AFLATOXIN B_1: CYTOTOXIC MODE OF ACTION EVALUATED BY MAMMALIAN CELL CULTURES

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Aflatoxin B_1 rapidly inhibits RNA synthesis in rat liver cells, slices or liver *in vivo*. Established human cell lines (kidney T-cells, HeLa S_3, Chang liver) and mouse fibroblast 3T3 are more slowly affected. Prolonged exposure of synchronized cell cultures to the agent show that cells are retarded in their passage through the S-phase and exhibit a decreased rate of DNA synthesis. Consequent to this, mitosis is also inhibited. Liver cells appear to convert aflatoxin B_1 to a more potent cytotoxin which can then affect normally non-susceptible cells. This may explain the susceptibility of liver to tumorogenesis by this carcinogen.

1. Introduction

Aflatoxin B_1 is possibly the most potent carcinogen of the closely related group of toxins produced by the fungus *Aspergillus flavus*. The liver is almost exclusively the organ of attack but susceptibility varies between species [1–3]. Aflatoxin B_1 is also cytotoxic to cells in culture at concentrations of a few micrograms per ml [1–6] and this action has been equated with its ability to bind to DNA and thus affect RNA transcription and translation [7–9] as well as affecting polyribosome profiles [10–11]. These effects are particularly marked in liver cells whereas cells from other tissues [4, 12] require higher concentrations or longer exposures to achieve the same results. This fact together with *in vitro* enzyme studies [10, 13, 14] has suggested that prior metabolic conversion of the toxin is involved in its action *in vivo*. However since the presently known metabolites are less active than aflatoxin B_1 itself, an unequivocal demonstration of this transformation is lacking. The present investigation has confirmed the relative susceptibility of liver cells to the toxin and moreover that it is rapidly converted by these cells into a more potent inhibitor capable of affecting normally resistant cells.

2. Experimental

Established cell lines of human origin kidney, T-cells, HeLa, S_3 and Chang liver and mouse 3T3 cells were grown as monolayers in LPC or BME medium containing 10% new-born calf serum. Synchrony was achieved as previously described using a double TdR block [15]. Inhibition studies were carried out in LPC medium as previously described [16] or in BME containing 6 mM tricine in place of bicarbonate, to facilitate the manipulations in the absence of CO_2. Rat liver cells were prepared from liver slices of 150–180 g male albino rats or from whole embryonic (15–17 days) livers by enzymatic dispersion with 0.5% trypsin in Hank's medium.

Crystalline aflatoxin B_1 was obtained from Makor Chemicals Ltd., Jerusalem. Stock solutions (2 mg/ml) were made in chloroform–propylene glycol (4:1) and kept in the dark. Aliquots were evaporated free of $CHCl_3$ immediately prior to the addition of aqueous media to avoid solubility problems. Aqueous solutions were used immediately and screened from direct illumination.

^{3}H-TdR, ^{3}H-CdR and ^{3}H-Urd (uridine) were obtained from the Radiochemical Centre, Amersham.

3. Results

The inhibition of nuclear RNA synthesis in different cell lines and in rat liver cells is shown in table 1. It will be seen that liver cells are particularly susceptible to inhibition and that this property is retained even in the established line of Chang liver cells. On the other hand mouse 3T3 fibroblasts and human kidney T-cells are relatively insensitive. DNA synthesis in both T-cells and Chang cells is less sensitive to the immediate action of aflatoxin B_1 than is RNA synthesis. Thus 10 μg/ml of toxin preincubated for 30 min under identical conditions still gives 98 and 88% of control T and Chang cell DNA synthetic rates, respectively. The interpretation of the results of exposing cells to aflatoxin for long periods is complicated by two factors. First, cell multiplication is retarded, but not completely blocked. Thus concentrations of 5–10 μg/ml aflatoxin reduce cell numbers by 20% after 24 hr and by 40% after 48 hr exposure. Hence inhibition studies based on the precursor incorporation per cell at these times can be misleading. Second, as will be discussed further, the aflatoxin-containing medium cannot be refreshed over the prolonged periods of contact, and even when the pH is maintained constant the rate of nucleic acid synthesis in the controls often begins to decrease markedly under these conditions. This is particularly noticeable for DNA synthesis in Chang liver cells resulting in apparently higher rates of synthesis in the inhibited cell cultures (table 2).

The early inhibition of RNA synthesis in Chang cells is dependent upon the preincubated aflatoxin-containing medium being left in contact with the cells during the precursor pulse. If this medium is removed and substituted with the original aflatoxin solution immediately prior to pulsing a much lower degree of inhibition is obtained (table 3). This effect tends to diminish as the inhibition becomes established with a prolonged period of exposure. This phenomenon suggests a metabolic conversion of the aflatoxin to a more potent inhibitor. Such a conversion should be evident by exposing cells directly to this preincubated

Table 1
Inhibition of RNA synthesis by aflatoxin B_1.

Cells	Preincubation time (hr)	Conc. (μg/ml)	Control (cpm/10^5 cells)	Treated (% of control)
Mouse 3T3	0.5	10	1,550	100
		20		98
Kidney T	0.5	10	1,670	96
		20		88
		40		65
HeLa S_3	0.5	10	1,790	89
		20		80
Chang liver	0.5	5	1,620	80
		10		64
Embryonic rat liver	1	10	1,100	80
Adult rat liver	1	10	4,400	44
Rat liver slices	1	10	11.5 (cpm/μg RNA)	59
Rat liver (*in vivo*)	3	(0.5 mg/kg)	15.5 (cpm/μg RNA)	26

Values are the mean of 3 separate determinations. Cell suspensions in LPC medium were pulsed with 0.5 μCi/ml of uridine-5-^{3}H for 30 min. Rat liver slices were incubated with Krebs-Ringer-phosphate medium [8] containing 1 mg/ml of glucose and were pulsed with 2 μCi/ml of cytidine-5-^{3}H. *In vivo* experiments were done with 100 g rats injected with aflatoxin B_1 (0.5 mg/kg, i.p.) in propylene glycol 3 hr prior to the injection of 50 μCi/kg of cytidine-5-^{3}H. After 30 min the animals were decapitated and liver cell nuclei isolated and the activity in the nuclear RNA [17] estimated by liquid scintillation counting.

Table 2
Effect of increasing exposure time to aflatoxin B_1 on the inhibition of RNA and DNA synthesis.

	Time (hr)	RNA			DNA		
		Control	Treated	%	Control	Treated	%
Chang	2	18,100	14,100	78	19,900	17,900	90
	16	11,000	8,800	80	6,390	6,250	98
	24	8,500	7,500	88	3,450	3,800	110
T-cells	24	13,600	14,300	105	23,400	12,900	55
	38*	23,000	19,800	86	26,600	13,600	51

Values are the means of two separate determinations.

* Synchronized cultures (15) treated with 5μg/ml of aflatoxin B_1 for 12 hr after the 1st TdR block, then for 24 hr during the 2nd TdR block then for 2 hr after the 2nd TdR block in normal LPC medium. All other cultures were asynchronous and were treated with 10 μg/ml aflatoxin. Cell progression is retarded by these concentrations of aflatoxin resulting in fewer cells in treated cultures (see text for discussion).

medium. In table 3 aflatoxin B_1-containing supernatant from 2 hr preincubated Chang cells has been transferred to fresh cultures of T or Chang cells. It is evident that pre-exposure of aflatoxin to Chang cells has increased its potency for inhibition of RNA synthesis. This is not a peculiarity of T or Change cells since HeLa cells also demonstrate the same phenomenon. However, 3T3 cells in keeping with their strong resistance to inhibition are hardly affected by the converted compound. The same conversion possibly also occurs after exposure to rat liver cells, but here the result is somewhat obscured by the depression of the precursor incorporation in controls due to pool dilution effects and the speed of penetration and binding of the inhibitor itself into liver cells.

The metabolic alteration of aflatoxin by Chang and liver cells proceeds in the absence of oxygen and is not prevented by cycloheximide. Chang cells kept under anoxia for 2 hr however show a rapid burst of RNA synthesis when returned to air. This burst of new activity is particularly susceptible to aflatoxin inhibition.

Since the above inhibitions of RNA synthesis in cells cannot be directly correlated with cytotoxic action, the aflatoxin-containing media exposed to Chang

Table 3
Effect of pre-exposure of aflatoxin to Chang liver cells on its RNA inhibiting capacity.

	Tested on (% of controls)				
	Chang	Time (hr)		HeLa	3T3
		2	6		
(2) Medium from Chang control	99	95	90	96	98
(3) Aflatoxin-containing medium	75	95	92	85	98
(4) Chang-exposed aflatoxin	59	77	49	79	94
(5) Preincubated Chang cells + fresh aflatoxin	78	–	–	–	–

(1) Controls were changed for LPC medium only. Chang cells were preincubated with 10 μg/ml aflatoxin B_1 for 2 hr then the medium transferred to the different cell cultures and pulsed immediately for 20 min. Values as the means of at least three separate determinations. The experimental procedures (1–5) are demonstrated in the scheme shown in fig. 2.

Table 4
Cytotoxic action of aflatoxin B_1 on T cells preincubated with Chang or rat liver cells.

	Mitotic index		Pyknotic cells	
	Chang	Rat liver	Chang	Rat liver
Controls	2.8	2.7	0.3	0.4
+ incubated medium only	2.3	1.5	1.5	4.8
+ aflatoxin only (10 μg/ml)	1.4	0.8	2.1	2.0
+ incubated aflatoxin medium (10 μg/ml)	0.7	0.6	3.9	6.5

Aflatoxin B_1 (10 μg/ml) was incubated with Chang liver cells for 3 hr then transferred to cover-slip cultures of T-cells for 24 hr, before fixing and scoring for mitotic index or pyknotic cells. Values are the means of two separate determinations. The experimental procedures (1–4) are demonstrated in the scheme of fig. 2. Suspensions of adult rat liver cells were treated in the same manner.

and rat liver cells have been evaluated for their ability to produce cell death and mitotic inhibition when compared to the original solutions. Table 4 shows that in both cases the metabolically altered aflatoxin was more cytoxic than the unexposed solution. Although T-cells are not immediately sensitive to the cytotoxic inhibitory action of aflatoxin, prolonged exposures eventually inhibit RNA and DNA synthesis. It was therefore of interest to evaluate aflatoxin B_1, in terms of its effect on the cell cycle using synchronized cell cultures. The results of fig. 1 show that mitosis was only weakly affected when the inhibitor was added at the beginning of the S-phase preceding that mitosis, but was powerfully inhibited when the cells had been exposed for a number of hours prior to that S-phase. The inhibition of mitosis can be attributed to a prolongation of the time exposed cells take to traverse the S-period. Thus in T-cells, 7 hr after the removal of the second TdR block used for synchrony, control cultures contained less than 20% of cells capable of incorporating ^{3}H-TdR whereas cultures exposed to 5 μg/ml of aflatoxin during the previous 24 hr still exhibited 95% of DNA-synthesing cells. However the degree of labeling in such inhibited cells, as judged by grain counts, is considerably reduced as it also is in asynchronous cultures. This fact is confirmed by direct measurements made on synchronous cultures, table 2. In Chang liver cells a similar exposure to 5 μg/ml of aflatoxin also delays cells in S-phase but leads to a more pronounced inhibition of DNA synthesis than in T-cells as judged from grain counts, in

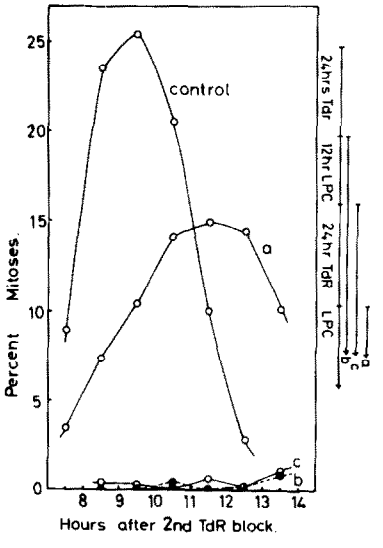

Fig. 1. Effect of aflatoxin B_1, addition to synchronized cultures of human kidney T-cells. Aflatoxin (5 μg/ml) was added at the periods indicated in the synchronization scheme in LPC medium.

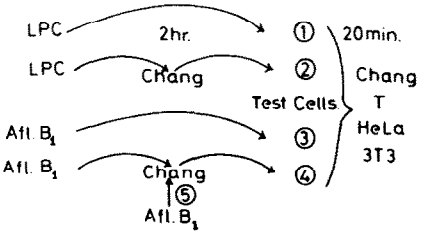

Fig. 2. Scheme for preincubation of aflatoxin B_1 with Chang liver cells (tables 3–4).

keeping with the more sensitive nature of these cells to aflatoxin.

4. Discussion

The different susceptibilities of mammalian cells in culture to the cytotoxic action of aflatoxin B_1, as measured primarily by the inhibition of RNA synthesis could be explained by variations in cell permeability as has been found for liver cells [2]. The present investigations using medium transfer however suggest that certain cells, notably liver cells, when exposed to aflatoxin for a short period produce a more potent cytotoxin than the original aflatoxin itself. Whether this product arises from the cells themselves as a result of exposure to aflatoxin, or is produced as a result of the metabolism of aflatoxin has not yet been determined. This observation may explain the singular susceptibility of the liver to the tumorigenic action of this carcinogen. Attemps were made to isolate this more potent inhibitor from aflatoxin solutions preincubated with rat liver cells. The cell-free supernatant was extracted with chloroform and the concentrated extract chromatographed by TLC silica gel plates in chloroform–acetone (4:1). Several metabolic fluorescent products with spectra different to aflatoxin B_1 were isolated from the plates by methanol extraction. None however showed any greater inhibitory capacity than did the original aflatoxin, which may only imply that the product is either chloroform insoluble or else is not fluorescent. The identification of this product thus awaits further investigation.

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