Effect of Aflatoxin B₁ on Cell Cultures

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ABSTRACT

GABLIKS, J. (Massachusetts Institute of Technology, Cambridge), W. SCHAFFER, L. FRIEDMAN, AND G. WOGAN. Effect of aflatoxin B₁ on cell cultures. J. Bacteriol. 90: 720–723. 1965.—Aflatoxin B₁, a metabolite of the mold Aspergillus flavus, is toxic to cell cultures. The toxic effect is evidenced by an inhibition of growth followed by progressive granulation, rounding, and finally sloughing of the cells from the glass. In studies with embryonated eggs, duck embryos were found to be four to five times more susceptible than chick embryos. In studies on Chang liver cultures, there were decreases in cell number, protein, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA) per culture with increasing aflatoxin B₁ concentrations. Since the cell number decreased and the protein, RNA, and DNA content per cell increased with increasing concentrations of aflatoxin, enlarged cells were suggested. These data are consistent with in vivo data of other workers who have found hypertrophic cells with enlarged nuclei in histological studies on the tissues of rats and ducklings fed toxic peanut meal.

In recent years, attention has been increasingly focused on a group of metabolites of Aspergillus flavus known collectively as aflatoxins. The outbreak of Turkey "X" disease in England in 1960, as well as the observed effects of aflatoxins on other domestic species, served to spotlight the hazardous nature of the aflatoxins.

Since 1960, more specific information on the toxicity of aflatoxins has been obtained, and many animal species have been shown to be susceptible. The toxin has been shown to produce hepatomas and renal injury in rats (Newberne, Carlton, and Wogan, 1964b), and hepatic-cell damage and bile-duct hyperplasia in ducklings (Newberne et al., 1964b), and has been implicated in the hepatitis of dogs (Aspin and Carnaghan, 1961) and swine (Loosmore and Harding, 1961).

Human susceptibility can only be inferred. However, Gabliks and Solotorovsky (1962) showed that cell cultures derived from the target tissue of susceptible host animals retain the susceptibility to diphtheria toxin in vitro. We therefore decided to examine cell cultures derived from two animals, each of known and different susceptibility, to compare their reactions with that of cell cultures derived from human tissue.

MATERIALS AND METHODS

The two established cell cultures used, HeLa cells derived from human cervical carcinoma and human Chang liver cells derived from normal tissue, were obtained from Microbiological Associates, Inc., Bethesda, Md. Two primary cell cultures, duck embryo and chick embryo, were also used. In addition, the toxicity of aflatoxin was studied in duck and chick embryonated eggs.

Established cell line procedures. Both HeLa and Chang liver stock cultures were grown at 37 C in 125-ml milk-dilution bottles in Eagle's basal medium supplemented with 10% calf serum, 100 units per ml of penicillin, and 100 μg/ml of streptomycin. The stock cultures were treated with 0.25% trypsin to remove the cells from the glass, and were then suspended in the growth medium at a concentration of 10⁴ cells per milliliter; 1 ml was planted in each of the screw-capped culture tubes used in experiments. The cultures were incubated for 3 days at 37 C to develop cell monolayers. At this time, the growth medium was decanted and replaced with 1 ml of fresh medium containing the desired quantity of aflatoxin B₁. The culture tubes were then incubated in a roller drum and observed daily for 2 to 3 days for any morphological changes.

At the termination of the experiment, the cultures were washed twice with Hank's balanced salt solution (BSS), and the cells were counted and analyzed for protein, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA).

Primary cell culture procedures. The primary cell cultures were prepared by the method of Merchant, Kahn, and Murphy (1964). Duck and chick embryos, 10 days old, were removed from the eggs, and after tissue disruption they were treated with 0.25% trypsin to release the individual cells. The cells were then counted and diluted to approximately 5 X 10⁴ cells per milli-
liter with growth medium which consisted of 0.5% lactalbumin hydrolysate and 5% calf serum in Hanks' BSS. Amounts of 1 ml were then planted in screw-capped culture tubes, and were grown for 48 hr; the medium was then decanted and replaced with 1 ml of the growth medium containing the desired quantity of aflatoxin B1. The cultures were then reincubated and examined daily for any cytopathogenic effects. At the end of 48 hr, the cultures were washed twice with Hanks' BSS, and the protein content was determined.

Embryonated egg procedures. A 0.5-ml portion of the desired dilution of aflatoxin B1 was inoculated into the chorioallantoic sac of embryonated eggs; 15-day-old duck and 10-day-old chick embryos (each one-half way through their development period) were used. The eggs were then incubated at 37 °C and candled daily. Dead embryos were autopsied and examined for gross pathological signs. The LD50 was calculated by the method of Reed and Muench (1938). One day prior to hatching, all remaining viable embryos were killed and autopsied.

Determination of cytopathogenicity. The degree of cytotoxicity, determined by microscopic examination of the cell cultures, was evaluated by use of a scale based on the ratio of destroyed-to-normal cells. Destruction or morphological damage of 75 to 100% of the cells was classified as 4+; 50 to 75% as 3+; 25 to 50% as 2+; and less than 25% as a 1+ reaction. The lowest concentration of aflatoxin causing a 2+ reaction during the incubation period was designated as the 50% toxic dose (TD50).

Biophysical testing procedures. Intact cells or nuclei were counted with a hemacytometer. When counting nuclei, the cells were suspended in a citric acid and crystal violet solution (Merchant et al., 1964). An increase in the number of cells per culture during a specified incubation period indicated growth of the culture.

The total amount of protein was determined by the method of Lowry et al. (1951) as modified by Oyama and Eagle (1956) for tissue cultures. Concentrations of the test substance which reduced the total cell protein per culture by 50% were designated as the 50% inhibitory dose (TD50). This value was calculated by the method of Litchfield and Wilcoxon (1949).

The total amount of DNA and RNA per culture was determined by the method of Schneider (1945, 1946), as modified by us for small tube cultures. The nucleic acids were extracted with 2.5 ml of 0.5 m trichloroacetic acid for 25 min at 90 °C. The extracted cell residues were removed by centrifugation at 2,500 rev/min. Samples of the remaining supernatant liquid were used for determination of DNA with diphenylamine reagent and of RNA with orcinol reagent.

Aflatoxin. The stock preparation contained 2 mg of purified aflatoxin B1 in propylene glycol. Dilutions of the aflatoxin stock preparation were made in growth medium. A dilution series of propylene glycol prepared in growth medium and

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<tr>
<th>Cell culture</th>
<th>Aflatoxin B1 (μg/ml)</th>
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<tr>
<td></td>
<td>TD50</td>
</tr>
<tr>
<td>Human Chang liver (normal tissue)</td>
<td>1.0</td>
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<tr>
<td>HeLa (human carcinoma of cervix)</td>
<td>5.0-7.0</td>
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<tr>
<td>Primary duck (whole embryo)</td>
<td>1.0</td>
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<td>Primary chick (whole embryo)</td>
<td>&lt;5</td>
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<td>Embryonated eggs</td>
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<td>Duck</td>
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<td>Chick</td>
<td>2.0-5.0</td>
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* Results of 48-hr test.

Table 1. Toxicity of aflatoxin in cell cultures*

Fig. 1. Effects of aflatoxin B1 on cell number, protein, RNA, and DNA in Chang liver cultures at 18 and 30 hr of incubation in the presence of toxin.

Fig. 2. Effect of aflatoxin B1 on cell number, protein, RNA, and DNA per culture and per cell. This illustrates the effect after 30 hr of incubation of Chang liver cells in the presence of the indicated toxin levels.

tested in cell cultures demonstrated that no culture toxicity existed in the range used in these experiments.

Results

Cell culture susceptibility. Table 1 shows the toxicity titration results obtained in the various
systems employed. Duck-embryo cells were found to be most susceptible (ID₅₀ = 1.0 μg/ml) followed by human Chang liver cells (ID₅₀ = 1 to 1.75 μg/ml), chick-embryo cells (ID₅₀ = 5.0 μg/ml), and HeLa cells (ID₅₀ = 5.0 to 7.0 μg/ml).

The first cytotoxic effects noted were evidenced by an inhibition of cell growth followed by progressive granulation, rounding, and finally sloughing of the cells from the glass. The minimal toxic dose in the above systems (the highest dilution of the aflatoxin eliciting a 1+ reaction) was: Chang liver, 0.1 μg/ml; duck embryo, 0.1 μg/ml; chick embryo, 1 to 5 μg/ml; and HeLa, 5 μg/ml.

**Duck- and chick-embryo susceptibility.** When aflatoxin B₁ was tested in embryonated eggs inoculated via the choioallantoic sac, duck embryos were found to be four to five times more susceptible than chick embryos. This is in agreement with the comparative cell culture susceptibilities and the relative susceptibility of young ducklings and chickens tested in vivo (Newberne et al., 1964a, b; Asplin and Carnaghan, 1961).

Examination of the duck and chick embryos which died during the course of the egg titration studies, or were killed one day prior to hatching, showed dwarfing of the embryos, underdeveloped and malformed livers containing light patches, and, at times, petechial hemorrhaging. In some cases, the livers were also found to be bile-colored.

**Effects on Chang liver cells.** To understand the data in Table 1, we undertook a study to correlate the following parameters: cell count, cell protein, RNA, and DNA. Human Chang liver cell cultures were exposed to aflatoxin B₁ in concentrations ranging from 0.1 to 5.0 μg/ml for various times from 18 to 30 hr. The data summarized in Fig. 1 were obtained at 18 and 30 hr.

There was a definite decrease with increasing concentrations of aflatoxin in all of the parameters studied, with the possible exception of DNA, which appeared to remain fairly constant.

Figure 2 summarizes the results obtained at 30 hr in terms of the values per culture as compared with the values per cell. There was an increase per cell of all of the parameters tested, and, further, this increase paralleled the increase in toxin concentration. Since we counted only the remaining viable cells in the population, the above finding may only be explained in terms of continued synthesis of cell protein, RNA, and DNA within the remaining viable cells, while cell division was inhibited. The comparative increase in cell numbers and cell protein is illustrated in Fig. 3.

**Discussion**

The comparisons of cytotoxicity indicate that human liver cells are in the same susceptibility range as the duck-embryo cells. HeLa and chick embryos are significantly less sensitive to aflatoxin.

Our findings concerning a decrease in cell division and a concomitant increase in the protein, RNA, and DNA per cell suggest the possibility of cell enlargement. The work of several other investigators presents analogous data. Legator and Withrow (1964) demonstrated the inhibitory effect of aflatoxin on mitotic division in cultured embryonic lung cells derived from human tissue. Newberne et al. (1964a, b) demonstrated swollen liver cells as one of the earliest pathological findings in ducklings and rats fed toxic peanut meal and crystalline aflatoxin B₁. In addition, these early pathological findings in the livers of ducks and rats fed toxic peanut meal or A. flavus extracts are consistent with ours in human liver cell cultures. Since aflatoxin is known to induce hepatomas in test animals, the possible susceptibility of humans to aflatoxin cannot be excluded, and more studies with a greater variety of human cell cultures are needed to clarify whether it is possible to predict human susceptibility by means of the techniques of cell culture.

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**Literature Cited**


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