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Response of Culture Human Skin Fibroblasts to Aflatoxin B_1

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Aflatoxin is the generic name for a mixture of highly toxic metabolites produced by certain strains of the mold *Aspergillus flavus* as they grow on groundnuts and other grains (LEGATOR et al. 1965, CLIFFORD & REES 1967). The chemical structures of these aflatoxins have been studied extensively (NESBITT et al. 1962, ASAO et al. 1963, 1965). Aflatoxin B_1 (AFB_1) has been shown to be the most abundant and most toxic species (MAHER & SUMMERS 1970).

Numerous *in vivo* studies have been undertaken to better understand the toxic effects of AFB_1. The potent, selective hepatocarcinogenic effects of AFB_1 are demonstrated most strikingly in rats (BUTLER & CLIFFORD 1965, CLIFFORD & REES 1967) as well as in other mammalian species (NEWBERNE & BUTLER 1969), some fish (ASHLEY et al. 1964) and fowl (LEE et al. 1964).

In vitro studies using aflatoxins in cell culture have served to elucidate *in vivo* findings. Many of these techniques have been studied for use as toxicity bioassays due to their ease, sensitivity and rapidity compared to *in vivo* testing (LEGATOR et al. 1965, MISHRA & DIMAYORCA 1974). In depth studies in cell culture have been done in some cell systems in an attempt to better isolate, identify and characterize the toxic effects of AFB_1 that are seen *in vivo*. For example, the effects of AFB_1 have been studied on rat liver (CLIFFORD & REES 1967, BAUSHER & SCHAEFFER 1974), human embryo liver (ZUCKERMAN et al. 1968), Hela cells (HARLEY et al. 1969), human embryonic lung (LEGATOR & WITHROW 1967) and *Xeroderma pigmentosa* cells in culture (STICT AND LAISHER 1975).

Animals dosed orally (NEWBERNE & BUTLER 1969) and intraperitoneally (WOGAN 1968) with AFB_1 rapidly develop hepatic lesions. However, comparable toxic effects in laboratory animals have not been demonstrated when aflatoxin is administered via the skin (PURCHASE & STEYN 1973).

The present study was undertaken to evaluate the toxic effect of AFB_1 on normal human skin fibroblasts and to expand previous biological findings in cell culture.

MATERIALS and METHODS

A 1 g specimen of normal human skin was minced into 1 mm^3 frag-

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ments under sterile conditions and incubated at 37°C under normal atmosphere pressure in 10 mL Eagle's Minimum Essential Media (Gibco, Grand Island, NY) supplemented with fetal calf serum and Penstrep (Gibco, Grand Island, NY). The specimen was refed every other day until there was significant outgrowth of fibroblasts for subculturing. This required about 17 days.

After the fourth subculture the cells were harvested in 0.25% Trypsin buffered with 25mM Tricine (Calbiochem, La Jolla, CA) in Hank's Balanced Salt Solution (Gibco) for 30 minutes at 37°C and plated into 240 thirty mm^2 wells so that the final concentration of cells equaled 4×10^4 fibroblasts per well in initial plating in each experiment. After the cells were allowed to adhere to the wells for 24 hours at 37°C, the media was removed and 48 wells each were refed with the following concentrations of AFB_1 in the media: 1.00, 0.50, 0.10 and 0.01 µg/mL. In addition, 48 wells of fibroblasts were refed with media which contained no AFB_1 as control wells.

Aflatoxin B_1, grade A, dried *in situ* (Calbiochem) was first dissolved in acetone (10 mg/mL). This solution was added to 500 mL of sterile water and the latter was gently heat-stirred for several hours. The resultant solution was stored at 10°C in darkness. Aliquots were tested for the level of aflatoxin. Twenty microliter samples were spotted on silica gel thin layer chromatography plates. Fluorescent spots were compared visually to reference samples spotted simultaneously using the official AOAC procedure (HORWITZ et al. 1975). Dilutions were made as necessary and quantitations were repeated in triplicate. The stock solution used in this study contained 5.00 µg AFB/mL of H_2O. All experimental dilutions were made from this source. All toxin analyses were completed at the Mycotoxin Laboratory of the Virginia Division of Consolidated Laboratory Services, Richmond, VA.

On each day of the 8-day growth period, the media was removed from 6 wells of each of the test concentrations and the controls. The cells were trypsinized in the manner described above. The fibroblasts from each well were then removed to a centrifuge tube with a Pasteur pipet. Each well was subsequently washed with 1 mL of media to remove any remaining cells and this wash was added to the respective tube. All the tubes were centrifuged for 10 min at 1800 rpms and the supernatent was decanted from each tube. The resultant cell pellet was resuspended in 1 mL of media and the cells were dispersed by gentle agitation. Three of the tubes of the test concentrations and the control cells were used for triplicate counting determinations in a hemacytometer. The remaining 3 tubes of the test concentrations and the control cells were tested for viability by adding an equal volume of 1.0% Trypan Blue (in 4.25% NaCl) to the cell suspension and counting the number of dead cells per 100 cells to obtain a percent viability.

A second, similar experiment was conducted on the same fibroblasts in the 5th subculture. This 5-day growth and viability study used 3 concentrations of AFB_1: 5.00, 2.50 and 1.25 µg/mL plus control fibroblasts. The cells were plated, counted and stained in the manner previously described.

RESULTS

There were no statistical differences in the viabilities (%) of any of the test fibroblasts compared to the control (untreated) cells in either the 8-day or the 5-day study.

The growth curves in both studies (Tables 1 and 2) show characteristic lag, log and stationary growth phases for the duration of the experiments. Daily visual examination of the test and control cells under phase contrast microscopy revealed no apparent morphologic changes in the gross appearance of the cells.

In the 8-day experiment (Table 1), the growth rate of test versus control fibroblasts showed decreased mitosis at 0.10 µg/mL and 0.01 µg/mL on days 1 and 2. On day 3, all test concentrations were significantly decreased compared to control cells; while on day 4, only the cells treated with 1.00 µg/mL AFB_1 showed a stitistical difference to the control cells as well as to the other test concentrations, and this was due to an increase in cell numbers. On days 5 and 6, the growth rate of cells in both test and control wells are similar. On day 7, the 0.50 µg/mL and 0.10 µg/mL are statistically different from the controls and there are differences among the test concentrations (Table 1). On day 8, the 0.10 µg/mL concentration is significantly elevated compared to the control cells while 1.00 µg/mL and 0.01 µg/mL concentrations are significantly depressed compared to 0.10 µg/mL.

In the 5-day experiment, all test concentrations were significantly depressed compared to the control cells on day 5. On day 2, the 5.00 µg/mL and 1.25 µg/mL were decreased compared to the control and to 2.50 µg/mL while on day 3, only the 1.25 µg/mL is significantly decreased compared to control cells.

In the 5-day experiment, there was no refeeding of cells, and so all cell numbers are decreasing on day 5. In the 8-day experiment, the cells were refed with appropriate concentrations of AFB_1 made up in fresh media after day 4.

DISCUSSION

Generally, the correlation between *in vivo* and *in vitro* findings concerning the cytotoxic and carcinogenic effects of AFB_1 are in agreement if the data are interpreted carefully.

TABLE 1. Growth Curve for Normal Human Skin Fibroblasts Treated With Low Levels of Aflatoxin B_1

Duration of Treatment (Days)	Mean Cell Counts and Standard Deviations x 10^5 for Each Aflatoxin Treatment Level				
	1.00 µg/mL	0.50 µg/mL	0.10 µg/mL	0.01 µg/mL	Control
1	0.267 ±0.095	0.275 ±0.156	0.250[a] ±0.043	0.217[a] ±0.080	0.350 ±0.025
2	0.483 ±0.146	0.592 ±0.088	0.292[a,b] ±0.080	0.358[a,b] ±0.976	0.600 ±0.087
3	0.442[a,c,d] ±0.163	0.735[a] ±0.256	1.120[a] ±0.080	1.220[a,b] ±0.050	2.340 ±0.180
4	2.670[a,b,c] ±0.120	1.170 ±0.260	1.130 ±0.580	1.120 ±0.150	1.420 ±0.400
5	2.070 ±0.230	1.440 ±0.470	2.090 ±0.290	2.080 ±0.420	1.920 ±0.410
6	2.030 +0.470	2.800 ±0.300	2.870 ±0.730	2.270 ±0.210	2.320 ±0.250
7	2.400[b,c] ±0.200	1.600[a] ±0.180	3.270[a,b] ±0.120	2.030[b,c] ±0.150	2.130 ±0.230
8	2.370[c] ±0.190	2.130 ±0.200	3.040[a,b] ±0.120	2.100[c] ±0.200	2.150 ±0.150

[a] $p<0.05$ compared to Control
[b] $p<0.05$ compared to 0.50 µg/mL
[c] $p<0.05$ compared to 0.10 µg/mL
[d] $p<0.05$ compared to 0.01 µg/mL

TABLE 2. Growth Curve for Normal Human Skin Fibroblasts Treated With High Levels of Aflatoxin B_1

Duration of Treatment (Days)	Mean Cell Counts and Standard Deviations x 10^5 for Each Aflatoxin Treatment Level			
	5.00 µg/mL	2.50 µg/mL	1.25 µg/mL	Control
1	0.367 ±0.063	0.333 ±0.123	0.316 ±0.125	0.258 ±0.020
2	0.483[a,b] ±0.138	0.733 ±0.063	0.542[a,b] ±0.076	0.875 ±0.066
3	1.100 ±0.055	1.030 ±0.100	0.652[a] ±0.080	1.500 ±0.043
4	1.460 0.080	1.670 0.072	1.270 0.052	1.370 0.010
5	0.424[a] ±0.031	0.476[a] ±0.021	0.530[a] ±0.074	0.780 ±0.036

[a] $p<0.05$ compared to Control
[b] $p<0.05$ compared to 2.50 µg/mL

Studies where liver lesions appeared after the chronic application of AFB_1 to rat skin (WEI et al. 1970) and rabbit skin (UNGAR & JOFFE 1969) were subsequently reevaluated when it was shown that the lesions were due to ingestion of the toxin rather than to absorption through the skin (PURCHASE & STEYN 1973).

The resistance of skin fibroblasts to the cytotoxic effects of AFB_1 as shown in the present study are consistent with the observations of other investigators. For example, ZUCKERMAN et al. (1968) demonstrated selective aflatoxin cytotoxicity to human embryonic liver hepatocytes, but not to liver fibroblasts. They postulated that different cell types within the liver varied in their ability to bind aflatoxin with DNA, that there were selective permeabilities of the different cell types to the toxin, or that the hepatocytes differed from the liver fibroblasts in their ability to metabolize, and thus be affected by the toxin. These 3 hypothesis are consistent with the findings of the present study.

In another study, the earliest effect of the toxin to cultured human embryonic lung cells is the almost immediate (after 6 hours exposure of cells to 0.10 µg/mL AFB_1) suppression of DNA synthesis and cell mitosis (LEGATOR 1966). This observation was not seen in the human skin fibroblasts used in this study, although when higher concentrations of AFB_1 were used, all cells showed significant depression in growth rates after 5 days in culture compared to control cells. Furthermore, consistent with the findings in the present study LEGATOR et al. (1965) reported no differences in viabilities of their cells in a 4-day growth study using 0.05 to 5.00 µg/mL AFB_1 except in the highest concentration of the toxin. This lack of depressed viability was explained by the presence of viable but non-dividing cells in the cultures.

Further resistance to the cytotoxic effects of AFB_1 in cell culture was demonstrated by BAUSHER & SCHAEFFER (1974). In their study, a cloned population of rat liver cells exhibited only 28% reduction of ^{14}C-uridine incorporated into RNA at 6 hours after treatment with 50 µg/mL of AFB_1, while an established rat cell line exhibited a 70% reduction of radioisotope incorporation into RNA. It should be noted that these rat liver cells, as well as the cells used in the present study, may have acquired resistance *in vitro* to the AFB_1 which is not demonstrated by the cells in their host environment *in vivo*. In addition, although cytotoxicity was depressed in the skin fibroblast line in this study compared to other cell types studied by other investigators, the carcinogenic effects on these cells in culture were not measured in the present study. Such effects could be demonstrated by chromosome analysis or labeled amino acid uptake.

Furthermore, PORTMAN et al. (1968) observed that the hepatocarcinogenic effects of AFB_1 may occur as the result of its

in vivo metabolism to a toxic nucleic acid reactant (i.e., an epoxide) through a hydroxylated derivative formed in the target organ, the liver, in a pathway similar to the activation of polycyclic hydrocarbons. This could explain the high susceptibility of liver cells *in vivo* and *in vitro* to the carcinogenic and cytotoxic effects of AFB_1. It is possible that certain cell types which show resistance to these effects *in vivo* and *in vitro* lack a key activating enzyme present only in specialized cells, which permits the cells to convert the toxin into a lethal form. It is further possible that some cells have a selective inability to transport, and thus, to metabolize the toxin. For example, subcutaneous injection of AFB_1 results in sarcoma and fibrosarcoma (DICKENS & JONES 1963) and kidney tumors in rats (EPSTEIN et al. 1969). Therefore, it appears that other tissues besides the liver may have the capacity for intracellular activation of the toxin. The reasons for resistance of the cells in the present study to the cytotoxic effects of AFB_1 have yet to be explained, but probably relates to the cell's ability to metabolize the aflatoxin.

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