FORMATION OF A FACTOR LETHAL FOR S. TYPHIMURIUM TA1530 AND TA1531 ON INCUBATION OF AFLATOXIN B_1 WITH RAT LIVER MICROSOMES

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SUMMARY

Strains TA1530 and TA1531, but not strains C207 and G46, of S. typhimurium (1) were killed when incubated with rat liver homogenate and aflatoxin B_1 but not with either component alone. The lethal effect required the microsomal fraction of liver, was dependent on O_2 and NADPH, and was inhibited by the addition of aniline, SKF-525A, or CO. Less of the lethal factor was formed with liver preparations from rats fed a low protein diet, and more was formed if the rats were administered phenobarbital. Aflatoxin M_1 (4-hydroxy aflatoxin B_1) was not toxic for these bacterial strains in the absence of liver preparations and was much less toxic than aflatoxin B_1 when incubated with rat liver.

Aflatoxin B_1, a metabolite of Aspergillus flavus, is the most potent known liver carcinogen for the rat (2). The available evidence suggests that aflatoxin B_1 is metabolized to an ultimate carcinogen in vivo, since hypophysectomy inhibits its hepatocarcinogenicity (3), its activity at other sites is much lower than in the liver (4), and there is a wide species difference in susceptibility to its carcinogenicity (5).

In the experiments reported here we have found that incubation of rat liver homogenates or fortified microsomes with aflatoxin B_1 produces a factor which is much more toxic than aflatoxin B_1 for certain Salmonella typhimurium histidine auxotrophs. It appears probable that the enzymatic conversion of aflatoxin B_1 to a highly toxic metabolite(s) is responsible for the lethal effect.

MATERIALS AND METHODS

Male CD-random-bred rats, 200-250 g, from the Charles River Breeding Laboratory (Wilmington, Mass.) were fed a diet containing 3 or 30% casein for at least 7 days prior to the experiments (6); in some cases the rats fed 30% casein also received drinking water containing 0.1% of sodium phenobarbital (7). The animals were guillotined, the livers washed in ice-cold isotonic saline, and 5 g portions of liver homogenized in 15 ml of 150 mM KCl with a Potter-Elvehjem-type homogenizer. Post-mitochondrial supernatant fractions were obtained by centrifugation at 9,000 g for 10 min; supernatants from 3 animals were pooled for each assay. In some cases microsome fractions were prepared by centrifugation of the post-mitochondrial supernatant fraction for 60 min at 100,000 g; the microsomal pellet was resuspended in 150 mM KCl so that 1 ml contained microsomes from 250 mg of liver.

Inocula of S. typhimurium G46 (a histidine-requiring missense mutant), C207 (a histidine-requiring frameshift mutant), TA1530 (G46 with a single deletion through the galactose operon, biotin operon, excision repair system for DNA (uvrB gene), and chlorate-resistance genes), or TA1531 (C207 with the same deletion as TA1530) (1) were kindly supplied by Dr. M. S. Legator, U. S. Food and Drug Administration, Washington. These inocula were grown overnight in 5 ml of Bacto nutrient broth (Difco Laboratories, Detroit, Michigan) in a shaking water bath at 37°. The bacteria were collected by centrifugation and resuspended in 1.5 ml of 0.9% NaCl; 0.1 ml of these suspensions contained 3-4 x 10^8 bacteria of strain TA1530, 4-6 x 10^6 for TA1531, and 7-8 x 10^8 for strains G46 and C207.

For the assays each flask contained 100 µmoles KCl, 20 µmoles glucose-6-phosphate, 25 µmoles $MgCl_2$, 300 µmoles sodium phosphate titrated to pH 7.4 with HCl, 1.5 µmoles NADP, 0.1 ml bacterial suspension, 0.1 µmoles aflatoxin B_1 (Calbiochem, Los Angeles, California) dissolved in 30 µl dimethyl sulfoxide or the solvent alone, and a predetermined amount of post-mitochondrial supernatant in a total volume of 3.0 ml. For the studies on the effects of various

inhibitors of the liver system the incubation medium contained microsomes equivalent to 25 mg of liver and 1 unit of glucose-6-phosphate dehydrogenase in place of the post-mitochondrial supernatant. SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride) was kindly supplied by Smith, Kline and French Laboratories, Philadelphia, Pa. For the studies on the cofactor requirements for the liver system the glucose-6-phosphate, NADP, and post-mitochondrial supernatant were omitted and the system was supplemented with 1.5 µmoles of NADPH or NADH and microsomes equivalent to 25 mg of liver.

All incubations were carried out in 10-ml Erlenmeyer flasks which were shaken in air at 100 strokes/min for 20 min in a 37° water bath in the dark. The reactions were stopped by placing the flasks on ice. The number of surviving bacteria was determined by plating serial dilutions in 50 mM potassium phosphate buffer, pH 6.7, onto Davis minimal agar (8) spread with 0.1 µmoles of biotin and 5 µmoles of histidine per plate. The plates were generally incubated for 72 hrs at 37° before the colonies were counted. The results are expressed as the ratio of the number of colony-forming bacteria in each test situation as compared to the number in control incubation flasks which contained dimethyl sulfoxide in place of aflatoxin B_1. All assays were performed in duplicate. Aflatoxin M_1 was isolated from the urine of rats injected i.p. with 5 mg/kg of aflatoxin B_1. A $CHCl_3$-methanol (85:15) extract of urine was fractionated by chromatography on 1.5 mm silica gel plates (Silica gel HF254, Merck AG, Darmstadt, Germany) with $CHCl_3$-methanol (90:10) as the developing solvent. The band with a R_f of 0.25, after elution with methanol, had the ultraviolet and mass spectra characteristic of aflatoxin M_1 (9,10).

RESULTS AND DISCUSSION

While aflatoxin B_1 does not affect the survival of S. typhimurium TA1530, TA1531, C207 or G46 under the conditions of our experiments, incubation of aflatoxin B_1 with rat liver post-mitochondrial supernatant appears to convert it to a toxic metabolite for strains TA1530 and TA1531. With a sat-

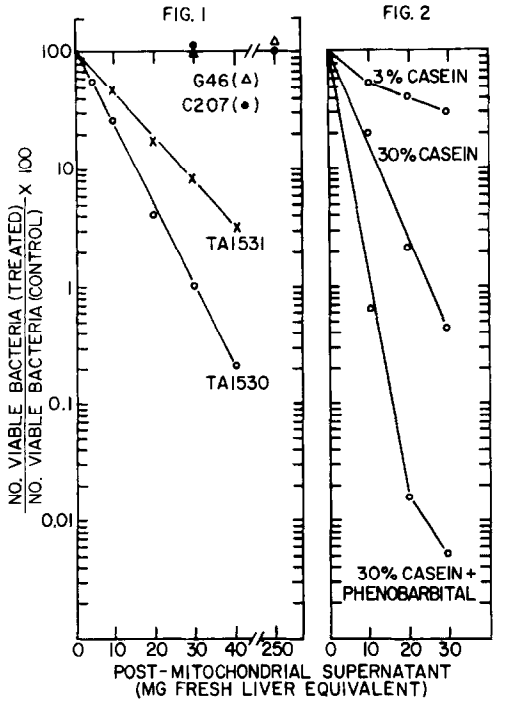

Figure 1. The effect of incubating strains TA1530, TA1531, G46 and C207 of S. typhimurium with aflatoxin B_1 and increasing amounts of post-mitochondrial supernatant from the livers of rats fed the 30% casein diet.

Figure 2. The effect of incubating S. typhimurium TA1530 with aflatoxin B_1 and the post-mitochondrial supernatant from the livers of rats fed 3% casein or 30% casein with or without phenobarbital.

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urating amount of aflatoxin B_1 the survival decreased exponentially with increasing concentrations of post-mitochondrial supernatant (Figure 1). Strains C207 and G46 are essentially unaffected by this factor whereas strains TA1530 and TA1531 are very sensitive. These two strains have been shown to be very sensitive to chemical mutagens (1), probably because they both have a deletion extending through the excision repair system for DNA.

Fractionation of the liver homogenate showed that the metabolic activity for production of the lethal factor(s) is localized in the microsomal fraction and requires a NADPH-generating system and O_2 (Tables 1 and 2). Production of the lethal factor is decreased if N_2 or CO/O_2 (80:20) replaces air as the gas

Table 1. The effect of incubation with various rat liver fractions and aflatoxin B_1 on S. typhimurium TA1530

Liver fraction	$\frac{\text{No. viable bacteria (treated)}}{\text{No. viable bacteria (control)}} \times 100$
Homogenate	0.5
Post-mitochondrial supernatant	0.9
Microsomes + NADPH-generating system	1.0
Post-microsomal supernatant	80

Each flask contained the fraction listed from 25 mg of liver from rats fed the 30% casein diet.

Table 2. The effect of incubations with aflatoxin B_1 and rat liver microsomes under various conditions on S. typhimurium TA1530

Exp. No.	Addition	$\frac{\text{No. viable bacteria (treated)}}{\text{No. viable bacteria (control)}} \times 100$
1	None	104
	NADPH	3
	NADH	39
	NADPH-generating system	1
	" " " + SKF 525A (0.2mM)	15
	" " " + aniline (8mM)	52
2	NADPH-generating system	8
	" " " (CO-O_2 gas phase)	18
	" " " (N_2 gas phase)	17

The NADPH-generating system contained NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase. Each flask also contained microsomes from rats fed the 30% casein diet, aflatoxin B_1 and other cofactors as indicated in the Materials and Methods.

phase, if the mixed-function oxidase inhibitor SKF-525A is added, or if aniline is added as a competitive substrate. The ability of the microsomes to produce the lethal factor is abolished by heating them in a 100° waterbath for 10 min.

The liver microsomes from rats fed a 3% casein diet were less active and the microsomes from rats administered phenobarbital were more active than those from rats fed the 30% casein diet without phenobarbital in the production of the lethal factor (Figure 2). The activities of the drug metabolizing enzymes in this liver fraction are similarly changed by these treatments (7). One metabolite of aflatoxin B_1 produced by the hepatic mixed-function oxidases is aflatoxin M_1 (4-hydroxy aflatoxin B_1); the yield of this metabolite is increased by prior administration of phenobarbital to rats (11). Aflatoxin M_1 appears not to be the toxic metabolite produced in our system since at a concentration of 12 µg/ml it caused no lethality of TA1530 when incubated with heat-denatured post-mitochondrial supernatant. When incubated with unheated post-mitochondrial supernatant equivalent to 250 mg liver, 12 µg/ml of aflatoxin M_1 permitted the survival of 80% of the bacteria while 3 µg/ml of aflatoxin B_1 permitted the survival of only 0.5%.

We have not observed revertants of TA1530 in this system. The lethal factor may not be mutagenic. Alternatively, the difference between the concentrations required to produce mutations and to kill the bacteria may be both small and critical or the factor may be so reactive that it is not able to reach the critical target for mutagenesis.

Scaife (12) has recently reported evidence that rat liver cells produce a cytotoxic metabolite of aflatoxin B_1 which can affect other types of cells not susceptible to aflatoxin B_1. Scaife's metabolite may be identical to that produced by the hepatic mixed-function oxidase in our system. Further studies on the identity and other biological properties of the lethal factor produced by rat liver microsomes are in progress in our laboratory.

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