BACTERIOLOGICAL REVIEWS, June, 1966 Vol. 30, No. 2
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Biological Effects of Aflatoxin in Cell Culture[1]

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INTRODUCTION

Comparatively little is known about the primary biological effects of this group of mycotoxins. In certain sensitive animal species, aflatoxin is acutely toxic at low concentrations, usually showing hepatotoxicity. In addition to its acute toxicity, aflatoxin B_1 is an exceedingly potent carcinogen in rats (2, 8) and trout (L. M. Ashley, J. N. Haver, and G. N. Wogan, Federation Proc. **23**:105, 1964). In simple biological systems, the major effort has been directed toward the development of bioassays to augment and confirm the chemical procedures of analysis. There have been few reports on the biological action of this group of toxins and only two reports on the action of aflatoxin in in vitro systems. Juhasz and Greczi (7) found that low concentrations of various groundnut samples destroyed cultured calf kidney cells, and Smith (15) found that the toxicant inhibited the incorporation of C^{14}-leucine into protein in various liver preparations. This review covers the biological activity of aflatoxin in cultured embryonic lung cells, reported in a series of papers (10, 11). The effect on cell growth, cell morphology, mitotic division, and deoxyribonucleic acid (DNA) synthesis will be reviewed. In addition to the work on cultured human embryonic lung cells, the effect of this mycotoxin as an inducing agent with lysogenic bacteria will be presented.

In tissue culture studies, a heteroploid human embryonic lung cell line, L-132, and a diploid human embryonic lung cell strain were both used. The cells were cultured in monolayer by use of Eagle basal medium with Earle's balanced salt solution (BSS) and 10% calf serum. Aflatoxin was added to the growth medium by use of one of the following procedures. (i) The chemical was dissolved in propylene glycol and added directly to the culture medium. The amount of solvent in the culture medium never exceeded 0.1%; this concentration was 5 to 10 times less than that needed to exhibit toxic effects on the cell in preliminary experiments. (ii) The aflatoxin preparation was dissolved in chloroform and added to the culture vessel. The chloroform was removed under nitrogen, and sterile medium was added. Usually, to achieve maximal solubility, the uninoculated medium containing the toxicant was sonically treated in a Branson S-75 unit for 30 sec. After sonic treatment the medium was inoculated with approximately 100,000 cells per milliliter of medium. The cells were incubated at 37 C in an atmosphere of 3% CO_2.

CELL GROWTH IN THE PRESENCE OF AFLATOXIN

To determine the effect of aflatoxin on cell growth, cell counts and protein and DNA determinations were made. An aflatoxin preparation (495) containing 15% aflatoxin B_1, 9% G_1, and less than 1% B_2 and G_2 was used. The final concentration added to the culture vessel was based on the known concentration of B_1 and G_1 in this preparation. Up to the 48th hr after the addition of the toxicant, cells exposed to 0.05, 0.1, 0.5, and 1.0 ppm increased in number; with 0.05 ppm concentration, growth differed little from that of the control. There was only slight increase in cell numbers between 48 and 93 hr at 0.05, 0.1, and 0.5 ppm; at 1.0 ppm there was a slight decrease in cell numbers. During this same period of incubation, the cells used for control purposes divided at a rapid rate. At the highest concentration used (5.0 ppm), there was an entirely different response; only a negligible amount of growth was

[1] A contribution to the symposium "The Aflatoxins" held at the Annual Meeting of the American Society for Microbiology, Atlantic City, N. J., 28 April 1965, with F. R. Roegner as convener, and F. R. Roegner and M. S. Legator as Consultant Editors.

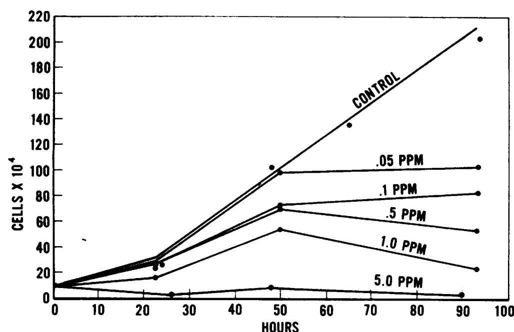

FIG. 1. *Effect of aflatoxin on cell growth. Cell-count determination (performed by use of a Coulter counter).*

found at any of the time intervals investigated. Figure 1 summarizes the results of this experiment.

DNA was determined according to the method of Webb and Levy (17), and protein was assayed by the method of Lowry et al. (13). The DNA and protein determinations essentially paralleled the results of the cell-count assays. Figure 2 indicates the effect of 0.5 ppm on protein content when the cultures were examined during an incubation period of 4 to 93 hr.

To determine the viability of the cells after various time exposures to aflatoxin, trypan blue was used as a vital stain. No difference was found in viability between the control and the cells exposed to the toxicant, except at a concentration of 5.0 ppm.

Morphology and Giant-Cell Formation

The L-132 cells exhibited a great deal of abnormal morphological patterns, including vacuolization and accumulation of cellular debris. Cell vacuolization was similar to the response reported by Smith (15) in monolayer growth of monkey kidney cells. In addition, there was an abnormally high number of giant cells. Figure 3 illustrates a typical giant cell, showing cell vacuolization after an exposure to 0.1 ppm of aflatoxin.

To determine the number of giant cells, harvested cells were fixed in acetic acid-ethyl alcohol (1:3) and stained with 1% Toluidine Blue. Approximately 4,000 cells were counted to determine the number of giant cells in the population. The normal population contained approximately 0.3% giant cells. After an exposure for 8 to 12 hr to 1 ppm of crystallized aflatoxin B_1, a 92% increase in giant cells was found when compared with the control.

To determine the persistence of giant cells after exposure to the toxicant, L-132 cells were exposed to 1 ppm of aflatoxin (495) for 24 hr. After this exposure period, the medium containing the toxicant was removed and fresh medium without toxicant was added. The number of giant cells was then determined at various time intervals after the removal of the chemical. After a 24-hr exposure to aflatoxin, followed by removal of the chemical, an 81 to 100% increase in giant cells was found for a period up to 10 hr. From 12 to 24 hr after the removal of the toxicant, proportionately fewer giant cells were seen. Table 1 depicts the results of this study.

Effect on Mitosis

For the study of the effect of aflatoxin on the mitotic frequency, three samples of 1,000 cells each were counted. The slide preparation was essentially the one used by Moorhead and Defendi (14) for chromosome studies. With the use of small Leighton tubes the same procedure was followed, except that the cells were not removed, but were stained and fixed *in situ*. The cells were counted at 100× magnification, and the number of cells in the metaphase per total cell population was recorded. Figure 4 illustrates a typical slide showing cells in metaphase.

Figure 5 records the effect of aflatoxin on suppression of mitosis in L-132 cells. A 55% reduction in mitosis was observed in these cells at 0.5 ppm when compared with the control. The control showed a mitotic index of 5.4%. When a sample of crystallized B_1 aflatoxin was evaluated by the same procedure, a 43% reduction in mitosis was observed at 0.5 ppm. With diploid embryonic lung cells in the 20th to 25th passage, activity essentially comparable with that of the L-132 heteroploid cells was found. In comparison with the control, the diploid cells exhibited a mitotic suppression of 50% with 0.1 ppm of aflatoxin (495). The L-132 heteroploid cells are somewhat easier to handle and are the cells of choice.

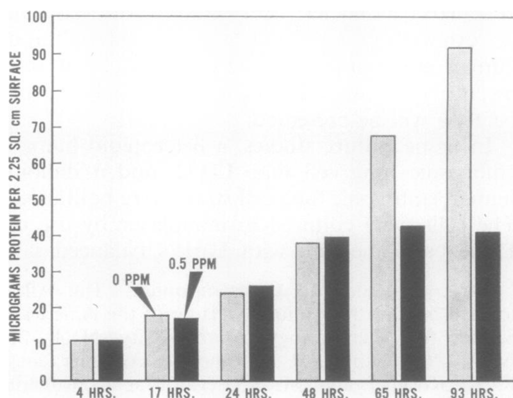

FIG. 2. *Effect of aflatoxin on cell growth. Total protein determination.*

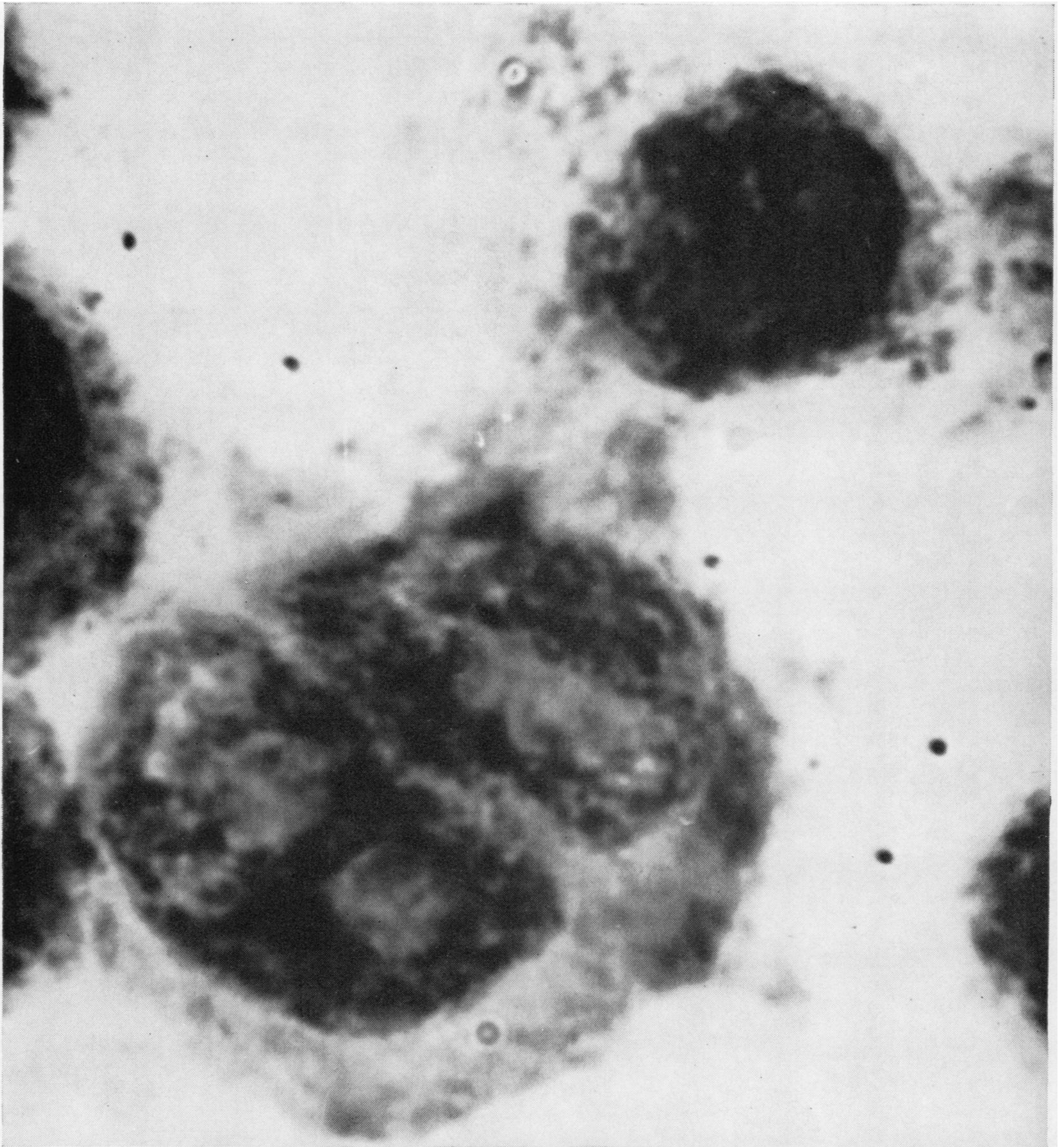

FIG. 3. *Giant-cell formation after exposure to 0.1 ppm of aflatoxin.*

TABLE 1. *Giant-cell formation after exposure to aflatoxin*

Time (hr) after removal of aflatoxin	Per cent increase over control
0	81
2	81
6	92
8	88
10	100
12	70
14	68
18	70
20	60
22	51
24	34

To determine the optimal time for mitotic suppression, the toxicant was added for varying lengths of time to the L-132 cells. In this experiment, colcemide was added for 1 hr prior to fixing. Figure 6 depicts the results of this study. The time recorded in the figure includes the 1-hr colcemide treatment. No effect on mitosis of the L-132 cells was found before 4 hr of exposure to the toxicant. After 6 hr of exposure to aflatoxin, a 35% suppression was observed, and at 8 hr a reduction of 64% in the cells undergoing mitosis was noted. Between 8 and 12 hr, there was only a slight increase in mitotic suppression. The results of this experiment indicate that aflatoxin affects mitosis

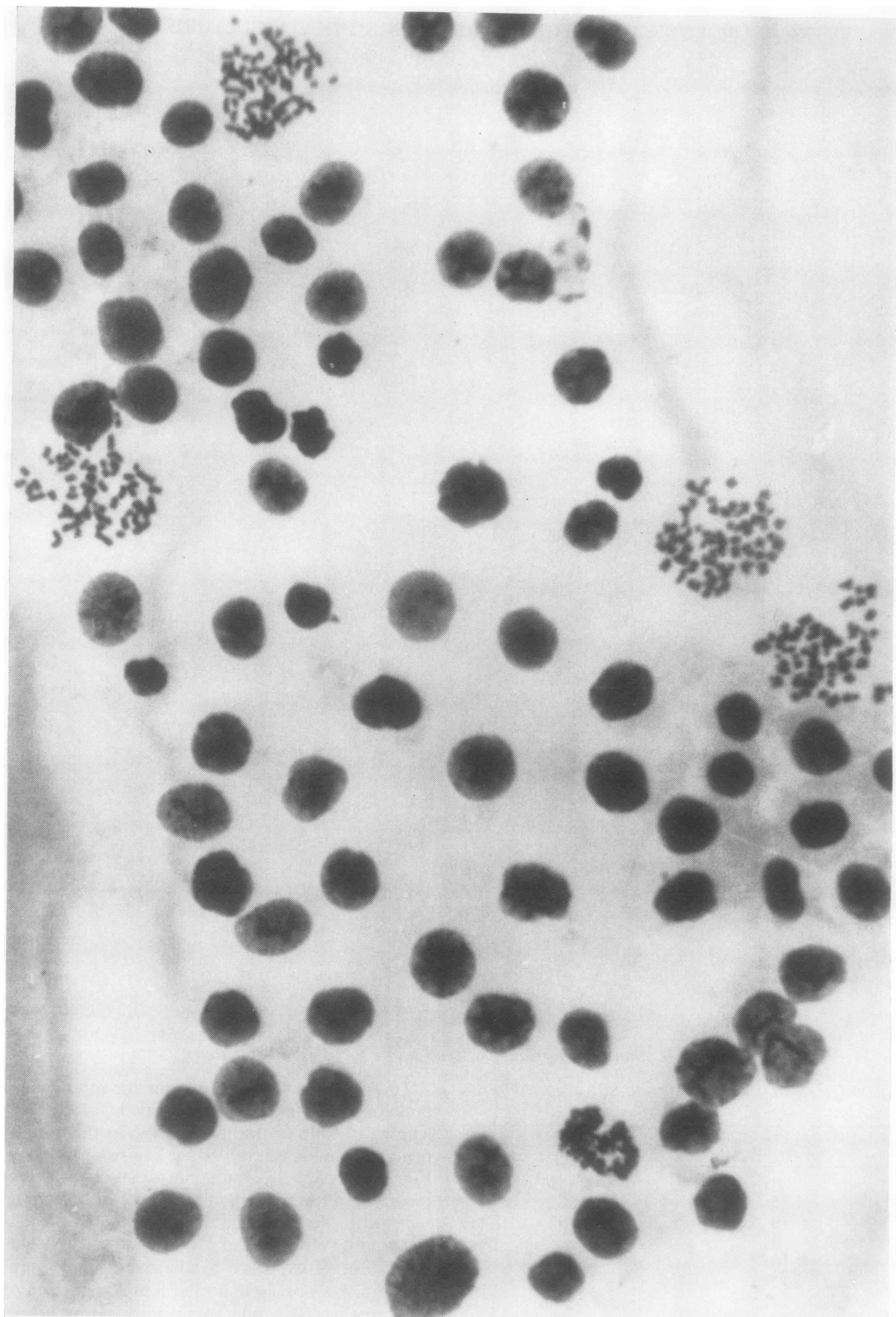

FIG. 4. *Determination of L-132 cells undergoing mitosis,* × *100. Note four cells in this field arrested at metaphase. Number of metaphase cells per total number of cells counted is used to determine mitotic frequency.*

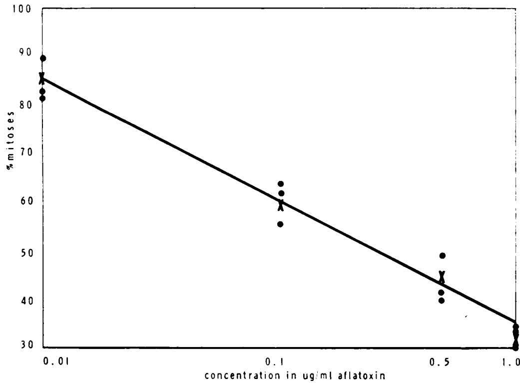

FIG. 5. *Effect of different concentrations of aflatoxin on mitosis. Symbols:* X, *average of three seperate determinations;* ●, *individual determinations.*

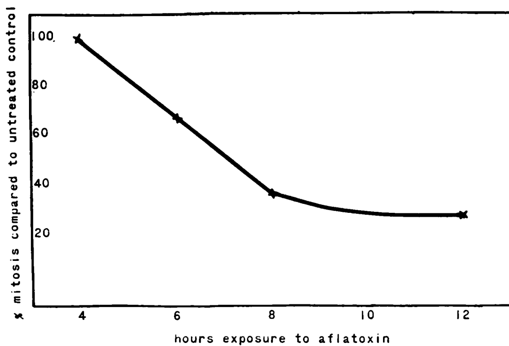

FIG. 6. *Effect of aflatoxin on mitosis, function of time.*

of the L-132 cells between 4 and 6 hr after exposure, with the maximal effect occurring between 8 and 12 hr.

AUTORADIOGRAPHIC STUDIES

To determine the effect of aflatoxin on DNA synthesis, tritiated thymidine was used. The labeling procedure was essentially that of Moorhead and Defendi (14). The cells were incubated in the presence of aflatoxin for 16 hr prior to "pulsing." The cells were then exposed for 20 min to tritium-labeled thymidine at a concentration of 0.5 μc/ml. The medium was removed and replaced with 50% fresh medium, and 50% medium was removed prior to pulsing. After an additional 12 hr, the cells were removed, fixed, and dipped in NTB2 liquid emulsion (Eastman Kodak Co., Rochester, N.Y.), and the slides were incubated for 10 days prior to developing. The number of labeled cells was determined with a minimum of 1,000 cells counted for each determination. In this study, the aflatoxin (495) and crystallized aflatoxin B_1 were both used. A concentration of 0.1 ppm of B_1 produced a 40% inhibition of DNA synthesis. More than 80% inhibition of DNA synthesis occurred with 1 ppm of aflatoxin (495). The crude aflatoxin in 1 ppm was more effective than 1 ppm of aflatoxin B_1. The results of this experiment are summarized in Fig. 7.

Figure 8 shows the inhibition of DNA synthesis after exposure of cells to 1 ppm of aflatoxin (495) for various time intervals from 4 to 12 hr. During this period, DNA synthesis was reduced by 4 to 84%.

INDUCTION OF BACTERIOPHAGE IN LYSOGENIC BACTERIA

"Radiomimetic" compounds are known to induce bacteriophage development in lysogenic

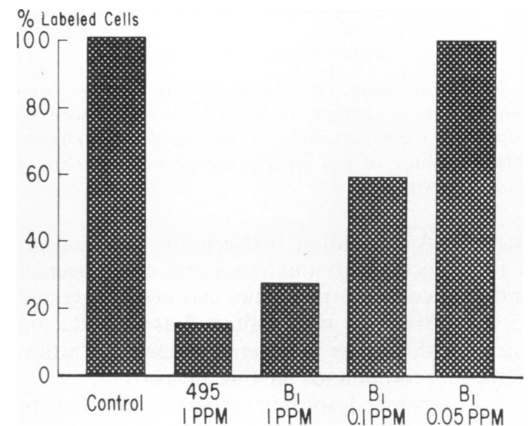

FIG. 7. *Effect of crystallized aflatoxin B_1 and crude aflatoxin (495) on incorporation of tritiated thymidine as compared with control.*

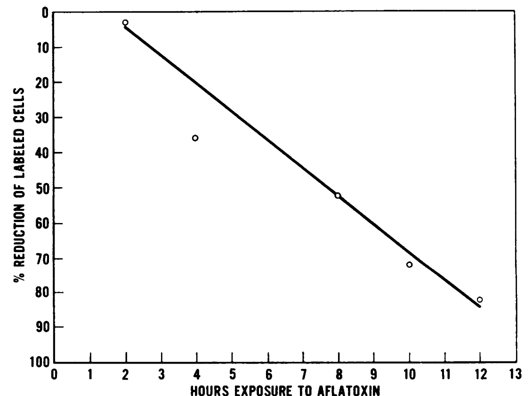

FIG. 8. *Inhibition of incorporation of thymidine-H^3 into DNA after exposure to 1 ppm of aflatoxin for various time intervals.*

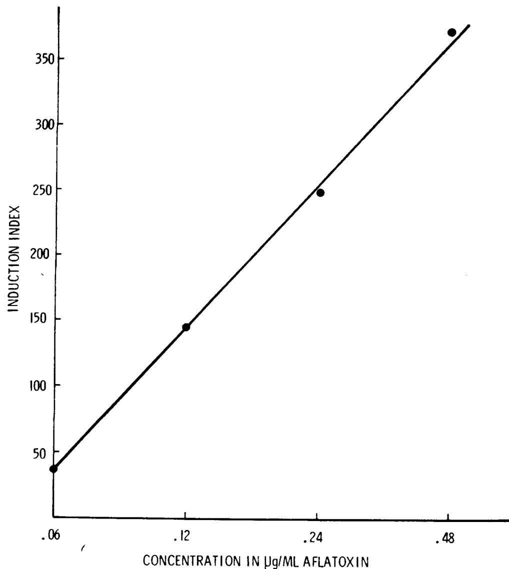

FIG. 9. *Increase in plaque-forming phage from Staphylococcus aureus (LM204) after exposure to aflatoxin. Induction index is the number of plaque-forming phage in test sample compared with that of negative control.*

bacteria. A correlation between the inducing activity of such agents and mutagenic, carcinogenic, and antineoplastic properties has been suggested (5, 12). Aflatoxin was evaluated as an inducing agent to determine whether it resembles "radiomimetic" compounds in this property.

Two separate lysogenic systems were used. In the first system, a lysogenic *Escherichia coli*, P4 λ 6, initially obtained from the Pasteur Institute, was used. This organism is lysogenic for λ phage, requires methionine, and is sensitive to streptomycin. The indicator culture W1485, initially isolated by Lederberg, was used. In the second system, a sensitive strain of *Staphylococcus aureus*, M204, and a lysogenic *S. aureus* strain, LM204, initially isolated from milk, were used. The procedure of Lein et al. (12) was followed, with a 1-hr induction period and a 3.5-hr incubation period.

A crude aflatoxin preparation was used as well as crystallized B_1. The results with both the *E. coli* and *S. aureus* lysogenic systems were comparable. Aflatoxin at a concentration as low as 0.06 μc/ml induced lysogenicity. Figure 9 illustrates the results of this study.

DISCUSSION AND SUMMARY

The investigation in tissue culture allows one to construct a probable sequence of events to account for the effect of aflatoxin in this biological system. The earliest effect of this toxicant is the suppression of DNA synthesis and mitosis; this effect is detectable within the first few hours after exposure of the cells to aflatoxin. The inhibition of all division and DNA synthesis leads to the survival of the cell in a nondividing stage, which can be quantitated after 48 hr of exposure to aflatoxin. The abnormal production of giant cells could well be accounted for by the enlargement of nondividing cells. The giant-cell formation seems to be of the nonsyncytial type, as seen in tissue culture cells infected with certain viruses, and is associated with arrested mitosis in metaphase (1).

The suppression of mitosis, inhibition of DNA synthesis, formation of giant cells, and induction of bacteriophage in lysogenic bacteria indicate that aflatoxin affects biological systems in a manner similar to alkylating agents. Alkylating agents are known to be mutagenic, carcinogenic, and antineoplastic (4). The carcinogenic effect may well be a manifestation of the mutagenic action (4, 16). Previous studies and the results reported herein indicate that aflatoxin is mutagenic as well as carcinogenic. Antineoplastic activity has yet to be demonstrated.

LITERATURE CITED

1. BARSKI, G., AND R. ROBINEZUS. 1959. Evaluation of *Herpes simplex* cellular lesions observed *in vitro* by phase contrast microcinematography. Proc. Soc. Exptl. Biol. Med. **101**:632–636.
2. DICKENS, F., AND H. E. JONES. 1964. Carcinogenic activity of a series of reactive lactones and related substances. Brit. J. Cancer **15**:85–100.
3. ENDO, H., M. ISHIZAWA, T. KAMIYA, AND S. SONODA. 1963. Relation between tumouricidal and prophage-inducing action. Nature **198**: 558–560.
4. HADDOW, A. 1958. Chemical carcinogens and their mode of action. Brit. Med. Bull. **14**:79–92.
5. HEINEMANN, B., AND A. J. HOWARD. 1964. Induction of lambda-bacteriophage in *Escherichia coli* as a screening test for potential antitumor agents. Appl. Microbiol. **12**:234–239.
6. HOWARD, D. H. 1962. Synthesis of DNA in normal and irradiated cells and its relation to chromosome breakage. Heredity (Suppl.) **6**:261.
7. JUHASZ, G., AND E. GRECZI. 1964. Extracts of mould infected groundnut samples in tissue culture. Nature **203**:861–862.
8. KRAYBILL, H. F., AND M. B. SHIMKIN. 1964. Carcinogenesis related to foods contaminated by processing and fungal metabolites. Advan. Cancer Res. **8**:191–248.
9. LANCASTER, M. L., F. P. JENKINS, AND J. M. PHILIP. 1961. Toxicity associated with cer-

tain samples of groundnuts. Nature **192**:1095–1096.

10. LEGATOR, M. S., AND A. WITHROW. 1964. Aflatoxin: effect on mitotic division in cultured embryonic lung cells. J. Assoc. Offic. Agr. Chemists **47**:1007–1009.
11. LEGATOR, M. S., S. M. ZUFFANTE, AND A. R. HARP. 1965. Aflatoxin: effect on heteroploid human embryonic lung cells. Nature **208**:345-348.
12. LEIN, J., B. HEINEMANN, AND A. GOUREVITCH. 1962. Induction of lysogenic bacteria as a method of detecting potential antitumor agents. Nature **196**:783–784.
13. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR., AND R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
14. MOORHEAD, P. S., AND V. DEFENDI. 1963. Asynchrony of DNA synthesis in chromosomes of human diploid cells. J. Cell Biol. **16**:202–208.
15. SMITH, R. H. 1963. The influence of toxins of *Aspergillus flavus* on the incorportation of C^{14} leucine into proteins. Biochem. J. **88**:50–51.
16. STRONG, L. C. 1948. The induction of mutations by a carcinogen. Proc. Intern. Congr. Genet. 8th, Stockholm, 1949. Suppl. to Hereditas, p. 486–499.
17. WEBB, J. M., AND H. B. LEVY. 1955. A sensitive method for the determination of deoxyribonucleic acid in tissues and microorganisms. J. Biol. Chem. **213**:107–117.