Vol. 81, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1978 Pages 965-972

METABOLIC ACTIVATION OF AFLATOXINS RELATED TO THEIR MUTAGENICITY[1]

H. L. Gurtoo, R.P. Dahms and B. Paigen
Departments of Experimental Therapeutics and Grace Cancer Drug Center (HLG, RPD) and Molecular Biology (BP)
Roswell Park Memorial Institute
New York State Department of Health
Buffalo, New York 14263

Received February 10, 1978

SUMMARY:

Rat hepatic microsome-mediated DNA-binding and mutagenesis to Salmonella typhimurium strain TA-100 by various aflatoxins and some mixed function oxygenase-mediated metabolites of aflatoxin B_1 were studied. The data indicated a good correlation between the DNA-binding and mutagenesis; a requirement for the intact C_2-C_3 double bond in parent aflatoxins B_1 and G_1; and relative inactivity of aflatoxin B_1 metabolites with an otherwise intact C_2-C_3 double bond.

INTRODUCTION:

Aflatoxins are toxic fungal metabolites. The aflatoxins, produced by certain strains of Aspergillus flavus and parasiticus, are potent hepatotoxic and hepatocarcinogenic agents in a number of animal species (1,2). The four naturally occurring aflatoxins, namely aflatoxin (AF)B_1, AFB_2, AFG_1, AFG_2, administered as a mixture were shown to be potent hepatocarcinogens (3-6) and also induced neoplasms in tissues and organs other than liver (3, 6-8). While AFB_1 and AFG_1 possess the C_2-C_3 double bond, AFB_2 and AFG_2 lack this double bond (Fig. 1). Carcinogenicity and toxicity studies have revealed that AFB_1 is the most potent of all aflatoxins and the order of potency is $AFB_1 > AFG_1 > AFB_2 > AFG_2$ (2,8); AFG_1 is relatively active but AFB_2 and AFG_2 are relatively inactive in most mammalian species (2,8).

Aflatoxins are common contaminants of several human and animal foods in various parts of the world (9,10,11), and have been implicated in the etiology of

[1] Supported by USPHS Grants ES-00993 from the National Institute of Environmental Health-Sciences, and by CA-13038 and CA-18542 from the National Cancer Institute

0006-291X/78/0813-0965$01.00/0

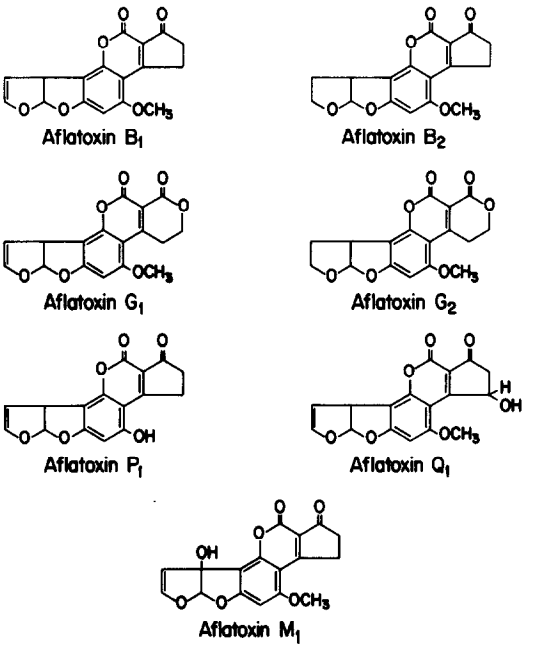

Figure 1: Structures of parent aflatoxins and some metabolites of aflatoxin B_1

human liver disease per se or as secondary insult to hepatitis virus-, and alcohol-induced liver disease (12-14). Recently, considerable evidence has accumulated to suggest that AFB_1 requires metabolic activation to become active and this activation involves the formation of AFB_1-2,3-oxide by the hepatic microsomal mixed function oxygenase (15-19). In addition to this, AFB_1 undergoes microsome-mediated biotransformations to other metabolites- AFM_1, AFQ_1, AFP_1- that do not involve oxidation at the C_2-C_3 double bond (Fig. 1). Since intact C_2-C_3 double bond in these metabolites could be a potential site for activation, as has been reported for some polycyclic aromatic hydrocarbons (20), it was of interest to study the microsome-mediated activation of these metabolites. In addition, the availability of various parent aflatoxins and some metabolites of AFB_1 provided an opportunity to evaluate correlation between hepatic microsome-mediated DNA-binding and bacterial mutagenesis and the biological significance of the intact C_2-C_3 double bond of the AFB_1 metabolites. This paper describes studies on the metabolic activation of various aflatoxins to DNA-binding and mutagenic derivatives.

MATERIALS AND METHODS:

Chemicals:

Chromatographically pure AFB_1, AFB_2, AFG_1 and AFG_2 were obtained from Calbiochem., San Diego, Calif. The purity of these chemicals was verified by thin layer chromatography (TLC) and by spectroscopy, as described previously (16,21). Before use the chemicals were rechromatographed, diluted in dimethyl sulfoxide and then stored at -70° as described previously (16,21). Native calf thymus DNA was purchased from Worthington Biochem. Corp., Freehold, N.J. Sources of other chemicals have been reported previously (16,21).

Metabolites of AFB_1

Unlabeled and ^{3}H-labeled AFM_1, AFQ_1 and AFP_1 were prepared from AFB_1 and ^{3}H-AFB_1. AFB_1 was tritiated by New England Nuclear Co., Boston, Mass. and was purified repeatedly by TLC as described previously (16,21). The ^{3}H-AFB_1 thus obtained was diluted with unlabeled AFB_1 to give a specific activity of 2-4 Ci/mole.

Various metabolites, AFM_1, AFP_1 and AFQ_1 were prepared as described below:

For the preparation of AFM_1 hepatic microsomes isolated from 3-methylcholanthrene treated C57BL/6J mice were used (22,23), whereas for the preparation of AFQ_1 and AFP_1 hepatic microsomes from phenobarbital-treated rats and mice, respectively, were used (22). Hepatic microsomes were incubated in 1 ml volume in potassium phosphate buffer (pH 7.4), containing AFB_1 (or ^{3}H-AFB_1) and an NADPH-generating system. Concentrations of various ingredients are given elsewhere (22). The incubations were carried out at 37° for 30 min. The reaction was stopped with 2 ml cold methanol and then centrifuged to remove the protein. The supernatants from several incubations were pooled and extracted 3 times with chloroform (v/v, 1:10). The chloroform extracts were pooled, evaporated under vacuum and the residue, dissolved in a small volume of methanol, was applied to a silica gel column and the metabolites were eluted with chloroform: isopropanol mixture (95:5). One ml fractions were collected and metabolites were

identified in a 50 μl sample applied to silica gel plates and developed in the same solvent system (22). Various metabolite peaks were identified by the UV inspection of the silica gel plates, pooled separately and then purified by repeated thin layer chromatography (TLC) in chloroform: acetone (80:20) and chloroform: isopropanol (95:5) solvent systems. Large batches of the metabolites were prepared in this way and stored in chloroform at -70°. Immediately before use, metabolites were purified by TLC. The concentration and the specific activity of various metabolites was determined by UV-spectroscopy on an Aminco DW-2 UV/visible spectrophotometer and by liquid scintillation counting. The following extinction coefficients in ethanol or methanol were used: AFM_1, 19,000 (362 nm); AFQ_1, 17,500 (366 nm); AFP_1, 15,400 (362 nm). AFM_1 and AFQ_1 were relatively stable, but AFP_1 was more labile.

DNA-binding Assay

Hepatic microsomes from phenobarbital-treated rats are more active in catalyzing DNA-binding from AFB_1 than those from control or 3-methylcholanthrene-treated rats (18). Therefore, hepatic microsomes from phenobarbital-treated rats were used in the DNA-binding assay which contained hepatic microsomes (2 mg), native calf thymus DNA (4 mg), aflatoxin (concentration indicated in the table) and an NADPH-generating system (prewarmed for 10 min at 37°) in a total of 4.5 ml buffer mixture. Incubation was carried out at 37° for 15 min in the case of AFB_1 and for 30 min in the case of other aflatoxins. At the termination of the incubation, the reaction mixture was extracted with phenol: cresol mixture, the DNA was precipitated from the aqueous phase, treated with RNAase and deproteinized by repeated extraction with chloroform: isoamyl alcohol (95:5) mixture. The final preparation had 260/280 ratio of about 1.9 and contained less than 3% protein or RNA. Details of this method have been described previously (18). Since various aflatoxins used were ^3H-labeled or unlabeled i.e. ^3H-AFB_1, ^3H-AFM_1, ^3H-AFQ_1, ^3H-AFP_1, AFB_2, AFG_1, AFG_2, binding was examined either by counting radioactivity of an aliquot or by spectroscopic method (18). The 362 nm extinction coefficients used for estimating the binding to DNA

treated with AFB_2, AFG_1 and AFG_2 were AFB_2, 21,415; AFG_1, 18,940; AFG_2 (365 nm), 16,400. Incubations were carried out in duplicate or triplicate and for each experimental incubation parallel control incubations were run that contained everything except the NADPH-generating system. The values obtained for the control incubations were subtracted from the experimental values.

Mutagenesis Assay

Mutagenesis of various aflatoxins and metabolites of AFB_1, all in the unlabeled form, to Salmonella typhimurium TA-100 was studied essentially by the methods reported by Ames and co-workers (24). This strain of bacteria was kindly provided by Dr. B.N. Ames (University of California, Berkeley, California). This strain, like the other strains used in mutagenesis studies, requires histidine for growth, and this requirement forms the basis of the assay. Strain TA-100 is derived from strain TA-1535 (detecting mainly base-pair mutagens) by the addition of a plasmid that increases the sensitivity to mutagens through error-prone recombinational repair (24,25). For these mutagenesis studies, 15,000 x g supernatant fraction of the livers isolated from phenobarbital-treated rats was used. Various controls for the liver supernatant, solvent (dimethyl sulfoxide 25 μl/plate), and aflatoxins were run simultaneously and each determination was done in duplicate or triplicate.

RESULTS AND DISCUSSIONS

The microsome-mediated DNA-binding and mutagenic activities of various parent aflatoxins and some metabolites of AFB_1 are summarized in Table 1. In the range 28 to 112 μM, DNA-binding activity of AFB_1 is linear with the substrate concentration. As reported previously by others (26-28) and as shown here, AFB_1 is a potent mutagen to various mutagenesis tester strains. However, AFB_2, a congener of AFB_1 which lacks C_2-C_3 double bond, does not bind to DNA and also is less than 1% as mutagenic as AFB_1. Recent studies have shown that *in vivo* binding to DNA as well as carcinogenic activity of AFB_2 is only about 1-2% that of AFB_1 and it has been suggested that AFB_2 has to be desaturated to AFB_1 before it can be activated (29,30). AFG_1 is a potent carcinogen but much weaker than AFB_1 (2,8). In the

TABLE 1

Rat liver hepatic microsome-mediated mutagenesis to Salmonella typhimurium and metabolism of various aflatoxins to DNA-alkylating metabolites (metabolic activation)

Aflatoxin	Metabolic Activation			Mutagenesis[a]		
					Strain TA100	
	μM	pmoles met. bound/μmole DNA-P	Relative potency	μg/dish	Revertants	Relative potency
AFB_1	28 56 112	767 1544 3053	100	0.4	538	100
AFB_2	280	N.D.[b,c]	0.0	3.0 6.0	11 61	0.3 0.7
AFG_1	280	1400[c]	18.3	1.0 2.0 3.0	111 267 271	8.2 9.9 6.7
AFG_2	280	N.D.[b,c]	0.0	2.0	30	1.0
AFM_1	112	78	2.6	1.0	30	2.3
AFQ_1	112	49	1.6	1.0	23	1.7
AFP_1	35 80	N.D.[b]	0.0	1.0	24	1.8

[a] Background reversions due to spontaneous mutation (130 revertants/plate) have been subtracted.
[b] Not detectable at this or lower concentrations.
[c] Binding of the metabolite to DNA was measured spectrophotometrically as described previously (18).

present studies AFG_1 was about 18% as active as AFB_1 in the DNA-binding assay and about 8-10% in the mutagenesis assay. On the other hand, AFG_2, a congener of AFG_1 which lacks C_2-C_3 double bond, was essentially inactive in binding to DNA and was as weak as AFB_2 in the mutagenesis assay. These data suggest that a good correlation between *in vitro* DNA-binding and mutagenesis exists and that the critical site in the molecule is the C_2-C_3 double bond. Several recent studies (15-19, 29) have established the significance of the C_2-C_3 double bond of AFB_1 in the metabolic activation. In accordance with the present data, studies by Wong and Hsieh (28) have shown that mutagenesis to Salmonella typhimurium strain TA-98 by the parent aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) depends on the presence of an intact double bond. Studies on the carcinogenicity of these aflatoxins have shown that

AFB_1 is more potent than AFG_1, whereas AFB_2 and AFG_2 are essentially inactive or at the most very weakly active (2,8).

The question of what happens to the metabolic reactivity of the C_2-C_3 double bond when the parent aflatoxin is metabolized by the microsomal mixed function oxygenase at other sites of the molecule, has been addressed in the present studies. The data in Table 1 show that in microsome-mediated DNA-binding and mutagenesis to the strain TA-100, AFM_1, AFQ_1 and AFP_1 are less than 5% as active as AFB_1. Previously, Wong and Hsieh (28) have reported that in microsome-mediated mutagenesis to Salmonella typhimurium TA-98, AFM_1, AFQ_1, and AFP_1 were less than 3.5% as active as AFB_1. In carcinogenesis studies, AFP_1 and AFQ_1 are essentially inactive, whereas AFM_1 has some activity (cited in 28).

The results reported here as well as the mutagenesis studies of Wong and Hsieh (28) and carcinogenesis studies of several other investigators lead to the following conclusions: (a) A good correlation exists between in vitro DNA-binding, mutagenesis and possibly carcinogenesis by aflatoxins; (b) the intact C_2-C_3 double bond in the parent aflatoxin is necessary for microsome-mediated DNA binding, mutagenesis and in vivo carcinogenesis; and (c) MFO-mediated metabolism at other sites in the AFB_1 molecule essentially represents a deactivation step.

ACKNOWLEDGEMENT

We acknowledge the skilled technical assistance of Ms. Martha Brown.

REFERENCES

1. Newberne, P.M. and Butler, W.H. Cancer Res. 29:236 (1969).
2. Wogan, G.N., Edwards, G.S. and Newberne, P.M. Cancer Res. 31:1936 (1971).
3. Butler, W.H. and Barnes, J.M. Br. J. Cancer 17:699 (1963).
4. Carnaghan, R.B.A. Nature 208:308 (1965).
5. Sinnhuber, R.O., Wales, J.H., Ayres, J.L., Englebrecht, R.H. and Amend, D.F. J. Natl. Cancer Inst. 41:711 (1968).
6. Wogan, G.N. and Newberne, P.M. Cancer Res. 27:2370 (1967).
7. Salmon, W.D. and Newberne, P.M. Cancer Res. 23:571 (1963).
8. Wogan, G.N. Methods in Cancer Res. 7:309 (1973).
9. Campbell, T.C. and Hayes, J. Tox. Appl. Pharmacol. 35:199 (1976).
10. Shank, R.C., Wogan, G.N. and Gibson, J.B. Fd. Cosmet. Toxicol. 10:51 (1972).
11. Alpert, M.E., Wogan, G.N. and Davidson, C.S. Gastroenterology 54:149 (1968).
12. MaCSween, R.N.M. and Scott, A.R. J. Clin. Path. 26:936 (1973).
13. Bagshawe, A.F., Parker, A.M. and Jindani, A. Br. Med. J. 1:88 (1971).
14. Vogel, C.L., Anthony, P.P., Mody, N. and Barker, L.F. Lancet 2:621 (1970).
15. Garner, R.C., Miller, E.C. and Miller, J.A. Cancer Res. 32:2058 (1972).

16. Gurtoo, H.L. and Dave, C. Res. Commun. Chem. Pathol. and Pharmacol. 5:635 (1973).
17. Swenson, D.H., Miller, J.A. and Miller, E.C. Biochem. Biophys. Res. Commun. 53:1260 (1973).
18. Gurtoo, H.L. and Dave, C. Cancer Res. 35:382 (1975).
19. Essigmann, J.M., Croy, R.G., Nadzan, A.M., Busby, W.F., Jr., Reinhold, V.N., Buchi, G. and Wogan, G.N. Proc. Natl. Acad. Sci. USA 7:1870, 1977.
20. Selkirk, J.K. J. Tox. Environ. Health 2:1245 (1977).
21. Gurtoo, H.L. and Campbell, T.C. Molec. Pharmacol. 10:776 (1974).
22. Vaught, J.B. and Gurtoo, H.L. Life Sci. 21:1497 (1977).
23. Gurtoo, H.L., Dahms, R.P., Kanter, P. and Vaught, J.B. J. Biol. Chem. submitted.
24. Ames, B.N., Lee, F.D. and Durston, W.E. Proc. Natl. Acad. Sci. USA 70: 782 (1973).
25. McCann, J., Spingarn, N.E., Kobori, J. and Ames, B.N. Proc. Natl. Acad. Sci. USA 72:979 (1975).
26. Ames, B.N., Durston, W.E., Yamasaki, E. and Lee, F.D. Proc. Natl. Acad. Sci. USA 70:2281 (1973).
27. Ong, T.M. Mutation Res. 9:615 (1970).
28. Wong, J.J. and Hsieh, P.H. Proc. Natl. Acad. Sci. USA 73:2241 (1976).
29. Swenson, D.H., Lin, J.K., Miller, E.C. and Miller, J.A. Cancer Res. 37: 172 (1977).
30. Roebuck, B.D. and Segal, W.G. Proc. Amer. Assoc. Cancer Res. 16:94 (1975).