Metabolism of Aflatoxin B₁ and Identification of the Major Aflatoxin B₁-DNA Adducts Formed in Cultured Human Bronchus and Colon¹

Herman Autrup,² John M. Essigmann, Robert G. Croy, Benjamin F. Trump, Gerald N. Wogan, and Curtis C. Harris

Human Tissue Studies Section, Laboratory of Experimental Pathology, National Cancer Institute, Bethesda, Maryland 20014; [H. A., C. C. H.; and Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (J. M. E., R. G. C., G. N. W.); and Department of Pathology, University of Maryland Medical School, Baltimore, Maryland 21201 (B. F. T.)

ABSTRACT

Aflatoxin B₁, and benzo(a)pyrene were activated by both cultured human bronchus and human colon as measured by binding to cellular DNA and protein. The binding of aflatoxin B₁ to DNA was dose dependent, and the level of binding was higher in cultured human bronchus than it was in the colon. When compared to aflatoxin B₁, the binding level of benzo(a)pyrene to both bronchial and colonic DNA was generally higher. The major adducts formed in both tissues by the interaction of aflatoxin B₁ and DNA were chromatographically identical to 2,3-dihydro-2-(N³-guanyl)-3-hydroxyaflatoxin B₁ (Structure I) with the guanyl group and hydroxy group in trans-position and an adduct which has been tentatively identified by other investigators as 2,3-dihydro-2-(N³-formyl-2',5',6'-tri-aminoo-4'-oxo-N²-pyrimidyl)-3-hydroxyaflatoxin B₁ (Structure II). Seventy % of the radioactivity associated with bronchial DNA was found in these two peaks, and the ratio of radioactivity between the peaks was nearly 1. In colonic DNA, the ratio between Structures I and II was approximately 2. These observations add aflatoxin B₁ to the list of chemical procarcinogens metabolized by cultured human tissues and in which the carcinogen-DNA adducts are similar to the adducts formed in animal tissue susceptible to the carcinogenic action of aflatoxin B₁.

INTRODUCTION

Aflatoxin B₁, a mycotoxin produced by certain strains of Aspergillus flavus, is a very potent liver carcinogen in several animal species (5, 43) and has also been shown to induce colonic cancer in rats fed a diet deficient in vitamin A (28, 29) and in 20% of rats fed a low level (2 ppm) until death (42). Epidemiological evidence indicates that it may also be an important factor in the etiology of human liver cancer (31, 32, 34). AFB₁ has been detected by fluorescence microscopy in the liver from a liver cancer patient (36). AFBI is a procarcinogen which requires metabolic activation to exert its carcinogenic and mutagenic effects (5, 16, 22, 42). AFBI binds covalently to cellular macromolecules both in vivo (6, 13) and after activation in vitro by liver microsomes (9, 11, 12, 14, 26, 38, 39). AFB₁-2,3-oxide has been identified as the active metabolite responsible for this binding. The major reaction product formed between AFB₁ and DNA both in vitro using rat liver microsomes and in rat liver in vivo is AFB₁-N³-Gua (6, 9, 25, 38).

It was shown previously that cultured human bronchus and colon are able to metabolize several classes of chemical carcinogens, such as polynuclear aromatic hydrocarbons and N-nitrosamines as measured by: (a) the level of binding to cellular macromolecules; (b) the profile of metabolites; and (c) the identification of adducts formed between the metabolites of the carcinogen and DNA (2, 3, 17-22).

The results in this paper indicate that AFB₁ can be activated by cultured human tissues and that the major adduct formed between DNA and AFB₁ is chromatographically identical to the adduct formed in rat liver, an organ which is susceptible to the carcinogenic action of AFB₁.

MATERIALS AND METHODS

Bronchial and colonic specimens were obtained at the time of either surgery or "immediate autopsy" (40). The specimens were immersed immediately in L-15 Medium (Grand Island Biological Co., Grand Island, N. Y.) at 4° until cultured as described previously (1, 20). The colonic tissues were cultured for 1 day and the bronchial tissues were cultured for 7 days prior to incubation with [³H]AFB₁ (0.5 μM or 1.5 μM; 13 or 45 Ci/mmol; Moravek Biochemical, City of Industry, Calif.) or [³H]BP (1.5 μM; 20 or 24 Ci/mmol; Amersham/Searle, Arlington Heights, Ill.) for 24 hr. After incubation, the mucosa was scraped from the supporting stroma. Mucosa from 5 explants were pooled and DNA was then isolated by phenol extraction and purified on a CsCl gradient as described previously (21). Proteins were isolated from the phenol layer and the binding levels assayed as described previously (2). One explant from each group of 5 was fixed in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate and was examined by high-resolution light microscopy for monitoring of cell viability (4).

For identification of the AFB₁-DNA adduct, the mucosae were pooled from 20 and 60 explants from the bronchus and the colon, respectively. The pooled mucosae were homogenized in Tris-sodium dodecyl sulfate buffer (pH 6.5), incubated with proteinase K (Merck, Darmstadt, W. Germany) for 2 hr, and extracted with water-saturated phenol (21). After centrifugation at 10,000 x g for 30 min, DNA was isolated from the upper phase by precipitation with ice-cold ethanol and kept overnight at -20°. After

¹ This work was supported in part by Contracts N01-CP 43265 and N01-CP-43237 from the NIH.
² To whom request for reprints should be addressed.
³ The abbreviations used are: AFB₁, aflatoxin B₁; AFB₂,2,3-oxide, 2,3-dihydro-2,3-epoxyaflatoxin B₁; AFB₁-N³-Gua, 2,3-dihydro-2-(N³-guanyl)-3-hydroxyaflatoxin B₁; BP, benzo(a)pyrene; HPLC, high-pressure liquid chromatography.
centrifugation, the DNA was redissolved in 0.1 M Tris-HCl buffer (pH 6.5), treated with RNase I (Worthington Biochemicals, Freehold, N. J.) at 37° for 1 hr, and extracted twice with chloroform/phenol (1/1). The DNA solution was kept at −20° until identification of the adduct.

**Identification of AFB1-DNA Adduct.** The pH of the DNA solution was lowered to 5.3 with 0.1 M potassium acetate (pH 5.0) and then heated at 100° for 15 min. Heat treatment of AFB1-modified DNA's in acidic buffer has been shown in an earlier study to result in liberation of virtually all of the AFB1-N7-Gua adduct (Structure I) (6). To facilitate chromatographic analysis, the denatured DNA skeleton, from which the adduct was removed, was subsequently hydrolyzed to mononucleotides using nuclease P1 (10 µg/mg DNA; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) at 37° for 6 hr (10). After deproteinization with an equal volume of chloroform, a portion of each hydrolysate was taken for an initial HPLC analysis. To release an AFB1-DNA adduct which was more acid stable, further hydrolysis was necessary by adjusting the HCl concentration to 0.15 M and heating at 100° for 30 min. The hydrolysate was then reanalyzed by HPLC.

The HPLC system consisted of a Micromeritics Model 7000 liquid chromatograph (Micromeritics Corp., Norcross, Ga.), equipped with a Model 730 universal injector and a Waters Model 440 detector (Waters Associates, Milford, Mass.) which monitored UV absorbance at 254 and 365 nm and using a 30- x 0.4-cm µBondapak C18 column (Waters Associates).

Portions of hydrolysates were analyzed by HPLC under 2 sets of chromatographic conditions. Gradient analysis was accomplished by using a linear methanol/water gradient ranging from 10 to 80% methanol over 40 min (6). Column flow rate was 1 ml/min, and temperature was 50°. The hydrolysates were also analyzed using isocratic elution conditions which provide for more complete resolution of the AFB1-dihydrodiol, a potential hydrolysis product, and the AFB1-N7-Gua adduct (Chart 1, Structure I) (25). The column was eluted at ambient temperature with 20% ethanol in 10 mM potassium acetate (pH 5.1) at 1 ml/min.

**RESULTS**

AFB1 was metabolized into an intermediate which bound to both colonic and bronchial DNA and protein. Binding of AFB1 to bronchial DNA was dependent on the concentration of AFB1 in the culture media (Table 1), and a wide individual variation was observed. At the higher concentration of AFB1 (1.5 µM), the binding level of AFB1 to DNA in bronchus (10 ± 9 pmol AFB1 bound per 10 mg DNA; mean ± S.D.; 6 cases) indicates a level of modification equal to 1 AFB1 moiety per 3.1 x 10^4 nucleotides. The binding level of AFB1 to DNA was generally lower than the binding of BP to bronchial DNA from the same patient. A slight negative correlation (r = −0.57; p > 0.05) between the binding level of AFB1 and BP to bronchial DNA was found. A severalfold lower binding level of AFB1 to colonic DNA was observed (Table 2; 2.8 ± 2.1; mean ± S.D.; 10 cases) that was equivalent to a modification of DNA by 1 AFB1 moiety per 8.5 x 10^4 nucleotides. However, a direct comparison between the binding level of AFB1 to DNA in colon and bronchus is not possible, because tissues from different donors were used. In addition, the higher concentration of AFB1 used with bronchus (1.5 µM) had a toxic effect on the colonic explants. Most of the AFB1 bound to DNA (nonde
detectable after 5 days) was removed when the bronchi were cultured for an additional 5 days after the exposure to the carcinogen, whereas the binding to cellular protein (72% remained after 5 days) appeared more persistent (Case B167).

The AFB1-DNA adducts released by hydrolysis of either colon or bronchus DNA appear to be identical. In bronchial DNA, 44% of the radioactivity eluted in the unretained HPLC
Metabolism of AFB1 and BP by cultured human colon

Human colon was cultured in a chemically defined medium for 24 hr before incubation with [3H]AFB1 (0.5 μM; 5 or 13 Ci/mmol) or [3H]BP (1.5 μM; 20 or 24 Ci/mmol) dissolved in dimethyl sulfoxide (0.5% final concentration) for another 24 hr. DNA and protein were purified as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Case</th>
<th>Segment of colon</th>
<th>AFB1 pmol</th>
<th>Protein pmol</th>
<th>BP pmol</th>
<th>Protein pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>C74</td>
<td>Descending</td>
<td>1.4</td>
<td>8.4</td>
<td>4.5</td>
<td>30.0</td>
</tr>
<tr>
<td>C80</td>
<td>Ascending</td>
<td>1.4</td>
<td>14.0</td>
<td>18.8</td>
<td>174.0</td>
</tr>
<tr>
<td></td>
<td>Transverse</td>
<td>4.1</td>
<td>10.6</td>
<td>18.0</td>
<td>95.0</td>
</tr>
<tr>
<td>C82</td>
<td>Ascending</td>
<td>0.7</td>
<td>2.3</td>
<td>3.0</td>
<td>26.0</td>
</tr>
<tr>
<td></td>
<td>Transverse</td>
<td>2.6</td>
<td>2.6</td>
<td>5.1</td>
<td>57.0</td>
</tr>
<tr>
<td>C83</td>
<td>Ascending</td>
<td>2.1</td>
<td>4.8</td>
<td>20.4</td>
<td>113.0</td>
</tr>
<tr>
<td></td>
<td>Transverse</td>
<td>5.4</td>
<td>1.8</td>
<td>12.5</td>
<td>44.0</td>
</tr>
<tr>
<td>C87</td>
<td>Transverse</td>
<td>0.9</td>
<td>10.7</td>
<td>14.1</td>
<td>163.0</td>
</tr>
<tr>
<td>C89</td>
<td>Sigmoid</td>
<td>6.9</td>
<td>2.9</td>
<td>2.0</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>Rectum</td>
<td>2.0</td>
<td>1.7</td>
<td>0.4</td>
<td>8.7</td>
</tr>
</tbody>
</table>

a Patient information: specimen number, sex, age, collection (C74, F, 60; C80, M, 16; IA; C82, M, 55; IA; C83, F, 75; IA; C87, M, 62; S; C89, M, 60, S). IA, immediate autopsy; S, surgery.

DISCUSSION

The metabolism of AFB1 has been investigated in the liver microsomal fraction from both humans and experimental animals (5, 24, 27, 41, 45). AFB1 is metabolized by mixed-function oxidases to hydroxylated derivatives, which generally are far less mutagenic and carcinogenic than is the parent compound (16, 23, 44), and an epoxide which has been implicated as the ultimate carcinogenic metabolite. Cytochrome P-450 has been suggested to be involved in the formation of the epoxide (14, 15), while different forms of cytochrome P-450 have been suggested for the various detoxification pathways (15). The initial evidence for the production of AFB1-2,3-oxide was the release of 2,3-dihydro-2,3-diol after hydrolysis of AFB1-2,3-oxide. The AFB1-modified DNA was isolated under mildly acidic conditions which minimize the artifactual alkali-induced conversion of Peaks I to II in DNA. To rule out the possibility that the method used in these experiments to isolate the DNA caused the conversion of AFB1 to AFB1-2,3-oxide, in vitro-modified AFB1-DNA (1 AFB1 molecule per 200 bases) was taken through the procedure. The isolation process did not have an effect, quantitatively and qualitatively, on the chromatogram of the hydrolyzed DNA; 89% of the radioactivity cochromatographed with Adduct I.
fact that the level of adductation in the S. typhimurium was of the same magnitude as those observed here with human DNA. The binding level of AFB, to DNA was also generally lower than the binding of BP to DNA isolated from the same patients; however, the opposite has been shown by in vitro incubation with liver microsomes and DNA, where AFB, has a 40-fold higher binding level than did BP (14). This difference could, in part, be explained by the fact that cytochrome P-448 is the predominant form of the “hemoprotein cytochrome P-450” in cultured tissues and cells, while the phenobarbital-induced form of cytochrome P-450 is responsible for the activation of AFB, into the reactive AFB-2,3-oxide. No clear difference was observed in the binding level of AFB, to either DNA or protein in the human tissues, whereas several authors have reported a higher level of binding of AFB, to DNA than to protein in animal tissues (38).

The adducts formed between AFB, and DNA in both human bronchus and colon were qualitatively similar to the adducts formed in rat liver, an organ highly susceptible to the carcinogenic action of AFB,. Over 70% of the AFB, binding in bronchus DNA was accounted for as 2 components, I and II, which were chromatographically identical to authentic AFB,-N'-Gua (Structure I) and an adduct tentatively identified as 2,3-dihydro-2-(N'-formyl-2',6'-triamino-4'-oxo-6'-pyrimidyl)-3-hydroxyaflatoxin B, (Structure II), respectively. Quantitatively, these DNA’s differed from other AFB,-modified DNA’s that we have examined, in that most of the incorporated radioactivity in DNA from human bronchus and colon was distributed almost equally between Structures I and II. In previously examined DNA specimens (6, 9, 35), the levels of Structure I far exceeded those of Structure II. We have not examined, in this initial study, the effects of experimental variables relating to incubation of AFB, with human tissues on the quantitative profile of adducts in DNA. Therefore, it is difficult to say whether these quantitative differences between DNA Adducts I and II in human tissues (exposed to AFB, for 24 hr) and rat liver (exposed for 2 hr) actually reflect biochemical differences between these respective models with regard to factors such as the relative rates of formation and removal of these adducts in DNA.

One can only speculate as to the carcinogenic or mutagenic significance of lesions caused by the binding of the AFB, epoxide to DNA. Formation of Adduct I in DNA induces a positive change in the imidazole ring of guanine, and this possibly alters the tertiary structure of DNA in the vicinity of the adduct. This substitution also substantially weakens the covalent bond between the adduct and the DNA backbone, which may facilitate its rapid chemical and/or biochemical removal. The adduct tentatively identified as Structure II is very probably derived from Structure I and, as indicated above, is also a major product in the DNA’s of these tissues. Unlike Structure I, however, Structure II would not carry a positive change in DNA, and its glycosidic bond would be chemically more stable than that of Structure I. Arylation of the N-7 position has also been reported for BP using benzo(a)pyrene diol-epoxide in a cell-free system (30). The major adduct formed with BP was at the N-2 position of guanine in both cultured human bronchus and colon (3, 22).

Another factor in organ susceptibility could be the ability to repair the modified DNA. Our results indicate that, 5 days after a single 24-hr exposure to AFB, only minor amounts of AFB, remained bound to DNA. Whether this disappearance is due to a cellular repair mechanism or simply a depurination reaction due to the instability of the glycoside bond is unknown. However, repair of DNA in human cells after AFB, exposure has been reported (33).

Although AFB, is known as a hepatocarcinogen, AFB, has also been found to induce colonic cancer in rats fed a diet deficient in vitamin A. The increase in the number of animals with colonic tumors correlated with a decrease in the number of animals with liver tumors (28, 29). It was suggested that a decrease in the activity of the drug-metabolizing enzymes in vitamin A-deficient animals caused this change in organ distribution of cancer. Similarly, more mutagenic metabolites of AFB, were found in the urine of rats fed a lipotrope-deficient diet than in urine of rats fed a normal diet (37). By correlating these observations to the human situation, one could consider the possibility that AFB, could be an effective colonic carcinogen in people whose ability to metabolize exogenous compound by the liver has been impaired. One clinical report has also tentatively suggested the involvement of AFB, in colonic cancer in humans (7). Unfortunately, no information on the epidemiology of colonic cancer is available from the regions of Africa where the dietary levels of AFB, have been determined (31, 32).

AFB, has not as yet been shown to induce lung cancer in experimental animals. This, however, does not exclude the possibility that AFB, is a carcinogen in human lungs. AFB, has been detected in lung tissue biopsied from a person working in a grain storage facility (8); this indicates that AFB, can be present in the working environment either free or associated with air-borne particulates. This finding, together with our observation that human bronchus metabolizes AFB, as does rat liver, suggests the possibility that AFB, may be a hazard for the human lung.

ACKNOWLEDGMENTS

We would like to thank Dr. M. Poirier, Dr. U. Saffiotti, and Dr. G. Stoner for valuable comments during the preparation of the manuscript. The technical assistance of Rochelle Schwartz, Gwen Meyers, Frank Jackson, and Maria Yamaguchi as well as the secretarial assistance of Maxine Bellman are gratefully acknowledged.

REFERENCES

25. Lin, J.-K., Miller, J. A., and Miller, E. C. 2,3-Dihydro-2-(guan-7-yl)-3- 
30. Osborne, M. R., Harvey, R. G., and Brookes, P. The reaction of trans- 
36. Stora, C. L’aflatoxine est presente dans des cancers primitifs du foie 
on conversion of aflatoxin B, to bacterial mutagen(s) by rats In Vivo and 
38. Swenson, D. H., Lin, J.-K., Miller, E. C., and Miller, T. A. Aflatoxin B,- 
2,3-oxide as a probable intermediate in the covalent binding of aflatoxins 
aflatoxin B, an acid hydrolysis product of an RNA-aflatoxin B, adduct 
formed by hamster and rat liver microsomes in vitro. Biochem. Biophys. 
40. Trump, B., McDowell, E., Barrett, L., Frank, A., and Harris, C. Studies of 
ultrastructure, cytchemistry and organ culture of human bronchial 
epithelium. In: E. Karbe and J. J. Park (eds.), Experimental Lung Cancer, 
41. Vaut, J. B., Klos, W., and Wogu, T. H. In vitro metabolism of 
112, 1975.  
43. Wogan, G. N. Aflatoxin carcinogenesis. Methods Cancer Res., 7: 309- 
45. Wong, Z. A., and Hsieh, D. P. H. Aflatoxicol: major aflatoxin B, metabo-
Metabolism of Aflatoxin B₁ and Identification of the Major Aflatoxin B₁-DNA Adducts Formed in Cultured Human Bronchus and Colon

Herman Autrup, John M. Essigmann, Robert G. Croy, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/39/3/694

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/39/3/694.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.