Modulation of Aflatoxin Metabolism, Aflatoxin-7'-guanine Formation, and Hepatic Tumorigenesis in Rats Fed Ethoxyquin: Role of Induction of Glutathione S-Transferases

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ABSTRACT

The effects of dietary administration of ethoxyquin (EQ) on aflatoxin B1 (AFB1) metabolism, DNA adduct formation and removal, and hepatic tumorigenesis were examined in male Fischer rats. Rats were fed a semipurified diet containing 0.4% EQ for 1 wk, gavaged with 250 µg of AFB1 per kg 5 times a wk during the next 2 wk, and, finally, restored to the control diet 1 wk after cessation of dosing. At 4 mo, focal areas of hepatocellular alteration were identified and quantitated by staining sections of liver for γ-glutamyl transpeptidase. Treatment with EQ reduced by >95% both area and volume of liver occupied by γ-glutamyl transpeptidase-positive foci. Utilizing the same multiple dosing protocol, patterns of covalent modifications of DNA by AFB1 were determined. EQ produced a dramatic reduction in the binding of AFB1 to hepatic DNA: 18-fold initially and 3-fold at the end of the dosing period. Although binding was detectable at 3 and 4 mo postdosing, no effect of EQ was observed, suggesting that these persistent adducts are not of primary relevance to AFB1 carcinogenesis. Analysis of nucleic acid bases by high-performance liquid chromatography revealed no qualitative differences in adduct species between treatment groups. The inhibitory effect of EQ on AFB1 binding to DNA and tumorigenesis appears related to induction of detoxification enzymes. Rats fed 0.4% EQ for 7 days showed a 5-fold increase in hepatic cytosolic glutathione S-transferase (GST)-specific activities. Multiple molecular forms of GST were induced, and concomitant elevations in messenger RNA levels coding for the synthesis of GST enzymes important to AFB1 detoxication, particularly glutathione S-transferase, leads such as GST, can lead to enhanced carcinogen elimination, as well as reductions of AFB1-DNA adduct formation and subsequent expression of preneoplastic lesions, and, ultimately, neoplasia.

INTRODUCTION

The aflatoxins are fungal metabolites, and the most biologically potent of these compounds, AFB1, is toxic, mutagenic, and hepatocarcinogenic in a wide range of organisms (1). This mycotoxin is a consistent contaminant of the human food supply and is epidemiologically linked to incidences of human liver cancer in Asia and Africa (2). The ultimate carcinogenic form of aflatoxin is thought to be the 8,9-oxide, which binds predominantly to the N7 atom of guanine in DNA, as well as to RNA and protein (1). Apart from binding to macromolecules, AFB1, 8,9-oxide can undergo hydrolysis to aflatoxin 8,9-dihydrodiol and conjugation with glutathione to form AFB-SG. This glutathione conjugate is a major biliary metabolite (3, 4) that is formed through the catalytic action of a family of isozymes, the glutathione S-transferases, and is considered to be a detoxication product. Hydrolysis of aflatoxin 8,9-oxide occurs spontaneously, and the involvement of epoxide hydrase in this metabolic step is uncertain (5, 6). Aflatoxin 8,9-dihydrodiol has toxic properties (7), although its role in AFB1, carcinogenicity is unknown. The known pathways involved in the metabolic activation of AFB1 are summarized in Fig. 1.

Although not extensively studied, experimental AFB1 hepatocarcinogenesis is amenable to modification by dietary manipulations, and chemoprotective interventions may offer a useful approach for reducing the incidence of human liver cancer in high-risk areas. Phenobarbital and β-naphthoflavone are potent inhibitors of AFB1 carcinogenesis in rats (8, 9). Both these agents induce cytochrome P-450 isozymes that accelerate Phase I metabolism of AFB1 to hydroxylated products which are considerably less genotoxic than either AFB1 or its 8,9-oxide (10, 11). Presumably, these inductions serve to alter the balance between metabolic activation and detoxification of aflatoxin. Dietary antioxidants, such as BHT, BHA, and ethoxyquin, also inhibit AFB1 hepatocarcinogenesis when fed simultaneously with the carcinogen (12, 13). Several mechanisms for the anticarcinogenic activity of antioxidants have been proposed. Because there is no simple relationship between protection and antioxidant potential [except against tumor promotion (14)], it appears unlikely that antioxidants are serving as direct scavengers of electrophilic carcinogens, thus protecting critical macromolecules from modification. Much recent evidence suggests that the protective effects may arise from enhanced carcinogen inactivation through selective induction of Phase II detoxication pathways which facilitate the clearance of activated metabolites through conjugation reactions (15).

In the present study, we describe a comprehensive and systematic approach for elucidating the mechanism of chemoprotection by the antioxidant ethoxyquin against AFB1, hepatocarcinogenesis. This strategy entails the integration of biochemical and biological endpoints that they may all be directly interrelated through overlapping experimental protocols. Under experimental conditions that approximate those used by Cabral and Neal (12) to demonstrate the protective effect of ethoxyquin against AFB1, hepatocarcinogenesis, we find that the selective induction of enzymes important to aflatoxin 8,9-oxide detoxication, particularly glutathione S-transferase, leads to enhanced biliary elimination of aflatoxin, dramatically reduced adduct formation of AFB1 binding to DNA, and a marked suppression in the hepatic burden of the putative preneoplastic GGT-positive foci.

3924

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MECHANISM OF CHEMOPROTECTION BY ETHOXYQUIN

MATERIALS AND METHODS

Animals and Diets. Male F344 rats, 75–100 g (Charles River Breeding Laboratories, Wilmington, MA), were housed singly in wire-bottomed cages under controlled conditions of temperature, humidity, and lighting. Food and distilled water were available ad libitum. Purified diets of the AIN-76A formulation (16) lacking the recommended addition of 0.02% ethoxyquin (17) were used, and fresh diet was provided to the animals at least every other day. Ethoxyquin was incorporated into the AIN-76A diet at a final concentration of 0.4% and was stored at 5°C. Rats were acclimated to the control diet for 1 wk before switching to the experimental diet. All rats were weighed weekly. The 0.4% ethoxyquin diet had no effect on animal growth rates.

Chemicals. Ethoxyquin was obtained from Pfaltz and Bauer (Stamford, CT), CDNB was from Eastman Organic Chemicals (Rochester, NY), and DCNB was from Aldrich Chemical Co. (Milwaukee, WI). AFB1 was purchased from Calbiochem (San Diego, CA). [3H]AFB1 (24 Ci/mmol) was from Moravek Biochemicals (City of Industry, CA), and [14C]stylene-7,8-oxide was from Amersham (Arlington Heights, IL). 32P-labeled nucleotides were purchased from New England Nuclear (Boston, MA). All other chemicals were of the highest quality obtainable commercially.

Enzyme Assays. Rats maintained on experimental diets were stunned and killed by cervical dislocation, and livers were removed and homogenized in 4 vol (w/v) of a 50 mM Tris-HCl buffer, pH 7.0, containing 0.25 M sucrose with a Polytron homogenizer. Homogenates were centrifuged at 105,000 × g, and the resulting supernatant fluid was used for glutathione S-transferase assays with CDNB and DCNB as substrates (18). Microsomes isolated from the same preparation were used for glutathione S-transferase assays with CDNB and DCNB as substrates (18). Microsomes isolated from the same preparation were used for the assay of epoxide hydrase using [14C]styrene-7,8-oxide as substrate (19). Protein was determined by a modified Lowry assay (20) using bovine serum albumin as standard.

Separation of Molecular Forms of Glutathione S-Transferase, RNA Isolation, and RNA Blot Analysis. Livers were homogenized in 12 ml of guanidine isothiocyanate stock solution, and RNA was purified by a method described by Chirgwin et al. (22). For RNA slot blots, 5 µg of total RNA were denatured in formaldehyde and serially diluted onto nitrocellulose paper (23). The glutathione S-transferase complementary DNA clones were radiolabeled with [32P]dATP and [32P]dCTP by standard nick translation to a specific activity of 1 × 107 cpm/µg (24). Clones pGTB38, pGTB42, and pGTA/C44 were the generous gift of Dr. Cecil B. Pickett and hybridized to mRNAs for rat glutathione S-transferase subunits 1 and 2 (25), 2 (26), and 3 (27), respectively. Filters were hybridized for 16 h at 37°C in 50% formamide, 3x Denhardt's (0.06% Ficoll, 0.06% bovine serum albumin, and 0.06% polyvinylpyrrolidone), 0.45 M sodium chloride-0.045 M sodium citrate (pH 7.4), 0.5% sodium dodecyl sulfate, and 10% dextran sulfate. After hybridization, filters were washed in 0.15 M NaCl-0.015 M sodium citrate-0.5% sodium dodecyl sulfate at 60°C. Washed filters were exposed overnight to Kodak XAR-5 film, and the level of hybridized RNA was determined by densitometric scanning of autoradiographs.

Aflatoxin Metabolism. All animals were anesthetized throughout the experiment with sodium pentobarbital, and the bile ducts were cannulated with PE10 tubing. Rats were infused with saline through the jugular vein and maintained in a humidified incubator at 37°C. Rats were gavaged with 250 µg of [3H]AFB1 (80 µCi/µmol) per kg, and bile was collected on ice in the dark in two sequential 1-h samples and stored at −80°C until analysis. Bile samples were thawed, diluted 1:1 with 50% methanol, and placed on ice for 30 min. After centrifugation at 12,000 × g for 1 min, samples were chromatographed using a DuPont Zorbax ODS column (7.2 mm x 25 cm) with 25% ethanol in 20 mM triethylammonium formate (pH 3.0) mobile phase at a flow rate of 1.1 ml/min. AFB-SG eluted at 7.5 min, and authentic standard was the kind gift of Dr. G. E. Neal.

Isolation of DNA and Analysis of Aflatoxin-DNA Adducts. After 1 wk on the experimental diets, rats received by gavage 250 µg of [3H]-AFB1 (80 µCi/µmol) per kg of body weight in 100 µl of tricaprylin (Sigma Chemical Co., St. Louis, MO) 5 days a wk for the next 2 wk (Days 0 to 4 and 7 to 11) at 8 a.m. One wk following cessation of dosing all animals were restored to the control diet. Animals were sacrificed on the indicated days at 10 a.m. (2 h after AFB1 dosing), and livers and kidneys were excised and minced in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.0) containing 0.25 M sucrose, and nuclei were isolated and purified by the method of Hymer and Kruff (28). DNA was purified (29) and hydrolyzed (30) as described previously. Nucleic acid hydrolysates were analyzed by reverse-phase chromatography using a 25-cm Beckman C18 5-µm Ultracore ODS column. Chromatography was performed at ambient temperature using a 25-min gradient of 10 to 18% ethanol/20 mM triethylammonium formate (pH 3.0) at 1.0 ml/min. The eluent was monitored at 365 nm, and 1-ml fractions were collected. The radioactivity in each fraction was determined by an LKB liquid scintillation counter using Scintiverse (Fisher Scientific, Pittsburgh, PA) as the scintillation cocktail. All samples were spiked with authentic standards synthesized and described (31).

Analysis of Foci. Rats were treated as described for the DNA adduct studies, but with unlabeled AFB1. Twelve wk after restoration of animals to control diets, they were sacrificed, livers were removed and weighed, and 4 liver samples were taken by the use of a 15-mm-diameter cork borer. Liver samples were then frozen in liquid nitrogen and stored at −80°C. Multimolecular forms of glutathione S-transferase were separated by ion-exchange column chromatography as described by Kitahara et al. (21). Briefly, 105,000 × g supernatants (40 mg/2.5 ml) dialyzed against 10 mM sodium phosphate buffer, pH 7.4, were applied to CM-Sephadex C-50 columns (2.5 × 8 cm) pre-equilibrated with the buffer and were eluted with 40 ml of the buffer and then 300 ml of the buffer with a linear gradient of NaCl to a final concentration of 80 mM. For RNA isolation, livers (0.75 g) were homogenized with a Polytron homogenizer in 12 ml of guanidine isothiocyanate stock solution, and RNA was purified by a method described by Chirgwin et al. (22). For RNA slot blots, 5 µg of total RNA were denatured in formaldehyde and serially diluted onto nitrocellulose paper (23). The glutathione S-transferase complementary DNA clones were radiolabeled with [32P]dATP and [32P]dCTP by standard nick translation to a specific activity of 1 × 107 cpm/µg (24). Clones pGTB38, pGTB42, and pGTA/C44 were the generous gift of Dr. Cecil B. Pickett and hybridized to mRNAs for rat glutathione S-transferase subunits 1 and 2 (25), 2 (26), and 3 (27), respectively. Filters were hybridized for 16 h at 37°C in 50% formamide, 3x Denhardt's (0.06% Ficoll, 0.06% bovine serum albumin, and 0.06% polyvinylpyrrolidone), 0.45 M sodium chloride-0.045 M sodium citrate (pH 7.4), 0.5% sodium dodecyl sulfate, and 10% dextran sulfate. After hybridization, filters were washed in 0.15 M NaCl-0.015 M sodium citrate-0.5% sodium dodecyl sulfate at 60°C. Washed filters were exposed overnight to Kodak XAR-5 film, and the level of hybridized RNA was determined by densitometric scanning of autoradiographs.

Statistical Analyses of the Data. The data were subjected to a one-way analysis of variance with comparisons among groups performed using the Neuman-Keuls procedure (35).
RESULTS

Induction of Glutathione S-Transferase by Ethoxyquin. Fig. 2, left, depicts the time-course for the induction of epoxide hydrase and glutathione S-transferase-specific activities in rat liver microsomes and cytosols, respectively, following addition of 0.4% ethoxyquin to the diet. Within 1 day on the ethoxyquin diet glutathione S-transferase activities were significantly elevated about 1.5-fold. Maximal induction with either DCNB (3.5-fold) or CDNB (4.5-fold) as substrate was observed after 1 wk of feeding the diet and persisted throughout the feeding period. Removal of ethoxyquin from the diet resulted in diminution of glutathione S-transferase activities such that base-line activities were reached within 10 days. A comparable pattern was observed for the induction of epoxide hydrase activity, although maximal induction was somewhat greater (6.5-fold). Dose-response curves for the induction of both enzymes are shown in Fig. 2, right. Rats fed ethoxyquin at levels as low as 0.05% showed significant elevations of enzyme activities after 2 wk on diets.

Ethoxyquin treatment also produced similar patterns of induction of glutathione S-transferase activity in the kidney. After 14 days on the experimental diet, DCNB- and CDNB-dependent activities were elevated by 3- and 4.5-fold, respectively. Activities returned to basal levels within 10 days following removal of ethoxyquin from the diet (data not shown).

There are at least 6 to 7 basic molecular forms of glutathione S-transferase in rat liver cytosol which are separable by CM Sephadex C-50 column chromatography. These isoforms exhibit different specificities towards DCNB and CDNB (21) as shown in Fig. 3, left. The administration of 0.4% ethoxyquin in the diet for 1 wk markedly induced the levels of forms 1-1, 1-2, and 3-3 and, to a lesser extent, forms 2-2, 3-4, and 4-4 (Fig. 3, right). In order to investigate the mechanisms by which ethoxyquin increases hepatic glutathione S-transferase activities, the time-course for increases in hepatic glutathione S-transferase mRNA levels was examined following introduction of ethoxyquin into the diet. Shown in Table 1, significant elevations in the levels of mRNA coding for subunits 1 and 2 were evident within 24 h; however, maximal increases of 6-fold were not observed until Day 7, and these levels were maintained through subsequent feeding to Day 14. More modest increases in the levels of mRNA specifying for subunit 3 were also noted. Overall, there appears to be a good concordance between the magnitude of the increase in glutathione S-transferase isoforms determined by ion-exchange chromatography and the respective increases in mRNA levels following ethoxyquin treatment.

Effect of Ethoxyquin on Hepatobiliary Elimination of Aflatoxin. Induction of Phase II enzymes, such as glutathione S-transferase, would be expected to facilitate the conjugation and elimination of xenobiotics like aflatoxin. Inasmuch as antioxidant treatment significantly reduces the accumulation of aflatoxin in liver following p.o. (data not shown) or i.p. administration (36), we examined the effects of ethoxyquin on the biliary elimination of aflatoxin. Bile ducts were cannulated in rats fed either control or ethoxyquin-supplemented (0.4%) diets for 1 wk. Chromatography was performed as described in "Materials and Methods." Values are units/ml.

Table 1 Time-course for the induction of hepatic glutathione S-transferase mRNA by ethoxyquin

<table>
<thead>
<tr>
<th>Days on ethoxyquin diet</th>
<th>Relative mRNA levels for glutathione S-transferase subunits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 and 2</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>1</td>
<td>2.7</td>
</tr>
<tr>
<td>3</td>
<td>4.1</td>
</tr>
<tr>
<td>7</td>
<td>6.0</td>
</tr>
<tr>
<td>14</td>
<td>6.4</td>
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</tbody>
</table>

Fig. 3. CM-Sephadex column chromatography of hepatic glutathione S-transferase from rats fed either control or ethoxyquin-supplemented (0.4%) diets for 1 wk. Chromatography was performed as described in "Materials and Methods." Values are units/ml.

Fig. 2. Time-course and dose-response for the induction of hepatic glutathione S-transferase (GST) and epoxide hydrase activities by dietary ethoxyquin. Specific activities are expressed as the ratio of ethoxyquin (treated) to control values at each time or dose. Left, time-course. Rats were placed on diet containing 0.4% ethoxyquin and sacrificed at the indicated times. After 14 days animals were restored to the control diet. Hepatic enzyme activities were determined as described in "Materials and Methods." Right, dose-response. Rats were maintained on diets containing the indicated concentrations of ethoxyquin for 1 wk prior to assay. Points, mean of duplicate determinations done on each of 4 animals. Bars, SE.
Table 2 Effect of ethoxyquin on the biliary elimination of aflatoxin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>nmol AFB-SG eliminated/2 h</th>
<th>% of administered dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.65 ± 0.12*</td>
<td>1.1</td>
</tr>
<tr>
<td>Ethoxyquin</td>
<td>2.71 ± 0.47</td>
<td>5.1</td>
</tr>
</tbody>
</table>

* Mean ± SE for 5 animals/group.

DNA Adduct Formation and Removal. It is well established that a single dose of AFB₁ is not an efficient carcinogenic regimen in rats; however, a dosing regimen of small repeated doses can induce a high incidence of hepatocellular carcinoma (1). Therefore, the effect of ethoxyquin on the kinetics of aflatoxin-DNA adduct formation and removal was examined in rats treated in a multiple-dosing protocol of 2 wk, as utilized by Croy and Wogan (37). This protocol would have been expected to yield a 100% incidence of hepatocellular carcinoma if dosing had continued for a total of 8 wk (38). The time-course for the formation and removal of total aflatoxin-DNA adducts in liver of rats receiving p.o. injections of 250 μg of AFB₁ per kg on each of Days 0–4 and 7–11 is shown in Fig. 4. Maximal binding levels were achieved following the second dose, and binding following the next 3 doses remained at a plateau level of about 140 pmol of aflatoxin equivalents bound per mg of DNA. Overall binding declined after cessation of the first dosing period; however, resumption of AFB₁ dosing produced only minor elevation of binding levels as the cycle of adduct formation and removal was renewed. This 50% diminution of aflatoxin-DNA binding during the second cycle presumably results from AFB₁-induced alterations in cytochrome P-450-mediated AFB₁ activation (37). Total DNA adduct levels dropped 5-fold in the first wk following cessation of dosing and continued to decline at a comparable rate over the next 4 mo to a level of 100 fmol of aflatoxin equivalents bound per mg of DNA at 133 days. Inclusion of ethoxyquin in the diet, beginning 1 wk prior to and extending to 1 wk beyond dosing with AFB₁, produced a dissimilar pattern of effects and yielded substantially lower binding levels during the early time period. At 2 h after the first AFB₁ dose approximately 18-fold less binding was observed in the ethoxyquin-treated animals. By Day 2 the difference declined to 6-fold and was about 3.5-fold throughout the second dosing cycle. The difference in binding levels diminished during the postdosing period such that binding levels in control and ethoxyquin rats were indistinguishable at Days 106 and 133.

A similar pattern for the effect of ethoxyquin on aflatoxin adduction to renal DNA was observed. However, initial levels of DNA adducts were about 10-fold lower than measured in liver. No aflatoxin-DNA adducts were detectable in DNA isolated from kidney after Day 49 (data not shown).

Liquid chromatographic analysis of hydrolyzed DNA from the livers of these animals revealed no remarkable qualitative differences in the adduct profile induced by ethoxyquin treatment. Fig. 5, top, depicts a chromatographic profile of aflatoxin-DNA adducts from control rat liver DNA isolated 2 h after the first dose, where 70% of the DNA adducts are found in the form of AFB-N₂-Gua. Decomposition products of this major adduct, namely, the formamido-pyrimidine derivatives [AFB-N²-FAPyr (major) and AFB-N²-FAPyr (minor)] and aflatoxin 8,9-dihydriodiol, account for most of the remaining aflatoxin-DNA adduct species. These data are in agreement with results previously reported by Croy and Wogan (37) using a different route of administration (i.p.). Also shown in Fig. 5, bottom, ethoxyquin treatment reduced the amount of the N²-guanine adduct by greater than 95%. The relative concentrations of the ring-opened formamido-pyrimidine adducts were also decreased to a comparable degree. The quantitative changes in individual adduct levels throughout the 5-mo experimental period are enumerated in Table 3. When integrated across time, ethoxyquin treatment reduced the accumulation of AFB-N²-Gua, AFB-N²-FAPyr (major), and AFB-N²-FAPyr (minor) adducts by 77, 71, and 76%, respectively. However, the temporal patterns for the different adducts were quite distinct. The levels of the two formamido-pyrimidine adducts remained constant over the 2-wk dosing period; approximately 40 and 10 pmol bound/mg of DNA for the major and minor derivatives, re-
Gua adduct was detectable after Day 21, indicating that this enzymatic process is rapidly removed from DNA by chemical and/or biological mechanisms.

The AFB-N7-Gua adduct decreased rapidly after Day 1, such that although the dominant species on the first day of dosing, levels of this adduct at these late time points. By contrast, adducts 2 h after gavage with a single dose of 250 μg of [3H]AFB1 per kg of body weight by gavage on Days 0 to 4 and 7 to 11.

**Table 3** Effect of ethoxyquin on concentrations of aflatoxin acid hydrolysis products in hepatic DNA of rats exposed to multiple doses of AFB1.

<table>
<thead>
<tr>
<th>Concentration (pmol AFB1 derivative/mg DNA)</th>
<th>AFB-N7-FAPyr (minor)</th>
<th>AFB-N7-FAPyr (major)</th>
<th>Diol</th>
<th>AFB-N7-Gua</th>
<th>Total</th>
</tr>
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<tr>
<td>Day</td>
<td>C</td>
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<td>C</td>
<td>EQ</td>
<td>C</td>
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<td>0*</td>
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<td>1*</td>
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<td>0.2</td>
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<tr>
<td>133</td>
<td>ND</td>
<td>0.1</td>
<td>ND</td>
<td>0.1</td>
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</tbody>
</table>

* C: control; EQ: ethoxyquin; ND: not detectable.
* Rats administered 250 μg of [3H]AFB1 per kg of body weight by gavage on Days 0 to 4 and 7 to 11.

**Table 4** Effect of ethoxyquin on aflatoxin-induced γ-glutamyl transpeptidase-positive foci in rat liver.

<table>
<thead>
<tr>
<th>Dietary treatment</th>
<th>Incidence at 16 wk</th>
<th>No. of foci/cm²</th>
<th>Foci vol (% of total liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12/12</td>
<td>1.60 ± 0.28</td>
<td>0.1097 ± 0.0527</td>
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<tr>
<td>Ethoxyquin</td>
<td>3/10</td>
<td>0.04 ± 0.02</td>
<td>0.0003 ± 0.0001</td>
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</table>

a Calculations of the number of foci per liver and the size of the foci by the methods of quantitative stereology (34) could not be performed because of too few foci in the ethoxyquin-fed animals. A mean of 490 ± 30 mm³ of liver was examined per rat in the controls and 7.75 ± 1.29 foci were detected, whereas, in the ethoxyquin-fed rats, 767 ± 25 mm³ of liver contained 0.30 ± 0.15 foci.

b Mean ± SE.

d Differs from control: P < 0.01.

DISCUSSION

We have previously observed that there is an excellent correlation between the degree of inhibition of overall aflatoxin-DNA binding in rat liver and the induction of glutathione S-transferase activities in this tissue by the administration of various antioxidants in the diet (36). The best inducer/inhibitor studied in this series was ethoxyquin, which after 2 wk in the diet stimulated a 4-fold increase in glutathione S-transferase activity and engendered a 90% inhibition in aflatoxin binding following a single, high dose of AFB1. Because a single dose of AFB1 is not an effective carcinogenic regimen in rats, we have examined the effect of ethoxyquin on the kinetics of DNA adduct formation and removal and altered foci formation in rats treated in a multiple-dosing protocol. The study of Cabral and Neal (12), who fed ethoxyquin at a level of 0.5% concurrently with feeding AFB1, at 4 ppm for 6 wk, demonstrated that ethoxyquin is an excellent chemoprotective agent. However, no studies on the mechanism of protection afforded by ethoxyquin have been described under experimental conditions that directly relate to reduction of neoplasia.

Feeding ethoxyquin to mice (40) or rats (Figs. 2 and 3) leads to the rapid and sustained elevation of glutathione S-transferase activities. Elevated activities in mouse liver transferases following treatment with antioxidants, such as BHA and ethoxyquin, are associated with marked increases in the concentration of immunoprecipitable enzyme protein. Additionally, mRNA specifying the synthesis of the major murine glutathione S-transferase activity and engendered a 90% inhibition in aflatoxin binding following a single, high dose of AFB1. Because a single dose of AFB1 is not an effective carcinogenic regimen in rats, we have examined the effect of ethoxyquin on the kinetics of DNA adduct formation and removal and altered foci formation in rats treated in a multiple-dosing protocol. The study of Cabral and Neal (12), who fed ethoxyquin at a level of 0.5% concurrently with feeding AFB1, at 4 ppm for 6 wk, demonstrated that ethoxyquin is an excellent chemoprotective agent. However, no studies on the mechanism of protection afforded by ethoxyquin have been described under experimental conditions that directly relate to reduction of neoplasia.

GGT-positive Lesions in AFB1-treated Rats. Table 4 shows the data obtained from quantitative 2- and 3-dimensional analyses of GGT lesions in livers of rats treated with 2 dosing cycles of AFB1 and sacrificed at 4 mo. In general accord with the findings of Appleton and Campbell (39), we observed 1.6 foci per cm² of liver in rats treated with aflatoxin and maintained on the control diet. Foci were seen in all animals in this group. By contrast, rats placed on the 0.4% ethoxyquin diet during the dosing period showed only a 30% incidence in hepatic foci and a mean number of <0.1 foci per cm² of liver. Coordinately, percentage volumes of liver occupied by GGT-positive foci were reduced by >95% in the ethoxyquin-treated animals from a level of 0.1% observed in rats on the control diet. Because the number of observed foci were too few in the ethoxyquin-fed rats, it was not possible to to calculate or statistically compare the 3-dimensional values of mean focal diameter and mean focal volume by the techniques of quantitative stereology.
catalytic and binding proteins that promote the conjugation of glutathione with electrophilic groups of a variety of molecules, including AFB$_1$. In rat liver, only 3 cystolic isoforms, 1-1, 1-2, and 2-2, catalyze aflatoxin conjugation to glutathione at appreciable rates (43). Levels of these molecular forms are elevated following ethoxyquin treatment (Fig. 3). Furthermore, the data presented in Table 1 directly demonstrate that mRNA levels for these rat liver transferase subunits are markedly elevated. The observed increase in catalytic activity of glutathione $S$-transferase isoforms demonstrating substrate affinity for aflatoxin 8,9-oxide should, in turn, facilitate aflatoxin conjugation in vivo. Indeed, measurement of the biliary elimination of AFB-SG shortly after administration of AFB$_1$ demonstrates that formation of this conjugate is accelerated in ethoxyquin-treated animals (Table 2) and could account, in part, for the lower concentration of aflatoxin seen in the livers of these animals.

Alteration in the balance of competing pathways of aflatoxin 8,9-oxide reactions should directly modulate the availability of the 8,9-oxide for binding to its presumed major critical nucleophilic target, the N$^7$ atom of guanine. As expected, ethoxyquin treatment dramatically reduces the formation of aflatoxin-DNA adducts in rat liver following both single (36) and repetitive dosings (Fig. 5). Other antioxidants, particularly phenolic antioxidants, have been shown to reduce the binding of aflatoxin to DNA in a variety of systems in vitro (44, 45) and in several cases this binding can be modulated by glutathione $S$-transferases (46). In the present study, the reductive effect of ethoxyquin was partially pronounced during the dosing period, ranging from 18-fold after the first AFB$_1$, dose to 3.5-fold throughout the second 5-day dosing period. Surprisingly, no differential effect on the persistent derivative of AFB-N$^7$-Gua, AFB-N$^7$-FAPyr (major), is observed between treatment groups from 1 to 4 mo after cessation of dosing. When coupled with our observation than an identical treatment protocol yields a greater than 95% reduction at 4 mo in the formation of GGT-positive foci, it is apparent that these persistent adducts are not good biochemical markers for quantifying risk for aflatoxin hepatocarcinogenesis. However, there is an excellent quantitative association between the degree of reduction in overall initial binding of AFB$_1$ (Fig. 5) as well as AFB-N$^7$-Gua adduct formation (Table 3) and chemoprotection as adjudged by reduction in GGT-positive foci. Both total binding and levels of AFB-N$^7$-Gua were diminished by 95% with ethoxyquin treatment on Day 0. Thus, the high-yield, rapidly removed AFB-N$^7$-Gua adduct may be of primary importance to the initiation of aflatoxin hepatocarcinogenesis. Supporting this view, Croy and Wogan (47) investigated aflatoxin-DNA adduct profiles in the liver and kidneys of the male F344 rat and the male Swiss mouse. These investigators have demonstrated a positive correlation between total adduct levels in the DNA and organotropic and species-dependent carcinogenic effects. DNA adduct formation has also been correlated with the mutation to 6-thioguanine resistance in human lymphoblast cells incubated with AFB$_1$ and an activating system (48). In both studies, the AFB-N$^7$-Gua adduct represented 80-90% of the total adduct species formed at the time of assay. N$^7$-purine adducts can induce depurination by destabilizing the N-glycosyl bond (49). Consistent with our hypothesis, Foster et al. (50) have examined base substitution mutations induced by metabolically activated AFB$_1$ in the lacI gene of Escherichia coli and demonstrated that the production of aguaninic sites may be a major result of this binding. In summary, the data presented in Table 1 directly demonstrate that persistent aflatoxin-DNA adducts may be of primary importance to the initiation of aflatoxin hepatocarcinogenesis. Supporting this view, Croy and Wogan (47) investigated aflatoxin-DNA adduct profiles in the liver and kidneys of the male F344 rat and the male Swiss mouse. These investigators have demonstrated a positive correlation between total adduct levels in the DNA and organotropic and species-dependent carcinogenic effects. DNA adduct formation has also been correlated with the mutation to 6-thioguanine resistance in human lymphoblast cells incubated with AFB$_1$ and an activating system (48). In both studies, the AFB-N$^7$-Gua adduct represented 80-90% of the total adduct species formed at the time of assay. N$^7$-purine adducts can induce depurination by destabilizing the N-glycosyl bond (49). Consistent with our hypothesis, Foster et al. 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MECHANISM OF CHEMOPROTECTION BY ETHOXYQUIN

Much attention has been recently focused on the development of rapid, sensitive, noninvasive techniques for measuring carcinogen-induced genomic damage in individuals (59). Immunoassays have been developed for quantifying aflatoxins, particularly AFB-N$^7$-Gua, in biological fluids, such as urine (60), and are applicable to biochemical epidemiological studies in humans (61). Based on the differential effects of ethoxyquin on the kinetics of aflatoxin-DNA adduct and GGT-positive foci formation, our findings support the concept that measurement of this major, rapidly excised adduct is an appropriate dosimeter for estimating exposure status and risk in individuals consuming this mycotoxin.

Some chemoprotective interventions merely serve to shift target organ sites. Williams et al. (62) have demonstrated reduction in liver neoplasia at the apparent expense of enhanced bladder tumorigenesis in rats concurrently fed N$^2$-acetylaminofluorene and BHT. However, this group has subsequently shown that feeding BHT or BHA during AFB$_1$ dosing had a marked protective effect on hepatocarcinogenesis without increasing neoplasia at other secondary sites of AFB$_1$ tumorigenesis, such as kidney (13). Both the present study as well as other studies in our laboratory have demonstrated that treatment with ethoxyquin, BHA, or BHT does not increase AFB$_1$ binding to DNA in kidney but, rather, produces reductions comparable to those observed in liver, albeit starting at a 10-fold lower initial level (35). Other potential limitations for chemoprotection with some antioxidants relate to their inherent toxicities. BHA, BHT, and ethoxyquin have mutagenic and/or carcinogenic properties and can act as tumor promoters at several organ sites (63–65). Thus, in practice, other agents may be required for human use. The systematic, integrative approach described in this paper should serve as a useful prototype for developing model systems for the evaluation of putative anticarcinogens. With this approach, facile quantitative comparison of different agents can be accomplished and new agents, devoid of untoward toxicities, readily evaluated.

REFERENCES


MECHANISM OF CHEMOPROTECTION BY ETHOXYQUIN


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Modulation of Aflatoxin Metabolism, Aflatoxin-$N^7$-guanine Formation, and Hepatic Tumorigenesis in Rats Fed Ethoxyquin: Role of Induction of Glutathione S-Transferases

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