


Effects of Aflatoxin on the Proportions of Peripheral Blood Leukocytes and Alpha-Naphtyl Acetate Esterase (ANAE) Positive Lymphocytes in the Mouse

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Summary

In this study detrimental effects of aflatoxin (AF) on the peripheral blood formula and the alpha naphthyl acetate esterase (ANAE) - positivity profile of peripheral blood lymphocytes (PBL) on the mice were determined. A total of 40 white mice were randomly divided in to 5 groups each having 8 animals. The first group served as a control and received standard diet. The remaining 4 groups were fed diets containing 200, 400, 800 and 1600 ppb AF, respectively. The results of this study have revealed that AF given by the food caused significant declines both in the proportion of PBL and in the percentages of ANAE-positive peripheral blood lymphocytes (T-lymphocytes) in a dose dependent manner. These results may be concerned as a haematological evidence for immuno-suppressive effect of AF on cell-mediated immunity.

Keywords: *Aflatoxin, Lymphocytes, Alpha-naphtyl acetate esterase*

Farelerde Aflatoksinin Perifer Kan Lökositleri ve Alfa-Naftil Asetat Esteraz (ANAE) Pozitif Lenfositlerin Oranlarına Etkisi

Özet

Bu çalışmada aflatoksinin (AF) farelerin lökosit formülü ve perifer kan lenfositlerinin alfa naftil asetat esteraz (ANAE) pozitifliği üzerindeki etkileri araştırılmıştır. Çalışmada 40 adet beyaz fare 8 fareden oluşan 5 gruba ayrıldı ve birinci grup kontrol grubu olarak standart diyetle, diğer 4 grup ise sırasıyla 200 ppb, 400 ppb, 800 ppb ve 1600 ppb AF içeren diyetle beslendi. Sonuç olarak aflatoksin içeren diyetle beslenen gruplarda doza bağlı olarak perifer kan lenfosit oranları ile ANAE pozitif lenfosit (T-lenfosit) oranlarında düşüşler ortaya çıktı. Bu sonuçlar hücresel immünite üzerinde aflatoksinlerin baskılayıcı etkisinin olabileceğini hematolojik olarak ortaya koymuştur.

Anahtar sözcükler: *Aflatoksin, Lenfosit, Alfa naftil asetat esteraz*


INTRODUCTION


Heterocyclic metabolites of the genus *Aspergillus*, especially *A. flavus* and *A. parasiticus*, are aflatoxin (AF) those can occur as natural contaminants of foods and foodstuffs. Among 18 different AF, only aflatoxin B₁, B₂, G₁ and G₂ have been identified as natural food contaminants. Compared with the other AF (AFB₂, AFG₁ and AFG₂), AFB₁ is the most frequently determined and biologically

active component and also it is an extremely potent carcinogen that induces hepatocarcinomas in most species¹⁻³.

The susceptibility to the toxic effects of AF varies in different animal species because of the differences in the drug metabolism systems of the animal, responsible

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for the activation. The most susceptible animal species are ducklings and rabbits, whereas horse, cattle and laboratory animals are known to be less sensitive⁴. The sensitivity is related with the hepatic microsomal Mixed Function Oxidase (MFO) enzyme systems which converts especially AFB₁ to AFB₁-8,9 epoxide known as the most toxic metabolites of AFB₁. Because, these metabolites bind to DNA and form AFB₁-DNA adducts which is thought to be responsible for its carcinogenic effect^{5,6}.

AF has detrimental effects on the immune system. The AFB₁-DNA adduct inhibits ribonucleic acid (RNA) synthesis which results in inhibition of synthesis of proteins such as enzymes and antibodies⁴. Leeson et al.¹ have reported that the lower doses of AFB₁ affect cellular immunity and its higher doses affect humoral immunity. Suppression of the phagocytic activity of macrophages and decrease in T-lymphocyte counts result in serious depression of cellular immunity^{7,8} whereas the liver damage has been assumed to be responsible for deficiency in the humoral immunity because severe liver damage cause depression of complement activity^{9,10}.

The low level AFB₁ have been shown to play a direct immune-suppressive effect on the cell-mediated immune (CMI) reactions² by inhibiting phagocytic and microbicidal activity of peritoneal macrophages, and decreasing peripheral blood T-lymphocyte counts in the chicken^{7,8,11}. AFB₁ selectively suppressed CMI reactions in growing rats at the 300 µg and 600 µg dose levels as measured delayed type hyper sensitivity assay in the report of Reddy et al.¹². Functional deficits in phagocytic cells caused by AFB₁ have been also shown by Jakob et al.¹³. They have found that inhalation exposure of rats to AFB₁ aerosols suppressed alveolar macrophage phagocytosis resulting in depression both local and systemic defenses.

Enzyme histochemical studies can be used to evaluate the functional development and maturation of the immune system^{14,15}. Alpha-naphthyl acetate esterase (ANAE) is a lysosomal enzyme^{16,17} of mononuclear leukocytes, especially lymphocytes and monocyte-macrophage cell lineage. Mueller et al.¹⁸ have claimed that ANAE plays important roles in antigen endocytosis, antigen degradation and cytotoxic effects of activated T cells. Enzymatic positivity for ANAE has widely used to differentiate T, B lymphocytes and monocytes in human¹⁹, chickens^{20,21}, the cattle²², dogs²³ and in mice¹⁸.

The aim of this study was to determine the detrimental effects of AFB₁ on the leukocyte formula and the ANAE-positivity profile of PBL on the mice.

MATERIAL and METHODS

Production of Aflatoxin

The AF was produced by the method of Shotwell et al.²⁴. Briefly, 150 g of sterile boiled and pounded rice was inoculated with 2 ml of resuspended spores (2×10^6 spore/ml) of *Aspergillus parasiticus* NRLL 2999 (USDA, Agricultural Research Service, Peoria IL) and placed in an incubator at 28°C for 2 d. Subsequently, inoculated rice samples were boiled and pounded, steamed to kill the fungi, dried and ground to a fine powder. The powder was then analysed for AF content by the method of Shotwell et al.²⁴ using thin Layer Chromatography (TLC) - densitometer equipped with fluorescence detector (Perkin Elmer MPF 43-A) at 365 nm excitation and 425 nm emission wave lengths and UV-VIS Recording Spectrophotometer (Schimadzu-UZ 2100) with standards. Adequate amount of the powder was mixed to obtain the experimental feed samples were also determined by means of TLC.

Animals and Experimental Groups

A total of 40 white mice at 60 days-old-age in equal numbers of both sexes, were randomly divided into 5 groups each having 8 animals. The animals were housed individually in the cages located in a pathogen free, temperature-and humidity-controlled colony room which is maintained under a 12- hour day-night illuminating cycle with free access to food and water. The first group served as a control and received standard diet. The remaining 4 groups were fed diets containing 200, 400, 800 and 1600 ppb AF, respectively.

Peripheral blood samples were collected via the coxygeal vein in heparinized (10 UI heparin/ml⁻¹ of the blood) tubes. From each sample, four smears were prepared, air dried, and fixed in glutaraldehyde acetone fixative for 3 min at -10°C. After fixation, the smears were rinsed three times in distilled water and then allowed to dry at room temperature (20°C) for 30 min²². Two of them were stained with May Grünwald-Giemsa to determine leukocyte formula of the sample. The remaining smears were used for the demonstration of alpha-naphthyl acetate esterase (ANAE)¹⁶.

The incubation solution used in the enzyme demonstration was prepared according to Knowles et al.¹⁶. Briefly, 40 ml of 0.067 M phosphate buffer, pH 5.0 was mixed with 2.4 ml of hexazotized pararosaniline [1.2 ml of pararosaniline (Sigma) and 1.2 ml of 4% sodium nitrite (Merck) in distilled water] and 10 mg of alpha naphthyl acetate (Sigma) dissolved in 0.4 ml of acetone. pH of the mixture was adjusted to 5.8 with 2N NaOH.

Pararosaniline solution was prepared by dissolving 1 g pararosaniline in 20 ml boiling distilled water, then 5 ml concentrated HCl was added and cooled.

After 4 h incubation at 37°C, the smears were rinsed three times with distilled water and counterstained with 1% methyl green (Merck) in 0.1 M acetate buffer (pH 4.2) for 10 min. The slides were dehydrated in increasing concentrations of ethanol, cleared in xylene and mounted with Entellan.

In ANAE-demonstrated blood smears, the cells with lymphocyte morphology and having 1-5 reddish-brown granules were scored as ANAE-positive lymphocyte, T-lymphocyte. The positivity rates were determined by counting 200 lymphocytes. Similarly, the leukocyte formula was also determined by counting 200 leukocytes on the May Grünwald-Giemsa stained smears.

Statistical Analyses

Proportion of the PBL, and ANAE positivities were analysed with one-way ANOVA (SPSS, 10.0) following arc-sin transforming²⁵. Results were considered as significant when p values were less than 0.05.

RESULTS

In control animals, lymphocytes had the highest (71.71%) proportion in the peripheral blood leukocytes. Neutrophils (19.57%), eosinophils (4.28%), monocytes (4.14%) and basophils (1.28%) followed them, respectively. Majority of the peripheral blood lymphocytes (61.86%) were ANAE-positive in the control group (Table 1).

Lymphocyte levels decreased significantly ($P<0.001$) in AF-treated groups compared to the control group. The higher AF doses resulted in the lower lymphocyte levels. AF treatment decreased significantly ($P<0.001$) the proportions of ANAE-positive peripheral blood lymphocytes. Monocyte levels did not change with AF treatment whereas the higher dose groups had the higher neutrophil levels. Eosinophil levels of the AF-

treated animals also decreased significantly ($P<0.001$), except 800 ppb AF-received group. AF-treatment did not cause any significant change in basophil levels, except 200 ppb AF-received group (Table 1).

DISCUSSION

Contamination of the food and foodstuffs with aflatoxins (AF) is a serious concern in both animal and human health. Although there are many adverse effects caused by AF such as hepatotoxicity, nephrotoxicity, cytotoxicity, mutagenity, genotoxicity and teratogenity²⁶⁻²⁹, the most important and the well documented effect of AF is immunotoxicity^{14,15,30}. Neldon-Ortiz and Qureshi⁷ and Çelik et al.⁸ reported that low levels of AFB₁ caused inhibition of phagocytic and microbicidal activity of peritoneal macrophages in the chicken. Moreover, Neldon-Ortiz and Qureshi³¹ showed that AFB₁ administered in the early embryonic period caused similar effects on the macrophages of hatching chickens. Giambone et al.³² reported that in chickens fed a diet of 2.5 mg/kg AF, the delayed-type hypersensitivity skin reactions to tuberculin were reduced. Ghosh et al.³³ showed a significant decline in skin sensitivity and graft versus host reaction, which were used to evaluate cell mediated immunity in broiler chickens, even when a non-toxic dose was administered. They also reported significant decreases in phagocytic activity of the splenic macrophages.

Enzyme histochemistry is one of the cheap and the easiest methods used to evaluate the functional development and maturation of the immune system³⁴. In the normal peripheral blood, there is a good correlation between the proportion of T-cells and percentage of ANAE positive lymphocytes in humans¹⁹, in the chicken²¹, in the cattle³⁵, in the mouse¹⁸ and in the dog²³. Mueller et al.¹⁸ have showed that ANAE- positive lymphocytes have higher frequency than negative cells in both lymph and lymphoid organs in mice. Similarly, the results of the esterase staining obtained from this study have showed that ANAE-positive lymphocyte proportions were higher when compared to ANAE-

Table 1. Leukocyte formula and ANAE-positivity of PBL of the control and experimental groups (mean±SE)

Tablo 1. Kontrol ve çalışma grubu farelerde perifer kan formül lökosit ve ANAE pozitiflik oranları (ortalama±standart hata)

Groups (n=8)	Proportion of ANAE positive PBL (%)	Proportion of lymphocytes (%)	Proportion of monocytes (%)	Proportion of neutrophils (%)	Proportion of eosinophils (%)	Proportion of basophils (%)
Control	61.86±2.91 ^a	71.71±3.09 ^a	4.14±0.90 ^a	19.57±3.82 ^d	4.28±1.25 ^a	1.28±0.48 ^a
200 ppb AF	39.57±4.16 ^b	68.42±1.71 ^a	3.57±0.53 ^a	22.71±2.69 ^{cd}	2.00±0.81 ^b	1.00±0.00 ^b
400 ppb AF	37.79±7.40 ^b	66.71±2.92 ^b	3.57±0.97 ^a	25.57±2.63 ^{bc}	2.57±0.97 ^b	1.57±0.53 ^a
800 ppb AF	34.64±5.40 ^b	64.85±2.91 ^b	4.71±1.60 ^a	28.28±2.56 ^b	3.00±1.29 ^{ab}	1.14±0.37 ^a
1600 ppb AF	34.14±6.04 ^b	57.42±1.51 ^c	4.42±2.14 ^a	33.28±1.71 ^a	2.42±0.53 ^b	1.85±0.89 ^a

^{a-d} Values within a column with no common superscripts are significantly different ($P<0.05$)

negative lymphocyte proportions.

Changes in the proportions of leukocytes reacting positively for various enzymes, preferentially those of the lymphocytes, are regarded as a useful tool not only for determining the functional capacity and maturational stage of the immune system but also to evaluate healthy status of humans and animals³⁰. Mycotoxin-induced immunosuppression may be determined as depressed cellular^{8,32} and humoral immune reactions³⁶. Çelik et al.³ have showed that AF depress cell functions besides decrease the number of ANAE positive lymphocytes (T-lymphocytes) in both peripheral blood and in lymphoid tissues. Ghosh et al.³³ have recorded that significant ($P<0.05$) decreases were observed in the chicks fed AFB₁ (1 mg/kg diet) at d 35 and 42 in ANAE-positive peripheral blood lymphocyte (T-lymphocyte) counts compared with the control group. Sur and Çelik³⁰ have observed that low level administration of AFB₁ in ovo at the beginning of early embryonic development decreased the ANAE positive peripheral blood lymphocyte percentages at the hatching period. In the present study, AF treatment decreased significantly ($P<0.001$) the proportions of ANAE-positive peripheral blood lymphocytes. This result is similar to the results of previous studies.

In this study, AF-treatment caused striking changes in the leukocyte formula of the experimental groups although any significant changes in monocyte levels did not occur in the AF treated groups. The neutrophile percentages were gradually increased whereas the lymphocyte and eosinophile proportions were decreased in increasing toxin doses in a dose-response manner (Table 1). The reason for the increasing of neutrophile percentages was considered due to general inflammation by toxication. However, the percentages of the peripheral blood monocytes were not affected by AF-administration.

The results obtained from this study give some hemathological evidence to explain the immunosuppressive effects of AF and reveal that AF given by the food is highly toxic molecule for immune system and causes significant declines both in the proportion of PBL and in the percentages of ANAE-positive, peripheral blood lymphocytes in a dose dependent manner. However these results are not sufficient to explain the exact cellular mechanism because the lymphocyte function assays have not been performed in this study. So, further experiments should be done to clarify the immunotoxic mechanism of the AF.

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