Effect of Aflatoxin on Phagocytosis of Aspergillus fumigatus Spores by Rabbit Alveolar Macrophages

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Rabbits were given daily doses of aflatoxin B₁ equivalents of 0.03, 0.05, 0.07, and 0.09 mg for a 2-week period. Macrophages were harvested at the end of the experimental period, and in vitro phagocytosis experiments were conducted using Aspergillus fumigatus spores as ingestion particles. Alveolar macrophages from rabbits given the above doses of aflatoxin had reduced phagocytic activity when compared with macrophages from control rabbits. Incorporation of serum from the aflatoxin-treated rabbits in the in vitro culture system resulted in less stimulation of phagocytosis by macrophages from control rabbits when compared with the same system incorporating control serum. Stimulation of phagocytosis by macrophages was least when both serum and macrophages from aflatoxin-treated rabbits were used in the in vitro system.

Aflatoxins induce a variety of effects on animal systems, including teratogenicity, mutagenicity, and carcinogenicity (8). The carcinogenic activity of aflatoxin was described in guinea pigs as early as 1963 (4). Some carcinogens inhibit certain aspects of immune processes (2, 23), including cellular immunity (9). Aflatoxin, a hepatotoxic carcinogen, affects immunity (14). These effects have included an inhibition of humoral antibody in mice and chickens (7, 23) but no significant change in antibody formation in turkey pouls vaccinated against fowl cholera (15). In agreement with the latter study, Adinarayanaiah et al. (1) found that aflatoxin did not significantly affect antibody production to Salmonella pullorum in chickens. In other studies aflatoxin did not inhibit antibody production with aspergillosis in turkey pouls (20) or with Brucella abortus antigen given to guinea pigs (25). Aflatoxin impaired the resistance to Pasteurella multocida infection in turkeys vaccinated against fowl cholera (15). The impairment was apparently not associated with agglutinating antibody because the resistance could be restored by giving injections of either normal or immune serum to vaccinated turkeys before challenge inoculation (16). Results from this last study seem to indicate that aflatoxin depressed some humoral substance other than antibody. As a result of this and previous studies in which hepatotoxins inhibited complement activity (17), another study was done which showed that aflatoxin acted much like other hepatotoxins in depressing complement activity in guinea pigs (26). Because complement is active in the promotion of phagocytosis by opsonization of particles to be ingested (11), a study of the effect of aflatoxin on the ingestion of particles (Aspergillus fumigatus spores) by rabbit alveolar macrophages was designed and is reported here.

MATERIALS AND METHODS

Rabbits. Rabbits were used in this experiment because they are quite susceptible to aflatoxin having a mean lethal dose of approximately 0.3 mg/kg with no differences in susceptibility found between sexes (13). Also, alveolar macrophages are easily obtained in large quantities from this species. Fifteen New Zealand white, female rabbits each weighing 2 kg were used in this study. They were given feed and water ad libitum throughout the experimental period. The rabbits were weighed at the beginning and at the end of each week of the 2-week experimental period.

Aflatoxin. Partly purified aflatoxin prepared with A. parasiticus NRRL 2999 was taken from the same lot used in previous studies (18, 21, 26). The partly purified aflatoxin was 34.8% B₁, 1.9% B₂, 23.8% G₁, and 1.1% G₂. For this study, aflatoxin G₁ was considered one-half as toxic as B₁, and aflatoxins B₂ and G₂ were negated in computing toxic equivalents of aflatoxin B₁, according to the relative toxicities of aflatoxins reported by Carnaghan et al. (5).

Administration of toxin to rabbits. Different concentrations of partly purified aflatoxin were prepared by dissolving sufficient quantities in chloroform to yield 0.03, 0.05, 0.07, and 0.09 B₁ equivalents/0.1 ml. Each concentration was used in preparing doses of aflatoxin by placing 0.1 ml of the solution in each of a
sufficient quantity of no. 5 gelatin capsules (Eli Lilly and Company, Chicago, Ill.) to provide enough daily doses for three rabbits for 2 weeks. After the chloroform was allowed to evaporate for 16 h, the capsules were filled with lactose to enhance taste and were stored in the dark at 4 C. The controls were given capsules filled with lactose only. Daily doses were administered to rabbits with a balling gun. Care was taken to insure that the rabbits had swallowed the capsules before they were returned to their cages.

**Spore suspension.** Spores of *A. fumigatus* were obtained as described by Richard et al. (19). The spores were suspended in medium 199 at a concentration which when the suspension was added to serum at a ratio of 2:1 for cell culture, the final concentration of spores was approximately $1.5 \times 10^5$ per ml. Spores were counted in a hemocytometer.

**Experimental design.** A 10-ml blood sample was taken from each rabbit 24 h before the end of the 2-week experimental period. Serum was collected from each sample and frozen overnight at -70 C.

The rabbits were killed by injecting 2 ml of succinyl choline into the marginal ear vein. The lungs were removed, and macrophages were washed from the lungs with Hanks balanced salt solution (BSS) by the method of Myrvik et al. (12). The cell button obtained after centrifugation of 30 ml of the lung washings from a rabbit was resuspended in 40 ml of BSS, and 0.2 ml of the suspension was placed in each of the eight cells of a tissue culture chamber/slide (Lab-Tek tissue culture chamber/slides, Lab-Tek products, Division of Miles Labs., Inc., Naperville, Ill.). After the macrophages were allowed to attach to the glass for 20 min at 37 C, the culture chambers were rinsed with medium 199, and culture systems were prepared as shown in Table 1. The cultures were incubated for 1 h at 37 C. Macrophages from control rabbits and from rabbits receiving 0.09 mg of B$_1$ equivalents of aflatoxin/day were tested for Trypan blue exclusion with 0.125% aqueous Trypan blue. Approximately 96% of the macrophages were viable after 1 h of incubation at 37 C. After 1 h of incubation, the cultures were rinsed with absolute methanol and fixed with absolute methanol for 1 h. The plastic culture chambers were removed from the glass slides, and the cells on the slides were stained with Wright-Giemsa stain. The number of macrophages containing spores was obtained by randomly counting 100 macrophages in each of the eight areas on the slide with a light microscope. Thus, 2,400 macrophages representing three rabbits were counted for each dose level.

**RESULTS**

Preliminary studies with 3-kg female rabbits indicated that alveolar macrophages required serum in the medium for maximum numbers of macrophages to ingest spores of *A. fumigatus* and that differences were not found when fresh or frozen rabbit serum was used (Table 2). However, the effect of the serum diminished when it was inactivated by heating at 56 C for 30 min (Table 2).

| Attached macrophages from: | Medium 199 containing spores* and 33% serum from: |
|-----------------------------|--------------------------------------------------|
| Group I. Normal rabbits (pooled from controls)* | Normal rabbits (pooled from controls)* |
| 0.03                        | 0.05                                             |
| 0.07                        | 0.09                                             |
| Group II. Normal rabbits (pooled from controls) | Treated rabbits$: ^{c}$ |
| 0.03                        | 0.05                                             |
| 0.07                        | 0.09                                             |
| Group III. Treated rabbits$^{c}$ | Normal rabbits (pooled from controls) |
| 0.03                        | 0.05                                             |
| 0.07                        | 0.09                                             |
| Group IV. Treated rabbits$: ^{c}$ | Treated rabbits$: ^{c}$ |
| 0.03                        | 0.05                                             |
| 0.07                        | 0.09                                             |

* Final spore concentration = $1.5 \times 10^5$/ml.

* Sufficient controls were included to insure that there were no differences between control rabbits and sera.

* An eight-chamber tissue culture slide was used for each of the three rabbits at each dose level. All dose values are expressed as milligrams of B$_1$ per day.

* Eight hundred macrophages were observed for spores for each rabbit.

* Only the serum from the same rabbit was used in each test; e.g., macrophages from rabbit A at dose 0.03 mg of B$_1$ per day plus serum from the same rabbit.

**Table 2. Ingestion of *A. fumigatus* spores by rabbit alveolar macrophages: effect of frozen and heated serum**

| Medium          | % Macrophages ingesting spores (avg)* |
|-----------------|--------------------------------------|
| M-199 only      | 32                                   |
| M-199 + fresh serum | 73                                |
| M-199 + frozen serum | 77                                |
| M-199 + heated serum* | 46                                |

* Eight replicates.

* Heated at 56 C for 30 min.

any of the doses of aflatoxin used in this study (group III, Table 1) had reduced phagocytic activity when compared with that of macrophages from the control rabbits (group I, Table
1), and the degree of reduction did not appear to be dose related (Fig. 1). Incorporation of serum from the aflatoxin-treated rabbits in the in vitro culture system of normal macrophages (group II, Table 1) resulted in less stimulation of phagocytosis by the macrophages and was dose related (Fig. 1). Phagocytosis was stimulated least when both serum and macrophages from the aflatoxin-treated rabbits (group IV, Table 1) were used in the in vitro culture system (Fig. 1). In the latter system, the reduced stimulation of phagocytosis was dose related, and only 15% of the macrophages ingested spores in 1 h when serum and macrophages from the rabbits given the highest dose level (0.09 mg of B<sub>1</sub> equivalents per day) of aflatoxin were used.

All the rabbits at all dose levels of aflatoxin had lower weight gains during the 2-week period than did the controls (Fig. 2). The rabbits at 0.05 mg of B<sub>1</sub> equivalents per day did not gain weight during the experimental period, and those at higher dose levels lost weight (Fig. 2). In preliminary trials, rabbits given 0.09 mg of B<sub>1</sub> equivalent per day for a 3-week period either died during the 3rd week or were debilitated at the end of the 3rd week; thus, all subsequent experiments were of 2 weeks duration. The rabbits at the two higher dose levels had a substantial increase in the icterus index of their serum at the end of the experimental period (Fig. 2). The rabbits at the highest dose level (0.09 mg/day) had pale livers. Upon histopathological examination of hematoxylin- and eosin-stained liver sections of the rabbits, those on the two highest dose levels (0.07 and 0.09 mg/day) had changes typical of those described for aflatoxicosis (13).

There were no visible lesions in the lungs of any of the rabbits, and no differences were noted in the ease of obtaining macrophages from any of these rabbits.

**DISCUSSION**

Although aflatoxin apparently affected phagocytosis by alveolar macrophages of rabbits, the effect was greatest in in vitro culture systems in which serum from the aflatoxin-treated rabbits was used. Aflatoxin impairs the function of the reticuloendothelial system in clearing colloidal carbon from the circulation in chickens (10). The diminution of phagocytosis in the experiments reported here could be due to a decrease in complement activity or some other opsonizing factor present in the sera. Others have suggested that complement is involved in phagocytosis, although the mechanisms in this phenomenon are unknown (3, 11). Previously, we have shown that aflatoxin reduces the complement activity in guinea pig sera (26). Attempts were made to determine complement titers on the rabbit sera used in the experiments reported here, and they indicated a lowering of complement activity. However, several problems led us to exclude the results of these titrations from this report.

In the experiment in which heated serum was compared with frozen or chilled serum and with no serum in the phagocytosis of spores by
normal alveolar macrophages, the number of macrophages ingesting spores was reduced 43% when no serum was present. However, the reduction was only 29% when heated serum was used. From this result one would expect that a heat-stable substance in serum assists in phagocytosis as well as the heat-labile substance, presumably complement. Macrophages ingesting spores were reduced approximately 38% when serum from the high dose (0.09 mg of B1/day) rabbits was used in culture systems of normal macrophages. Perhaps this level of aflatoxin consumption interfered with the formation or activity of the heat-stable substance present in normal sera in addition to the heat-labile substance, or perhaps some inhibiting factor is present in serum of rabbits receiving this level of aflatoxin. Aflatoxin affects serum factors in guinea pigs other than the major complement part (25).

Carcinogenic substances other than aflatoxin inhibit cellular immunity (9); and aflatoxin B1, when added to phytohemagglutinin-stimulated cultures of human lymphocytes, reduced the uptake of tritiated thymidine when compared with the reduction by control cultures (22). Because macrophages apparently play a significant role in cellular immunity (6) and we have shown an effect of aflatoxin on ingestion of particles by alveolar macrophages, aflatoxin may cause defects of other tissue macrophages, such as in the handling of antigens on their surfaces, and thus interfere with mechanisms of cell-mediated immunity.

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