Drug resistance patterns and susceptibility to aflatoxin B₁ of strains of *Escherichia coli* and *Staphylococcus aureus*

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Summary. The antibacterial properties of aflatoxin B₁ have been evaluated against antibiotic-resistant clinical isolates of *Escherichia coli* and *Staphylococcus aureus*. The inhibition of growth ranged from 11.5 to 60.0% and 4.5 to 18.5% in the strains of *S. aureus* and *E. coli*, depending on the extent of drug resistance. Aflatoxin-B₁ binding varied with toxin concentration, the presence of surfactants (Tween-80 or EDTA) as well as with the antibiotic-resistance pattern; binding was maximal in antibiotic-sensitive strains and least in the most resistant strains. Binding of aflatoxin B₁, correlated with growth inhibition. Aflatoxin B₁ also caused leakage of cell contents and decrease in inulin uptake, effects which were also concentration dependent.

Introduction

Micro-organisms have been used for bioassay (Clements, 1968; Jayaraman *et al.*, 1968; Boutibonnes and Jacquet, 1976) and detoxification (Ciegler *et al.*, 1966) of aflatoxins. The lethal effects of aflatoxins on micro-organisms include: growth inhibition (Arai *et al.*, 1967; Burmeister and Hessel-tine, 1966); biochemical alterations in *Bacillus megaterium* (Lillehoj and Ciegler, 1968; Beuchat and Lechowich, 1971); inhibition of oxygen uptake (Nezval *et al.*, 1970); induction of lysogenic strains (Wragg *et al.*, 1967); inhibition of enzyme induction or biosynthesis (Black and Altschul, 1965; Chiids and Legator, 1966; Lillehoj and Ciegler, 1970; Anand, 1972); and alteration of RNA metabolism and decrease in cell numbers, protein concentration, RNA and DNA content in cell cultures (Gabliks *et al.*, 1965).

Aflatoxins are also mutagenic and carcinogenic (Legator, 1966; Lijinsky, 1968; Kinoshita *et al.*, 1968) and mimic the action of actinomycin D and mitomycin C (Wragg *et al.*, 1967; Nezval and Bosenberg, 1970). Despite so much research, the cytotoxic action of aflatoxins is poorly understood. In the present investigation we examined the relationship between resistance of micro-organisms to antimicrobial agents and their susceptibility to aflatoxin B₁ to clarify the mode of action of the latter.

Materials and methods

Organisms

Clinical isolates of *Staphylococcus aureus* and *Escherichia coli* were obtained from the Post-Graduate Institute of Medical Education and Research, Chandigarh 160 012, as also were *S. aureus* strain NCTC 6571 and *E. coli* strain NCTC 10418. Strains were grown for 24 h at 37°C on slopes of Trypticase Soy Agar and then held at 4°C and subcultured fortnightly. Antibiotic susceptibilities were determined by the disk technique (Stokes, 1975).

Aspergillus parasiticus strain ATCC 15517 was grown on Sabourauds Agar for 5 days at 28°C and held at 4°C. Basmati rice was used as the substrate for aflatoxin production at 28 ± 1°C for 6 days (Shotwell *et al.*, 1966). Aflatoxin B₁ was purified from crude aflatoxin on thin chromatographic plates (Pons and Goldblatt, 1965) and quantitated spectrophotometrically by the procedure of Nabney and Nesbitt (1965).

Effect of aflatoxin B₁ on growth

To 3 ml of sterile Trypticase Soy Broth were added aflatoxin B₁ 10 µg/ml and 0.1 ml of a 100-fold dilution of a 24 h culture of the test strain. The broths were then incubated for 24 h at 37°C. The control (without toxin) was processed in the same manner. Growth was monitored by measuring changes in absorbance at 540 nm. The percentage inhibition of growth was calculated as follows.

$$\text{Percentage inhibition} = \frac{\text{Control OD} - \text{Test OD} \times 100}{\text{Control OD}}$$

Preparation of cell suspensions

Test strains were grown for 24 h at 37°C in trypticase broth. Cells were centrifuged at 10 000 *g* for 15 min. The packed cells were washed twice with phosphate-buffered saline (PBS; 0.02 M, pH 7.2) with intermittent suspension in PBS and centrifugation. The cells were finally suspended in PBS to an absorbance of 1.0 or 1.5 at 540 nm.

Binding studies

Aflatoxin B₁ 60 µg/ml was added to test cell suspensions of *E. coli* strain B111Q (5 ml/tube) and, after mixing, incubated for 2 h at 37°C. The controls without toxin were processed likewise. After incubation the cells were centrifuged; packed cells and supernate were used for estimation of bound and free toxin respectively. Binding of aflatoxin B₁ by test bacteria was also studied in the presence of Tween-80 0.05% v/v or EDTA 0.05% w/v in the same manner. The packed cells were washed twice

and to aflatoxin B₁. Thus the bacteria resistant to most antibiotics were least susceptible to aflatoxin B₁. However, in the strains of *E. coli* examined the same degree of relationship between antibiotic resistance and action of aflatoxin B₁ was not observed because the organism grew at this low concentration of aflatoxin B₁ (10 µg/ml).

Binding of aflatoxin B₁ by bacteria decreased progressively with the development of resistance to an increasing number of antibiotics (table II). More aflatoxin was bound by strains of *S. aureus* (5.9–10%) than by strains of *E. coli* (6.5–8.8%). Although the differences in bound aflatoxin B₁ between strains of *S. aureus* (1.3–4.2%) and *E. coli* (0.1–2.2%) were less marked, a definite relationship between bound toxin and the extent of drug resistance could still be observed.

Table I. Susceptibility to antibiotics of strains of *E. coli* and *S. aureus*

Antibiotic	Concentration (µg/disk)	<i>S. aureus</i> strain					<i>E. coli</i> strain					
		NCTC 6571	12988	12911	12878	SD	NCTC 10418	5994	13919	5995	13802	13804
Ampicillin	10	—	—	—	—	—	—	—	±	—	+	±
Cephaloridine	30	—	+	+	—	±	—	—	—	—	+	+
Chloramphenicol	30	—	—	—	—	+	—	—	—	—	+	+
Gentamicin	10	—	—	+	—	—	—	—	—	—	—	—
Kanamycin	30	—	—	±	+	—	—	—	—	+	+	+
Methicillin	50	—	—	+	+	+	—	—	—	—	—	—
Streptomycin	10	—	—	—	+	+	—	—	±	+	+	+
Tetracycline	30	—	—	—	+	+	—	—	—	—	+	+

+ = Resistant; — = sensitive; ± = moderately sensitive.

with PBS. The amount of aflatoxin B₁ estimated in washings represented loosely bound toxin. Firmly bound toxin was extracted from packed cells with chloroform.

Permeability studies

Aflatoxin B₁ 20–80 µg/ml and inulin 8 µg/ml were added to test cell suspensions of *E. coli* strain B111Q (5 ml/tube). Control cells suspension contained the same amount of inulin but no toxin. After incubation for 2 h at 37°C, the cells were centrifuged at 10 000 *g* for 15 min and the supernate was assayed for residual aflatoxin B₁ (Nabney and Nesbitt, 1965), inulin (Corcoran and Page, 1939) and cell contents leakage was measured by UV absorbance at 260 and 280 nm.

Results

The susceptibilities to antibiotics and aflatoxin B₁ of the strains of *E. coli* and *S. aureus* studied are shown in tables I and II. Strains of *S. aureus* were generally more susceptible to aflatoxin B₁ than strains of *E. coli* but in both instances there was a direct relationship between resistance to antibiotics

Table II. Aflatoxin B₁ growth inhibition of and binding to strains of *E. coli* and *S. aureus*

Organism	*Resistance pattern			Percentage of toxin bound	Percentage growth inhibition by aflatoxin B ₁ 10 µg/ml
	S	MS	R		
<i>S. aureus</i>					
Reference (NCTC 6571)	8	—	—	10.13	60.00
12988	7	—	1	8.40	58.80
12911	3	1	3	6.20	33.00
12878	4	—	4	5.90	27.30
SD	1	1	4	5.90	11.50
<i>E. coli</i>					
Reference (NCTC 10418)	6	—	—	8.80	18.50
5994	6	—	—	8.70	18.00
13919	4	2	—	7.60	15.80
5995	4	—	2	7.10	14.80
13804	1	1	4	6.50	11.50
13802	1	—	5	6.56	4.50

*S = Sensitive; MS = moderately sensitive; R = resistant.

Binding of aflatoxin B₁ by suspensions of *E. coli* increased from 7 to 30 µg as the concentration of toxin in the medium was raised from 20 to 80 µg/ml, however, as shown in table III, the percentage of bound toxin remained within a narrow range (35–38%). At a concentration of 60 µg/ml, the percentage of aflatoxin B₁ bound by *E. coli* increased by 9.8% in the presence of Tween-80 0.05% and by 21.3% in the presence of EDTA 0.05% as shown in table IV. Inulin uptake by *E. coli* decreased from 72.5 to 18% when the toxin concentration in the suspending medium was increased from 0 to 80 µg/ml. The supernate from a toxin-treated (20–80 µg/ml) suspension of *E. coli* strain B111Q showed increasing absorbance at 260 nm and 280 nm (figure) which paralleled increases in toxin concentrations in the suspending medium (20–80 µg/ml).

Discussion

Changes in functional and structural properties of tumour cell mitochondria (Pedersen, 1978), formation of aberrant cells (Beuchat and Lechowich, 1971), preferential inhibition of gram-positive organisms by aflatoxin B₁ and higher susceptibility of protoplasts in comparison to parent strains

Table III. Binding of aflatoxin B₁ to cell suspensions of *E. coli* strain B 111Q

Toxin concentration (µg/ml)	Percentage of toxin bound	Amount bound (µg)
20	35.00	7.00
40	35.35	14.02
60	36.18	21.71
80	38.40	30.72

Absorbance (OD) of organism suspension adjusted to 1.5 at 540 nm.

Fig. Effect of aflatoxin B₁ (20–80 µg/ml) on inulin uptake (■—■) and intracellular contents leakage measured by OD at 260 nm (●—●) and 280 nm (○—○) in *E. coli* strains B 111Q.

(Tiwari *et al.*, 1984) suggested the cell membrane as a possible target-site for the action of aflatoxin B₁. Bacterial resistance to antibiotics has been associated with decreased permeability (Okamoto and Mizzuno, 1964) and reduced binding by resistant cells (Salton and Tomasz, 1974). Strains of bacteria varying in susceptibility to antimicrobial agents were selected to study the effect of aflatoxin B₁, the assumption being that these strains would also vary in binding of and permeability and susceptibility to the aflatoxin.

The direct relationship between bound toxin and susceptibility to antibiotics in strains of *E. coli* and *S. aureus* supports the above hypothesis and relates to permeation of cells by aflatoxin. The increased

Table IV. Binding of aflatoxin B₁ to cell suspension of *E. coli* strain B 111Q in presence of Tween-80 0.05% v/v or EDTA 0.05% w/v in TSB

Aflatoxin concentration (µg/ml)	Effector	*Loosely bound toxin (µg)	**Firmly bound toxin (µg)	Percentage of toxin bound
60	—	6.9	3.9	17.2
60	Tween-80	9.2	7.0	27.0
60	EDTA	13.3	9.8	38.5

Cell suspension absorbance (OD) adjusted to 1.0 at 540 nm.

* Loosely bound represents toxin released in washings.

** Firmly bound represents toxin extracted with chloroform after washings.

binding of aflatoxin B₁ by *E. coli* strain B111Q as toxin concentrations increased from 20 to 80 µg/ml, and the enhancement of this effect by Tween 80 or EDTA, provides further evidence that the cell membrane is the target-site. The susceptibility of strains of *S. aureus* compared to strains of *E. coli*, despite similar degrees of toxin-binding suggests that cell membranes of the former are more permeable to aflatoxin B₁. Studies with *B. megaterium* suggest that the cytotoxic effects of aflatoxin may be mediated by a single locus (Tiwari *et al.*, 1985).

The decreased uptake of inulin and increased leakage of UV-absorbing cell contents with increase in aflatoxin-B₁ concentration, provides further confirmation of the cell membrane as the locus for

aflatoxin B₁ action. Decrease in oxygen uptake in prokaryotes in the presence of aflatoxin (Nezval *et al.*, 1970) also provides indirect evidence of cell membrane involvement. Deficiency in cell membrane functions can be manifest in several metabolic functions, e.g., inhibition of growth, enzyme production or induction, decrease in cell numbers, DNA to protein ratio and alteration of RNA metabolism (Gabliks *et al.*, 1965; Arai *et al.*, 1967; Lillehoj and Ciegler, 1970; Stark, 1980; Niranjani *et al.*, 1982). Hence, it is concluded that the primary effect of aflatoxin appears to be on cell membranes and lethality in micro-organisms is partially due to leakage of cell contents and decreased uptake of essential nutrients.

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