IN VIVO COVALENT BINDING OF AFLATOXIN B₁ AND AFLATOXIN M₁ TO LIVER DNA OF RAT, MOUSE AND PIG

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SUMMARY

[¹⁴C] Aflatoxin B₁ (AFB₁) was isolated from cultures of Aspergillus parasiticus grown on [1-¹⁴C] sodium acetate. Covalent binding of AFB₁ to liver DNA of rat and mouse was determined 6–8 h after oral administration. The effectiveness of covalent binding, expressed as DNA binding per dose in the units of a ‘Covalent Binding Index’ (CBI), (µmol aflatoxin/mol DNA nucleotides)/(mmol aflatoxin/kg animal), was found to be 10 400 for rats and 240 for mice. These CBI partly explain the different susceptibility of the two species for the incidence of hepatic tumors.

The corresponding values for pig liver DNA, 24 and 48 h after oral administration, were found to be as high as 19 100 and 13 300. DNA-binding has not so far been reported for this species although it could represent an appropriate animal model for studies where a human-like gastrointestinal tract physiology is desirable.

Aflatoxin M₁ (AFM₁) is a metabolite found in the milk of cows that have been fed AFB₁-contaminated diet. [¹⁴C] AFM₁ was also found to be produced by cultures of A. parasiticus giving a yield of about 0.3% of the total aflatoxins. A test for covalent binding to rat liver DNA revealed a CBI of 2100 showing that AFM₁ must also be regarded as a strong hepatocarcinogen. It is concluded that AFB₁ contaminations should be avoided in dairy feed.

INTRODUCTION

Aflatoxins are highly toxic mould metabolites and frequent contaminants of groundnut cake used in animal nutrition. The main representative of this

Abbreviations: AF, aflatoxin; AFB₁, aflatoxin B₁; AFM₁, aflatoxin M₁; CBI, Covalent Binding Index
class of compounds, AFB₁ (I), is known to be the most potent hepatocarcinogen for animals [1,2]. Epidemiological studies suggest that the prevalence of human liver cancer in certain tropical areas of the world is correlated with the intake of aflatoxins [3].

The mode of carcinogenic action of AFB₁ probably involves a covalent interaction of a reactive metabolite, most likely the 8,9-epoxide* [4, 5, and references therein], with biological macromolecules in the target organ [6, and references therein]. Since DNA seems to be the most critical site of attack for the initiation of a tumor [for a review, see Ref. 7], the extent of such a covalent interaction of chemicals with DNA appears to be a useful quantitative indicator in the process of chemical carcinogenesis.

The susceptibility of man to the carcinogenic activity of AFB₁ is not known and must be extrapolated from animal data. It would be advantageous to base upon an animal model with pharmacokinetics similar to that of man. An animal species which resembles man at least with respect to the physiology of the gastrointestinal tract and the rate of basal metabolism is the pig. Its susceptibility to the carcinogenic action of aflatoxins is estimated in this report on the basis of covalent binding of AFB₁ to pig liver DNA. It will also be shown that the known difference in the susceptibility between the rat and the mouse is indeed reflected by different degrees of DNA binding.

AFM₁ (II) is a metabolite of AFB₁ formed in all mammals studied so far. About 1% of orally ingested AFB₁ is secreted as AFM₁ in the milk of cows [8]. Low levels of AFM₁ are therefore regularly detected in milk when groundnut cake has been fed, and it would be important to know more about its carcinogenicity. AFM₁ has been studied in the rainbow trout and was found to be strongly hepatocarcinogenic [9,10]. Due to lack of material, only preliminary studies have been performed with mammals [11,12] and no definite conclusions on its potency could be drawn.

We were able to purify [¹⁴C]AFM₁ from a culture of A. parasiticus and to measure the covalent binding of AFM₁ to rat liver DNA after oral administration. The results indicate a strong carcinogenicity of AFM₁ in mammals.

*Previously called the 2,3-oxide but renumbered according to IUPAC recommendations.
MATERIALS AND METHODS

Materials
Reagents without specified distributor were of the highest purity available from Merck, Darmstadt, F.R.G.

$[^{14}C]$AFB₁. It was prepared biosynthetically from $[^{1-^{14}C}]$sodium acetate with *A. parasiticus* ATCC 15517 as outlined by Hsieh and Mateles [13]. Purity and identity of the compound obtained was checked with thin-layer chromatography by comparison with authentic AFB₁, supplied by Senn Chemicals, Dielsdorf, Switzerland. The incorporation efficiency of the various batches varied between 1% and 2%. The specific activity of AFB₁ used for the different binding experiments was determined to be 20.3 or 15.5 mCi/mmol, and a radiochemical purity of $>95\%$ was achieved.

$[^{14}C]$AFM₁. It was found in various biosynthetic preparations that *A. parasiticus* ATCC 15517 produced small amounts of AFM₁ (0.3% of the total AF production). $[^{14}C]$AFM₁ was obtained as a by-product of biosynthetically prepared $[^{14}C]$AFB₁ and purified by repeated chromatography on silicagel G (solvent systems: chloroform/acetone (88:12) and chloroform/isoamyl alcohol/acetone (80:10:10). UV- and mass spectra as well as chromatographic properties of the purified compound were completely identical with the corresponding data of authentic AFM₁ (supplied by Senn Chemicals, Dielsdorf, Switzerland). The radiochemical purity was $>90\%$ and no other AF was detectable with thin-layer chromatography at a limit of detection of 0.2% AFB₁. The specific activity of the three samples used was 31.5, 15.5 and 3.7 mCi/mmol.

Animals and treatments

Isolation of DNA. Male rats (ZUR:SIV-Z), male mice (ZUR:ICR-Z) and female pigs (Hampshire x Deutsches Edelschwein) were obtained from the Kantonales Tierspital, Zürich, Switzerland. The weights are given in Tables I and II. The aflatoxins were administered by gavage in 10% aqueous ethanol.

The isolation and purification of DNA was performed according to Markov and Ivanov [14] with the modifications as described before [15].

Control experiments for binding of $[^{14}C]$AF-radioactivity to DNA without enzymatic activation. 63 000 dpm AFB₁ was shaken for 1 h at 37°C with the total homogenate of 7.5 g rat liver in 50 ml lysing medium (0.24 M sodium phosphate (pH 6.8) 8 M urea, 1% sodium dodecylsulfate, 0.01 M EDTA) [14]. The gross radioactivity of 3.1 mg DNA isolated after this incubation was 40.3 cpm in an integral $^{14}C$ channel and did not significantly differ from the 40.4 cpm of 2.4 mg DNA from an inactive liver. A similar incubation of 33 700 dpm AFM₁ with 10.2 g liver in 50 ml lysing medium for 4.5 h at room temperature yielded a sample of 2.6 mg DNA with 21.3 cpm (narrow channel) as compared with 22.1 cpm from 2.4 mg DNA of an inactive liver.
RESULTS

The binding experiments with AFB₁ and AFM₁ are compiled in Tables I and II, respectively. The effectiveness of covalent binding is expressed in the CBI units used throughout our DNA binding experiments.

\[ \text{CBI} = \frac{\text{\(\mu\)mol AF bound/mol DNA nucleotide}}{\text{mmol AF administered/kg body wt.}} \]

This equation shows how many molecules of the test compound are bound per \(10^6\) nucleotides after the theoretical administration of 1 mmol/kg animal.

Most literature data on DNA binding by aflatoxins are based upon intraperitoneal injections [6, and references therein]. Since the oral route should be preferred for a toxicological evaluation of a food contaminant, we determined in one experiment whether the route of administration has any effect on the CBI. The first three lines of Table I reveal that the CBI obtained after i.p. injection was similar to the mean CBI obtained after oral administration. The three values were therefore combined and a mean of 10 400 was obtained for AFB₁ and rat liver DNA.

The CBI for mouse liver DNA was 240 whereas the pig revealed a higher effectiveness of binding than the rat, even at 48 h after the administration. The radiobirosynthesis of AFM₁ has never been described before, nor a DNA-binding experiment ever reported. The amount of \([^{14}\text{C}]\text{AFM}_1\) obtained was small, but each of the three batches was sufficient to determine the covalent binding to liver DNA in one rat (Table II). The individual difference between the three animals was marked, but the order of magnitude was clearly around 2000. This relatively high CBI shows that the hydroxylation of AFB₁ to AFM₁ reduced its effectiveness of covalent DNA binding by a factor of five only.

The presence of radioactivity on the DNA of a treated animal does not, a priori, prove covalent DNA binding. The radioactive molecule might have undergone non-covalent interaction with DNA, or a radioactive fragment might have been incorporated biosynthetically. However, the control experiments showed, that intercalated aflatoxins are completely removed from the DNA in the purification process. Secondly, it was shown that the \(^{14}\text{C}\)-label from [1-\(^{14}\text{C}\)]acetate is incorporated into metabolically stable positions of AF [16]. Only 0.5% of the radioactivity is recovered as \(^{14}\text{C}\)carbon dioxide if a dose of \([^{14}\text{C}]\text{AFB}_1\) is administered to a rat [17]. In addition, the biosynthetic incorporation of one-carbon fragments into DNA is much less efficient than the binding of AFB₁. The incorporation of radioactivity of \([^{14}\text{C}]\)methanol into mouse liver DNA, 12 h after oral administration, was only about 25, when expressed in the same CBI units as used for the aflatoxins (unpublished data). It is therefore evident that the radioactivity measured on the DNA obtained from the aflatoxin experiments represents true covalent adduct formation.
### Table I

**Covalent Binding of AFB₁ to Liver DNA of Rat, Mouse and Pig**

<table>
<thead>
<tr>
<th>Species</th>
<th>Animal wt (g)</th>
<th>Route admin.</th>
<th>Time of exposure (h)</th>
<th>Dose admin. (μg/kg)</th>
<th>Radioact. admin. (dpm/kg)</th>
<th>% total dose in liver at time of sacrifice</th>
<th>Spec. act. of DNA (dpm/mg)</th>
<th>Effectiveness of covalent binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>205</td>
<td>i.p.</td>
<td>6</td>
<td>17.8</td>
<td>2.6 \times 10^4</td>
<td>ND</td>
<td>89</td>
<td>3.4 \times 10^{-3}</td>
</tr>
<tr>
<td>Rat</td>
<td>188</td>
<td>p.o.</td>
<td>6</td>
<td>14.2</td>
<td>2.1 \times 10^4</td>
<td>ND</td>
<td>43</td>
<td>2.1 \times 10^{-3}</td>
</tr>
<tr>
<td>Rat</td>
<td>275</td>
<td>p.o.</td>
<td>6</td>
<td>10.9</td>
<td>1.6 \times 10^4</td>
<td>9.1</td>
<td>73</td>
<td>4.6 \times 10^{-3}</td>
</tr>
<tr>
<td>Mouse</td>
<td>45</td>
<td>p.o.</td>
<td>8</td>
<td>29</td>
<td>3.2 \times 10^4</td>
<td>2.2</td>
<td>2.9</td>
<td>9.0 \times 10^{-7}</td>
</tr>
<tr>
<td>Mouse</td>
<td>42</td>
<td>p.o.</td>
<td>8</td>
<td>24</td>
<td>2.6 \times 10^4</td>
<td>2.1</td>
<td>1.7</td>
<td>7.0 \times 10^{-7}</td>
</tr>
<tr>
<td>Pig³</td>
<td>15 \times 10³</td>
<td>p.o.</td>
<td>24</td>
<td>3.1</td>
<td>4.2 \times 10³</td>
<td>13.0b</td>
<td>26.1</td>
<td>6.2 \times 10^{-2}</td>
</tr>
<tr>
<td>Pig³</td>
<td>15 \times 10³</td>
<td>p.o.</td>
<td>48</td>
<td>3.1</td>
<td>4.2 \times 10³</td>
<td>12.9b</td>
<td>18.1</td>
<td>4.3 \times 10^{-3}</td>
</tr>
</tbody>
</table>

*Both pigs had received the same dose 3 and 5 weeks before this 3rd administration. Some residual radioactivity on the DNA from these early doses cannot be excluded. It can, however, be expected that this contribution does not considerably alter the CBI.

bMore than 80% covalently bound to macromolecules.
TABLE II

<table>
<thead>
<tr>
<th>Animal wt. (g)</th>
<th>Dose admin. (μg/kg)</th>
<th>Radioact.剂 (dpm/kg)</th>
<th>% total dose in liver at time of sacrifice</th>
<th>Spec. act. of DNA (dpm/mg)</th>
<th>Effectiveness of covalent binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>265</td>
<td>3.6</td>
<td>7.7 · 10^6</td>
<td>ND</td>
<td>1.9</td>
<td>2.4 · 10^-6</td>
</tr>
<tr>
<td>231</td>
<td>6.2</td>
<td>6.6 · 10^6</td>
<td>ND</td>
<td>7.0</td>
<td>8.2 · 10^-6</td>
</tr>
<tr>
<td>282</td>
<td>25.9</td>
<td>5.2 · 10^5</td>
<td>5.0</td>
<td>4.9</td>
<td>9.5 · 10^-6</td>
</tr>
</tbody>
</table>

DISCUSSION

AFB₁. The finding that the route of administration does not alter the CBI for liver DNA is a clear indication that the liver takes over most of the metabolism of AFB₁. This is in agreement with the fact that the carcinogenic activity of AFB₁ is directed almost exclusively towards this organ. It is interesting to note that, on a weight basis, there is even a strong accumulation of AFB₁ bound to liver DNA. For instance, line 1 of Table I shows that after a dose of 2.6 · 10⁶ dpm/kg body weight a specific activity of 89 · 10⁶ dpm/kg DNA resulted. A similar accumulation can be observed with the methylation of liver DNA by dimethylnitrosamine [7] and is an indication of strong carcinogenicity of the compound under investigation.

The binding of AFB₁ to liver DNA was lower by a factor of 40 in mice than in rats. This reflects the different susceptibility of the two species to the hepatocarcinogenic activity of AFB₁, which is at least 100 times lower in mice than in rats [18]. While this manuscript was in revision, similar species differences for the binding of AFB₁ to hepatic macromolecules were reported [19]. These authors found a difference of a factor of 700 between rat and mouse liver DNA, 1.5 h after i.p. injection. The discrepancy to our results can in part be due to the use of other strains, in part to the choice of a different time between administration and sacrifice. 1.5 h might have been too short for a maximum binding to occur in the mouse because it was reported that the transport of AFB₁ into hepatic cells is slower in the mouse than in the rat [20]. This assumption is substantiated by the fact that the fraction of the radioactivity in whole mouse liver amounted to 0.7% in their experiment as opposed to more than 2% in ours (Table I), at 8 h after oral administration. The corresponding values for rat liver, 11.1% and 9%, respectively, correspond much better.

Several earlier studies have dealt with the time dependence for the DNA-aflatoxin adducts. The amount of DNA-bound carcinogen rises steeply after an i.p. administration, reaches a maximum value around 2 h [6,21] and decreases thereafter with an approximate half-life of 10 h [21] to 15 h [22]. Other studies report a time of 6 h for maximum binding [23] or a plateau level between 2 h and 6 h [22]. We chose a time lag of 6–8 h in order to
account for a slower distribution after oral administration. It is possible that these time points yield CBI values that are a few percent off the maximum. This will not, however, markedly affect a discussion of the relative susceptibility of rat and mouse.

Our DNA binding experiments with the pig are, to our knowledge, the first studies with this species which is an appropriate animal model for the study of processes for which a human-like physiology of the gastrointestinal system might be desirable. The CBI of more than ten thousand, at 24 h and 48 h after the administration, indicate that the pig might be highly susceptible to the carcinogenic activity of AFB$_1$. The difference to the rat becomes even more evident if it is considered that the CBI for the rat has decreased to 1500 and 1100 at 24 h and 48 h, respectively [6]. The high CBI obtained with the pig could therefore be due to low DNA repair capability but it would be premature for a firm statement on the basis of only two determinations. First of all, it is obvious from data with the rats that appreciable individual variability is observed for CBI obtained under identical conditions, and secondly, it is probable that the maximum DNA binding is reached later in the bigger animal.

AFM$_1$. The CBI for rat liver DNA has been shown to correlate semi-quantitatively with the carcinogenic potency of a compound [7]. It was shown that a CBI of $10^3$–$10^4$ stands for strong hepatocarcinogens, a CBI of $10^2$ for moderate, and of about ten for weak hepatocarcinogens. An extensive discussion [7] of the predictive value of a CBI revealed that a comparison of structurally related chemicals like AFB$_1$ and AFM$_1$ can very well be based quantitatively upon measurement of DNA binding.

The difference between AFB$_1$ and AFM$_1$ in hepatoma incidence in the trout was found to be of a factor of 4 [9], the difference in the Salmonella/microsome mutagenicity test was about 30 : 1 in one report [24] and about 3 : 1 in another [25]. The rate data on carcinogenicity in the rat cannot be used for a quantitative comparison of the two compounds [11,12]. Our binding values with rats suggest that AFM$_1$ will be somewhat less carcinogenic than AFB$_1$, the ratio being about 1 : 5. AFM$_1$ must therefore still be classified with the strong hepatocarcinogens and, due to the carry-over of aflatoxins from feed to the milk, extreme caution should be exerted in the feeding of milk cows with AFB$_1$ contaminated feed.

ACKNOWLEDGEMENTS

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