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Aflatoxin Inhibition of Viral Interferon Induction

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The inhibitory effect of four basic aflatoxins on interferon induction by influenza virus in LLC-MK_2 cell monolayers follows a structure-activity series with decreasing potency in the order aflatoxin $B_1 > G_1 > B_2 \approx G_2$. Of the four aflatoxins, B_1 was the most deleterious to both cell growth and the viability of cells in confluent cultures. The fact that higher levels of influenza virus growth were attained in aflatoxin-treated cells than in normal cell monolayers was related to increased aflatoxin concentration in association with decreased interferon production. The ability of interferon to confer cellular resistance against viral infection, however, was not altered by aflatoxin. The inhibitory activity of aflatoxin on interferon production may be a factor contributing to impairment of host resistance to viral infections.

Information available on mycotoxin-induced changes of immunity and impairment of resistance to disease has been obtained largely from studies with aflatoxins, a group of closely related, secondary toxic metabolites produced by certain strains of *Aspergillus*. Aflatoxins have been reported to decrease significantly serum complement activity (21, 36), decrease or impair phagocytosis (26), increase susceptibility to and mortality from certain infectious agents (8, 17), interfere with acquired resistance (23), or inhibit cellular immune processes (24, 29). However, considerable variation of these effects on immunity have been encountered which, in part, may be the consequence of various animal species metabolizing diverse mycotoxins at different rates (22). The major effects of mycotoxins on immunological mechanisms, however, appear to be related to nonspecific humoral factors and to cellular aspects of immunity (27).

The interferon system is an important component of the host's defense mechanism. The belief prevails that the biological significance of interferon must be greater than that of a mere antiviral substance (28). Although first described as an antiviral protein, interferon is now considered to be a group of glycoproteins of diverse cellular origins which may also inhibit proliferation of normal and malignant cells and regulate or modulate various aspects of the immune response (9). Steroids, hormones, carcinogenic hydrocarbons, arsenicals, metals, mineral dust, asbestos fibers, and bacteriocins have been reported to affect interferon synthesis adversely (5, 7, 10-13, 16, 34, 37). To our knowledge, the in vitro activity of mycotoxins on the interferon system has not been investigated. This report describes studies related to the effect of aflatoxins on viral interferon induction and virus multiplication.

MATERIALS AND METHODS

Viruses. The A_o/PR8/8/34 influenza and parainfluenza 1 (Sendai) virus strains employed in this study were obtained from the American Type Culture Collection, Rockville, Md. Stock virus pools of each strain were prepared from embryonated chicken eggs in the manner described previously (14). Influenza and Sendai virus pools contained 10^7 and 10^9 cell-infecting units of virus per ml, respectively, when assayed by the immunofluorescent cell-counting procedure (14).

Cell cultures. Rhesus monkey kidney (LLC-MK_2) and human Chang conjunctival (clone 1-5c-4) cell lines obtained from the American Type Culture Collection were used for induction and assay of interferon, respectively. Cell lines were propagated in plastic tissue culture flasks (25 cm^2) with Eagle minimum essential medium containing 10% fetal bovine serum and maintained with minimal essential medium plus 0.5% fetal bovine serum.

Aflatoxin. Crystalline aflatoxins AFB_1, $-B_2$, $-G_1$, and $-G_2$ were obtained commercially (Aldrich Chemical Co., Milwaukee, Wis.).

Interferon induction. Duplicate experiments were performed, and the procedure generally employed to study the effects of aflatoxin on interferon induction was carried out as follows: 1 to 100 μg of aflatoxin suspension in a 5-ml volume of maintenance medium was added to 25-cm^2 plastic flasks containing complete LLC-MK_2 cell monolayers which were then incubated at 35°C for 24 h. Residual medium was decanted and 1 ml of influenza virus, which had been inactivated by ultraviolet irradiation for 45 s at a distance of 76.2 mm and wavelength of 253.7 nm, was added onto cell monolayers that were then incubated at 35°C for 2 h. The multiplicity of infection was approximately 1.0. Inoculum was removed and 5 ml of maintenance me-

dium was added to each flask. After incubation, at 35°C from 22 to 24 h, supernatant fluid was decanted and centrifuged at 100,000 × *g* for 1 h and dialyzed against HCl-KCl buffer, pH 2.0, at 4°C for 24 h. Dialysis was continued against two changes of phosphate-buffered saline, pH 7.1, at 4°C for 24 h. Fluids were passed through Millex filters (0.45 μm) (Millipore Corp., Bedford, Mass.) to obtain sterile preparations. Samples were stored at −70°C until they were assayed for interferon activity. Preparations with antiviral activity possessed the biological and physical properties ascribed to viral interferons (20). Controls consisting of cell monolayers which were not treated with aflatoxin were handled exactly as described above.

Interferon assay. Samples of interferon were assayed in duplicate. An immunofluorescent cell-counting assay of interferon that has been described previously (15) was employed. Interferon-treated cell monolayers were challenged with 10^4 cell-infecting units of Sendai virus, and infected cells were visualized by the direct fluorescent antibody staining. The reciprocal of the interferon dilution that reduced the number of infected cells to 50% of the control served as the measure of interferon activity, i.e., 50% infected cell-depressing dilution ($ICDD_{50}$). With this assay system, 0.8 international reference human (69/19) interferon unit assayed as 1 U.

RESULTS

The survival of "nondividing" LLC-MK_2 cells in confluent monolayers incubated for 24 h with different amounts of either AFB_1, $-B_2$, $-G_1$, or $-G_2$ was a requisite to investigating the effect of aflatoxin on interferon induction and viral replication. AFB_2, $-B_1$, and $-G_2$ in 500-μg concentrations did not appear to affect cell viability as determined by trypan blue dye exclusion procedure (Table 1). The same quantity of AFB_1 diminished the number of viable cells in confluent monolayers by approximately 50%. At a concentration of 100 μg of AFB_1, cell viability was minimally affected.

The growth of cells as determined by the number of viable cells arising from cell suspensions incubated with varied concentrations of aflatoxins was found to be markedly affected by both AFB_1 and AFG_1. In comparison to the number of cells in control cell cultures, 500 μg of either AFB_1 or AFG_1 limited cell replication to 6 and 32%, respectively. The same quantity of either AFB_2 or AFG_2 did not affect cell growth. At a concentration of 10 μg of AFB_1, cell replication was still restricted to less than 50%. Of the four aflatoxins, AFB_1 was the most deleterious to cell growth as well as to the viability of cells in confluent monolayers.

Different quantities of the four aflatoxins, ranging from 1 to 500 μg, were tested to determine their effect on interferon induction by influenza virus in confluent LLC-MK_2 cell monolayers. Results (Table 2) show that AFB_1 was

TABLE 1. *Effect of different quantities of aflatoxins on viability of LLC-MK_2 cells*

Aflatoxin concn (μg)	Surviving fraction of cells in presence of aflatoxin:[a]			
	B_1	B_2	G_1	G_2
500	0.552 ± 0.004	1.000 ± 0.000	0.980 ± 0.007	1.000 ± 0.000
250	0.630 ± 0.007	0.981 ± 0.004	0.972 ± 0.018	1.000 ± 0.000
100	0.921 ± 0.084	1.000 ± 0.000	0.979 ± 0.020	0.965 ± 0.003
50	0.950 ± 0.009	0.974 ± 0.014	0.967 ± 0.017	0.970 ± 0.017
10	0.988 ± 0.015	0.992 ± 0.004	1.000 ± 0.000	1.000 ± 0.000
0 (control)	1.000	1.000	1.000	1.000

[a] Results are expressed as mean surviving fraction ± standard error. Surviving fraction of cells is computed by dividing number of living cells (trypan blue dye exclusion) in aflatoxin-treated cell monolayers by number of living cells in controls (5×10^6).

TABLE 2. *Effect of aflatoxin concentration on inhibition of viral interferon induction by PR8 influenza virus*

Aflatoxin concn (μg)	Interferon inhibited by aflatoxins[a]			
	B_1	B_2	G_1	G_2
500	ND[b]	74.7 ± 1.5	82.9 ± 1.3	78.5 ± 2.6
100	95.6 ± 0.9	39.7 ± 4.7	62.0 ± 1.5	29.5 ± 3.8
10	65.1 ± 1.6	33.4 ± 3.2	50.0 ± 2.2	21.0 ± 2.9
1	36.6 ± 6.3	23.9 ± 4.7	27.0 ± 4.7	17.9 ± 3.1
0 (control)	0	0	0	0

[a] Results are expressed as mean percent interferon inhibition ± standard error. Percent interferon inhibition was computed by dividing interferon titers from aflatoxin-treated LLC-MK_2 cell cultures by control interferon titer of 315 ($ICDD_{50}$) and subtracting from 100%. By using analysis of variance (arc sine transformation) we noted the following significant differences: $B_1 > B_2$ and G_2, $G_1 > G_2$ ($P < 0.01$); $B_1 > G_1$, $G_1 > B_2$ ($P < 0.05$); $B_2 > G_2$ ($P < 0.05$) levels.

[b] ND, Not determined because of cytocidal activity.

more effective in depressing interferon yields than the other aflatoxins tested. The highest percentage (95.6) of interferon inhibition was noted with 100 μg of AFB_1. This magnitude of interferon inhibition was not achieved with as much as 500 μg of either of the other aflatoxins. Although not as effective as 1 μg of AFB_1, 1 μg of the other aflatoxins was also capable of depressing interferon yields. AFG_1, however, was more active than either AFB_2 or AFG_2 in diminishing interferon production. Statistical analysis of the inhibitory activity of the four aflatoxins on interferon production showed ranking with decreasing potency in the order aflatoxin $B_1 > G_1 > B_2 \approx G_2$.

The inhibition of interferon production was dependent on the sequence of aflatoxin and viral inducer administration onto cell monolayers. When cells were treated with either 50 μg of AFB_1 or AFG_1 for at least 20 h before the addition of viral inducer, interferon was depressed 66 and 38%, respectively. When virus and aflatoxin were administered together, interferon yields were still reduced by AFB_1 and AFG_1 by as much as 40 and 27%, respectively. Aflatoxins added 2 h after the viral inducer had a negligible influence on interferon production, suggesting that once the sequence of viral interferon induction is initiated, aflatoxin does not impair subsequent events.

A series of experiments was performed to obviate the possibility that diminished interferon production might be the consequence of aflatoxin activity on virus infectivity, interferon degradation, or the induction of a subtle interferon antagonist. Aflatoxin did not affect integrity or the ability of influenza virus to infect cells. When virus was assayed in the presence of either AFB_1, $-B_2$, $-G_1$, or $-G_2$, at concentrations that ranged from 100 to 0.1 μg of aflatoxin, all virus assay values showed less than a twofold difference as compared with that of controls. The findings also imply that neither the adsorption process of virus to cells, including virus attachment and penetration, nor the ability of virus to multiply in cells were impeded by aflatoxins.

Interferon potency was not adversely affected by aflatoxin. When interferon of known titer was mixed with AFB_1, ranging in quantities from 10 to 100 μg, or with control medium, assays of the preparations revealed no decline in interferon potency.

That the interaction of aflatoxin with cell monolayers might result in the formation of an interferon antagonist was investigated. This could account for the low levels of interferon noted. To test this, equal volumes of maintenance medium or undiluted low-titer interferon preparations obtained from aflatoxin-treated cell cultures were added to interferon preparations of known potency. The mixtures were then assayed for interferon activity. The potency of the known interferon preparation was not diminished under these conditions, indicating the absence of an interferon antagonist.

Experiments designed to study the replication of influenza virus concomitant with interferon production in both untreated and AFB_1 (50 μg)-treated cells revealed similar rates of virus growth (Fig. 1). However, virus multiplied to a level that was approximately fourfold higher in AFB_1-treated cells than in untreated control cells. Minimal amounts of interferon were detected in AFB_1-treated cells, whereas as high as 88 $ICDD_{50}$ units of interferon per ml was obtained from untreated cells. In subsequent experiments, the levels of virus multiplication in cells treated with 10 μg or 1 μg of AFB_1 were only twofold higher or less, respectively, than that in control cell cultures. Interferon yields increased correspondingly. The maximal level of virus growth achieved in cell cultures treated with 50 μg of AFG_1 was only twofold higher than in untreated cultures. These data suggest that the higher level of virus multiplication attained in aflatoxin-treated cell cultures was dependent on the concentration of aflatoxin and its capacity to depress interferon production.

To determine whether aflatoxin could alter

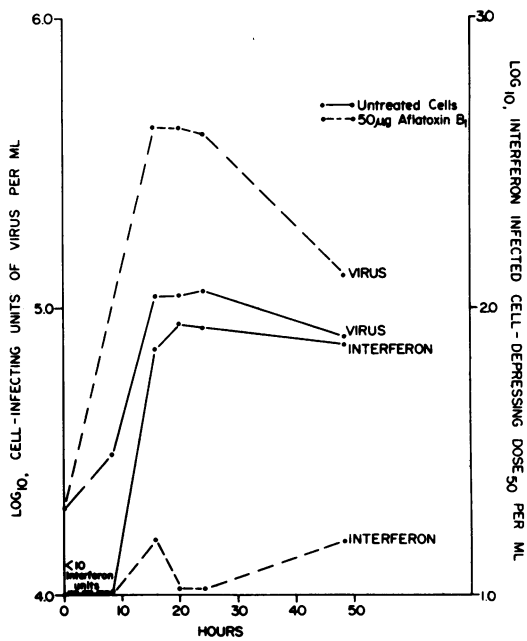

FIG. 1. *Growth curves of PR8 influenza virus concomitant with interferon production in untreated and pretreated aflatoxin LLC-MK2 cells in monolayer. Multiplicity of cell infection by virus was 1.0.*

the response of cells to the protection afforded by interferon against virus infection, an interferon preparation of known potency was assayed in the usual manner on clone 1-5c-4 cell monolayers that had been pretreated 20 h earlier with 100 μg of either AFB_1, AFG_1, or the appropriate control medium. Results revealed that interferon assay values of 105 and 100 $ICDD_{50}$ per ml for AFB_1 and AFG_1, respectively, were attained. These values were similar to that of the control, 107 $ICDD_{50}$ per ml. The process by which resistance is conferred by interferon was not apparently impaired by these aflatoxins.

DISCUSSION

The capacities of the aflatoxins to depress influenza virus-induced interferon in cell cultures were demonstrated in this study. The inhibitory effect on interferon production followed a structure-activity series with decreasing potency in the order aflatoxin $B_1 > G_1 > B_2 \approx G_2$. The designated order of biological activity is in general agreement with previous demonstrations relative to aflatoxin toxicity (acute) in animals and in cell cultures, altered biochemical processes in vitro, mutagenicity, and carcinogenicity (1-3, 33). It has been substantiated that both the dihydrofurofuran moiety and cyclopentenone ring are important determinants of aflatoxin biological activity (3, 6, 25). The varied biological effects induced by different aflatoxin derivatives is attributed to alterations of these structures. This circumstance may also account for the capacities of different aflatoxin derivatives to depress viral interferon induction.

Early virus-cell interactions were not impaired by aflatoxin. When influenza virus was assayed by using cells previously treated with either of the four aflatoxins, virus assay values were comparable to those of controls. The implication is that the integrity of cell membranes in relation to the process of virus integration (attachment and penetration) into cells was not adversely affected by the presence of aflatoxin. In subsequent experiments, virus growth rates in aflatoxin-treated and normal cells were comparable, but the levels of virus concentration attained were from two- to fourfold higher in cell cultures previously treated with aflatoxin. The magnitude of virus growth was related to the concentration and the capacities of aflatoxin derivatives to depress interferon production. This phenomenon of enhanced viral growth linked to the inhibitory activity of a carcinogen, i.e., AFB_1 on interferon production, has been demonstrated also with other carcinogenic agents (5, 16, 37).

The ability of interferon to confer cellular resistance against viral infection was not impaired when cells were pretreated with aflatoxin. In the interferon-treated cell, virus protein synthesis is inhibited while the majority of host protein syntheses are apparently unaffected (32, 37). The process by which interferon confers cellular resistance, presently considered to be the inhibition of the translation of viral ribonucleic acid (RNA) in the interferon-treated cell (18), apparently was not deterred by aflatoxin.

Protein synthesis is a requisite for viral interferon production. Inhibition of protein synthesis through transcriptional and translational processes, as well as inhibition of nucleic acid synthesis by affecting the transcription process, is a biochemical effect induced by AFB_1 and AFG_1 (3). Depending on the concentration and the aflatoxin derivative, we have shown that viral interferon synthesis may be partially or almost completely inhibited. One microgram of aflatoxin was able to limit interferon production. Less than 1 μg of aflatoxin has been reported to inhibit cell growth or to induce diverse cell abnormalities (4, 19, 38). Although the mechanism is unknown, aflatoxin may exert its adverse effect on interferon induction through (i) the formation of the inducing molecule, (ii) activation or depression of the interferon gene, or (iii) the transcription and translation of the interferon messenger RNA. The latter may be the more likely possibility in view of the inhibitory activity of aflatoxin on RNA synthesis.

Despite the inhibitory effects attributed to aflatoxin on certain macromolecular activities of host cells, we demonstrated that virus infectivity and viral replication were unaffected by the presence of aflatoxin. Possible explanations for this phenomenon may reside in the multiplication of influenza virus which differs from that of most other RNA viruses in its sensitivity to various inhibitors and the diverse activity of inhibitors under experimental conditions. For example, known inhibitors of protein and RNA syntheses, i.e., cycloheximide and 1-β-D-ribofuranosylbenzimidazole, have been shown to induce interferon synthesis in cell cultures (38). Inhibition of protein synthesis has been demonstrated in rat liver preparations exposed in vitro to aflatoxins. However, when the process was studied in vivo, the toxin inhibited the synthesis of only a few specific proteins (inducible enzymes), and total liver protein synthesis appeared to be largely unaffected (38). Actinomycin D has no apparent effect on the in vitro activities of the influenza virion- or cell-associated polymerases, but in vivo this reagent as well as other inhibitors of cell deoxyribonucleic acid function may block transcription (31). Em-

phasis has been placed upon the parallelism between the biochemical actions of AFB_1 and actinomycin D in inhibition of nuclear RNA synthesis, in vitro binding of deoxyribonucleic acid, and inhibition of enzyme induction. The significant similarities in action of the two compounds, however, does not imply that they act through analogous mechanisms or that all of the biochemical effects of AFB_1 can be attributed to those pathways apparently shared by both substances (38). Studies with other inhibitors and physical treatment of cells indicate that a functional cell nucleus or a deoxyribonucleic acid-dependent host function, or both, is needed for the multiplication of influenza viruses, but this function has not been identified (30). Some of the puzzling aspects of aflatoxin activity on host-cell functions as well as gaps in our knowledge concerning the role of the host cell in the initiation and maintenance of influenza virus RNA synthesis need to be resolved to explain influenza virus multiplication in aflatoxin-treated cells.

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