Metabolic Basis for the Protective Effect of the Antioxidant Ethoxyquin on Aflatoxin B<sub>1</sub> Hepatocarcinogenesis in the Rat

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ABSTRACT

The effect of dietary administration of 0.5% ethoxyquin (EQ) on the <i>in vitro</i> induction of enzymes and effect on aflatoxin B<sub>1</sub> (AFB<sub>1</sub>)-DNA binding in liver and the consequent <i>in vitro</i> metabolism of AFB<sub>1</sub> by male Fischer F344 rat liver-derived fractions have been examined. EQ increased microsomal cytochrome P-450s, in particular those isozymes classified as phenobarbital inducible, and the <i>in vitro</i> rate of metabolism of AFB<sub>1</sub>. The formation of the presumed detoxified metabolites, aflatoxins M<sub>1</sub> and Q<sub>1</sub>, was enhanced to a greater extent than was the formation of the active metabolite, aflatoxin B-8,9-epoxide (assessed by the level of aflatoxin B-8,9-dihydrodiol). Prolonged feeding with EQ was accompanied eventually by a reduction in the initially elevated cytochrome P-450 content, but this was not reflected in any significant decrease in the rate of AFB<sub>1</sub> metabolism <i>in vitro</i>. EQ increased the glutathione S-transferase activity of the liver cytosol fractions as assessed with the model substrate 1-chloro-2,4-dinitrobenzene. The capacity of these fractions specifically to catalyze the conjugation of AFB<sub>1</sub> with glutathione was induced to a far greater extent than was the conjugation of 1-chloro-2,4-dinitrobenzene. γ-Glutamyl transpeptidase was induced in the periportal areas of the liver lobule. Reduced <i>in vivo</i> binding of [<sup>3</sup>H]AFB<sub>1</sub> to DNA of liver and kidney was found to result from EQ treatment. It is concluded that the reduced hepatocarcinogenesis which results from feeding EQ simultaneously with AFB<sub>1</sub> is due to the reduction in DNA-adduct formation which in turn is due at least in part to increased detoxifying metabolism in the microsomal, cytosolic, and plasma membrane compartments of the liver cells.

INTRODUCTION

The aflatoxins are a group of mycotoxins, produced as secondary metabolites by species of <i>Aspergillus</i>. There is epidemiological evidence that they play a role in the high incidence of human liver disease in certain areas of the world (1–3). The aflatoxins, of which AFB<sub>1</sub>, is the most abundant naturally occurring form and the most potent, require metabolic activation in order to produce most of their biological effects in experimental systems (4–6). Some of the pathways involved in this metabolism are illustrated in Fig. 1. There is considerable interest in factors which, by affecting the balance between toxifying and detoxifying metabolism, could affect the susceptibility of individuals to these toxins. Of particular importance in this area are the possible effects of dietary constituents. It was previously reported from this laboratory that ingestion of the antioxidant EQ by the aflatoxin-sensitive male Fischer F344 rat affords protection against the hepatocarcinogenic action of the toxin (7). In the present study we have examined the effect of EQ ingestion on the metabolic pathways illustrated in Fig. 1. At the stage of completion of these studies, Kensler et al. (8) communicated the results of their study which was directed to examine the same question. Although in areas which overlapped similar results were obtained, differences in experimental design and approaches provided additional information on the mechanism of protection of EQ against hepatic carcinogenesis from AFB<sub>1</sub>.

MATERIALS AND METHODS

Materials

Ethoxyquin was obtained from Koch-Light Laboratories, Colnbrook, United Kingdom. CDNB, DMSO (spectroscopic grade), and arachis oil were products of British Drug Houses, Poole, United Kingdom. [3H]AFB<sub>1</sub> (30 Ci/mmol) was obtained from Moravek Biochemicals, Brea, CA. AFB<sub>1</sub> was obtained from Makor Chemicals, Jerusalem, Israel. Rabbit reticulocyte lysate was obtained from Amersham International, Aylesbury, Bucks, United Kingdom. [35S]Methionine (800 Ci/mmole) was from New England Nuclear, Slough, United Kingdom. Other chemicals were obtained from Sigma Chemical Co., Poole, United Kingdom or Boehringer Corp., Lewes, United Kingdom and were of the highest purity available.

Animal and Tissue Procedures

Male Fischer F344 rats (60–100 g), bred on site, were housed in wire-bottomed cages (5 rats/cage) under controlled lighting and temperature. Control animals were fed a diet of powdered Medical Research Council 41B rat and mouse diet (containing no added antioxidant) to which was added 2% arachis oil (v/w). Treated animals were fed as above except that EQ was blended into the diet with the arachis oil to give a final level of 0.5% (v/w). Diets were stored at 4°C and were administered for periods of up to 6 weeks. Previous studies had demonstrated no serious adverse health effect on the rats during periods of feeding with the EQ-containing diet similar to those used in the present study. Animals received water <i>ad libitum</i>. Animals were killed by decapitation (or ether in the case of livers used for preparation of RNA samples), exsanguinated, and the livers and kidneys rapidly removed and placed on ice. For preparation of microsomal or cytosol fractions for enzyme assay, portions of the livers from 2 or 3 rats were homogenized on ice-cold 150 mM KC1 (5 g tissue/20 ml KC1). Fractions were prepared by differential centrifugation as previously described (9, 10); 100,000 × g supernatant fractions were used as the cytosol preparations in incubations after storage at −70°C. Microsomal fractions were resuspended in 150 mM KC1, resedimented at 100,000 × g, and finally resuspended in 150 mM KC1 (0.625 g liver/ml suspension). Aliquots of this suspension (400 µl) were used in incubations following storage at −70°C. Slices of liver and kidney (2–3 mm) were fixed immediately in ice-cold acetone before paraffin wax embedding and sectioning for GHT histochemistry as described previously (11).

Analytical Procedures

Protein and Cytochrome P-450 Determinations. Protein contents of the fractions were determined by the method of Lowry et al. (12) using...
bovine serum albumin as standard. Cytochrome P-450 content of microsomal suspensions was determined essentially by the method of Omura and Sato (13). The difference in absorption at 450 and 500 nm was used to quantify the cytochrome P-450 concentration using an extinction coefficient of 91 mmol/liter/cm.

Microsomal Metabolism of AFB. Aliquots of the microsomal fraction (400 μl equivalent to 0.25 g wet weight of liver) were incubated with 128 nmol AFB, (dissolved in 20 μl DMSO) at 37°C under O2 in a shaking water bath for 15 or 30 min. The cofactors added were 50 μmol KCl, 15.5 μmol MgCl2, 0.5 units glucose 6-phosphate dehydrogenase, 10 μmol glucose 6-phosphate, and 0.75 μmol NADP in a total volume of 1.9 ml. Samples were prepared and subsequently analyzed by HPLC on Magnusphere 5-μl octadecyl silane (Magnus Scientific Instrumentation, Aylesbury, Bucks, United Kingdom) reverse phase columns (100 x 4.6 mm) using a 15% methanol/water (v/v) to 40% methanol/water (v/v) gradient containing 0.01% H3PO4 and 8% aconitrate (v/v) in all solvents as previously described (9, 10, 14, 15).

GSTase Assay. Enzyme activity using CDNB as a substrate was measured by the procedure of Habig et al. (16). Cytosols were diluted to give a Δ absorbance of 0.02–0.05/min. Enzyme activity was calculated using an extinction coefficient of 9.6 mmol CDNB/liter/cm.

Conjugation of AFB, was determined using quail liver microsomes as the activating system in the presence of the appropriate rat liver cytosols (1 ml) and 5 mM reduced GSH (9). Subsequent assays of AFB-GSH were by HPLC as previously described (9, 17).

GGT Assay. GGT activity was assayed in liver samples essentially by the fluorimetric method of Smith et al. (18) as described previously (19) using L-(+)-glutamyl-7-amino-4-methyl coumarin as substrate. Tissue homogenates were diluted in 0.1 M ammonium/HCl buffer, pH 8.5, and 100 μl of tissue homogenates were incubated with 250 μM substrate (200 μM) in ammonium buffer containing 20 mM glycylglycine and 0.1% (v/v) Triton X-100 at 37°C for 10 min. The reaction was stopped by adding 2 ml ice-cold 0.05 M glycine buffer, pH 10.4. Fluorescence was read at 440 nm with an excitation at 370 nm.

DNA-binding Studies. Rats received an i.p. injection of 1 mg/kg [3H]AFB1 (10 μCi/kg) dissolved in 0.5 ml DMSO, and were killed with ether anesthesia 2 h later. Livers were removed and nuclei isolated essentially as described by Croy et al. (20). The crude nuclear pellet was suspended in 2 ml homogenizing buffer and stored at -70°C, and the DNA was subsequently extracted. DNA was purified essentially by the procedure of Hertzog et al. (21).

RESULTS

Data in Table 1 show that feeding with 0.5% EQ in the diet resulted in an increase in the cytochrome P-450 content of the liver microsome fraction of >200% by the ninth day on the diet. The concentration of cytochrome P-450 subsequently declined so that by day 42 the elevation in cytochrome content was approximately 100%. Despite the elevation in cytochrome P-450, there were no detectable changes in the total protein levels of either the microsomal or the cytosol fractions during this time. When total microsomal proteins from control, PB-, MC-, and EQ-treated livers were run on a 7.5% polyacrylamide-SDS gel and stained with Coomassie blue, the cytochrome P-450 protein banding pattern in EQ-treated livers was similar to that from PB-induced liver (data not shown). The higher molecular weight bands characteristic of MC-induced liver were...
obtained when proteins run on 9% gels were blotted onto nitrocellulose and probed with antibodies against specific isozymes of cytochrome P-450 (26, 27), although by this method it was possible to see a modest induction of an MC-type cytochrome P-450 (Fig. 2). However the most marked inductions were seen with anti-PB,a and -PB2c. The complexity of the blot probed with anti-PB,a is probably associated with the multiplicity of this gene family (27). Results with anti-MC,b are not shown since no change in the level of this protein was observed in EQ-treated livers compared to controls.

After in vitro translation of mRNA from control liver only PB-type cytochrome P-450 proteins were immunoprecipitated by the polyclonal antibodies used (anti-PB,a,-PB2c,-PB3a,-MCia, and -MCib). However, when translation products of 11-day EQ-treated mRNA were used, not only was it possible to immunoprecipitate induced levels of at least 2 distinct isozymes of PB-type cytochrome P-450s (Fig. 3, b and c), but also immunoprecipitation with anti-MCia cytochrome P-450 antibody gave a band at the expected molecular weight for this isozyme (data not shown). PB2c-type cytochrome P-450 appeared to be elevated at least 3-fold in 11-day treated translation products compared to control, and PB3a-type cytochrome P-450 was even more strongly induced.

When the in vitro AFB1-metabolizing capacity of microsome fractions was examined (Table 2), the increase in cytochrome P-450 was paralleled by an increased metabolism of AFB1. After 30 min of incubation, there was an increase in the formation of AFB1-dhd, but the greatest induction in terms of formation of soluble AFB1 metabolites was in the production of AFQ1 and AFM1. There was no significant change in the level of AFP1 formation. These findings were confirmed in a separate experiment (Fig. 4) in which the assays were carried out after a 15-min incubation. The rate of AFB1 metabolism was linear over this period (14).

Induction of GSTase activity by EQ was determined using the model substrate CDNB (16). A rapid induction of activity was observed, and the elevated level (approximately, 450% increase over control level) was maintained during the 6-week EQ feeding period used (Fig. 5). The ability of the cytosol fraction specifically to conjugate activated AFB1 with GSH was also determined. A considerable enhancement of this activity was detected (Table 3), with the percentage increases over control levels of the formation of AFB1-GSH being almost 100-fold in contrast to a 5-fold increase in CDNB-conjugating activity.

Hepatic GGT activity was rapidly induced by EQ with almost a 100-fold induction by 6 weeks (Fig. 6). The increased activity was localized in periportal hepatocytes extending eventually to some midzonal regions of the lobule (Fig. 7). The drop in activity at 2 weeks of feeding was a consistent result observed in several different experiments. Histochemically a reduction both in area occupied by positive hepatocytes and in the intensity of stain was observed at this time.

Feeding with EQ led to a substantial decrease in binding of AFB1 to DNA in vivo in both liver and kidney (Table 4). A higher percentage of reduction in binding to DNA of liver was observed compared to that for kidney in both 2- and 14-day EQ-fed samples, and the control level of binding in the kidney was only 10% that in the liver. Examination of the 0.1 M HCl DNA hydrolysates by HPLC showed that >75% of the adducted AFB1 in these liver samples (2 h after dosing) was in the form

| Table 1 Protein and cytochrome content of subcellular fractions isolated from control or EQ-fed rats.

<table>
<thead>
<tr>
<th>EQ feeding (days)</th>
<th>Microsomal protein (mg/g liver)</th>
<th>nmol/mg microsomal protein</th>
<th>Cytosol protein (mg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>35.3 ± 1.6*</td>
<td>9.83 ± 0.92</td>
<td>0.302</td>
</tr>
<tr>
<td>1</td>
<td>33.5 ± 2.5</td>
<td>8.01 ± 0.75</td>
<td>0.239</td>
</tr>
<tr>
<td>3</td>
<td>33.9 ± 3.2</td>
<td>17.25 ± 1.5</td>
<td>0.508</td>
</tr>
<tr>
<td>7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>40.0 ± 2.8</td>
<td>31.76 ± 2.7</td>
<td>0.794</td>
</tr>
<tr>
<td>11</td>
<td>ND</td>
<td>ND</td>
<td>70.5 ± 2.9</td>
</tr>
<tr>
<td>14</td>
<td>36.3 ± 3.5</td>
<td>22.48 ± 1.8</td>
<td>0.619</td>
</tr>
<tr>
<td>28</td>
<td>39.3 ± 4.2</td>
<td>24.59 ± 2.2</td>
<td>0.626</td>
</tr>
<tr>
<td>42</td>
<td>35.0 ± 2.7</td>
<td>18.87 ± 1.3</td>
<td>0.539</td>
</tr>
</tbody>
</table>

* Mean ± SD of triplicate determinations.

ND, not determined.

Fig. 2. Immunoblots of microsomal proteins probed with anti-cytochrome P-450 polyclonal antisera. Microsomal proteins (15 ng/lane) were electrophoresed and blotted on to nitrocellulose as described in "Materials and Methods." Lanes 1, purified isozyme as standard; lanes 2, control liver; lanes 3, PB treated; lanes 4, MC treated; lanes 5, 72 h EQ; lanes 6, 2-week EQ; lanes 7, 4-week EQ; lanes 8, 6-week EQ. Blots were probed with anti-P-450 antisera as indicated (27). The lower bands are artefacts found on all blots with different antisera.

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**DISCUSSION**

EQ has been recognized for some time as an inducer of microsomal metabolism, mainly of cytochrome P-450 (28) with induction of cytochrome P-448 less pronounced in the rat (29). It was also shown to induce GSTase in the mouse (30, 31). The present report provides at least a partial explanation for the role of these and other effects of EQ in the reduction of AFB₁ hepatocarcinogenicity in the rat, previously observed in this laboratory (7). Although AFB₁ is not a single-dose carcinogen, the *in vivo* hepatic DNA binding data following a single administration of AFB₁ could be expected to correlate with the situation at individual stages in the continuous administration of AFB₁ necessary to provoke the carcinogenic response. It may well be that this response could be modified during the process of continuous administration of AFB₁, and further experiments are needed to investigate this possibility.

**Table 2. Effect of EQ on the in vitro metabolism of AFB₁ by rat liver microsomes**

In incubations of microsomal fractions from each liver sample (400 µl microsomal suspension) with AFB₁ (128 nmol in 20 µl/DMSO) and subsequent HPLC analysis of the residual AFB₁ and soluble AFB₁ metabolites, the recoveries of AFB₁ guanine, with the remainder in the form of the ring-opened adduct 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxy AFB₁ and AFB₁-dhd (results not shown).

<table>
<thead>
<tr>
<th>EQ feeding (days)</th>
<th>AFB₁ metabolized (µg/g liver)</th>
<th>AFB₁-dhd</th>
<th>AFB₁ metabolites produced (µg/g liver)</th>
<th>% recovery of AFB₁</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>AFQ₁</td>
<td>AFM₁</td>
</tr>
<tr>
<td>0</td>
<td>56.8</td>
<td>6.68</td>
<td>4.92</td>
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</tr>
<tr>
<td>3</td>
<td>99.2</td>
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<td>17.96</td>
<td>12.04</td>
</tr>
<tr>
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<td>10.56</td>
<td>16.80</td>
<td>9.96</td>
</tr>
<tr>
<td>28</td>
<td>82.0</td>
<td>7.36</td>
<td>17.76</td>
<td>9.36</td>
</tr>
</tbody>
</table>

**Table 3. Effect of EQ on conjugation of microsomally activated AFB₁ with GSH catalyzed by rat liver cytosol fractions**

AFB₁ (128 nmol) was activated by quail liver microsomes (400 µl suspension containing 250 mg quail liver equivalent/incubation) in incubations containing cofactors as in Table 2 and 1 ml rat liver cytosol (equivalent to 225 mg rat liver) for 30 min at 37°C. Analysis of AFB₁-GSH by HPLC was essentially as described by Moss and Neal (9). Results are means ± SD of triplicate assays. Comparable activity for mouse liver cytosol is 94.28 ± 2.3 µg AFB₁-GSH/g liver.

<table>
<thead>
<tr>
<th>EQ feeding (days)</th>
<th>AFB₁-GSH formed (µg/g rat liver)</th>
<th>(µg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.12 ± 0.01</td>
<td>1.77 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>10.34 ± 0.63</td>
<td>127.7 ± 7.8</td>
</tr>
<tr>
<td>9</td>
<td>16.91 ± 0.45</td>
<td>206.7 ± 5.4</td>
</tr>
<tr>
<td>28</td>
<td>15.13 ± 0.32</td>
<td>209.3 ± 4.4</td>
</tr>
</tbody>
</table>

**Fig. 4.** Effect of EQ on in vitro microsomal metabolism of AFB₁. A, AFB₁; B, AFB₁-dhd; C, AFQ₁; D, AFM₁. Cl, control feeding; III, EQ, 1 day; III, EQ, 3 day; III, EQ, 9 day; III, EQ, 14 day; III, EQ, 42 day. Results are expressed as a percentage of control values. Incubations were for 15 min; otherwise analyses were carried out as in Table 1. Results are means of duplicate assays; variation <10% of means.

**Fig. 5.** Effect of EQ on the capacity of rat liver cytosol to catalyze conjugation of CDNB with GSH. Results are means ± SD (bars) of 3 determinations.
METABOLIC BASIS FOR EQ PROTECTION VERSUS AFLATOXIN

The effect in vivo of increased AFBl detoxification pathways resulting from EQ feeding was seen in the reduced covalent binding of [3H]AFBl to liver and kidney DNA (Table 4). The reduced binding to liver DNA can probably be correlated with the reduced hepatocarcinogenesis (7). A reduction in covalent binding to liver DNA was also reported by Kessler et al. (8, 32). In their experiments using multiple p.o. dosing with 250

and AFQ1. These metabolites have considerably less toxic potential than the parent compound (33, 34). There was also an increased rate of AFBl-dhd formation from AFBl, indicating an increased rate of formation of the carcinogenic epoxide (Fig. 1). In both experiments which examined the metabolism of AFBl but using different incubation times (Fig. 4 and Table 1), however, a much lower induction of the epoxidation pathway than that of the hydroxylation activity was detected. An EQ-induced increase in microsomal cytochrome P-450 content, particularly of the PB-type cytochrome P-450s, was observed in contrast to a minimal change in total protein contents of microsomal or cytosolic fractions. The total cytochrome P-450 levels increased from between days 1 and 3 of treatment with EQ up to at least day 9, but from 2 weeks onwards levels declined significantly. There was, however, only a small decrease in certain of the metabolic pathways of AFBl, (AFM1, production, Fig. 4D and Table 2, and possibly AFBl-dhd, Table 2), and it is apparent therefore that the total cytochrome content and rate of AFBl metabolism are not quantitatively closely coupled. This has been the finding in other experiments using rodent and avian species (15) and requires further study. The reasons for the decline in cytochrome P-450 levels after prolonged EQ feeding are not known at present but again indicate the complexity of the tissue response to the antioxidant. It is possible that changes in the nature of the induced microsomal membranes may take place during prolonged exposure to the antioxidant but at this stage this is merely speculation. Further experimentation is required to clarify these points. The metabolic pathways observed in the present study, in which the formation of AFQ1 and AFM1 are both substantially stimulated by EQ treatment are of interest since from use of other cytochrome P-450-inducing compounds, e.g., PB and 3-MC, the formation of AFQ1 appears to be predominantly mediated via a PB-inducible cytochrome P-450 system and AFM1 by a 3-MC-inducible cytochrome P-448 (35). The present results indicate that EQ may best be classified as a mixed inducing substance, although the antibody studies, in agreement with a previous report (29), indicate a preferential induction of PB-type cytochrome P-450s, and the patterns of hepatic microsomal AFBl metabolite formation resemble most closely those seen to result from pretreatment of rats with PB (35).

The second factor which apparently is involved in the resistance to AFBl is the pronounced induction of GSTase activity. Results obtained using the model substrate CDNB were consistent with the induction of a spectrum of isoenzymes of GSTase in agreement with the results of Kessler et al. (8, 32). The data in Table 3, however, emphasize that EQ treatment resulted in a greater percentage increase in the activity of those isoenzymes capable of catalyzing the conjugation of activated AFBl compared with those catalyzing conjugation of CDNB. AFBl-conjugating activity has been demonstrated to be principally due to the basic isoenzymes of GSTase (36). This induction is consistent with the increased conjugation of AFBl with GSH observed by Kessler et al. (8) in the bile of EQ-treated animals. The increased hepatic GST activity is likely to result in more efficient conversion of AFBl-GSH to AFBl-cysteinyl-glycine and may facilitate removal from the liver (37).

The effect in vivo of increased AFBl detoxification pathways resulting from EQ feeding was seen in the reduced covalent binding of [3H]AFBl to liver and kidney DNA (Table 4). The reduced binding to liver DNA can probably be correlated with the reduced hepatocarcinogenesis (7). A reduction in covalent binding to liver DNA was also reported by Kessler et al. (8, 32). In their experiments using multiple p.o. dosing with 250
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\[ \mu \text{AFB}_1/\text{kg body weight (25\% of the dose used in the present study), feeding with EQ at a level of 0.4\% in the diet reduced the DNA binding in the initial stages of dosing by >90\%, compared to 97\% in the present study (see Table 4). Reduced binding to kidney DNA was also induced by feeding with EQ. However, we found (38) that while treatment with EQ protected against AFB_1-induced hepatocarcinogenesis, it also resulted in kidney lesions, a finding also reported by other workers (39, 40). Perhaps the lower percentage of reduction in DNA binding in kidney compared with liver seen after feeding with EQ is related to this observation. Further work is in progress to examine this factor.}

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REFERENCES


\[ ^3 \text{J. R. P. Cabral, M. M. Manson, and G. E. Neal, manuscript in preparation.} \]
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