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Impairment of Phagocytosis by Heterophils from Chickens During Ochratoxicosis†

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The effect of graded concentrations of dietary ochratoxin A (0, 0.5, 1.0, 2.0, 4.0, and 8.0 μg/g of diet) on the in vitro phagocytic, locomotory, and bactericidal capacities of heterophils from broiler chickens was investigated. Both the percentage and the mean phagocytic activities were decreased significantly ($P < 0.05$) at 4.0 and 8.0 μg/g. Both directed and undirected locomotion of heterophils was impaired significantly at the same concentrations. A crossover experiment revealed that the reduced percentage of phagocytosis was associated with the heterophil itself and not with a serum factor such as complement. Heterophils from birds that consumed 4.0 μg/g were not impaired in ability to kill engulfed bacteria.

Of the myriad effects of mycotoxins, the ability to impair the immune system is of profound interest to the scientist and the animal husbandryman. For example, aflatoxin, probably the best known mycotoxin, impairs reticuloendothelial activity (13), primary immune response (23), complement system (21), phagocytic activity of leucocytes (4) and alveolar macrophages (19), and cell-mediated immunity as measured by the graft-versus-host reaction and delayed hypersensitivity skin reactions to tuberculin (9). The activity of other mycotoxins on the immune system has not been studied as thoroughly.

Ochratoxins, a group of toxic metabolites produced by *Aspergillus* and *Penicillium* species (2, 5, 10, 22) of which ochratoxin A (OA) is the most potent, have been considered primarily as nephrotoxins (11). Richard et al. (20), in a pioneering study, reported that OA given to guinea pigs at the rate of 0.45 mg/day for a 4-week period did not affect the complement activity or the antibody response to *Brucella abortus* antigen, although OA significantly lowered the level of β-globulin in serum of guinea pigs. On the other hand, secondary aerosacculitis was a fairly constant feature of field outbreaks of ochratoxicosis in poultry (Huff and Hamilton, unpublished data) which suggested that the immune system was weakened during the disease. The objective of our study was to investigate the effect of dietary OA on the phagocytic abilities of circulating leukocytes from chickens, the heterophil in particular, as an indication of the status of nonspecific host defense. The abilities pertinent to phagocytosis included humoral factor(s), engulfment, directed and undirected locomotion, and bactericidal activity.

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MATERIALS AND METHODS

Animal husbandry. Ochratoxicosis was induced by incorporating known amounts of pure OA (0, 0.5, 1.0, 2.0, 4.0, and 8.0 μg/g) into a commercial broiler starter ration free of any medication and feeding it to broiler chickens (Shaver × Shaver) from 1 day to 3 weeks old, at which time the experiment was terminated. The chicks were raised in electrically heated batteries under continuous illumination, with feed and water available ad libitum. There were four groups of 10 male birds at each of the treatments, and the experimental design was completely randomized. Analyses of variance were done on group means as outlined in Bruning and Kintz (3).

Ochratoxin production. OA was produced by inoculating *Aspergillus ochraceus* NRRL 3174 into wheat under static conditions for 10 days by Trenk et al. (24). OA was extracted from the wheat (25) and partially purified on preparative thick-layer chromatography plates of Silica Gel G (8). OA was removed from the silica gel by repeated washings with benzene-acetic acid (9:1). The benzene-acetic acid extracts were evaporated, and the residue was dissolved in benzene, from which OA was crystallized. Crystalline OA was dissolved in 0.1 M $NaHCO_3$ and mixed with a small amount of the feed which was dried and mixed with the remaining portion of the feed.

Measurement of phagocytosis. Phagocytosis was measured by the direct counting procedure (14) with the phagocytic particle *Enterobacter cloacae* grown on brain heart infusion agar (BBL Microbiological Systems) and quantitated by pour plate count before killing with Formalin. Heparinized (10 U/ml) whole blood from individual birds was pooled on a group basis before calculating the total phagocytes from the total (16) and differential (12) leukocyte counts. Bacteria in 0.1 ml of 0.85% NaCl were added to 0.9 ml of blood to give an initial 10:1 ratio of bacteria to total

phagocytes, and the mixture was incubated at 41°C (body temperature of chickens) in a water bath shaker (60 cycles/min) with a 7.5-cm traverse. Blood smears made at timed intervals from the incubation mixture were stained with Wright stain. Then 100 heterophils per slide were counted to determine the percentage of heterophils phagocytizing. The mean phagocytosis was determined by counting the number of bacteria engulfed per heterophil exhibiting phagocytosis (25 phagocytizing heterophils were counted per slide).

Locomotory properties. Heterophils for assay of directed (chemotactic) locomotion were obtained by the density gradient centrifugation method of Noble and Cutts (17). The purified heterophils were suspended in pooled, fresh, normal chicken serum at a concentration of 5×10^6/ml. The chemotactic stimulus was prepared (26) by growing *E. cloacae* on brain heart infusion agar (BBL Microbiology Systems) for 24 h at 37°C and washing from the surface with pooled, fresh, normal chicken serum. The suspension was centrifuged to sediment the bacteria. The supernatant layer was removed and used as the stimulus after diluting it with additional chicken serum to produce a migration of approximately 100 heterophils per microscope field as described below.

Directed locomotion by heterophils was measured with Boyden chambers by the method of Clark and Kimball (6). The chemotactic stimulus (0.2 ml) was placed in the lower compartment, and the same volume of heterophil suspension was placed in the upper compartment. The compartments were separated by a polycarbonate membrane filter of 3-μm pore size (Bio-Rad Laboratories, Richmond, Calif.). The chambers were then sealed with a plastic film to prevent evaporation and incubated at 41°C for 90 min. Next, the filters were removed, placed bottom side up on a microscope slide, fixed with methanol, and stained with Wright stain before examination at 400× magnification. Heterophils which had migrated through the membrane were counted in 10 random fields. Directed locomotory activity was expressed as the mean number of cells per field.

Assays for undirected (passive or spontaneous) locomotion were the same as those described for directed locomotion except that normal chicken serum was substituted for the chemotactic stimulus in the lower compartment of the Boyden chamber.

Heterophil viability. The viability of heterophils was equated with their ability to exclude trypan blue (7). One hundred percent of the heterophils in heparinized whole blood were viable after 1 h of incubation at 41°C. About 90% of the heterophils separated by density gradient centrifugation were viable after 1 h of incubation at 41°C. The presence of ochratoxin in the diet had no effect on the viability of heterophils from birds consuming the diet.

Bactericidal activity. The method for bactericidal assays was adapted from Quie et al. (18). The indicator organism was *E. cloacae* grown as described above, washed twice in Seligmann salt solution (17), and suspended at a concentration of 5×10^7/ml of solution. Heterophils were obtained as described above at 5.5 $\times 10^6$/ml of chicken serum. Phagocytic mixtures of 0.9 ml of heterophils and 0.1 ml of bacteria provided a 1:1 ratio of bacteria to heterophils. The mixture in siliconized tubes (13 by 100 mm) was shaken at 60 cycles/min at 41°C. At timed intervals total viable counts were done in pour plates of MacConkey agar (Difco) after lysing the heterophils (0.0025 ml) in sterile distilled water (1.0 ml) to free the engulfed bacteria.

RESULTS

The effect of graded levels of dietary OA on the percentage of heterophils phagocytizing *E. cloacae* after different times of incubation is shown in Fig. 1. Analysis of variance revealed that OA at 8.0 μg/g decreased significantly ($P < 0.05$) the percentage of phagocytic activity of heterophils at 10 min of incubation which was the shortest time interval measured. A significant effect with 4.0 μg/g also was observed, but an incubation period of 30 min or longer was required. The lower concentrations of OA did not depress phagocytosis significantly from control values. The percentage of phagocytosis at all dose levels reached a plateau by 30 min of incubation.

Another measurement of phagocytosis is the mean number of particles engulfed per phagocyte. The mean phagocytosis by heterophils was also reduced significantly by 4.0 and 8.0 μg/g (Table 1) to about 45% of the control value.

A crossover experiment was done to determine whether a cellular or humoral factor(s) was impaired. Heterophils from birds fed 4.0 μg of OA per g were suspended in normal serum, heterophils from control birds were suspended in serum from birds fed OA (4.0 μg/g), and their phagocytic activities were compared with cells in their homologous serum. The results of this 2 × 2 factorial experiment are shown in Table 2. Analysis of variance revealed one main effect, indicating a cellular factor was impaired during ochratoxicosis but not a humoral factor.

The effect of dietary OA on both directed and

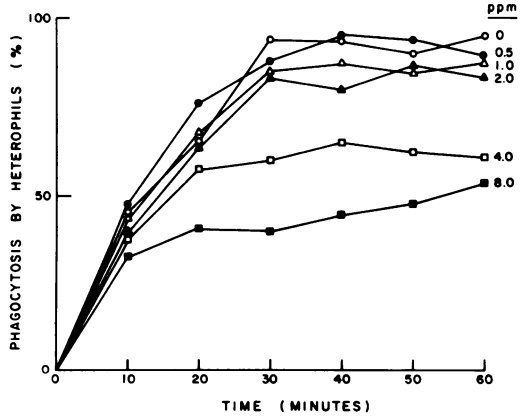

FIG. 1. *Percentage of phagocytosis by heterophils from broiler chickens fed graded concentrations of dietary OA.*

TABLE 1. *Mean phagocytic activity of heterophils during ochratoxicosis*

Ochratoxin (μg/g)	Phagocytic activity (bacteria/heterophil)
0	9.1 ± 1.0[a]
0.5	9.8 ± 0.7[a]
1.0	8.3 ± 0.8[a]
2.0	7.6 ± 0.9[a]
4.0	4.1 ± 0.3[b]
8.0	3.5 ± 0.4[b]

[a,b] Values with different superscripts differ significantly ($P < 0.05$). Values are the number of *E. cloacae* ± standard error engulfed per heterophil in 30 min at 41°C with reciprocating agitation (60 cycles/min) from whole chicken blood.

TABLE 2. *Effect of dietary OA on cellular and humoral phagocytic factors of heterophils from chickens*

Cells	Serum	Activity of heterophils (% phagocytizing)
Normal	Normal	90[a]
Normal	Ochratoxin	87[a]
Ochratoxin	Normal	63[b]
Ochratoxin	Ochratoxin	64[b]

[a,b] Values with different superscripts differ significantly ($P < 0.05$). Analysis of variance showed only cellular effect. Birds were fed 0 or 4.0 μg/g. Tabular values are means of four groups of 10 birds and were obtained by counting 100 heterophils per bird.

undirected locomotion of heterophils is given in Table 3. Both types of locomotion were inhibited significantly by 4.0 and 8.0 μg/g, and both types were inhibited by about one-half.

Bactericidal activity of heterophils toward engulfed bacteria was measured, but showed no significant difference between control birds and birds fed 4.0 μg of toxin per g.

DISCUSSION

It is clear from the data that heterophils from birds with ochratoxicosis had altered phagocytic capacities with the percentage and mean phagocytosis decreased (Fig. 1, Table 1). Reasons for this impaired phagocytic activity cannot be assigned at this time; however, the impairment is at the cellular and not at the humoral level (Table 2). The data in Fig. 1 on inhibition of the cellular factor and particularly the extended plateaus suggest basically an all-or-none phenomenon with two main populations, one capable of phagocytosis and the other incapable. The reduction by OA of mean phagocytosis in heterophils capable of phagocytosis (Table 1) suggests, on the other hand, a more complex situation. The all-or-none hypothesis could be retained if it is assumed that OA is removing from action finite, independent sites or processes for the engulfment of individual bacteria. Such an assumption agrees with the slight but statistically insignificant change in rate observed (Fig. 1).

From present data it is tempting to speculate that the cellular phagocytic factor(s) impaired during ochratoxicosis are related simply to movement or motility of heterophils because both directed and undirected movement had about the same minimal inhibitory dose and extent of inhibition as the percent and mean phagocytosis. Both modes of locomotion are important to the innate immunity of animals since contact with particles and consequent phagocytosis would not occur without locomotion except as the result of chance encounters (1). The reduction in locomotory action during ochratoxicosis could be explained by the all-or-none hypothesis derived above; however, this explanation also founders on the reduction observed in mean phagocytosis. An alternative hypothesis to explain the effect on mean phagocytosis would be inhibition of rate of locomotion; however, this hypothesis would seem to demand significant rate effects on percentage of phagocytosis which were not seen. These contradictions appear to be best resolved at the present time by assuming that the effect on locomotion is separate from the impaired cellular factor revealed in the crossover experiment (Table 2). Despite this uncertainty about the exact role of impaired locomotory abilities during ochratoxicosis, it appears safe to say that ochratoxin can induce a "lazy leukocyte" syndrome such as occurs in certain diseases of immune deficiency (15).

Although the mechanism for the impairment of phagocytosis by heterophils during ochratoxicosis is not understood, it is simple compared with that of aflatoxicosis which affects a humoral factor (complement) and the bactericidal process as well as the locomotory processes and the rate, mean, and percentage of phagocytosis (4). It should be noted that the present findings do not disagree with those of Richard et al. (20) on

TABLE 3. *Impairment of directed and undirected locomotion by heterophils during ochratoxicosis*

Ochratoxin (μg/g)	(Cells migrating/high-power field)	
	Directed	Undirected
0	98.5 ± 6.1[a]	30.3 ± 3.6[a]
0.5	99.2 ± 4.2[a]	32.5 ± 5.1[a]
1.0	90.0 ± 4.5[a]	28.3 ± 3.2[a]
2.0	84.0 ± 5.3[a]	24.8 ± 3.4[a]
4.0	51.3 ± 13.9[b]	14.3 ± 2.6[b]
8.0	47.0 ± 7.3[b]	13.0 ± 2.1[b]

[a,b] Values in a column with a different superscript differ significantly ($P < 0.05$). All values are mean ± standard error of four groups of 10 birds.

OA in guinea pigs. Additional work on the impairment of the immune system during aflatoxicosis and ochratoxicosis is needed to predict their consequences, although it appears reasonable on the basis of present data to expect secondary infections during outbreaks of these two nutritional toxicoses.

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