EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 36, No. 4, 351-357, August 2004

Effect of diet on aflatoxin B_1-DNA binding and aflatoxin B_1-induced glutathione S-transferase placental form positive hepatic foci in the rat

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Accepted 24 July 2004

Abbreviations: AFAR, AFB_1-aldehyde reductase; AFB_1, aflatoxin B1; CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione S-transferase; GST-P, GST-placental form; PB, phenobarbital; PC, Purina Chow

Abstract

Effects of diets on hepatic aflatoxin B_1 (AFB_1)-DNA binding and AFB_1-induced glutathione S-transferase placental (GST-P) form positive hepatic foci have been examined in young male Fischer rats. Animals were fed either AIN-76A or Purina Chow (PC) diet for 1 wk before AFB_1-DNA binding studies *in vivo* and *in vitro*. Animals were injected *i.p.* with AFB_1 (1 mg/kg body wt) and 3 days later were given either AIN-76A or PC diet with or without 0.1% phenobarbital (PB) in their drinking water. All animals were sacrificed 10 wks after AFB_1 dosing for analysis of AFB_1-induced GST-P positive hepatic foci by immunochemistry. Two h after *i.p.* injection of AFB_1, hepatic AFB_1-DNA binding in AIN-76A fed rats was twice as much as those in PC fed animals without affecting GSH levels. There was no significant effect of diet on either cytochrome P-450 content, GSH levels or microsomal cytochrome P-450 mediated AFB_1-DNA binding to exogenous DNA. There was a 40% increase in cytosolic GSH S-transferase activity with 1-chloro-2,4-dinitrobenzene as a substrate in PC fed animals compared to those given AIN-76A diet. The number and area of AFB_1-induced GST-P positive hepatic foci were twice and fivefold as much in AIN-76A fed compared to those in PC fed rats. The number of AFB_1-induced GST-P positive foci was increased 5-10 fold in the presence of PB in both groups. In summary, the present data indicate that feeding of PC diet compared to AIN-76A diet inhibits the initiation phase whereas AIN-76A stimulates the promotion phase of AFB_1 hepatocarcinogenesis in rats by inhibiting AFB_1-DNA binding and increasing AFB_1-induced hepatic foci respectively.

Keywords: AFB_1; AFB_1-DNA binding; diet; GST-P positive foci; hepatocarcinogenesis

Introduction

Epidemiological studies have established that contamination of foods with aflatoxin B_1 (AFB_1), a mycotoxin, is one of the important risk factors responsible for human liver cancer (Wogan,1992). Among laboratory animals, rat is the most susceptible to AFB_1 hepatocarcinogenesis (Newberne and Butler, 1969; Eaton and Gallagher, 1994). Metabolic activation of AFB_1 occurs via cytochrome P-450 enzyme system to form an ultimate reactive metabolite, AFB_1-epoxide, which interacts covalently with cellular macromolecules including DNA. This covalent interaction of DNA with AFB_1 is considered to be the first step responsible for the initiation of AFB_1 carcinogenesis (Miller and Miller,1981); AFB_1-DNA adduct is considered to be the only intermediate responsible in the AFB_1 pathogenesis of hepatocarcinogenesis (Eaton and Gallagher, 1994). Various isoforms of glutathione S-transferases (GSTs) and AFB_1-aldehyde reductase (AFAR) play an important part in modulation of AFB_1-DNA binding and AFB_1 carcinogenesis in various susceptible and resistant species by inactivation of the reactive AFB_1-epoxide by conjugating with either GSH or proteins (Eaton and Gallagher,1994; Manson *et al.*, 1997).

Dietary factors have been shown to modify various stages of chemical carcinogenesis in several animal

models. Thus, low protein diets inhibited initiation and promotion stages whereas high protein diet stimulated the promotion phase of AFB_1 hepatocarcinogenesis in the rat model (Appleton and Campbell, 1983). Earlier studies indicated that high dietary fat content increased both initiation and promotion of AFB_1-induced hepatic lesions (Rogers and Newberne, 1969) whereas later studies suggested that high fat content was important for initiation but not for promotion (Baldwin and Parker, 1987). Induction of hepatic and colon tumors by several different carcinogens also is enhanced in rats fed a high fat diet (Rogers and Newberne, 1975). Numerous studies have demonstrated that dietary restriction of laboratory animals reduced the incidence of chemically-induced tumors (Pariza and Boutwell, 1987; Sugie *et al*., 1993),due to inhibition of metabolic activation of chemical carcinogens (Chou *et al*., 1993).

Commercial diet such as Purina Chow (PC) contains many plant ingredients which induce xenobiotic metabolizing enzymes including GSTs (Salbe and Bjeldanes, 1989; Wattenberg, 1992). These commercial diets also demonstrate a lot of seasonal variability in response to the carcinogenic process. Therefore, a semi-synthetic diet such as AIN-76A diet has been established to minimize variations in the carcinogenic process. This AIN-76A diet has been used in several carcinogenicity studies with many types of chemical carcinogens (Russell *et al*., 1987; Hendrich *et al*., 1988; Hecht *et al*., 1989). In this report, effects of commercial diet, Purina Chow (PC) and a semi-synthetic diet, AIN-76A are compared for their effect on hepatic AFB_1-DNA binding and AFB_1-induced GST-placental (GST-P) positive hepatic foci in rats. Marked differences are observed in both of these parameters examined.

Materials and Methods

Chemicals

Non-radioactive AFB_1, DMSO, GSH, calf thymus DNA, NADPH and diaminobenzidine were purchased from Sigma Chemical (St. Louis, MO). 1-Chloro-2,4-dinitrobenzene (CDNB) was a product of Eastman Kodak, Rochester, NY. Phenobarbital (PB) was obtained from Amend Drug and Chemical Co., Irvington, NJ. Radioactive [^{3}H] AFB_1 (Sp. Activity, 30 Ci/mmol) purchased from Moravek Biochemicals, Brea, CA was diluted with non-radioactive AFB_1 in DMSO to obtain the required specific activity. Scintillation fluid 'Scintiverse II' was purchased from National Diagnostic Co., Manville, NJ. Vectastain ABC kit for immunohistochemical staining was bought from Vector Laboratories, Burlingame, CA. Rat GST-P antibody in rabbit was kindly provided by Dr. Kiyomi Sato's laboratory, Hirosaki, Japan. AIN-76A diet (Second Report, 1980) was purchased from Dyets Inc., Bethlehem, PA. AIN-76A diet contained casein, 20% by wt, cornstarch 15%, sucrose 50%, fiber 5%, corn oil 5%, DL-methionine 0.3%, vitamin mix 1.0% and mineral mix 3.5%. PC diet obtained from Ralston Purina Co., St. Louis, MO, consisted of ground yellow corn, soybean meal, beet pulp, fish meal, ground oats, brewers dried yeast, alfalfa meal, cane molasses, wheat germ meal, dried whey, meat meal, wheat middlings and animal fat. PC diet contained protein 23.0% by wt, fat 5%, fiber 6.0%, starch 40%, sucrose 5%, lactose 2.5% and mineral mix 2.5%.

Animals and treatment

Sixty-six male Fischer rats (6-7 wk old, 90-100 g body wt, Charles River Breeding Laboratories, Wilmington, MA) were housed in plastic cages with hardwood chips for bedding in an air-conditioned room at 23 ± 2^{o}C with a 12:12-h light-dark cycle.

Where indicated, one set of 18 animals was used for AFB_1-DNA binding studies *in vivo* and subcellular studies *in vitro* including microsome-mediated AFB_1-DNA binding to exogenous DNA. The animals were randomly divided in two groups. One group was maintained on PC diet and another group on AIN-76A diet for a wk. Three animals from each group on PC diet or AIN-76A diet were sacrificed by exsanguination for isolation of hepatic microsomes and cytosol. Other six animals from each group were injected *i.p.* with [^{3}H] AFB_1 (40 μg AFB_1 containing 10 μCi [^{3}H] AFB_1 dissolved in 0.2 ml DMSO/100 g body wt) 2 h before sacrifice for hepatic AFB_1-DNA binding studies.

Where indicated, another set of 48 animals was on PC diet for a wk before *i.p.* administration of either DMSO or AFB_1 (1 mg/kg body wt) dissolved in DMSO. Three days after either DMSO or AFB_1 administration, each group of six animals was given either PC or AIN-76A diet with or without 0.1% PB in their water. All animals were sacrificed 10 wks after AFB_1 dosing for analysis of AFB_1-induced GST-P positive hepatocytes and foci.

Liver microsomes and cytosols were prepared as described previously (Lotlikar *et al*., 1989).

Microsome-mediated binding of AFB_1 to exogenous DNA

The incubation medium was as described previously (Lotlikar *et al.,* 1989). After isolation of DNA by the published procedure (Daoud and Irving, 1977), isolated DNA was centrifuged, washed twice with cold ethanol and was finally dissolved in 1 ml of 10 mM Tris buffer, pH 7.4 containing 1 mM EDTA. One aliquot was used for UV spectrophotometry for quantifying DNA (Lotlikar *et al*., 1989). Two aliquots of iso-

lated DNA were taken for radioactivity measurements by using 5 ml of Scintiverse II. Radioactivity in samples was counted in LKB 1219 Racbeta liquid scintillation spectrometer with an efficiency of 45% for [^{3}H] counting. Results of [^{3}H] AFB$_1$ binding to DNA were corrected for DNA recovery and are expressed as pmol [^{3}H] AFB$_1$ binding/mg DNA/30 min. Variations in duplicate samples were less than 5%.

Hepatic AFB$_1$-DNA binding *in vivo*

Animals were sacrificed by exsanguination 2 h after AFB$_1$ injection and livers were excised, homogenized in 10 mM Tris-HCl buffer, pH 7.4 containing 2 mM $CaCl_2$ and 0.25 M sucrose. The homogenates (10%) were centrifuged at 600 *g* for 10 min and the nuclear pellet was extracted to isolate DNA according to the published procedure (Daoud and Irving, 1977) and dissolved in Tris-buffer and radioactivity counted as described above. Aliquots of DNA were taken colorimetric determination of DNA (Burton,1956). Results of [^{3}H] AFB$_1$ binding to nuclear DNA were corrected for DNA recovery and are expressed as pmol [^{3}H] AFB$_1$ bound/mg DNA.

AFB$_1$-induced GST-P positive hepatocytes and foci

Ten weeks after either DMSO or AFB$_1$ dosing, all animals were sacrificed by exsanguination under CO_2 euthanasia. Livers were excised, cut into 2- to 3-mm thick sections and were fixed in 10% phosphate-buffered formalin for immunohistochemical staining method of Hsu *et al* (1981). The sites of peroxidase binding were detected by the diaminobenzidine method (Graham and Karnofsky, 1966). By taking one section from each lobe, about 4-6 cm^2 of liver from each animal was examined for immunohistochemical staining. GST-P positive hepatocytes and hepatic foci were counted with the use of a light microscope. Diameters of these foci were determined with an eyepiece micrometer. Areas of these foci were calculated from diameters of these foci.

Other assays

Hepatic GSH levels were determined colorimetrically (Ellman, 1959) using a calibration curve. The values were expressed as μmol GSH/g liver. GSH S-transferase activity in liver cytosols using CDNB as a substrate was assayed spectrophotometrically by the published method (Habig and Jakoby, 1981). Cytochrome P-450 content in liver microsomes was determined from the CO-difference spectra of dithionite reduced samples using an extinction coefficient 91/mM/cm between 450 and 490 nm (Omura and Sato, 1964). Protein in microsomes and cytosols was determined by the Lowry method with bovine serum albumin as the standard (Lowry *et al*., 1951). The significance of the data was statistically evaluated by using Student's *t*-test; $P < 0.05$ was considered statistically significant.

Results

Two hours after *i.p.* injection of AFB$_1$, hepatic GSH levels and AFB$_1$-DNA binding were examined in rats fed either PC or AIN-76A diet for a week (Table 1). AFB$_1$-DNA binding in AIN-76A fed rats was twice as much as those in PC fed animals. Hepatic GSH levels in AIN-76A fed rats was decreased by about 10% compared to those in PC fed rats; however, this decrease was not statistically significant.

Effect of diet was examined *in vitro* studies with microsomal cytochrome P-450 mediated AFB$_1$-binding to exogenous DNA and other parameters (Table 2). Even though AFB$_1$-DNA binding to exogenous DNA was slightly higher (15%) in animals fed AIN-76A diet than those in PC diet, this difference was not statistically significant. Similarly, there were no significant differences either in cytochrome P-450 content or

Table 1. Effect of diet on hepatic GSH levels and AFB$_1$-DNA binding in rats.

Diet	AFB$_1$-DNA Binding (pmole/mg DNA)	GSH Levels (μmol/g liver)
PC	42.2 ± 6.3	6.5 ± 1.2
AIN-76A	96.2 ± 12.1[a]	5.8 ± 0.5

Each group consisted of 6 animals that were fed either PC or AIN-76A diet for a wk before AFB$_1$ administration. All animals were injected *i.p.* with [^{3}H] AFB$_1$ (40 μg AFB$_1$ containing 10 μCi [^{3}H] AFB$_1$ dissolved in 0.2 ml DMSO/100 g body wt) 2 h before sacrifice. Hepatic GSH levels were assayed colorimetrically (Ellman, 1959). Hepatic nuclear DNA was isolated by the published procedure (Daoud and Irving, 1977) and quantitated by the colorimetric procedure (Burton, 1956). Radioactivity in duplicate samples was counted in a scintillation spectrometer with an efficiency of 45% for [^{3}H] counting. Results of [^{3}H] AFB$_1$ binding to DNA were corrected for DNA recovery and are expressed as pmol [^{3}H] AFB$_1$ bound/mg DNA. Results are given as means ± SD of 6 animals in each group. [a]Data are considered significant with value of $P < 0.05$ when compared with respective data of animals on PC diet.

Table 2. Effect of diet on hepatic microsomal cytochrome P-450 content, cytochrome P-450 mediated AFB_1-binding to exogenous DNA, GSH levels and cytosolic GSH S-transferase activity.

Diet	Cytochrome P-Content (nmol/mg protein)	AFB_1 Binding to Exogenous DNA (pmol/mg DNA/30 min)	GSH levels (μmol/g liver)	GSH S-transferase activity with CDNB (nmol/min/mg protein)
PC	0.64 ± 0.08	60 ± 7.0	6.0 ± 0.5	737 ± 75
AIN-76A	0.75 ± 0.12	69 ± 10	5.1 ± 0.7	528 ± 63[a]

Each group consisted of 3 animals that were fed either PC or AIN-76A diet for a wk before sacrifice. Isolation of liver microsomes, cytosols and incubation medium for microsome-mediated binding of AFB_1 to exogenous calf thymus DNA were as described (Lotlikar *et al.*, 1989). Isolation of DNA was by the published procedure (Daoud and Irving, 1977) and quantitation of DNA by UV spectrophotometry (Lotlikar *et al.,* 1989). Radioactivity in duplicate samples was counted in a scintillation spectrometer. Results of $[^3H]AFB_1$ binding to DNA were corrected for DNA recovery and are expressed as pmol $[^3H]AFB_1$ binding/mg DNA/30 min. Microsomal cytochrome P-450 content was determined from the CO-difference spectra of dithionite reduced samples using an extinction coefficient 91 mM/cm between 450 and 490 nm (Omura and Sato, 1964). GSH S-transferase activity in liver cytosols using CDNB as a substrate was assayed spectrophotometrically (Habig and Jakoby, 1981). Results are given as means ± SD of 3 animals in each group. [a]Data are considered significant with value of $P < 0.05$ when compared with respective data of animals on PC diet.

Table 3. Promoting effects of diet and PB on AFB_1-induced GST-P positive hepatic foci in rats at 10 weeks.

Diet	AFB_1 (1 mg/kg)	PB	GST-P Positive Hepatocytes			
			Single cells	Mini Foci (2-9 cells) (No/cm^2)	Foci (100-300 μm)	Area of Focia (mm^2/cm^2)
PC	-	-	0.04 ± 0.09	0.03 ± 0.08	0	0
	-	+	0.09 ± 0.22	0.15 ± 0.14	0.21 ± 0.22	0.002 ± 0.002[a]
	+	-	0.14 ± 0.12	0.11 ± 0.11	0.43 ± 0.32[b]	0.013 ± 0.010[b]
	+	+	1.08 ± 0.33[a]	2.47 ± 1.05[a]	3.80 ± 1.24[a]	0.156 ± 0.051[a]
AIN-76A	-	-	0	0	0	0
	-	+	0.05 ± 0.09	0.08 ± 0.09	0.14 ± 0.19	0.001 ± 0.002
	+	-	0.38 ± 0.18[b,c]	0.73 ± 0.77	0.91 ± 0.20[b,c]	0.066 ± 0.014[b,c]
	+	+	1.05 ± 0.77	4.28 ± 0.85[a,c]	3.82 ± 0.90[a]	0.275 ± 0.065[a,c]

Each group consisted of 6 animals. All animals were on PC diet for 1 wk before *i.p.* administration of either DMSO or AFB_1 (1 mg/kg body wt) dissolved in DMSO. Three days after either DMSO or AFB_1 administration, each group was started on different combination of diet and water with or without 0.01% PB. All animals were sacrificed 10 wks after AFB_1 dosing. Livers were excised, cut into 2-3 mm thick sections and were fixed in 10% phosphate-buffered formalin for immunohistochemical detection of GST-P positive hepatocytes and hepatic foci by immunohistochemical staining method (Hsu *et al.,* 1981). The sites of peroxidase binding were detected by the diaminobenzidine method (Graham and Karnofsky, 1966). About 4-6 cm^2 of liver from each animal was examined for immunohistochemical staining. GST-P positive hepatocytes and hepatic foci were counted with the use of a light microscope. Diameters of these foci were determined with an eyepiece micrometer. Areas of these foci were calculated from diameters of these foci. Results are given as means ± SD of 6 animals in each group. [a]Data are considered significant with value of $P < 0.05$ when considered with respective data of animals without PB treatment. [b]Data are considered significant with value of $P < 0.05$ when compared with respective data of animals without AFB_1 dosing. [c]Data are considered significant with value of $P < 0.05$ when considered with respective data of animals on PC diet.

hepatic GSH levels in these two groups. There was, however, a 40% increase in cytosolic GSH S-transferase activity with CDNB as a substrate in PC fed animals compared to those given AIN-76A diet.

Data on promoting effects of diet and PB on AFB_1-induced GST-P positive foci in rats are summarized in Table 3. The number and area of AFB_1-induced GST-P positive foci were twice and fivefold as much in AIN-76A fed rats compared to those in PC fed animals. The number of AFB_1-induced GST-P positive hepatic foci was increased several fold in the presence of PB in both groups of rats. However, the area of foci was 75% higher in AIN-76A fed rats than those in PC fed diet.

Discussion

Data presented in this report indicate that feeding of AIN-76A diet compared to PC diet increased both hepatic AFB_1-DNA binding and AFB_1-induced GST-P positive hepatic foci in rats.

Since AFB_1-DNA binding is considered to be the first step responsible for the initiation of AFB_1 hepatocarcinogenesis (Miller and Miller, 1981; Eaton and Gallagher, 1994), data of increased AFB1-DNA binding presented in this report (Table 1) suggest that feeding of AIN-76A diet instead of PC diet during initiation may increase the initiation of AFB_1 hepatocarcinogenesis in the rat.

AFB1 is metabolized *via* cytochrome P-450 enzyme system not only to active AFB_1-epoxide but also to noncarcinogenic ring-hydroxylated metabolites (Eaton and Gallagher, 1984). Various diets have been shown to affect the levels and activities of P-450 enzymes (Yang *et al*., 1992). In the present study, however, data on hepatic P-450 levels and microsome-mediated AFB1-DNA binding to exogenous DNA suggest that microsomal P-450 system cannot account for differences in AFB_1-DNA binding observed *in vivo* (Table 1).

Hepatic cytosolic enzymes such as GSTs and AFAR also play an important role in modulation of AFB_1-DNA binding (Eaton and Gallagher, 1994; Manson *et al*., 1997). Various cruciferous vegetables, antioxidants and PC diet have been shown to induce hepatic GSTs and AFAR in rodents (Salbe and Bjeldanes, 1989; Wattenberg, 1992; Manson *et al*., 1997). In the present study, there was 40% more hepatic GST activity in animals fed PC diet compared to those fed AIN-76A diet (Table 2). This may account for less hepatic AFB_1-DNA binding observed *in vivo* studies in animals fed PC diet compared to those fed AIN-76A diet (Table 1) and are compatible with less hepatic AFB_1-DNA binding observed in rats given dietary Brussel sprouts which induce GST activity (Salbe and Bjeldanes, 1989). Concomitant analysis of AFB_1-GSH conjugate in bile as shown earlier (Jhee *et al*.,1989), could have provided direct evidence for the involvement of GSTs in modulation of AFB_1-DNA binding *in vivo* studies. Similarly, analysis of hepatic cytosolic inhibition of microsome-mediated AFB_1-DNA binding to exogenous DNA with the formation of AFB_1-GSH conjugate (Raj *et al*., 1984) or direct assay of AFB_1-GSH conjugate with hepatic cytosols and synthetic AFB_1-epoxide as a substrate as reported previously (Gopalan *et al*., 1992; Chen *et al*., 1995) may have implicated GSTs in modulation of AFB_1-DNA binding.

Hepatic GSH plays an essential role for the activity of GSTs. It has been shown earlier that in addition to modulation of GSTs activity, modulation of hepatic GSH levels by GSH depleting agents increased hepatic AFB_1-DNA binding in rodents (Gopalan *et al*., 1994). However, in the present study since hepatic GSH levels did not change significantly (Table 1), it appears that hepatic GSH levels in both groups did not play any significant role in modulation of AFB_1-DNA binding.

It has been demonstrated that rats fed various antioxidants in their diets induce hepatic AFAR activity that inactivates AFB_1-epoxide (Manson *et al*., 1997). Thus, there is a possibility that PC or AIN-76A diet could have shown differences in AFAR activity that may have accounted for differences in AFB_1-DNA binding *in vivo*.

We and others have shown that acute response of animals toward rather high levels of AFB_1 *via i.p.* administration are relatable to the biological responses of extended cumulative low dose 1.6 ppm AFB_1 that is likely to occur in the contaminated food consumption (Hiruma *et al*., 1997 and references therein). In addition, such a single high *i.p.* dose used in the present study enables one to study the carcinogenic process in a relatively shorter time (10 wks compared to 50-70 wks). Recent studies from our laboratory with AFB_1 (Gopalan *et al*., 1993; Hiruma *et al*., 1997) have lent support to the hypothesis that early GST-P positive hepatocytes induced by diethylnitrosamine are the precursors for neoplastic foci and nodules (Satoh *et al*., 1989; Dragen *et al*., 1994). The present data suggest that AIN-76A diet fed during the promotion phase enhanced the promotion of AFB_1 hepatocarcinogenesis in the rat. In the present study, dietary PB induced the number and area of AFB_1-induced hepatic foci several fold with both AIN-76A and PC diets similar to those observed earlier with diethylnitrosamine-induced hepatic foci in rats (Russell *et al*., 1987). PB has been shown not only to stimulate the proliferation of phenotypically altered hepatocytes *in vivo* but also to inhibit apoptosis of these altered cells (Schulte-Hermann *et al*., 1990; Warner and Schrenk, 1996; Pitot, 1998). The higher number and area of AFB_1-induced hepatic foci in rats fed AIN-76A diet compared to PC diet observed in the present study are in accord with previous studies performed with diethylnitrosamine-induced hepatic foci in rats (Russell *et al*., 1987; Hendrich *et al*., 1988). Similar dietary modulations of azaserine-induced pancreatic cancer and nitrosamine-induced lung tumors in rodents have been demonstrated with AIN-76A and PC diets (Longnecker *et al*., 1981; Hecht *et al*., 1989).

The presence of high sucrose in AIN-76A diet has been implicated as a promoting factor in altered hepatic foci induction in diethylnitrosamine hepatocarcinogenesis in rats; in those studies, the selective enhancement of proliferation in enzyme altered foci was also observed (Hei and Sudilovsky, 1985). Dietary fructose has been demonstrated to enhance N-nitosomorpholine-induced preneoplastic hepatic lesions and hepatocarcinogenesis in rats by affecting predominantly carbohydrate metabolism (Enzmann *et al*., 1989). In the present study, sucrose and possibly its intestinal hydrolysis product, fructose may also be

responsible for the promotion of AFB_1-induced hepatic foci in rats.

In summary, the present data indicate that feeding of PC diet compared to AIN-76A diet inhibits the initiation phase whereas AIN-76A diet stimulates the promotion phase of AFB_1 hepatocarcinogenesis in rats by inhibiting AFB_1-DNA binding and increasing AFB_1-induced hepatic foci respectively.

Molecular mechanisms of dietary effects on initiation and promotion phases of AFB_1 hepatocarcinogenesis in rats need further investigation.

Acknowledgment

We are indebted to Faye Lotlikar for the excellent assistance in preparing and editing of this manuscript. Rat GST-P antibody in rabbit was kindly provided by Dr. Kiyomi Sato's laboratory in Hirosaki, Japan. This research was supported by USPHS grant R01 CA 31641 awarded to PDL by the National Cancer Institute, Department of Health and Human Services.

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