Natural chlorophyll inhibits aflatoxin B1-induced multi-organ carcinogenesis in the rat

Michael T. Simonich 1, Patricia A. Egner 1, Bill D. Roe buck 2, Gayle A. Orner 1, Carole Jubert 1, Cliff Pereira 3, John D. Groopman 4, Thomas W. Kensler 5, Roderick H. Dashwood 1,2, David E. Williams 1,2 and George S. Bailey 1,2.

1 Linus Pauling Institute, 2 Environmental and Molecular Toxicology Department, and 3 Department of Statistics, Oregon State University, Corvallis, OR 97331, USA. 4 Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205, USA and 5 Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH 03755, USA

Email: george.bailey@oregonstate.edu
Fax: 541-737-7966;
To whom correspondence should be addressed. Tel: 541-737-3164;
Advance Access publication February 8, 2007
doi:10.1093/carcin/bgm027
Carcinogenesis vol.28 no.6 pp.1294–1302, 2007

Chemo prevention by chlorophyll (Chl) was investigated in a rat multi-organ carcinogenesis model. Twenty-one male F344 rats in three gavage groups (N = 7 rats each) received five daily doses of 250 µg/kg [3H]-aflatoxin B1 ([3H]-AFB1) alone, or with 250 mg/kg chlorophyllin (CHL), or an equimolar amount (300 mg/kg) of Chl. CHL and Chl reduced hepatic DNA adduction by 42% (P = 0.031) and 55% (P = 0.008), respectively, AFB1–albumin adducts by 65% (P < 0.001) and 71% (P < 0.001), respectively, and the major AFB–N7-guanine urinary adduct by 90% (P = 0.0047) and 92% (P = 0.0029), respectively. To explore mechanisms, fluorescence quenching experiments established formation of a non-covalent complex in vitro between AFB1 and Chl (Kd = 1.22 ± 0.05 µM, stoichiometry = 1Chl:1AFB1) as well as CHL (Kd = 3.05 ± 0.04 µM, stoichiometry = 1CHL:1AFB1). The feces of CHL and Chl co-gavaged rats contained 137% (P = 0.0003) and 412% (P = 0.0048) more AFB1 equivalents, respectively, than control feces, indicating CHL and Chl inhibited AFB1 uptake. However, CHL or Chl treatment in vivo did not induce hepatic quinone reductase (NAD(P)H:quinone oxidoreductase) or glutathione S-transferase (GST) above control levels. These results are consistent with a mechanism involving complex-mediated reduction of carcinogen uptake, and do not support a role for phase II enzyme induction in vivo under these conditions. In a second study, 30 rats in three experimental groups were dosed as in study 1, but for 10 days. At 18 weeks, CHL and Chl had reduced the volume percent of liver occupied by GST placental form-positive foci by 74% (P < 0.0001) and 77% (P < 0.001), respectively compared with control livers. CHL and Chl reduced the mean number of aberrant crypt foci per colon by 63% (P = 0.0026) and 75% (P = 0.0004), respectively. These results show Chl and CHL provide potent chemoprotection against early biochemical and late pathophysiological biomarkers of AFB1 carcinogenesis in the rat liver and colon.

Introduction

Chronic exposure to aflatoxin B1 (AFB1), a major cause of the high prevalence of hepatocellular carcinoma (HCC) in Asia and sub-Saharan Africa (1,2). Prevention measures that limit human exposure to AFB1, and its adverse health effects, are important public health goals in these geographies. Research in experimental chemoprevention has received little study. While Chl is potently anti-mutagenic (reviewed (22), only a few studies have suggested that natural Chl might have cancer preventive properties in whole animals. In the rainbow trout model of dietary carcinogenesis, hepatic DNA-adduct formation resulting from 200 ppm dibenz[a]pyrene exposure was reduced 66% by co-exposure to 3000 ppm Chl in the diet (23). Moreover, the adduct inhibition by Chl was nearly identical to the protection afforded by a similar dietary co-exposure to CHL (23). In the rat colon, dietary spinach or an equimolar amount of Chl equally inhibited cytotoxicity and colonoocyte proliferation induced in animal models such as the rat have demonstrated more than a score of natural and synthetic chemopreventive agents that can dramatically inhibit the formation of aflatoxin biomarkers and the resulting incidence of putative pre-neoplastic lesions and HCCs (3–9).

One such agent, chlorophyllin (CHL), a water soluble, sodium–copper salt of chlorophyll (Chl) (Figure 1), was tested in a clinical chemoprevention trial on a human population in eastern China with chronic, unavoidably high aflatoxin exposure and a high incidence of HCCs (10). Administration of 100 mg dietary CHL three times per day led to a highly significant, 50% reduction in the level of aflatoxin–N7-guanine (AFB–N7-guanine) in the urine of participants (11). Elevated urinary output of this hepatic DNA adduct biomarker in humans is clearly associated with increased risk of liver cancer (12), and diminished levels of AFB–N7-guanine have been associated with reduced HCC risk in several animal studies (3–5,8). Thus, diet supplementation with CHL might reduce human liver cancer risk from dietary AFB1.

Protection by CHL, however, is not limited to AFB1. Mechanistic studies in mammals and fish suggest that CHL acts as a carcinogen-blocking agent, at least in part, by forming tight complexes with AFB1, as well as heterogeneous amine and polycyclic aromatic hydrocarbon carcinogens (13–15). Complex formation appears to block carcinogen absorption, thereby reducing bioavailability to the target tissue resulting in less DNA adduction and, ultimately, lower tumor incidence (16). Additional mechanisms of CHL protection have been proposed including inhibition of bioactivating enzymes, induction of detoxifying enzymes and direct antioxidant activity (16).

While CHL is widely manufactured, purity among different suppliers varies greatly (17) and its use as a dietary supplement entails some expense and inconvenience. More importantly, CHL chemoprevention is not without some potential risk. Promotion by a post-initiation CHL exposure has previously been reported in the rat where 0.1% CHL in the drinking water for 20 weeks after initiation increased the incidence of 1,2-dimethylhydrazine (DMH)-induced colon tumors from 10 to 47% (18). Post-initiation treatment with 0.001% CHL in the drinking water for 47 weeks also significantly increased colon tumor multiplicity in response to DMH (19), and the same CHL dose by water for 16 weeks after 2-amino-3-methylimidazo[4,5-f] quinolone (IQ) initiation significantly increased colon aberrant crypt foci (ACFs)/colon in the rat (20).

A potential alternative to CHL is natural Chl, which is without known toxicity in mammals. In the one study where post-initiation effects of Chl on colon carcinogenesis were examined, Chl suppressed rather than promoted azoxymethane- (a metabolite of DMH) and IQ-induced ACFs (20). An additional attraction is the potential to achieve protective doses of Chl without supplementation. Dietary Chl intake comparable with the 100 mg per meal (0.13 mole per meal) daily doses of CHL administered in the human intervention trial is obtainable by moderate/high consumption of green vegetables. Spinach leaves, for example, average 0.12 mmole Chl per 100 g wet weight, so consumption of 100 g of spinach, or equivalent vegetable, per meal would approximate the above dose of CHL. However, in comparison with CHL, the chemopreventive properties of natural Chl have received little study. While Chl is potently anti-mutagenic (reviewed in 21), and was recently shown to induce mammalian phase 2 detoxifying enzymes in vitro (22), only a few studies have suggested that natural Chl might have cancer preventive properties in whole animals. In the rainbow trout model of dietary carcinogenesis, hepatic DNA-adduct formation resulting from 200 ppm dibenz[a]pyrene exposure was reduced 66% by co-exposure to 3000 ppm Chl in the diet (23).

Abbreviations: ACF, aberrant crypt focus; AFB1, aflatoxin B1; ANOVA, analysis of variance; Chl, chlorophyll; CHL, chlorophyllin; DMH, 1,2-dimethylhydrazine; GST, glutathione S-transferase; GST-P, glutathione S-transferase placental form-positive foci; HCC, hepatocellular carcinoma; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; NQO, NAD(P)H:quinone oxidoreductase; TEG, triethylene glycol.
Natural Chl inhibits AFB1-induced multi-organ carcinogenesis in the rat

Fig. 1. Chemical structures of Chl and CHL.

by heme, a red meat, iron-containing pro-oxidant correlated with increased risk of colon cancer (24,25). In addition, the Chl-containing diet largely blocked formation of a cytotoxic heme metabolite (24). The authors speculated that green vegetables may decrease colon cancer risk from dietary heme through the protective effects of Chl.

Although these studies suggest a potential role for dietary Chl in cancer prevention, there are at present no animal data demonstrating its efficacy. The present study used the male F344 rat gavage model of AFB1 carcinogenesis (4,5) to compare the effects of Chl and CHL co-exposure against early biomarkers of genome damage including AFB1–DNA adduction, serum albumin adduction and urinary AFB1–N7-guanine excretion. We also examined AFB1 interactions with these two agents in vitro, as well as the in vivo response of hepatic phase 2 enzymes to Chl and CHL alone and in co-gavage with AFB1. A second study compared the effects of Chl and CHL on AFB1-induced pre-neoplastic lesions in the liver and colon. The results demonstrate that Chl and CHL have similar potency to reduce AFB1 uptake, biomarker response and tumor initiation in the rat. They do not support the idea that induction of phase II enzymes by Chl or CHL is an operative protective mechanism in vivo in this model.

Materials and methods

Materials

CHL, AFB1, tricaprylin and triethylene glycol (TEG) were from Sigma Chemical Co. (St Louis, MO). [3H]-AFB1 (21.8 Ci/mmol) was from Moravek Biochemicals (Brea, CA). The purity and concentration of AFB1 was confirmed by absorbance in ethanol at 362 nm (ε362 = 2.18 × 105 M⁻¹ cm⁻¹). The chloride content of CHL was based on the manufacturer’s assay of 4.3% copper and assertion that all copper was present as copper chlorins (Lot # 14H002, 51.3% Cu chlorin content, additional material is water soluble Na salts). Highly purified Chl was prepared as described below.

Purification of Chl

A comprehensive description of the Chl purification will be presented elsewhere (C.Jubert and G.S.Bailey, in preparation). Briefly, organic spinach was homogenized with cold water, freeze-dried, washed twice with petroleum ether (boiling point 30–60°C) and the solids extracted twice using methanol/petroleum ether (3:1, v/v). The combined extracts were washed with saturated sodium chloride, filtered and evaporated in vacuo (temperature < 30°C). This crude Chl extract (90% pure by high-performance liquid chromatography) was further purified by counter current chromatography using an Ito multilayer coil separator–extractor (P.C., Potomac, MD). Analyses of counter current chromatography fractions were performed by high-performance liquid chromatography, mass spectrometry and proton nuclear magnetic resonance spectroscopy. A minor impurity was detected at δ 5.40 consisting of 0.35 protons upon integration, and the only other extra peaks were the NMR solvent (δ 2.05) and the two signals due to H2O and deuterated water (δ 2.78 and 2.75). Purity was estimated to be >95% compared with Chl-a standards (Sigma Chemical Co.), which were 90–92% pure based on spectroscopic measurements. Thirty grams of freeze-dried spinach yielded 225 mg of Chl-a and 75 mg of Chl-b for a total yield of 300 mg Chl, or 1% by dry weight. Chl preparations used in all experiments were a recombined 3:1 mixture of Chl-a:Chl-b.

Preparation of test solutions

Concentrated stocks (>1 mg/ml) of AFB1 were first prepared in dichloromethane and diluted to working concentrations in ethanol prior to dilution in the tricaprylin gavage vehicle. Final ethanol concentration in gavage solutions was <1% (v/v). Concentrated solutions of CHL were prepared in water and diluted to the administered concentration in tricaprylin gavage vehicle. CHL is virtually insoluble in water; thus, CHL solutions were prepared in petroleum ether, diluted to a working stock in ethanol and diluted to the administered concentration in tricaprylin gavage vehicle. Gavage solutions were monophasic with the exception of the AFB1–CHL co-gavage in which the non-chlorin salt fraction was insoluble (see Materials and methods).

Animals and diets

For the adduct study, male F344 rats, 75–100 g (Simonsen Laboratories, Gilroy, CA), were housed individually in metabolism cages, for easy collection of separate urines and feces, and maintained at 22°C on a 12-h light–dark cycle. For the liver and colon foci study, male F344 rats, 75–100 g, were housed two per cage in standard barrier cages (see details below). Rats were acclimated to AIN-93G diet (Dyets, Bethlehem, PA) without ethoxyquin antioxidant and fed ad libitum for 1 week prior to the period of carcinogen exposure.

Adduct study

After 1 week on the acclimation diet, the rats were randomly assigned to one of three treatment groups. The experimental treatments are summarized in Table I. Group 1 (N = 7) received by gavage 250 μg of [3H]-AFB1 (260 μCi/μmol) per kg of body wt (0.208 μCi/g body wt) in 3 μl of tricaprylin per gram of body wt. Group 2 (N = 7) received the same [3H]-AFB1 dose mixed with 250 mg CHL (as Cu chlorin) per kg body wt. Group 3 (N = 7) received the same [3H]-AFB1 dose mixed with 300 mg Chl per kg body wt. The amount of Chl administered was equimolar with the amount of CHL administered and, based on in vitro binding measurements, was calculated to complex at least 90% of the AFB1 in the gavage solutions at 25°C. All seven rats per group were gavaged on days 0, 1 and 2. Two hours after the day 2 gavage, four rats from each group were euthanized for tissue collection. The remaining three rats per group were gavaged on days 3 and 4, and 2 h after the day 4 gavage the remaining rats were euthanized for tissue collection. Rats were weighed daily throughout the experiment.

Isolation of DNA and analysis of hepatic aflatoxin–DNA adducts

Rats were euthanized with CO2 on the indicated days 2 h after gavage dosing. The liver was excised, cut into pieces of ~100 mg, which were divided equally between two 50 ml centrifuge tubes and immediately frozen in liquid N2 for later use. Approximately 1.2 g of frozen liver pieces were placed...
in 20 volumes of DNAzol reagent (Molecular Research Center, Cincinnati, OH) and homogenized with a few pulses of an Ultra-Turrax T8 homogenizer (IKA Works, Wilmington, NC). DNA extraction using DNAzol was according to the manufacturer’s protocol, and quantification of DNA yield was done with PicoGreen reagent (Molecular Probes, Eugene, OR). A known mass of purified DNA (average ≈1 mg) from each liver was directly counted in a Beckman 7500 liquid scintillation counter as a measure of total aflatoxin equivalents per milligram of DNA (signal/noise ratio >100 for all samples).

**Quantification of aflatoxin—serum albumin adducts**

Blood (≈1 ml) was collected from each rat on gavage day 2 or 4 at necropsy. The blood was centrifuged at 300g for 5 min and the resultant serum was removed and stored at −80°C until analysis. The serum albumin was purified by the method of Chapot and Wild (26) and quantitated using Bradford dye reagent and albumin standards (Bio-Rad Laboratories, Hercules, CA). The samples were prepared for liquid scintillation counting by mixing 0.5 ml of purified albumin sample (1–2 mg albumin) with 1 ml Soluene 350 tissue reagent and albumin standards (Bio-Rad Laboratories, Hercules, CA). The mixture at 55°C for 1 h. After cooling, 10 ml of Hionic Fluor LSC fluid (Perkin Elmer, Shelton, CT) in a glass scintillation vial and heating the mixture at 55°C for 1 h. After cooling, 10 ml of Hionic Fluor LSC fluid (Perkin Elmer) was added to the solubilized samples and total AFB1 equivalents were determined radiochemically in a Beckman 7500 liquid scintillation counter (signal/noise ratio >100 for all samples).

**Urinary AFB–N7-gua and fecal AFB equivalents**

Twenty-four hour urine samples were normalized using a spectrophotometric creatinine kit (TECO Diagnostics, Anaheim, CA). Urine aflatoxin metabolites were recovered using an aflatoxin-specific preparative monoclonal antibody immunoaffinity column as described previously (27–29). Metabolites were identified and quantitated by electrospray mass spectrometry as described (30). Twenty-four hour fecal samples, ≈1 g, were weighed and homogenized in 10 volumes of water, and 0.5 ml aliquots were decolorized overnight at room temperature with 30% hydrogen peroxide. A 0.1 ml aliquot of each sample (2 mg feces) was mixed with 10 ml of Hionic Fluor LSC fluid and counted in a Beckman 7500 liquid scintillation counter (signal/noise ratio >100 for all samples).

**AFB1 interactions with CHL and Chl in vitro**

Formation of a non-covalent complex between AFB1 and CHL or highly purified Chl was assessed by quenching of AFB1 fluorescence as described previously in detail for quenching of AFB1 (AFB1 structural analogue) fluorescence by CHL (31). Dissociation constant (Kd) determinations could not be carried out in the tricaprylin gavage solvent due to the strong fluorescence of that compound. We instead used a close approximation to tricaprylin, triethyleneglycol (TEG, pH 8.0), an amphipathic solvent that provided appropriate linearity of AFB1 fluorescence and sensitivity for monitoring quenching. TEG was not used for gavage co-solvent due to its dehydrating effect on rats. The initial concentration of AFB1 (substrate) was 10 μM in a 3 ml stirred quartz cuvette. CHL or Chl was added in 1.33 μM increments up to 31.92 μM Chl or Chl, with negligible increase in assay volume from lipid additions. Fluorescence was monitored at 428 ± 8 nm with excitation at 368 ± 8 nm, and was recorded 2 min after each ligand addition on a SLM 8000 photon counting spectrofluorometer (SLM Aminco, Urbana, IL). The fluorescence quenching data were iteratively fitted to 1:1 and 2:1 AFB1:CHL or Chl models of binding stoichiometry as described in (31).

**Phase 2 enzyme responses**

Two additional treatment groups indicated in Table 1 (N = 2 rats each) were gavaged concurrently with the rats in the adduct study and were used to measure phase 2 responses due solely to CHL or Chl exposure. These groups were gavaged on days 0–2 only, with 250 mg/kg CHL (as CuEChl) in tricaprylin or with 300 mg/kg Chl in tricaprylin. Approximately 0.5 g of frozen rat liver from each rat in the adduct study was homogenized in nine volumes of 3 mM Tris-Cl, pH 7.4, with 0.25 M sucrose and 1 mM ethyleneglycol-bistaminoethylether(5)-tetraacetic acid and then centrifuged at 100 000 g to yield microsomal-free cytosol. The cytosolic protein concentration of each sample was determined using the Bradford dye reagent with albumin standards. Cytosols were stored as 200 μl aliquots at −80°C for enzyme assays. The Prochaska bioassay (32) was used to measure the activity of NAD(P)/Quinone oxidoreductase (NQO) using dilute cytosol in triplicate assays. Afterward, the rate of each reaction was normalized to the sample protein content as determined by crystal violet staining (32). The activity of glutathione S-transferase (GST) was similarly assayed, but at 340 nm at 25°C with 1-chloro-2,4-dinitrobenzene as the substrate. One milliliter of 0.1 M potassium phosphate, pH 6.6, reaction buffer contained 50 μl 20 mM glutathione, 50 μl 20 mM 1-chloro-2,4-dinitrobenzene (ethanolic stock) and dilute cytosol. The reaction rate was measured at 1 min intervals for 5 min on a Beckman Coulter DU800 spectrophotometer.

**Late pathophysiological marker study**

Forty-two male F344 rats (mean weight 140 g) were arranged into six treatment groups. The experimental treatments are summarized in Table I. Groups 1–3 (N = 10 rats each) were experimental groups, and groups 4–6 (N = 5, 4 and 3 rats each, respectively) were controls. Group 1 received 250 μg/kg body...
wt AFB1 in tricaprylin. Group 2 received 250 μg/kg AFB1 plus 250 mg/kg body wt CHL (as Cu chlorin content) in tricaprylin. Group 3 received 250 μg/kg AFB1 plus 250 mg/kg Chl in tricaprylin. Group 4 received tricaprylin only and group 5 and group 6 received 250 mg/kg CHL and Chl, respectively. All treatments were administered by gavage (3 μl/kg body wt) five times per week for 2 weeks. One rat in group 1 died at week 5 and thus, group 1 ended with N = 9 rats. After 18 weeks, all rats were killed by CO2 asphyxiation, the livers removed and weighed. For glutathione S-transferase placental form-positive foci (GST-P) quantification, two 3 mm sections were cut by hand from the median lobe of the liver and fixed in acetone. Sections were processed for histology using the AMeX fixation and processing procedure (33). Slides were stained for expression of GST-P and examined by light microscopy as described previously (34). The volume percent of liver occupied by GST-P-positive foci is considered the least biased and most predictive estimate of eventual tumor burden (35); therefore, this was the primary endpoint evaluated. All samples were coded so that the individual analyzing them was blinded to the treatment group. ACFs quantification was done according to Ormer et al. (36).

Briefly, colons were removed, washed with cold phosphate-buffered saline, fixed mucosal side up in 10% phosphate-buffered formalin, stained with 0.2% methylene blue and ACF scored as described previously (37). The samples were also coded for blinded analysis.

### Statistical analyses

Data for the DNA adducts, serum albumin adducts, fecal AFB1 equivalents and phase 2 enzyme responses were analyzed by comparing treatment means with the control group mean by a standard analysis of variance (ANOVA) model (homogeneous variance and Dunnett’s adjustment to minimize type I errors).

For the urinary data, heterogeneous variance in statistical comparisons of treatment means necessitated natural log (ln) transformation of the raw data. The variance of the ln-transformed data was first tested for homogeneity using ANOVA of the squared deviations from the treatment means for each metabolite. This comparison indicated that variance among the treatments within each metabolite category was homogenous (P > F value), and that treatment means of the ln-transformed data could be compared by r-test. Dunnett’s adjustment for multiple comparisons was applied to the r-tests to minimize type I errors. All calculations were performed using SAS version 9.1 (SAS Institute).

Statistical analysis of the GST-P-positive foci data was by ANOVA, followed by a Bonferroni multiple comparison test to determine differences between individual groups. Statistical analysis of the ACF data was by ANOVA and post hoc Fisher’s PLSD test.

### Results

### Chl and CHL effects on AFB1-DNA adduction in vivo

The effects of CHL and Chl on hepatic AFB1-DNA adduction in rats that received 250 μg [3H]-AFB1 per kg by gavage for 5 days are shown in Figure 2 (panel A). Liver samples were taken on two separate days at 2 h post-dosing, the time of maximal AFB–N7-guanine levels in the rat liver (38). The overall DNA adduction level, measured as [3H]-AFB1 equivalents, among the three treatments was slightly higher on day 2 than day 4. This is consistent with previous studies in which the maximum levels of carcinogen binding to hepatic DNA are observed following the first few doses of AFB1, and decline thereafter, despite continued exposure (6,39). Co-gavage with 250 mg/kg CHL or the molar equivalent dose of 300 mg/kg Chl reduced hepatic DNA adduction by 33% (P = 0.003) and 47% (P = 0.001), respectively, relative to the control group by day 2. At 2 h after the day 4 gavage, the control group adduct level had declined somewhat from day 2, but protection by CHL and Chl remained substantial, with adducts decreased by 42% (P = 0.031) and 55% (P = 0.008), respectively, relative to the control group.

### Chl and CHL effects on AFB1-serum albumin adduction

Serum albumin is the major blood protein to be adducted following metabolic epoxidation of AFB1 (5,40,41). The levels of aflatoxin–serum albumin adduction were measured radioactively and the impact of CHL and Chl on albumin binding is shown in Figure 2 (panel B). The temporal pattern of albumin-adduct formation and the degree of chemoprotection is similar to that seen in the DNA. Day 2 showed an overall higher level of adduction and both the CHL and Chl co-treatments significantly reduced adducts by 52% (P = 0.038) and 62% (P = 0.022), respectively, relative to the positive control group. On day 4, as we observed for DNA adduction, the degree of protection by CHL and Chl remained significant, with 65% (P < 0.001) and 71% (P < 0.001) respective reductions in albumin adducts. Also, as seen with DNA adducts, CHL and Chl protection on day 4 appeared slightly greater than on day 2, though not significantly so.
Chl and CHL effects on urinary and fecal elimination of aflatoxin metabolites

Previous studies have shown that, in mammals, the major AFB1–N7-guanine adduct is rapidly excised and excreted via the urine (42,43), and that CHL-impeded absorption of AFB1 results in lower levels of excised DNA adducts in the urine, and correspondingly higher AFB1 equivalents in the feces (7). Table II shows the mean levels of the AFB1–N7-guanine adduct and two major less-toxic aflatoxin metabolites, AFM1 and AFP1, in 24 h urine samples taken on day 4 (N = 3 rats per treatment). Statistical comparisons were made on natural log-transformed data and reported on the original (e^x) scale. The level of the AFB1–N7-guanine adduct excreted in the urine was significantly reduced by 90% (P = 0.0047) and 92% (P = 0.0029) by co-gavage with CHL and Chl, respectively, compared with the control group. AFM1 in the urine was significantly reduced by 63% (P = 0.0173) and 81% (P = 0.0016) by CHL and Chl, respectively. AFP1 excretion to the urine was also significantly reduced by 90% (P = 0.0003) and 92% (P = 0.0002) by CHL and Chl, respectively. The relative amounts of protection by CHL and Chl against the urinary AFB1–DNA repair product and AFP1 metabolite were identical. CHL and Chl appeared slightly less effective in reducing the level of AFM1 in urine, but the basis for this is unclear.

Conversely, the day 4, 24 h feces from the CHL and Chl co-gavaged rats contained 137% (P = 0.0003) and 412% (P = 0.0048) more AFB1 equivalents, respectively, than did feces of the control group (Figure 3). The fecal elimination of AFB1 equivalents in the Chl-co-treated rats was 2-fold greater (P = 0.0212) than the elimination by CHL-co-treated rats, a larger difference between CHL and Chl effects than seen with any other parameter except urinary AFM1 excretion.

Chl and CHL interactions with AFB1 in vitro

We previously reported that CHL formed a strong non-covalent 1:1 complex with AFB1 and AFB2 (8,9 position saturated analogue) in vitro (Kd = 1.4 and 1.92 µM, respectively) (13,31). Importantly, those studies also noted that inclusion of CHL at sufficient concentration to achieve ≥99% calculated complexation of aflatoxin strongly and significantly reduced hepatic DNA adduction, aflatoxin uptake and biodistribution. We sought here to compare the ability of natural Chl and CHL to complex with AFB1, under similar co-solvent conditions, as a possible mechanism of Chl chemoprotection. The tricaprylin complex with AFB1 and AFB2 (8,9 position saturated analogue) provided a suitable approximation to tricaprylin (see Materials and methods). Titration of AFB1 in TEG with CHL or Chl in 1.33 M TEG provided a suitable approximation to tricaprylin (see Materials and methods). Titration of AFB1 in TEG with CHL or Chl in 1.33 M TEG provided a suitable approximation to tricaprylin (see Materials and methods).

To quantify CHL and Chl quenching of AFB1 fluorescence, the fractional fluorescence change (ΔF/F0) at 428 nm emission with each titration was plotted and a model assuming a 1:1 ratio of CHL or Chl to AFB1 binding and zero fluorescence yield of bound AFB1 was fitted (Figure 4B, CHL data not shown). This model provided an excellent fit to the data for CHL and Chl binding (r² = 0.9999) and yielded a Kd = 3.05 ± 0.04 µM for CHL and Kd = 1.22 ± 0.05 µM for Chl. Modeling assuming a 2:1 AFB1:CHL or Chl-binding stoichiometry yielded a poor fit to the data (not shown). In sum, with conditions approximating the gavage solvent, the in vitro binding of Chl to AFB1 was ~2.5 times stronger than the binding of CHL to AFB1.

Chl and CHL effects on hepatic phase 2 enzyme responses

Induction of phase 2 enzymes is an important and, in some cases, a sufficient detoxification response to block chemical carcinogenesis (44,45). Table III shows the activity levels of GST and NQO on gavage days 2 and 4 in each treatment. Neither CHL nor co-treatment significantly increased the basal activity of either enzyme above that seen in the AFB1 control liver (P > 0.05). The standard ANOVA model indicated evidence of decreased GST activity relative to AFB1 on day 4 in the CHL (P = 0.037) and Chl (P = 0.0043) co-treatments. Treatment with CHL or Chl alone (measured from day 2 only) did not induce GST or NQO. In sum, these data provide no evidence for induction of hepatic phase II enzymes in vivo following CHL or Chl treatment.

Chl and CHL effects on AFB1 pre-neoplastic lesions in the rat liver and colon

Rats administered AFB1 showed an 11% slower growth rate (P < 0.05), regardless of co-treatment, for the first 4 weeks of the experiment compared with the non-AFB1-treated rats (data not shown). By the fifth week of the 18 week study, all rats had attained the same growth rate. Treatment with vehicle + CHL or Chl had no effect on growth relative to treatment with vehicle only.

The volume percent of liver occupied by GST-P-positive foci is considered the least biased and most analogous focal predictor of final tumor burden (35) and, therefore, was the primary end point evaluated. CHL and Chl equally and significantly (P < 0.001) reduced the volume percent of liver occupied by GST-P-positive foci (Figure 5, panel A). GST-P-positive foci were seldom observed in vehicle and Chl or CHL control rats. The focal density in the liver (foci per cubic centimeter) was the same between the three aflatoxin-treated groups. CHL co-treatment significantly (P < 0.01) reduced mean focal diameter compared with AFB1 exposure alone, and the effect of CHL on this parameter approached significance (P = 0.084, data not shown).

The effect of CHL and Chl co-treatments on colon ACFs is shown in Figure 5 (panel B). AFB1 treatment resulted in 2.4 ± 0.6 (mean ± standard error) ACFs per colon, but co-treatment with CHL or Chl significantly reduced this ratio to 0.9 ± 0.3 (P = 0.0026) and 0.6 ± 0.3 (P = 0.0004), respectively. The number of crypts per ACF (focus size) did not differ significantly among the treatments.

Discussion

The present studies demonstrate substantial protection by natural Chl against AFB1 carcinogenesis in the rat liver and colon. Hepatic DNA

<table>
<thead>
<tr>
<th>Table II. Effect of Chl on AFB1 metabolites in rat urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gavage treatment</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>250 µg/kg AFB1</td>
</tr>
<tr>
<td>250 µg/kg AFB1 +</td>
</tr>
<tr>
<td>250 µg/kg AFB1 +</td>
</tr>
</tbody>
</table>

Statistically significant differences from the control are indicated with one or more asterisks: *P < 0.05, **P < 0.005 and ***P < 0.0005.

The multiplicative difference [e(control–treatment)] between control and treatment mean of the logged data: i.e. AFB1 alone produced 10.1 times and 12.65 times more AFB1–N2-guanine adducts than did co-treatment with CHL and Chl, respectively. The 95% confidence intervals (CIs) on the multiplicative differences are in brackets.
Natural Chl inhibits AFB₁-induced multi-organ carcinogenesis in the rat

adduct and serum albumin adduct burdens were dramatically reduced in Chl-co-exposed rats compared with controls. The hepatic and colonic burdens of presumptive pre-neoplastic foci were also dramatically reduced by Chl co-exposure.

A large body of mechanistic studies on rodents and trout indicates that the degree of attenuation of AFB₁-DNA adducts by dietary chemopreventive agents is closely correlated with reduced incidence of pre-neoplastic lesions and reduced tumor incidence (4,46–48) (reviewed in 7,16). A previous study in rats showed that gavage with 100 mg of CHL immediately followed by gavage of 10 μg of [3H]-AFB₁ resulted in a 45% decrease in the level of hepatic DNA adducts (7). This level of protection was mirrored by commensurate decreases in serum albumin adducts and urinary excretion of aflatoxin equivalents (7). The present study extends those findings by demonstrating that CHL co-treatment also protects against AFB₁-induced pre-neoplastic lesions in liver as well as colon.

While the precise mechanism of Chl protection during co-exposure with AFB₁ was beyond the scope of the present study, it appears that Chl does not protect by eliciting phase 2 enzyme detoxication of AFB₁ when co-administered with the carcinogen. Analysis of hepatic NQO and GST activity levels in rats co-exposed to AFB₁ and CHL or Chl indicated no increase in these phase 2 activities relative to the AFB₁-exposed livers. This result was unexpected in light of the recent report by some of us that CHL, Chl and related tetrapyrroles induced NQO activity in cultured murine hepatoma cells (22). In that study, induction was significant, though Chl was not a potent inducer with a concentration required to double NQO activity (CD) of 250 μM. CHL, however, was nearly 10 times as potent an inducer of NQO (CD = 30 μM). The relatively poor inducing potency for Chl in vitro may reflect poor uptake into these cells. Data on the metabolism of Chl in the whole animal gut as well as uptake of Chl or its oxidation products from the gut are very limited, but for decades the central assumption has been that Chl uptake in vivo is not significant. Poor bioavailability, however, is an unlikely explanation for our finding that CHL co-exposure did not induce NQO. The bioavailability of CHL components, especially chlorin e₄ ethyl ester, is well established (49), and the ethyl ester component (CD = 20 μM) is 50 times more potent an NQO inducer in vitro than Chl (22). Our NQO results with CHL co-exposure may reflect the inherent gap between cell culture and whole animal studies. Alternatively, differences in the dose and timing of CHL administration may account for different phase 2 induction. Dingley et al. (50) showed that rats given dietary CHL (10 g/kg diet) for 2 weeks prior to a single low-dose co-gavage of two heterocyclic amine carcinogens had a 1.6-fold increase in NQO activity relative to control animals 24 h after the carcinogen dose, but no change in GST activity. There are, however, substantial protocol differences in that study (a 40-fold higher concentration of CHL in the diet, administration of CHL for 2 weeks prior to the carcinogen) that might account for the difference in NQO induction. A whole-animal study comparing multiple doses of CHL and Chl and pre-versus co-initiation protocols may help shed light on the relative importance of phase 2 metabolism in CHL and Chl chemoprevention.

Both Chl and CHL given co-initiation (during carcinogen exposure) were quite effective in reducing putative pre-neoplastic foci in the colon and the liver. Consumption of chemoprotectants by humans is likely to involve these agents acting on previous as well as concurrent initiation events. Thus, the post-initiation effects of CHL and Chl are important. Our companion study in the rainbow trout model (M.T. Simonich et al., in preparation) included examination of the co-versus post-initiation effect of 2000 ppm dietary CHL on dietary dibenz[a,l]pyrene-initiated tumors in multiple organs. Post-initiation CHL for 9 months significantly promoted swim bladder tumor incidence from 10 to 38%, and promotion approached significance in the stomach. Co-initiation CHL had no effect in the swim bladder, but significantly inhibited tumor incidence by 30% in the liver and stomach. In the rat, post-initiation treatment with 0.1% CHL in the drinking water for 20 weeks reportedly increased the incidence of DMH-induced colon tumors from 10 to 47% (18). In another study, post-initiation treatment of rats with 0.001, 0.01 or 0.1% CHL in the drinking water for 47 weeks significantly increased the multiplicities of DMH-initiated colon tumors only at the 0.001% CHL dose (19). In that same study, the same CHL regimen inhibited in a dose-related manner dietary IQ-initiated liver tumors, but had no effect on IQ-initiated colon tumors (19). A follow-up study to (19) reported that 0.001% CHL in the drinking water for 16 weeks after IQ gavage significantly increased IQ-induced ACFs (20). Importantly, that study also included an experiment that showed that oral administration of 0.08% dietary natural Chl (approximately equivalent to 0.1% in the drinking water) inhibited azoxymethane- (a metabolite of DMH) and IQ-induced ACFs (20). Collectively, the pattern of post-initiation effects of CHL and Chl appears to depend heavily on several factors including concentration and duration of post-initiation exposure and the initiating carcinogen. Future work with Chl must carefully examine these factors to evaluate its true utility as a chemopreventive agent.

The present study provides limited evidence that Chl inhibits uptake of AFB₁ from the rat stomach, and that it does so with equal or greater efficacy than CHL. In pharmacokinetic compartments outside the gastrointestinal tract such as the liver, serum and urine, the adduct burden was reduced over a 2- to 13-fold range by Chl co-exposure, whereas in the feces of the same animals, roughly 5-fold more AFB₁ equivalents were eliminated relative to the control animals. Thus, Chl co-exposure largely restricted AFB₁ to the gastrointestinal tract. Moreover, substantial protection by CHL against ACF development in the colon suggests that, in addition to restricting AFB₁ to the gastrointestinal tract, CHL treatment reduced AFB₁ metabolism to toxic intermediates in the colon. One simple mechanism to explain inhibition of both uptake and colon metabolism of the carcinogen could be formation of a molecular complex between Chl and AFB₁ in the gut. Tight Chl complexation with AFB₁, some heterocyclic amine carcinogens and dibenz[a,l]pyrene is easily demonstrated in vitro (13–15) using both fluorescence and absorbance spectrophotometry. We show here that Chl is also able to form an AFB₁ complex of ~2.5 times greater stability in vitro. Interestingly, the chemoprotection afforded by Chl against AFB₁-induced DNA damage, serum...
Fig. 4. Spectrofluorometric titration of AFB1 with Chl. (A) Effect of Chl on the AFB1 emission spectrum from 380 to 550 ±9 nm (excitation 368 ± 8 nm) with AFB1 (substrate) concentration at 10 μM. Chl (ligand) was added in 1.33 μM increments up to 31.92 μM (some titrations omitted from the figure for clarity) and the spectrum was recorded 2 min after each addition. (B) Quantification of Chl quenching of AFB1 fluorescence at 428 ± 8 nm recorded from the above spectra. Data were normalized by converting fluorescence units to ΔAF/FO and the data were fitted to a 1:1 Chl:AFB1 complexation model.

Table III. Effect of Chl on hepatic phase 2 enzyme activities

<table>
<thead>
<tr>
<th>Gavage treatment</th>
<th>Activity (nmol/min/mg)</th>
<th>GST</th>
<th>NQO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
<td>Day 4</td>
<td>Day 2</td>
</tr>
<tr>
<td>250 μg/kg AFB1</td>
<td>294.5 ± 17.60</td>
<td>333.2 ± 26.67</td>
<td>26.7 ± 4.81</td>
</tr>
<tr>
<td>250 μg/kg AFB1 + 250 mg/kg CHL</td>
<td>328.0 ± 8.63</td>
<td>282.2 ± 7.83*</td>
<td>28.3 ± 2.06</td>
</tr>
<tr>
<td>250 μg/kg AFB1 + 300 mg/kg Chl</td>
<td>250.0 ± 4.60</td>
<td>261.6 ± 6.29**</td>
<td>27.3 ± 1.69</td>
</tr>
<tr>
<td>250 mg/kg CHL</td>
<td>313.8 ± 9.80</td>
<td>24.6 ± 1.42</td>
<td>25.1 ± 5.58</td>
</tr>
<tr>
<td>300 mg/kg Chl</td>
<td>271.2 ± 10.83</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Groups significantly different than the AFB1 control group are indicated with an asterisk (+P < 0.05 and ++P < 0.005).

*Liver tissue samples were collected as described in the legend to Figure 1. The day 2 and day 4 responses are mean responses ± standard errors for N = 4 animals per treatment on day 2 and N = 3 animals per treatment on day 4 (exception for Day 2 NQO activity, N = 3 for AFB1 treatment). Assays were done in triplicate for all groups. Only day 2 tissues were collected from the CHL and Chl control groups from N = 2 animals each.

2G GST activity in 100 000 g cytosol was assayed once per minute for 5 min at 25°C as described in Materials and methods. NQO activity in 100 000 g cytosol was assayed at the end of a 5 min reaction at 25°C as described.
natural Chlorophyll inhibits AFB1-induced multi-organ carcinogenesis in the rat

Acknowledgements

We especially thank Mandy Louderback for her excellence in rat husbandry and necropsy, and Adam Sinner for his diligent work on the enzyme assays as part of his training project. We thank Dr Michael Schimerlik of the Department of Biochemistry and Biophysics for help with modeling the fluorescence binding data. We also thank Yonghai Li and Cody Olsen of the Department of Statistics for their help with data analyses. Partly supported through National Institutes of Health grants CA90890, ES00210, ES03850, CA100608 and CA65525.

Conflict of Interest Statement: None declared.

References


Received July 31, 2006; revised January 4, 2007; accepted January 29, 2007