because of the impaired function of complement proteins and lower concentrations of IgG.[40–42] Based on the results of the present study, a dysfunction of acquired immunity could be the result of disorders of cellular and molecular components of innate immunity, particularly phagocytic cells. Therefore, a significant lower response of DTH and HA titers might be associated with the dysfunction of phagocytic cells such as antigen-presenting cells.[34]

An analysis on cytokine profiles in supernatants of splenocytes indicated significantly less IFN-γ, which, along with unaffected concentrations of IL-4, suggests effects on the production and differentiation of Th_1, which was caused by the suppression of DTH.[43] The lower concentration of IL-10 confirmed the inhibitory effects of AFM_1 on the propagation of the cell-mediated immune system toward Th_1. This could be a result of greater concentrations of IL-10 being released from regulatory T cells.[44,45]

Overall, the data obtained in the present study suggest that AFM_1 suppressed innate and acquired immunity. The presence of AFM_1 in milk and dairy products is an important issue, especially for children and infants who are more susceptible than adults. Therefore, regarding consumer safety, it is extremely important to further control the level of AFM_1 in milk, and this should be considered as a precedence for risk management actions. Concisely, by adopting good harvesting practices and physical removal methods, as well as by upgrading analytical facilities and implementing strict regulations, this would avoid or reduce the presence of these natural contaminants in milk and ensure the safety of milk and milk products for consumers.[46]

ACKNOWLEDGEMENTS

The authors thank the Vice Chancellor of Research, Mashhad University of Medical Sciences and Iran National Science Foundation (INSF), for financial support. Professor J P Giesy was supported by the Canada Research Chair program, the 2012 'High Level Foreign Experts' (#GDT20143200016) program, funded by the State Administration of Foreign Experts Affairs, the PR China to Nanjing University and the Einstein Professor Program of the Chinese Academy of Sciences, and a Distinguished Visiting Professorship in the School of Biological Sciences of the University of Hong Kong. The authors declare that they have no conflicts of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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