Conditions for Induction of Bacteriophage from Lysogenic Bacillus megaterium with Aflatoxin B₁

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Received for publication 21 December 1978

The present study was conducted to determine whether or not aflatoxin B₁ was an effective inducing agent for lysogenic bacteria and to characterize some of the parameters involved in induction. A lysogenic strain of Bacillus megaterium (NRRL-B-3698) and an indicator strain of this species (NRRL-B-3694) were used. Cultures of the lysogenic strain were incubated for various periods of time in the presence of aflatoxin B₁. Plaque-forming units as well as colony-forming units were then determined. Results of the present study indicated that bacteriophage lysogenizing B. megaterium could be induced with aflatoxin B₁. The optimum concentration for induction was 25 μg of toxin per ml of early-log-phase culture. Evidence suggested that: (i) higher concentrations of aflatoxin B₁ formed hydrophobic complexes which would not efficiently induce B. megaterium; (ii) the toxic effect of aflatoxin B₁ severely limited the number of cells which could be induced prior to killing action of the toxin; and (iii) concentrations less than 25 μg of aflatoxin B₁ per ml were not efficient inducers of bacteriophage production nor did they demonstrate the toxic effect observed at higher concentrations.

Researchers have found that certain chemical agents which will induce bacteriophage development in lysogenic bacteria act as carcinogenic, mutagenic, or carcinostatic agents (1, 6, 10, 14, 15). Procedures involving production of infective phage from lysogenic bacteria as a method to screen for chemical compounds that are carcinogenic, carcinostatic, or mutagenic have been described (5, 10, 12-14). Studies on the mechanism of induction demonstrate that inducing compounds have in common the ability to inhibit host cell deoxyribonucleic acid (DNA) metabolism (16, 17).

Aflatoxin B₁ is recognized as one of the most potent hepatocarcinogens in animals (3). There is evidence that DNA synthesis is affected in the presence of this compound (3, 7-9).

Currently, there is controversy as to whether aflatoxin B₁ or an aflatoxin B₁ metabolite (formed by a mammalian mixed function oxidase system) is responsible for inducing bacteriophage development or mutagenesis. Lilley and Ciegler (16) demonstrated that aflatoxin B₁ will induce bacteriophage development in a strain of Bacillus megaterium (used in the presently reported study). However, since these authors did not use a commercial preparation of aflatoxin B₁, Goze et al. (12) implied that the induction observed by the former authors was due to impurities in the toxin. Goze et al. (12), working with a different genus of bacteria (Escherichia coli), demonstrated that induction could only occur with an aflatoxin B₁ metabolite.

Maher and Summers (18) found that purified B₁ can act as a mutagenic agent whereas Ames et al. (2) stated that "the principal limitation of any bacterial system for detecting carcinogens as mutagens is that bacteria do not duplicate mammalian metabolism in activating carcinogens," and hence B₁ metabolism by a mammalian liver homogenate is required for mutagenicity.

The purpose of this study was to determine whether or not B₁ is an effective inducing agent and to characterize some of the parameters involved in induction.

MATERIALS AND METHODS

Bacterial cultures and growth conditions. The present studies were performed with a lysogenic strain of B. megaterium (NRRL-B-3698) and an indicator strain of this species (NRRL-B-3694). The cultures were obtained from L. K. Nakamura of the Northern Regional Research Center, Peoria, Ill. Lysis experiments were carried out in tryptone (5 g/liter)-glucose (1 g/liter)-yeast extract (5 g/liter)-potassium phosphate (1 g/liter) broth (TGY) (pH 7.0 adjusted with HCl). Side-arm flasks (500 ml) containing 25 ml of the previously described medium were inoculated with 0.2 ml of an overnight culture and allowed to grow to 30 to 36 Klett units (Klett-Summerson colorimeter,
The cells were grown in a New Brunswick Scientific Gyrotory incubator shaker (model G25) at 34°C. When cultures reached a value of 30 to 36 Klett units, 25 ml of media containing the appropriate concentration of aflatoxin B1 was added. This addition of 25 ml of media decreased the Klett reading to 15 to 18 units (early log phase of growth). After various periods of incubation, plaque-forming units (PFU) were determined. Appropriate dilutions were made, and 0.1 ml of sample was added to 0.1 ml of indicator cells and incubated for 30 min at 34°C. This was followed by addition of 2.0 ml of soft agar (TGY, 0.9% NaCl, 0.65% agar) and by plating on TGY plates which were then incubated at 34°C for 12 h. Finally, the number of plaques was determined at each dilution. This procedure was also used to determine the number of colony-forming units (CFU), omitting the indicator cells and the 30 min of incubation at 34°C.

**Toxin procedure.** Aflatoxin B1 was purchased as a reagent grade preparation (Calbiochem). Purity was confirmed via melting point and thin-layer chromatography determinations prior to utilization. It was dissolved in acetone (stock solution, 2,000 μg/ml) and added in the appropriate concentration to 25 ml of growth medium. The acetone was then aseptically removed under vacuum.

**Extraction of aflatoxin B1 from aqueous media.** Into a 125-ml separatory funnel, 10 ml of TGY containing toxin was added along with 10 ml of chloroform and thoroughly mixed. The chloroform layer was removed, and the procedure was repeated. Then the chloroform layer was transferred to an 18-by-150-mm test tube and dried under nitrogen. The residue was then redissolved in a benzene-acetonitrile solution (49:1, vol/vol).

The concentration of aflatoxin B1 was determined spectrophotometrically by use of a Beckman DU-2 spectrophotometer set at a wavelength of 360 nm (Beckman Instrument). The absorbance was then compared with a previously established standard curve. Aflatoxin B1 purity was determined by thin-layer chromatography (19) using plates (20 by 20 cm) coated with G-HR silica gel (Brinkmann Instrument) to a thickness of 250 μm. The developing solvent used was chloroform-acetone (4:1, vol/vol).

**RESULTS**

Preliminary experiments (data not shown) indicated that a Klett unit value between 15 and 18 was early (beginning) log phase of growth and that the concentration of acetone used to dissolve the aflatoxin B1 could be successfully removed from the medium without adversely affecting either medium components or cell growth.

It is evident from the data presented in Fig. 1 that the greatest extent of lysis of *B. megaterium* NRRL-B-3695 occurred at a concentration of 25 μg of aflatoxin B1 per ml of medium and to a smaller extent at other concentrations (10 μg/ml and 50 μg/ml). Lysis was found to be due to induction of bacteriophage as evidenced after plating appropriate dilutions of supernatant on indicator *Bacillus* cells (Table 1). When cells were incubated with 25 μg of toxin per ml of medium, a significant increase in PFU could be demonstrated. At this concentration of toxin, the number of PFU was increased by 138 times when compared with the control and 27 times when compared with the 10-μg/ml concentration. At 50 μg of toxin per ml, fewer PFU were observed than found in controls. Further experimentation using various concentrations of toxin demonstrated that at a range of 15 to 35 μg/ml, increased numbers of PFU were obtained when compared to controls. However, it was demonstrated that 25 μg/ml was the optimum concentration for induction.

A time study to determine (i) rate of formation of PFU, and (ii) rate of cell death due to aflatoxin B1 was performed. *B. megaterium* indicator cells (NRRL-B-3694) were used to determine the rate of death of cells due to various concentrations of aflatoxin B1. As the concentration of aflatoxin B1 increased, cellular growth decreased, with the greatest inhibitory effect occurring at a toxin concentration of 50 μg/ml.

The effect of aflatoxin B1 upon the number of CFU of the indicator strain of *B. megaterium* is...
shown in Table 2. The data indicate that the greatest increase in CFU per milliliter occurred in the first hour of incubation. In control cultures, the number of CFU per milliliter increased approximately threefold for every 0.5 h of incubation. However, in the presence of aflatoxin B₁, the number of CFU increased for the first hour of incubation but subsequently decreased.

A parallel experiment was performed with lysogenic B. megaterium cells (NRRL-B-3695). Table 3 indicates the relative increase in number of PFU per milliliter found after certain periods of incubation in the presence of various concentrations of aflatoxin B₁. The data indicate that, for control cultures (0 µg of aflatoxin B₁ per ml), there was a continuous increase in PFU during incubation. In cultures incubated in the presence of either 10 or 25 µg of aflatoxin B₁ per ml, the most significant increase in PFU occurred between 0.5 and 1 h of incubation. However, the greatest increase in PFU from cultures incubated in the presence of 50 µg of toxin per ml occurred between 1 and 1.5 h of incubation.

The final aflatoxin B₁ concentration in the TGY culture medium was determined at the end of the experiment involving different concentrations of aflatoxin B₁ and their effects on B. megaterium NRRL-B-3695. This was done to determine if certain concentrations of toxin would form hydrophobic aggregates. The results are presented in Table 4. The recovered toxin was checked for purity and for the possible presence of metabolites using the thin-layer chromatography method previously cited. The Rᵢ values and fluorescent properties of the recovered toxin were identical to those of the standard, and no metabolites were observed on thin-layer chromatography plates.

Possible interaction between aflatoxin B₁ molecules at a concentration of 50 µg/ml is indicated by data presented in Fig. 2. When the nonionic surfactant Triton X-100 was preincubated with toxin and subsequently added to cells, there was approximately an eightfold increase in the number of PFU per milliliter when compared to cells incubated in the presence of toxin alone. There was no significant difference in the numbers of PFU per milliliter from control cells when compared to cells incubated in the presence of Triton X-100.

**DISCUSSION**

Results from the present study indicate that lysogenic B. megaterium NRRL-B-3695 can be induced to produce bacteriophage by optimum concentrations of aflatoxin B₁. Both turbidometric data and the increase in PFU indicate that lysis of cells occurs. This evidence supports such work as that done by Lillegaard and Ciegler (16). They demonstrated lysis in B. megaterium using aflatoxin that was not pretreated with mammalian liver homogenate.

The mechanism whereby aflatoxin B₁ induces formation of bacteriophage within a lysogenic cell is still unknown. Gelderman et al. (10) stated that lysis appeared to be enhanced by agents which inhibited DNA synthesis and interfered

### Table 1. Effect of toxin concentration upon induction of lysogenic B. megaterium

<table>
<thead>
<tr>
<th>Aflatoxin B₁ (µg/ml)</th>
<th>PFU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.3 x 10⁷</td>
</tr>
<tr>
<td>10</td>
<td>6.7 x 10⁷</td>
</tr>
<tr>
<td>25</td>
<td>1.8 x 10⁸</td>
</tr>
<tr>
<td>50</td>
<td>1.1 x 10⁹</td>
</tr>
</tbody>
</table>

* Cultures were incubated for 2 h at 34°C in the presence of toxin.

### Table 2. Effect of toxin concentration and incubation time on B. megaterium indicator cells

<table>
<thead>
<tr>
<th>Aflatoxin B₁ (µg/ml)</th>
<th>CFU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3 x 10⁷</td>
</tr>
<tr>
<td>10</td>
<td>1 x 10⁷</td>
</tr>
<tr>
<td>25</td>
<td>3 x 10⁷</td>
</tr>
<tr>
<td>50</td>
<td>9 x 10⁷</td>
</tr>
</tbody>
</table>

* Incubation time in hours.

### Table 3. Effect of toxin concentration and incubation time upon induction of lysogenic B. megaterium

<table>
<thead>
<tr>
<th>Aflatoxin B₁ (µg/ml)</th>
<th>% PFU/ml*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>25</td>
<td>4,400</td>
</tr>
<tr>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

* Results are expressed as the percent increase in PFU per milliliter compared to the number of PFU per milliliter after the first 0.5 h of incubation.

### Table 4. Isolation of toxin from inoculated and incubated TGY culture medium

<table>
<thead>
<tr>
<th>Initial concn (µg/ml)</th>
<th>Toxic recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>76</td>
</tr>
<tr>
<td>25</td>
<td>74</td>
</tr>
<tr>
<td>50</td>
<td>75</td>
</tr>
</tbody>
</table>

* In uninoculated TGY medium, when 10, 25, and 50 µg of toxin per ml were added, an average of 85% was subsequently recovered.
with the normal replication cycle of a bacterial chromosome.

The actual binding of aflatoxin B₁ to DNA has been documented. It has been demonstrated to bind to both native and denatured DNA as shown by ultraviolet and visible spectrophotometry and equilibrium dialysis (7, 18, and 20). The mode of action of aflatoxin B₁, both in vitro and in vivo, involves inhibition of DNA-directed ribonucleic acid synthesis and DNA synthesis (4, 18). Thus, it seems likely that aflatoxin B₁ inhibits or in some manner interferes with the normal cellular chromosomal replicative pattern in such a way that induction of bacteriophage is stimulated.

In the present study, a concentration of 25 μg of toxin per ml of medium appeared to be the optimum concentration for induction of B. megaterium phage. It is possible that the lowered efficiency of induction at greater than optimum concentrations of aflatoxin B₁ can be explained by either of two mechanisms.

First, aflatoxin B₁ is nonpolar and, thus, hydrophobic (11). It is possible that, at certain concentrations, an aflatoxin B₁ molecule in an aqueous medium would tend to interact with other molecules of aflatoxin and form complexes (micelles or aggregates) of such size that they could not interact chemically with B. megaterium cells. The cell walls of this microorganism contain a hydrophilic exterior (lipophobic) which may resist interaction with large aflatoxin B₁ complexes, thus preventing induction. Research done by Fujimoto and Ohba (7) indicated the possibility of aflatoxin B₁ aggregate formation. In the present study, data indicated (Table 4) that, after incubating cells with 50 μg of toxin per ml of medium, the percentage of aflatoxin B₁ that could be recovered from the medium was equal to that recovered when other aflatoxin concentrations were used. This could be indicative of an aflatoxin B₁ complex formation that could interact with cells physically but that could not enter the cell or possibly could not interact chemically due to the large size of the aggregate formed.

The second possible explanation for the inefficiency of 50 μg of toxin per ml to cause induction could be due to the effect of the toxin upon cells and its rate of killing cells. All concentrations of aflatoxin B₁ inhibited B. megaterium growth (Table 2). There appeared to be no lysis. Thus, growth inhibition of B. megaterium NRRL-B-3694, a nonlysogen, was not due to bacteriophage production. Growth inhibition may be due to inhibition of DNA-directed DNA or ribonucleic acid synthesis. As the concentration of toxin increased, so did the growth inhibi-

tion, and the final number of CFU decreased (Table 2). At a toxin concentration of 50 μg/ml, the number of CFU began to decrease earlier and with greater intensity as compared to lower concentrations of aflatoxin B₁ (Table 2). Also, at a concentration of 50 μg of toxin per ml, the largest increase in PFU occurred after 1.5 h of incubation as compared to 1 h of incubation with either 10 or 25 μg/ml. These data imply that the rate of cell killing may be so rapid that there is not enough time for efficient prophage induction or that the toxin limits growth of the bacillus to such an extent that the inducible Bacillus population is reduced. Even with the same percentage of induction, there are significantly fewer cells to be induced.

Data in Table 4 also indicate that, when toxin is added to uninoculated medium, incubated, and extracted, an average of 15% of the original toxin cannot be recovered. This may be compared to an average of 25% which could not be recovered from inoculated and incubated medium. However, it is hypothesized that the latter value is due in large part to interaction of toxin with bacterial metabolites secreted into the surrounding medium since an average of 22% could not be recovered when toxin was added to filter-sterilized medium which had been previously inoculated and incubated. This would indicate that, if a metabolite is involved in induction, either it cannot be detected and extracted from the media using the procedures of the presently reported study or it is within the cell biomass.

Data in the present study also appear to indicate the possible formation of hydrophobic micelles or aggregates which are either dispersed or have their formation prevented in the presence of Triton X-100 (Fig. 2). It is also possible that micelles can enter cells in the presence of Triton X-100 because Triton X-100 may alter cellular permeability. This was supported by the observation that the induction efficiency of 50 μg of toxin per ml was significantly enhanced in the presence of this surfactant.

It may be argued that the Triton X-100 only acts to free bacteriophage non-covalently bound to membrane and lipid components (Fig. 2). If so, then a greater number of PFU would be expected in curve B than in curve A. This was not found to be the case. Also, the addition of Triton X-100 to 25 μg of toxin per ml resulted in approximately the same number of PFU as did 25 μg of toxin per ml without Triton X-100 (unpublished data). Both of these findings indicate that there is not a significant increase in release of bacteriophage from noncovalent binding mechanisms by the use of this surfactant alone.
Fig. 2. Effect of aflatoxin B<sub>1</sub> on lysogenic B. megaterium in the presence or absence of Triton X-100. Cells were incubated at 34°C in TGY broth containing either 50 μg of toxin per ml plus 0.002% (wt/vol) Triton X-100 or toxin alone. (A) Control, 1.2 × 10<sup>8</sup> PFU/ml; (B) Triton, 8.3 × 10<sup>8</sup> PFU/ml; (C) Toxin, 3.7 × 10<sup>8</sup> PFU/ml; (D) Toxin + Triton, 2.8 × 10<sup>8</sup> PFU/ml.

LITERATURE CITED


