MECHANISM OF INHIBITORY EFFECTS OF AFLATOXIN B₁ IN *ESCHERICHIA COLI* AND *SALMONELLA TYPHI*

R. P. TIWARI, C. K. DHAM, L. K. GUPTA, S. S. SAINI,
T. C. BHALLA AND D. V. VADEHRA

*Department of Microbiology, Panjab University,
Chandigarh-160014, India*

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The inhibitory effects of aflatoxin B₁ on growth, respiration and cell viability were studied in *Escherichia coli*, *Salmonella typhi*, L forms of *S. typhi* B-34-6 and protoplasts of *E. coli* and *S. typhi*. Tween-80 (0.05% v/v) or EDTA (0.05% w/v) accentuated the lethal effects of aflatoxin B₁ in test organisms. The protoplasts and L forms were more sensitive than the parent strain to the inhibitory effects of aflatoxin B₁. Cells from all growth phases were equally susceptible to the growth inhibition. The *E. coli* and *S. typhi* cells bound approximately 10–24% more aflatoxin B₁ with Tween-80 (0.05%) or EDTA (0.05%) added than with aflatoxin B₁ alone. Aflatoxin B₁ caused a substantial decrease in oxygen uptake in test preparations and this decrease was greatest in the L forms of *S. typhi*, followed by protoplasts, protoplast membranes, whole cells and cell debris.

Micro-organisms have been used extensively to detect and assay aflatoxins (1–7) because of the speed and simplicity of the methods. BURMEISTER and HESSELTINE (8) found gram positive microbes to be more sensitive to aflatoxin inhibition than gram negative ones. *Flavo bacterium auranticum* removes aflatoxins from solutions but lacked corresponding degrading enzymes (9). Aflatoxin binds to intact cells, protoplasts and protoplast membranes with and without nucleic acid (10). Aflatoxins inhibit metabolic and biosynthetic activities in micro-organisms including respiration (6), DNA synthesis (11), histidase mRNA transcription (12), penicillinase induction (13), and decrease in DNA to protein ratio (11). Aflatoxins are proved “hepatocarcinogens” despite some analogies and differences with antibiotics (14, 15). Despite such a large amount of literature on aflatoxins, not much information is available on the mode of action of aflatoxin B₁ in prokaryotes. The present study was done to explore the mode of inhibition of aflatoxin B₁ in *S. typhi* and *E. coli*.

MATERIALS AND METHODS

Aspergillus flavus NRRL-2999, a potent aflatoxin producer procured from Peoria, U. S. A., was used to produce aflatoxin B₁, using rice as the substrate (16). The culture was grown for 5 days at 28° on Sabourad's agar, then maintained at 4°. Subculturing was done every month.

E. coli, *S. typhi* and the L forms of *S. typhi* B 34-6 were taken from departmental culture collections. *E. coli* and *S. typhi* were grown on trypticase soy agar for 24 hr at 37° and maintained at 4°. They were subcultured fortnightly. The L forms were also maintained at 4°.

Protoplasts of *E. coli* and *S. typhi* were prepared by penicillin G treatment as described previously (17).

Effect on growth. Aflatoxin B₁ (20 µg/ml) and Tween-80 (0.05% v/v) or EDTA (0.05% w/v) were added to presterilized trypticase soy broth (TSB) at under 15 psi at 121°. The tubes were then inoculated with 0.1 ml of 16 hr old diluted (1: 100) culture. The inhibitory effects of aflatoxin B₁ on *E. coli*, *S. typhi* and L forms of *S. typhi* B-34-6 were monitored at every 4 hr of incubation at 37° by measuring the increase in absorbance at 540 nm. The controls (without toxin) were also treated in the same manner.

The inhibitory effects of aflatoxin B₁ on growth were also studied by viable counting. *S. typhi* (10⁴/ml) and *E. coli* (10⁵/ml) were suspended in TSB containing an appropriate amount of aflatoxin B₁ (8–150 µg/ml) or aflatoxin B₁ (8–32 µg/ml) and Tween-80 (0.05% v/v) and incubated at 37° for 24 hr. Samples were withdrawn aseptically at 4-hr intervals and the viable count was determined by standard plate count.

Effect on cells from different growth phases. *S. typhi* and *E. coli* were grown in TSB at 37° for 2, 4 or 8 hr. At the end of each incubation period, the cells were centrifuged at 10,000 × *g* for 15 min. The packed cells were then transferred to TSB containing aflatoxin B₁ (20 µg/ml) alone or together with Tween-80 (0.05% v/v) or EDTA (0.05% w/v) so as to give an initial absorbance of 0.05 units at 540 nm. These cultures were incubated for 24 hr at 37°. The effect on growth was monitored by measuring changes in absorbance at 540 nm after 24 hr. The percentage of inhibition (PI) was calculated using the formula:

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

Effect on viability of protoplasts. Aflatoxin B₁ (20 µg/ml) was added to *S. typhi* and *E. coli* protoplast preparations (10⁶/ml) in 5% sucrose containing 100 units of penicillin and 0.1% magnesium sulphate. The mixture was incubated at 37° and the protoplast viability was checked hourly for four hours. Controls were also processed in the same manner.

Binding studies. Cells grown overnight at 37° in TSB were centrifuged (10,000 × *g* for 15 min), then washed three times with phosphate buffer saline (PBS,

Fig. 1.

Fig. 1. The effects of aflatoxin B₁ (20 µg/ml) in the absence (●---●) and presence of Tween-80 (△---△) or EDTA (●---●) in *S. typhi*. Controls for *S. typhi* (○—○) and the L forms (■—■) are shown in solid lines. ■---■ represents the growth in tests (aflatoxin B₁ µg/ml) with the L forms of *S. typhi* B-34.

Fig. 2.

Fig. 2. Effects of aflatoxin B₁ (20 µg/ml) in the absence (●---●) and presence of Tween-80 (0.05% v/v △---△) and EDTA (0.05% w/v ●---●) on the growth of *E. coli* in TSB at 37°. ○—○ represents the control.

pH 7.2, 0.02 M). They were finally suspended in PBS to give an OD of 1.0 at 540 nm. Aflatoxin B₁ (60 µg/ml) was added to the cell suspensions and incubated for 2 hr at 37°. The cells were centrifuged again at the end of the incubation period. The packed cells were given three washings with PBS (10 ml) with intermittent mixing and centrifugation. The supernatant from each washing represented toxin released in the 1st, 2nd and 3rd washings. Finally the cells were sonicated at 25 kHz for 15 min. The toxin binding was also studied in the presence of Tween-80 or EDTA. Extraction and estimation of aflatoxin was done spectrophotometrically as described earlier (18). A similar method was followed for aflatoxin B₁ binding to protoplasts except that the incubation was done in 5% sucrose containing 0.1% magnesium chloride and aflatoxin B₁ (40 or 80 µg/ml). The protoplasts were not washed.

Effect on respiration. Cell suspensions of *E. coli*, *S. typhi* and the protoplasts were prepared as described in the toxin binding experiment. The washed cell suspension was sonicated at 25 kHz for 15 min and centrifuged at 10,000 × *g* for 15 min. The pellet (cell debris) was suspended in PBS to give an absorbance of 1.0 at 540 nm. The effect of aflatoxin B₁ (20 µg/ml) alone and in the presence of Tween-80 (0.05% v/v) was studied with a Warburg Apparatus using the standard procedure of UMBRET *et al.* (19).

Fig. 3.

Fig. 3. Growth inhibition at 0, 2, 4 and 8 hr in cells of *E. coli* by aflatoxin B₁ alone (A) and in the presence of Tween-80 (0.05% B) and EDTA (0.05% C) in TSB at 37°. The respective values for *S. typhi* cells are shown in solid bars.

Fig. 4.

Fig. 4. Effect of aflatoxin B₁ (16–150 µg/ml) in the presence and absence of Tween-80 (0.05% v/v) on the viable count of *S. typhi* in trypticase soy broth. Control (●) and tests with 16 µg (▲), 32 µg (○), 48 µg (□), 100 µg (■) and 150 µg (◐) of aflatoxin B₁ are shown in solid lines. Tests with Tween-80 and aflatoxin B₁ are shown in broken lines.

RESULTS

The aflatoxin B₁ effect on the growth of *E. coli*, *S. typhi* and the L forms of *S. typhi* B-34-6 and shown in Figs. 1 and 2. Slight growth inhibition was observed in *E. coli* and *S. typhi* in the presence of aflatoxin B₁. The addition of Tween-80 (0.05% v/v) resulted in a further decrease in the growth rate. The growth rate was almost 0 in the test L forms of *S. typhi* compared to the control.

Aflatoxin B₁ (20 µg/ml) inhibited growth equally in *E. coli* and *S. typhi* cells grown for 2, 4 or 8 hr in toxin free TSB and then transferred to TSB containing aflatoxin B₁. Addition of Tween-80 (0.05% v/v) or EDTA (0.05% w/v) accentuated the effect of aflatoxin B₁ on *E. coli* and *S. typhi* cells irrespective of the growth phase (Fig. 3).

The viable count decreased progressively with increase in aflatoxin B₁ concentration in the medium. Tween-80 (0.05%) accentuated the lethal effects of aflatoxin B₁ at all concentrations (Fig. 4). The viable count decreased by 4 and

Fig. 5. Effect of aflatoxin B₁ (20 µg/ml) on the viability of protoplasts of *E. coli* (●) and *S. typhi* (▲). Controls are shown in solid lines and tests in broken lines.

Fig. 6. Effect of aflatoxin B₁ on oxygen uptake in: a) L forms of *S. typhi* B-34 (●) and cell debris (○). b) Protoplasts of *E. coli* (□) and *S. typhi* (▲). c) *E. coli* (■) and *S. typhi* (●) cells.

The respective controls and tests are shown in solid and broken lines respectively. Δ and $\times$ represent the effects of aflatoxin B₁ in the presence of EDTA (solid lines) and Tween-80 (--- broken line with dots) in *S. typhi* and *E. coli* cells respectively.

7.5 log cycles in *S. typhi* and *E. coli* respectively when the toxin concentration was 32 µg/ml and Tween-80 (0.05% v/v). The death rate was much faster at the higher toxin concentration (48 µg/ml) in the presence of Tween-80 (0.05%) where no viable count was observed even after 12 hr of incubation. The viability of protoplasts decreased by 5 log cycles just after 4 hr of incubation at 37° (20 µg/ml aflatoxin B₁). The decrease in viable count was observed in *E. coli* and *S. typhi* protoplasts at 20 µg/ml aflatoxin B₁ whereas the parent strains were found refractile to this toxin concentration (Fig. 5).

Oxygen uptake was only 50% in the presence of aflatoxin B₁ in protoplasts as compared to the control (without toxin). The oxygen uptake in test cells

Table 1. Binding of aflatoxin B₁ (40–80 µg/ml) in absence and presence of Tween-80* (0.05% v/v) or EDTA** (0.05% w/v) to the cells of *E. coli*, *S. typhi* and to their protoplasts.

Organism	Aflatoxin B ₁ conc. (µg/ml)	Amount of toxin		Total bound aflatoxin (µg)	% Toxin bound
		Released in 4 washings (µg)	Extracted with CHCl ₃ (µg)		
<i>E. coli</i>	60	6.9	3.4	10.3	17.2
	60*	9.2	7.0	16.2	27.0
	60**	13.3	9.8	23.1	38.5
<i>E. coli</i> protoplast	40	—	2.9	2.9	7.0
	80	—	5.6	5.6	7.2
<i>S. typhi</i>	60	6.0	2.3	8.3	13.8
	60*	9.2	7.0	16.2	27.0
	60**	13.0	9.2	22.2	36.6
<i>S. typhi</i> protoplast	40	—	5.1	5.1	12.5
	80	—	10.8	10.8	13.5

Table 2. Effect of aflatoxin B₁ (5–15 µg/ml) on protoplast reversion in *S. typhi* and *E. coli*.

Exposure time with aflatoxin B ₁ (min)	Aflatoxin B ₁ conc. (µg/ml)	<i>S. typhi</i>		<i>E. coli</i>	
		Control CFU/ml	Test CFU/ml	Control CFU/ml	Test CFU/ml
0	0	1.0×10^8	1.0×10^8	1.0×10^7	1.0×10^7
30	5	5.6×10^7	<1	7.1×10^7	<1
30	10	5.6×10^7	<1	7.1×10^7	<1
30	15	5.6×10^7	<1	7.1×10^7	<1
60	10	1.0×10^8	<1	1.0×10^7	<1
120	10	9.2×10^7	<1	8.5×10^6	<1
180	10	8.5×10^7	<1	1.0×10^8	<1

decreased further on addition of Tween-80 (0.05% v/v) or EDTA (0.05% w/v). A similar trend was observed with cell debris suspensions, protoplasts and the L forms of *S. typhi*. The maximum decrease was observed in the L forms followed by protoplasts, protoplast membranes, whole cells and cell debris (Fig. 6).

E. coli and *S. typhi* cell suspensions bound more aflatoxin B₁ in the presence of Tween-80 or EDTA with the maximum (36–38%) occurring in the presence of EDTA. The protoplasts of *E. coli* and *S. typhi* bound less aflatoxin B₁ compared to the parent strain (Table 1).

The protoplast reversion to the parent strain was almost nil even after 30 min of contact at 5 µg/ml aflatoxin B₁ concentration (Table 2).

DISCUSSION

Despite the large amount of literature available on the biological activities of

aflatoxins, including mutagenicity and carcinogenicity, the mode of action still remains obscure. The mechanism most commonly suggested is its binding to DNA (20). The earlier work done by BURMEISTER and HESSELTINE (8) and in this laboratory found gram positive organisms comparatively more susceptible to aflatoxin B₁ inhibition than gram-negative organisms. This information along with the information on the formation of aberrant cells in *B. megaterium* (21) led us to hypothesize that the cell wall and cell membrane have a role in the aflatoxin B₁ mode of action. It is obvious from growth inhibition studies that the cell wall in *E. coli* and *S. typhi* somehow blocks the access of aflatoxin B₁ to the cell membrane (the most probable target), since the protoplasts of the same strains were far more susceptible even at a low aflatoxin B₁ (5 µg/ml) concentration while the parent strains were refractile to aflatoxin B₁ growth inhibition even at a higher toxin concentration (20 µg/ml). Thus it is possible that access of aflatoxin to the cell membrane for permeation into the cells depends more critically on aflatoxin B₁ binding to the cell wall or on its accumulation in periplasmic space than on merely the chemical composition of the cell wall or the cell membrane, since both parent strains and protoplasts can bind aflatoxin B₁. The protoplasts bound less aflatoxin B₁ but were much more sensitive to it than the parent strains, thus confirming the role of the cell wall as a barrier to the access of aflatoxin to the cell membrane. The decreased oxygen uptake by the L form protoplasts, protoplast membranes, whole cells and cell debris amply proves the involvement of cell membrane in aflatoxin B₁ action. The role was further confirmed when EDTA, a chelator known to affect permeability, released cell wall polysaccharides with little or no loss of other cell components (22). This accentuated the effects of aflatoxin B₁ probably by increasing the permeation of aflatoxin B₁ into the cells, resulting in further growth inhibition, decreased oxygen uptake and more toxin binding in test preparations. The results of decreased oxygen uptake and toxin binding are in agreement with the earlier findings (8, 13). Therefore, it appears that the lipopolysaccharides in *S. typhi* and *E. coli* may be a barrier to aflatoxin access to the cell membrane, since the protoplasts reverted to 0 even at 5 µg/ml of aflatoxin B₁ after only 30 min contact, whereas the parent strain were refractile even at 20 µg/ml of aflatoxin B₁. Hence, it is concluded that the aflatoxin B₁ effects on procaryotic cells is related to:

- (a) The binding of aflatoxin to the cell wall and/or its accumulation in periplasmic space for permeation.
- (b) Ultimately the permeation of aflatoxin B₁ into the cells and the resulting observed effects reported in the literature decreased DNA to protein ratio (11), inhibition of growth (8, 10, 21) and cell division as well as DNA replication seem to be after effects only. It is thus possible that aflatoxin B₁ effects the cell membrane as the primary target rather than DNA per se.

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