Aflatoxin $B_1$ Induction of Lysogenic Bacteria

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Received for publication 6 July 1970

A technique for biological verification of aflatoxin $B_1$ was developed based on toxin-mediated induction of lysis in a lysogenic strain of Bacillus megaterium NRRL B-3695. Reduction of culture turbidity was determined at various concentrations of toxin. Incubation of $1.1 \times 10^{-4}$ g (dry weight) of cells/ml of growth medium containing 25 $\mu$g of $B_1$ per ml at 37 C reduced initial turbidity 0.20 absorbance units in 4 hr. If the bacterial lysate of the lysogenic strain, after a 2-hr incubation with 25 $\mu$g of $B_1$ per ml, was plated with a sensitive B. megaterium strain (NRRL B-3694), plaque-forming units increased approximately 150 times relative to the control. Comparable testing of the effects of aflatoxin on the nonlysogenic, sensitive strain demonstrated that 75 $\mu$g of $B_1$ per ml neither induced lysis nor plaque-forming units. Although induction is not an exclusive property of aflatoxin $B_1$, the differential response of the lysogenic and sensitive Bacillus strains to $B_1$ offers a unique and rapid technique for biological verification of the toxin.

Several physical and chemical agents induce bacteriophage development in lysogenic bacteria. This induction ability attributed to a particular substance has been related to carcinogenic, carcinostatic, or mutagenic properties of the agent (1, 7, 9-10, 15, 25, 30-31). Several reports have described procedures involving production of infective phage from lysogenic bacteria as a screening mechanism for compounds that cause tumors, mutations, or are tumoricidal (8, 11, 14, 19, 27). Mechanism studies of inducing substances have shown that they have in common the characteristic of inhibiting host cell deoxyribonucleic acid (DNA) metabolism (20-21, 26).

Aflatoxin $B_1$ is recognized as one of the most potent hepatocarcinogens in rats (3) and also has certain antineoplastic properties (12). Although evidence is mounting to support the concept that the toxin acts through suppression of messenger ribonucleic acid (RNA) synthesis, several studies demonstrate that DNA synthesis is also affected (4). Investigation of the antibiotic action of aflatoxin demonstrated that the toxin exhibited certain radiomimetic properties against Flavobacterium aurantiacum, including preferential inhibition of DNA synthesis (22, 24). The activity of aflatoxin $B_1$ in various biological systems indicated that the toxin might function as an induction agent. This property was demonstrated in preliminary reports (17, 18). Furthermore, Jemmali (16) showed that incubation of a phage of Streptococcus lactis with aflatoxin before exposure to the sensitive bacteria inhibited the infective process.

Clements (5, 6) utilized the sensitivity of Bacillus megaterium to aflatoxin as a confirmatory test for the toxin. This antibiotic disc test can detect 1-$\mu$g quantities of aflatoxin $B_1$. Since some lysogenic bacteria are more sensitive to the action of antibiotics than corresponding indicator strains (28), the lysogenic and indicator bacilli used in the lytic test were examined for their relative sensitivity to various toxins in the antibiotic disc technique. Bacilli were chosen as the test organisms since preliminary experiments with Staphylococcus strains (16, 18) showed that they were not readily adapted to function as the test bacteria in a toxin-verification method.

MATERIALS AND METHODS

Bacterial cultures and growth conditions. The studies were performed with a lysogenic strain of B. megaterium (NRRL B-3695) and an indicator strain of this species (NRRL B-3694). The cultures were obtained from J. T. Wachsman, Dept. of Microbiology, University of Illinois, Urbana. Lysis experiments were carried out in tryptone-glucose-yeast extract broth (TGY) as described by Haynes et al. (13). Tubes were inoculated with $1.1 \times 10^{-4}$ g (dry weight)/ml of exponentially growing cells followed by incubation at 37 C on a reciprocal shaker. Growth was determined at 540 nm in a Beckman B spectrophotometer. After various periods of incubation, plaque-forming units were determined in samples cleared of cellular components by centrifugation. First, the supernatant was diluted to the appropriate level, and then 0.1 ml of the sample was added to 0.9 ml of the indicator strain followed by incubation at 37 C for 30 min and subsequent plating on TGY
agar petri plates. The number of plaque-forming units was determined after 12 hr of incubation at 30 C.

**Toxin procedures.** Aflatoxin, sterigmatocystin, and penicillic acid were produced and recovered by the methods described by Shotwell et al. (32), Lillehoj and Ciegler (23), and Bentley and Keil (2). T-2 toxin (4β, 15 - diacetoxy - 8α(3 - methylbutyryloxy) - 12, 13 - epoxycrotothec-9-en-3α-ol), patulin, and alternariol were supplied by H. Burmester (Northern Regional Laboratory), T. M. McCalla (University of Nebraska, Lincoln), and R. Thomas (Imperial College of Science and Technology, London), respectively. Toxin concentrations were determined chromatographically on thin-layer plates (32) and spectrophotometrically (29). After toxins in organic solvents were added to the growth medium, the solvent was removed under vacuum and proper dilution was made before inoculation.

**Antibiotic disc assay.** Antibiotic disc tests were carried out in petri plates containing TGY agar seeded with growing Bacillus cells. Antibiotic substances in various solvents were added to filter paper discs (.25 inch in diameter, ca. .64 cm); solvent was removed under a stream of warm air. The treated discs were placed on the seeded agar, and the plates were incubated for 12 hr at 30 C.

### RESULTS

**Lytic effect of toxins on strains of B. megaterium.** Aflatoxin B1-induced lysis of a lysogenic strain of B. megaterium (NRRL B-3695) was followed by measurement of the variation in turbidity of a growth medium containing various levels of the toxin (Fig. 1). Although 10 μg of B1 per ml inhibited growth approximately 80% after 4 hr, a dramatic decrease in turbidity was only observed in the presence of 25 μg of the toxin per ml. The initial turbidity decreased 0.20 absorbance units after 4 hr of incubation in 25 μg of B1 per ml.

Turbidity curves indicate that bacterial lysis in the presence of aflatoxin B1 was caused by induction of phage development in the lysogenic strain. This aspect was examined by plating the supernatants of 2 - and 3-hr cultures on the indicator Bacillus (Table 1). The bacterial lysate obtained after a 2-hr exposure to 10 μg of B1 per ml contained 7 × 10⁶ plaque-forming units per ml compared to 2 × 10⁶ infective centers in the control. Although growth determinations (Fig. 1) of the lysogenic strain in 10 μg of B1 per ml did not demonstrate any reduction in turbidity compared to the initial level, plaque count showed that this level of toxin induced release of the plaque-forming units from lysogenic bacteria. Incubation for 2 hr in 25 μg of the toxin per ml increased the yield of infective centers 160 times, whereas a 3-hr culture at this

![Fig. 1. Turbidimetric determination of the effect of aflatoxin B1 on development of a lysogenic strain of Bacillus megaterium (NRRL B-3695). TGY broth inoculated with 1.1 × 10⁻⁴ g (dry weight) per ml of exponentially growing cells and incubated at 37 C on a shaker.](attachment:fig1.png)

**TABLE 1. Aflatoxin B1 induction of plaque-forming units in Bacillus megaterium (NRRL B-3695)**

| Aflatoxin B1 | Plaque-forming units per ml² | 2 hr | 3 hr |
|--------------|------------------------------|------|------|
| μg/ml        |                              |      |      |
| 0            | 2 × 10⁴                       |      | 10⁷  |
| 1            | 2 × 10⁴                       |      | 10⁷  |
| 5            | 7 × 10⁴                       |      | 3 × 10⁷|
| 10           | 21 × 10⁴                      |      | 14 × 10⁷|
| 25           | 320 × 10⁴                     |      | 210 × 10⁷|

*² Plaque counts were determined on supernatants from cultures incubated in tryptone-glucose-yeast extract broth (TGY) at 37 C on a shaker. Samples of the supernatant were diluted and added to 0.9 ml of the indicator strain (NRRL B-3694) followed by incubating for 30 min at 37 C on shaker and plating on TGY agar. These petri plates were incubated at 30 C for 12 hr.
level of toxin increased plaque-forming units 210 times.

The effect of aflatoxin B₁ on the development of the nonlysogenic, indicator strain of *B. megaterium* (NRRL B-3694) is presented in Fig. 2. Although 25 μg of B₁ per ml modified growth only slightly, 50 and 75 μg of the toxin per ml inhibited development about 40 and 75%, respectively, after 4 hr of incubation. Besides reduced sensitivity of the indicator strain to aflatoxin, no plaque-forming units were observed in the supernatants of cells cultured in the presence of 75 μg of toxin per ml for 4 hr.

Since induction ability is not a unique property of aflatoxin B₁, a test was carried out to determine the effect of some other common mycotoxins in turbidometric evaluation of lysis (Table 2). Aflatoxin G₁, M₁, penicillic acid, sterigmatocystin, T-2 toxin, and patulin did not decrease initial turbidity of the lysogenic *Bacillus* after 4 hr of incubation, whereas alternariol reduced the initial absorbance.

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**Fig. 2.** Turbidometric determination of the effect of aflatoxin B₁ on development of an indicator strain of *B. megaterium* (NRRL B-3694). TGY broth inoculated with 1.1 × 10⁻⁴ g (dry weight) per ml of exponentially growing cells and incubated at 37°C on a shaker.

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**Table 2. Influence of various compounds on development of lysogenic Bacillus megaterium (NRRL B-3695)*

| Compound             | Amount (μg/ml) | Absorbance units |
|----------------------|---------------|------------------|
| Control              |               | Increase | Decrease |
| Aflatoxin B₁         | 25            | 0.60      | 0.20     |
| Aflatoxin G₁         | 75            | 0.35      |          |
| Aflatoxin M₁         | 75            | 0.55      |          |
| Penicillic acid      | 75            | 0.15      |          |
| Sterigmatocystin     | 75            | 0.48      |          |
| T-2 toxin            | 50            | 0.58      |          |
| Patulin              | 50            | 0.02      | 0.15     |
| Alternariol          | 50            |           |          |

* Variations in turbidity after 4 hr of incubation, determined in TGY broth initially inoculated with 1.1 × 10⁻⁴ g (dry weight) of exponentially growing cells.

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**Table 3. Antibiotic assay of a lysogenic and nonlysogenic strain of Bacillus megaterium*

| Compound             | Minimum inhibitory amount (μg/disc) |
|----------------------|-------------------------------------|
|                      | Lysogenic                          | Nonlysogenic                       |
|                      | NRRL B-3695                        | NRRL B-3694                        |
| Aflatoxin B₁         | 2                                   | 2                                 |
| Aflatoxin G₁         | 4                                   | 4                                 |
| Aflatoxin M₁         | >120                                | >120                               |
| Penicillic acid      | 60                                  | 30                                |
| Sterigmatocystin     | 120                                 | 60                                |
| T-2 toxin            | 200                                 | 100                               |
| Patulin              | 0.5                                 | 1                                 |
| Alternariol          | 10                                  | 15                                |

* Filter paper discs (¾ inch diam) containing antibiotic were placed on surface of TGY agar seeded with *Bacillus* strain. Petri plates were incubated for 12 hr at 30°C.

**Antibiotic disc assay.** When the effect of aflatoxin B₁ on lysogenic and indicator strains was compared in broth culture, the lysogenic strain was distinctly more sensitive to the action of the toxin than the indicator strain (Fig. 1, 2). Since antibiotic disc tests had been proposed as a detection technique for aflatoxins (5, 6), the method was tried on the lysogenic and indicator *Bacillus* strains as test organisms. The minimum quantities of various mycotoxins required to block growth of the strains visibly are listed in Table 3. Although a 2-μg amount of aflatoxin B₁ could be detected with this technique, the method did not demonstrate enhanced sensitivity of the lysogenic strain to the toxin. Furthermore, evaluation of the antibiotic activity of other mycotoxins by the disc method showed that there
was no pattern of sensitivity discriminating between the lysogenic and indicator test organisms.

DISCUSSION

Since a lysogenic strain of B. megaterium lysed in the presence of aflatoxin B₁ and releases plaque-forming units, whereas the indicator strain does not, the difference can provide a method for verification of the toxin. Although the turbidity test requires larger quantities of toxin than the standard antibiotic method, the test does provide an enhanced selective facility to identify the toxin because many compounds effective in the antibiotic test do not induce lysis. However, because other compounds can also initiate induction, this test does not determine the presence of B₁ absolutely. Therefore, induction suitably serves as a verification tool after initial detection of the toxin by thin-layer chromatography or other methods. Initial chromatographic detection of aflatoxin provides an estimate of toxin concentration, information which in turn can be used in preparing the solutions required for the lysis test. A differential response in turbidity between the lysogenic and indicator strains can be readily validated as an induction process by determination of the release of plaque-forming units.

An interesting facet of the induction test as a tool in screening mold metabolites for toxic substances is the correlation between the ability that a particular compound demonstrates as an induction agent and as a carcinogenic, carcinostatic, and mutagenic material (1, 7, 9–10, 15, 25, 30–31). Since many inducing agents are capable of initiating dramatic changes in tissues of higher animals, the positive identification of an unknown by induction indicates that the substance should be examined further. Therefore, the technique provides an additional, desirable tool not available in routine antibiotic screening methods.

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