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Effect of Aflatoxins on Rat Peritoneal Macrophages

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Phagocytosis, intracellular killing of *Candida albicans*, and superoxide production by rat peritoneal macrophages exposed to aflatoxins B_1, B_2, G_1, G_2, B_{2a}, and M_1 at several times and concentrations were analyzed to evaluate the intensity of a depressive effect for each mycotoxin. All aflatoxins used at very low concentrations had a depressive effect on the functions of macrophages. The biggest impairment of phagocytosis, intracellular killing, and spontaneous superoxide production was observed in macrophages exposed to aflatoxins B_1 and M_1.

The immunological consequences of exposure to mycotoxins are the suppression of humoral and cell-mediated immunity and an increase of susceptibility to disease. Evidence for suppression of immunity in humans by mycotoxins comes from epidemiological and postmortem studies (7). Direct evidence of suppression of immunity comes from experimental studies in animals (10–12). Not all animals are equally susceptible to mycotoxins. The effects of mycotoxins are related quantitatively to the amount, length of exposure, and natural sensitivity of the host to the mycotoxins. Experimental studies in susceptible animals showed, in relationship to the humoral immunity factors, depression of antibody formation and complement activity and decreasing interferon production and bactericidal activity (6, 14). Further investigation of the effects of mycotoxins, aflatoxins in particular, indicated that the greatest effect on immunity probably is related to cell-mediated processes; thymic and bursal involution in poultry, impaired phagocytosis, suppression of lymphablastogenesis, and suppression of delayed hypersensitivity and of the graft-versus-host have been reported in the literature (1–4, 9, 13).

The experiments described in this paper were carried out to analyze further the immunological consequences of exposure to mycotoxins. In our previous studies (data not shown), we found that the alfatoxins were detected in food with a higher incidence than other mycotoxins. However, we studied the effects of aflatoxins and some of their metabolites on several functions of rat peritoneal macrophages. Phagocytosis, intracellular killing of *Candida albicans*, and superoxide production by resident peritoneal macrophages exposed to aflatoxins at several times and concentrations were analyzed to evaluate the intensity of the depressive effect for each toxin and its relative metabolites.

MATERIALS AND METHODS

Animals. Norvegicus strain rats (12 weeks old) of either sex were used in all experiments.

Aflatoxins. Aflatoxins B_1, B_2, G_1, G_2, B_{2a}, and M_1 powder (Sigma Chemical Co.) were dissolved in methanol at a concentration of 1 mg/ml, divided into aliquots, and stored at −20°C. Further dilutions in RPMI 1640 medium were prepared from this solution. The same batch of each aflatoxin was used throughout the experiments.

Peritoneal cells. Resident peritoneal cells were obtained from rats by washing the abdominal cavity with RPMI 1640 medium; they were then repeatedly washed, counted, and suspended in RPMI 1640 at the appropriate concentration before being tested in different functional assays.

Peritoneal cells diluted to 10^6/ml were plated on 35-mm-diameter plastic petri dishes specifically treated for tissue culture (Nunc, Roskilde, Denmark) and allowed to adhere for 1 h at 37°C in a 5% CO_2 atmosphere. Dishes were carefully washed to eliminate nonadherent cells. Adherent cells were then incubated with several concentrations of each mycotoxin (10, 1, and 0.1 pg/ml) for 2 and 24 h at 37°C in 5% CO_2 atmosphere. The number of adherent cells in each plate was carefully determined after adhesion to dishes by microscopy, using an eyepiece equipped with a grid. Cytotoxicity tests, using trypan blue staining, were performed on peritoneal macrophages exposed to several concentrations of each aflatoxin; the highest cytotoxic dilutions were 1,000 pg/ml for B_1 and 2,000 pg/ml for the others. To exclude that the possible depressive effect was due to cytotoxicity toward the peritoneal cells, the highest concentration used was 10 pg/ml, which is 100-fold lower than the cytotoxic dose.

Phagocytosis and intracellular killing of *C. albicans*. A micromethod based on vital staining of phagocytized yeast with acridine orange (15) was used to assess phagocytosis and killing of *C. albicans* by peritoneal macrophages. Adherent cells after incubation for 2 and 24 h were washed with RPMI 1640 and incubated for 1 h at 37°C in a humidified 5% CO_2 atmosphere with *C. albicans* suspension containing 5×10^6 yeast cells per ml. The *C. albicans* suspension previously was opsonized for 30 min at 37°C with homologous serum to facilitate the phagocytosis. Cells were then stained with an acridine orange solution (1.44 mg/100 ml). Under UV light, viable *C. albicans* and cell nuclei stain green, whereas dead *C. albicans* cells stain reddish yellow. A differential count of cells containing yeasts were performed, and the intracellular killing activity was expressed as the percentage of positive cells containing dead yeast cells. At least 200 macrophages were scored on each slide, and all experiments were carried out in triplicate.

Superoxide production. Release of O_2 was determined spectrophotometrically by superoxide dismutase-inhibitable reduction of cytochrome *c* (type III; Sigma). This assay was performed as originally described by Johnston et al. (5, 8) with modifications. The mixture contained 80 μM cytochrome *c* and 130 U of superoxide dismutase (Sigma). Phorbol myristate acetate (Sigma) was added to the mixture as a stimulating agent at a concentration of 5 μg/ml. After incubation for 2 and 24 h, the peritoneal macrophages were washed with RPMI 1640 and then incubated with the mixture

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TABLE 1. Effect of different doses of aflatoxins on phagocytic activity of rat peritoneal macrophages

Pretreatment for:	Aflatoxin dose (pg/ml)	% Phagocytizing macrophages (mean ± SD; $n = 4$)[a]						
		Control	B_1	B_2	B_{2a}	G_1	G_2	M_1
2 h	0	67 ± 8.1						
	0.1		38.5 ± 4.0◆	51.2 ± 3.8◆	41.0 ± 2.6◆	61.5 ± 3.9*	63.8 ± 4.0*	50.0 ± 5.4◆
	1		34.5 ± 3.0◆	47.5 ± 3.7◆	36.5 ± 4.5◆	58.2 ± 4.2*	52.8 ± 5.4◆	41.8 ± 3.3◆
	10		20.5 ± 3.7◆	36.5 ± 2.9◆	30.8 ± 4.9◆	53.8 ± 1.7◆	50.8 ± 2.2◆	26.5 ± 4.5◆
24 h	1	80 ± 5.1						
	0.1		31.2 ± 4.1◆	42.2 ± 3.3◆	34.2 ± 7.0◆	51.2 ± 3.1◆	56.2 ± 4.1◆	33.2 ± 4.9◆
	1		25.8 ± 3.8◆	40.2 ± 3.3◆	31.2 ± 2.5◆	47.5 ± 2.1◆	49.5 ± 2.6◆	29.2 ± 3.0◆
	10		20.0 ± 2.2◆	31.5 ± 3.9◆	27.8 ± 4.9◆	47.0 ± 1.8◆	48.5 ± 2.6◆	25.0 ± 2.6◆

[a] $P < 0.05$ is considered significant. Symbols: ◆, $P < 0.05$; *, $P > 0.05$.

at 37°C in 5% CO_2 for 60 min. Incubation was carried out under gentle shaking. The reduction of cytochrome *c* was measured at 550 nm with a reference wavelength of 468 nm.

Statistical analysis. Results are expressed as means ± standard deviation. The significance was evaluated by analysis of variance. A value of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Effect of aflatoxin pretreatment on phagocytic activity of peritoneal macrophages. The phagocytic activity of rat peritoneal macrophages pretreated for 2 h was significantly lower than that in control macrophages at almost all tested doses of each aflatoxin (Table 1). Only incubation with 1 and 0.1 pg of aflatoxin G_1 and 0.1 pg of aflatoxin G_2 per ml did not significantly modify phagocytosis. If pretreatment with aflatoxin was protracted for 24 h, the phagocytic activity of peritoneal macrophages was significantly lower than in the control at all tested doses of each toxin; impairment of phagocytosis by macrophages pretreated for 24 h was higher than that by macrophages pretreated for 2 h, but the difference was not significant. At higher concentrations of each aflatoxin, the depressive effect after both pretreatments was higher.

Effect of aflatoxin pretreatment on microbicidal activity by peritoneal macrophages. The intracellular killing of *C. albicans* by rat peritoneal macrophages pretreated with aflatoxins for 2 h was significantly lower than in the control at all tested doses of each aflatoxin (Table 2). If pretreatment with aflatoxin was protracted for 24 h, the intracellular killing of *C. albicans* by peritoneal macrophages was significantly lower than in control macrophages at all tested doses of each toxin; impairment of intracellular killing by macrophages pretreated for 24 h was higher than that by macrophages pretreated for 2 h, but the difference was not significant. At higher concentrations of each aflatoxin, the depressive effect on killing was higher.

Effect of aflatoxin pretreatment on spontaneous and phorbol myristate acetate-induced O_2 production by peritoneal macrophages. The spontaneous O_2 produced by peritoneal macrophages pretreated with aflatoxins for 2 h was significantly lower than in control macrophages at almost all tested doses of each toxin (Table 3). Only incubation with 0.1 pg of aflatoxin G_1 per ml did not significantly modify O_2 production. At higher concentrations of each aflatoxin, the decrease in superoxide production was higher. But pretreatment for 2 h with different doses of aflatoxins did not significantly modify the phorbol myristate acetate-induced superoxide production by macrophages. Aflatoxin B_1 produced the biggest impairment of phagocytosis and intracellular killing. The lowest spontaneous superoxide production was observed after pretreatment with aflatoxin M_1. The smallest depressive effect on all tested macrophage functions was observed after pretreatment with aflatoxin G_1. Significant differences were not observed between superoxide anions spontaneously produced by peritoneal macrophages which were pretreated with each aflatoxin. Percent killing differences were significant only between B_1 and B_2, B_1 and G_1, B_1 and G_2 at a dose of 10 pg/ml with pretreatment for 2 h and between aflatoxins B_1 and B_2 (10 and 0.1 pg/ml), B_1 and G_1 (at all tested doses), B_1 and G_2 (10 and 0.1 pg/ml), B_2 and B_{2a} (at all tested doses), B_{2a} and G_1 (at all tested doses), and B_{2a} and G_2 (10 and 0.1 pg/ml) with pretreatment for 24 h. The phagocytosis assay showed significant differences only between B_1 and B_2, B_1 and G_1, B_1 and G_2, M_1 and G_1, and M_1

TABLE 2. Effect of different doses of aflatoxins on microbicidal activity of rat peritoneal macrophages

Pretreatment for:	Aflatoxin dose (pg/ml)	% Phagocytizing macrophages (mean ± SD; $n = 4$)[a]						
		Control	B_1	B_2	B_{2a}	G_1	G_2	M_1
2 h	0	28.5 ± 5.8						
	0.1		13.5 ± 4.2◆	18.2 ± 3.3◆	14.5 ± 2.9◆	20.2 ± 2.2◆	17.0 ± 3.4◆	16.2 ± 2.5◆
	1		10.2 ± 4.9◆	14.2 ± 4.1◆	10.2 ± 2.2◆	17.0 ± 4.2◆	15.2 ± 3.3◆	12.2 ± 4.1◆
	10		6.0 ± 2.2◆	12.2 ± 3.4◆	9.5 ± 2.1◆	14.8 ± 3.3◆	13.0 ± 3.2◆	9.2 ± 2.5◆
24 h	0	32.0 ± 7.5						
	0.1		11.0 ± 2.9◆	16.8 ± 4.1◆	10.5 ± 2.9◆	17.0 ± 3.4◆	15.5 ± 3.7◆	13.0 ± 2.9◆
	1		10.0 ± 1.8◆	14.5 ± 3.7◆	9.5 ± 1.3◆	15.0 ± 3.7◆	14.5 ± 2.9◆	11.8 ± 2.6◆
	10		5.5 ± 1.3◆	11.0 ± 2.6◆	7.5 ± 1.3◆	12.5 ± 2.9◆	11.2 ± 1.7◆	9.2 ± 3.3◆

[a] Percent containing dead *Candida* cells. $P < 0.05$ is considered significant. Symbols: ◆, $P < 0.05$; *, $P > 0.05$.

TABLE 3. Effect of different doses of aflatoxins on O_2 production by rat peritoneal macrophages pretreated for 2 h

Production	Aflatoxin dose (pg/ml)	nmol of O_2 per 10^6 cells (mean ± SD; $n = 4$)[a]						
		Control	B_1	B_2	B_{2a}	G_1	G_2	M_1
Spontaneous	0	11.8 ± 1.4						
	0.1		6.5 ± 2.6◆	6.3 ± 2.3◆	6.0 ± 2.8◆	7.0 ± 3.8*	6.7 ± 2.8◆	6.4 ± 2.4◆
	1		5.6 ± 3.8◆	5.7 ± 3.0◆	5.2 ± 3.1◆	6.0 ± 3.8◆	6.0 ± 3.0◆	5.5 ± 1.5◆
	10		2.9 ± 2.4◆	3.4 ± 2.5◆	3.2 ± 2.0◆	5.0 ± 3.2◆	4.8 ± 3.6◆	3.0 ± 1.1◆
PMA induced[b]	0	29.0 ± 9.1						
	0.1		29.0 ± 8.9	29.8 ± 7.0	29.7 ± 8.8	30.8 ± 9.5	29.7 ± 6.4	29.5 ± 2.5
	1	*	25.5 ± 8.0	28.0 ± 7.0	29.2 ± 8.7	29.1 ± 11.0	28.3 ± 5.4	29.3 ± 10.4
	10		24.7 ± 9.5	26.3 ± 8.1	26.0 ± 6.1	29.5 ± 9.1	28.8 ± 6.8	25.8 ± 11.7

[a] $P < 0.05$ is considered significant. Symbols: ◆, $P < 0.05$; *, $P > 0.05$.
[b] PMA, Phorbol myristate acetate.

and G_2 at all tested doses after macrophage pretreatment for 2 and 24 h.

The results obtained by using an experimental pattern of rat peritoneal macrophages pretreated in vitro with aflatoxins confirm experimental data obtained by using animals fed with fodder contaminated by aflatoxins. Our findings are important since the mononuclear phagocytis system represents a major defense mechanism against various pathogens.

Impairment of macrophages by aflatoxins could lead to a particular susceptibility of the host to infectious diseases. The mechanism(s) by which aflatoxins affect the macrophage biology remains to be determined. This paper gives further information on the danger to human health caused by aflatoxin contamination.

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