Aflatoxin B[subscript 1]-DNA adduct formation and mutagenicity in livers of neonatal female B6C3F1 mice

Aflatoxin B_1-DNA adduct formation and mutagenicity in livers of neonatal male and female B6C3F1 mice.

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Short Title: Aflatoxin adducts and mutation

ABSTRACT

Exposure to genotoxic chemicals at a young age increases cancer incidence later in life. Aflatoxin B_1 (AFB_1) is a potent genotoxin that induces hepatocellular carcinoma (HCC) in many animal species, and in humans. Whereas adult mice are insensitive to aflatoxin-induced carcinogenesis, mice treated with AFB_1 shortly after birth develop a high incidence of HCC in adulthood. Furthermore, the incidence of HCC in adult male mice treated as infants is much greater than in females, reasons for which are unclear. In this study, treatment with AFB_1 produced similar levels of DNA damage and mutations in the liver of newborn male and female *gpt* delta B6C3F1 mice. Twenty four h after dosing with AFB_1 (6 mg/kg) the highly mutagenic AFB_1-FAPY adduct was present at twice the level of AFB_1-N^7-guanine in liver DNA of males and females. A multiple dose regimen (3 x 2 mg/kg), while delivering the same total dose, resulted in lower AFB_1 adduct levels. Mutation frequencies in the *gpt* transgene in liver were increased by 20- to 30-fold. The most prominent mutations in AFB_1-treated mice were G:C to T:A transversions and G:C to A:T transitions. At this 21-day time point, no significant differences were found in mutation frequency or types of mutations between males and females. These results show that infant male and female B6C3F1 mice experience similar amounts of DNA damage and mutation from AFB_1 that may initiate the neoplastic process. The gender difference in the subsequent development of HCC highlights the importance of elucidating additional factors that modulate HCC development.

Keywords: Aflatoxin, neonatal mouse, hepatocarcinoma, mutation, gpt delta mouse

INTRODUCTION

Animals treated with chemical carcinogens during the perinatal period typically experience higher tumor incidence and shorter latency of tumor emergence (Anderson *et al.* 2000; Rice 1981). A well-documented example is the induction of hepatocellular carcinomas (HCC) by aflatoxin B_1 (AFB_1) in mice, which strongly varies with age of exposure. Brief exposures to large doses of aflatoxin during the neonatal period result in a high incidence of HCC in adulthood, while adult mice exposed to the same doses fail to develop HCC at any age (Vesselinovitch *et al.* 1972).

Despite an abundance of experimental data, the mechanistic basis for the high sensitivity of infant animals to AFB_1 and other genotoxic carcinogens is not fully understood. A related unanswered question is why exposure during infancy induces higher incidence of HCC in males than in females in adulthood. Treatment of newborn B6C3F1 mice with single or multiple doses of AFB_1 induced HCC by 82 weeks of age in >90% in males, compared to <10% of similarly treated females (Vesselinovitch *et al.* 1972). Similar experiments showed that female mice treated with diethylnitrosamine (DEN) develop HCC much less frequently than males (Nakatani *et al.* 2001a). Two possible explanations are: (i) in female mice, carcinogen activation may be less effective or inactivation and DNA repair more effective, resulting in less genetic damage and thus fewer tumor-initiating mutations than male mice, or (ii) similar amounts of genetic damage may occur in both sexes and subsequent hormonal and/or other environmental factors differentially modulate HCC development. Prior studies showed that hormonal status had a profound effect on tumor development in animals treated during the neonatal period. For example, orchidectomy of neonatal male mice dosed with DEN delayed tumor onset and reduced tumor yield (Vesselinovitch 1990). Estrogen-mediated inhibition of inflammatory responses in the liver following DEN treatment has also been shown to attenuate the post-initiation development of HCC in female mice (Naugler *et al.* 2007b). Little information exists, however, on the relative importance of genotoxic damage in neonates as compared to post-initiation events that determine differences in HCC incidence between males and females later in life.

In mice, AFB_1 is metabolized by cytochromes P450 1A2 and 3A4 to the 8,9-epoxide, which reacts with cellular DNA, producing the predominant AFB_1-N^7-guanine adduct (Eaton and Gallagher 1994). DNA adduct formation by the AFB_1-8,9-epoxide can be diminished by formation of AFB_1-glutathione conjugates, mediated by alpha class glutathione-S-transferases (GSTs) (Hayes *et al.* 1992). Increased expression of GSTs during the postnatal period is believed to be largely responsible for the diminished sensitivity of older mice (Shupe and Sell 2004b). Furthermore, in the rat, it has been shown that chemoprotective chemicals such as dithiolethiones, that induced GSTs and inhibited formation of AFB_1-DNA adducts also reduced the number of hepatic preneoplastic lesions and prevented tumor development (Roebuck *et al.* 2003b). Importantly, epidemiological studies have validated aflatoxin-DNA adducts as biomarkers of risk of HCC from aflatoxin exposure (Groopman *et al.* 2002; Groopman *et al.* 2008). An additional molecular connection between AFB_1 exposure and human HCC was established by the observation that G:C to T:A transversions characteristic of those induced by AFB_1-DNA adducts were found at high frequencies in codon 249 of the p53 gene in HCC of patients residing in geographical areas in which AFB_1 exposure is an established risk factor (Hussain *et al.* 2007).

The overall objectives of our study were to quantify aflatoxin adduct levels, characterize the frequency and spectrum of mutations induced in the liver of male and female *gpt* delta B6C3F1 mice, and assess relationships of these parameters to the known sensitivity of this strain of mice to AFB_1-induced HCC. The mutagenic potency of AFB_1 is well established in bacteria and mammalian cells, and G:C to T:A transversions are the most frequent base substitution mutation induced by AFB_1 DNA adducts. A prior study with similar objectives carried out in Big Blue *lacI* transgenic mice showed that AFB_1 is a potent liver mutagen in neonatal animals, but much less potent in the adult (Chen *et al.* 2010), in which fewer AFB_1-DNA adducts were previously found (Shupe and Sell 2004a). We carried out the study in the B6C3F1 mouse, which is extensively used as a bioassay animal for testing chemicals for carcinogenic activity and for which a large carcinogenesis literature exists. This work is an early stage of a continuing effort

to define biochemical markers related to age, gender and strain capable of identifying key biochemical processes that underlie sensitivity and resistance to carcinogens.

MATERIALS AND METHODS

Caution: *Aflatoxin B_1 is toxic, mutagenic and carcinogenic. This compound should be handled using appropriate precautions.*

Animals. C57BL/6 *gpt* delta transgenic mice were obtained from Takehiko Nohmi (Nohmi *et al.* 1996). The *gpt* delta B6C3F1 mice used in our experiments were generated by breeding female *gpt* delta C57BL/6J mice, which harbor an estimated 80 copies of the *gpt* gene on chromosome 17 (Nohmi *et al.* 1996), with male C3H/HeJ mice purchased from the Jackson Laboratories (Bar Harbor, Maine). All experiments were conducted in accordance with protocols approved by the MIT Committee on Animal Care.

AFB_1 treatment for adduct analysis. In the time-course study of adduct formation, male and female transgenic B6C3F1 mice were injected intraperitoneally (ip) on postnatal day 4 with a single dose of 6 mg/kg AFB_1 in 10 µl of dimethylsulfoxide (DMSO), both obtained from Sigma-Aldrich (St. Louis, MO). Mice were euthanized and livers collected 2, 4, 8, 12, 24, and 48 hours after treatment; livers were collected from a minimum of 3 male and 3 female animals at each time point. To assess adduct formation induced by a multiple dose regimen, male and female mice were injected ip on postnatal days 4, 7, and 10 with a dose of 2 mg/kg AFB_1 in 10 µl DMSO. Twenty-four hours after the last dose, animals were euthanized and livers were collected from 13 mice of each sex.

AFB_1 treatment for gpt mutation assay. Parallel experiments were conducted in which the single- or multiple dose AFB_1 regimens were employed. In the former protocol, as described above male and female mice were injected ip on postnatal day 4 with a single dose of 6 mg/kg AFB_1 in 10 µl DMSO or 10 µl DMSO alone. Animals were euthanized 21 days after the single dose was administered and livers were

collected from a minimum of 4 male and 4 female mice. In the second treatment schedule, male and female mice were injected ip on postnatal days 4, 7, and 10 with either 2 mg/kg AFB_1 in 10 µl DMSO or DMSO alone. These animals were euthanized 21 days after the last dose and livers were collected from a minimum of 4 male and 4 female mice.

***Isolation of liver DNA and hydrolysis of AFB_1-DNA adducts*.** DNA was isolated from livers of AFB_1-treated mice and DNA isolated using previously described procedures (Groopman *et al.* 1980; Kensler *et al.* 1986)). For adduct analysis, AFB_1-DNA adducts were released by hydrolysis in 1.0 N HCl at 95^{o}C for 15 min (Groopman *et al.* 1981). Internal $^{15}N_5$-guanine-derived standards for both AFB_1-N^7-guanine and AFB_1-FAPY were added after hydrolysis of AFB_1-DNA adducts to permit quantitative analysis by isotope dilution mass spectrometry.

DNA adduct analysis. Ultra-high performance liquid chromatography (UPLC) was used to separate AFB_1-DNA adducts prior to measurement by isotope-dilution mass spectrometry as previously described (Egner *et al.* 2006). UPLC was carried out on an Acuity C18 1.7 µm 1.0 x 150 mm column, and the composition of the initial mobile phase was 14% methanol, 1% acetonitrile, 0.1% formic acid and 85% water. An 8 minute linear gradient was employed, reaching a final mobile phase consisting of 37% methanol, 2% acetonitrile, 0.1% formic acid and 61% water. Flow rate was 120 µl per minute. The hydrolyzed DNA solution was diluted into the initial UPLC mobile phase and then injected for MS/MS analysis of AFB_1-DNA adduct levels. The protonated parent ion of the AFB_1-N^7-guanine adduct (*m/z* 480.1) was selected and subjected to collision-induced fragmentation producing a *m/z* 152 product ion that was monitored to quantify adduct levels. The AFB_1-FAPY adduct was quantified by selection of the *m/z* 498 parent ion and monitoring the collision-induced product ion *m/z* 452.

Gpt mutation assay and sequencing analysis. The liver of each animal was pulverized in liquid nitrogen and divided into aliquots of approximately 25 mg. Genomic DNA was extracted from 25 mg liver tissue using RecoverEase DNA Isolation Kit

(Agilent Technologies, Santa Clara, CA); subsequently, λ–EG10 phages were packaged *in vitro* from the genomic DNA using Transpack Packaging Extract (Agilent Technologies) following the manufacturer's instructions. The 6-thioguanine (6-TG) selection assay was performed as previously described (Nohmi *et al.* 1996). Briefly, *E. coli* YG6020 expressing Cre recombinase was infected with λ–EG10 phages rescued from murine genomic DNA and incubated on selective media containing either chloramphenicol (Cm) at 25 μg/ml or Cm (25 μg/ml) plus 6-TG (25 μg/ml) for 72 h until the appearance of colonies. Confirmation of the recovered 6-TG-resistant phenotype was achieved by restreaking mutant colonies on selective media containing Cm plus 6-TG. DNA was isolated from confirmed 6-TG-resistant mutants using a Miniprep Kit (Qiagen) according to the manufacturer's instructions. Sequencing of the *gpt* gene was performed at the Biopolymers Facility at Harvard Medical School (Boston, MA) using AMPure beads (Agencourt) and a 3730xL DNA Analyzer (Applied Biosystems) using the forward primer: 5'- TCTCGCGCAACCTATTTTCCC -3'. Sequences were aligned with the *E. coli gpt* gene (GenBank M13422.1) using NCBI Nucleotide Blast. Duplicate identical mutations from the same tissue sample were excluded from calculation of mutation frequency (MF) to avoid bias attributable to clonal expansion of sibling mutations. The MF was calculated by first taking the number of confirmed mutants and multiplying by the ratio of independent mutants to the total number of analyzed (sequenced) mutants and then dividing this product by the total number of colonies on the Cm only (control) plate. Samples from 33 mice were sequenced. Mutation frequencies and spectra were subjected to statistical analysis.

Statistical analysis Student's two-tailed *t* tests using PRIZM (Graphpad Software, Inc,) were used to determine the significance of differences in DNA adduct levels and mutation frequencies between experimental groups. Mutational spectra were compared using the Adams-Skopek test (Adams and Skopek 1987; Cariello *et al.* 1994).

RESULTS

***Time Course of AFB_1 DNA adducts in neonatal male and female mice*.** Newborn *gpt* delta B6C3F1 mice were administered AFB_1 using two regimens adapted from those previously shown to induce a high incidence of HCC (Figure 1) (Vesselinovitch *et al.* 1972). A time course analysis of levels of AFB_1-N^7-guanine in liver DNA was first performed in 4-day old animals after administration of a single 6 mg/kg intraperitoneal dose of AFB_1. Figure 2 shows the levels of AFB_1-N^7-guanine in male and female mice at intervals up to 48 h after dosing. The average levels of AFB_1-N^7-guanine were uniformly lower in females than in males, but due to inter-individual variation the differences were not statistically significant. The highest levels of AFB_1-N^7-guanine in liver DNA of both sexes (male, 9.4 ± 2.9; female 8.3 ± 4.3 adducts/10^6 nucleotides) were found at the earliest time point (2 h) after dosing. As previously observed in other species, adduct levels rapidly declined between 2 and 10-12 h. A second phase of slower adduct removal occurred between 10 and 48 h, with approximately 10% of the peak level of AFB_1-N^7-guanine attained at 2 h remaining at 48 h. This decline in AFB_1-N^7-guanine could have resulted from its conversion to AFB_1-FAPY (the imidazole ring-opened form of AFB_1-N^7-guanine), depurination or enzymatic removal by repair enzymes. These results showed that there were no major gender differences in metabolic activation of AFB_1 by P450s or other biochemical process that determine the fate of the AFB_1-N^7-guanine adduct in neonatal liver DNA. Further experiments were designed to examine whether levels of DNA adducts induced by the single or multiple dose regimens resulted in a gender difference in initial mutations that tracked with eventual tumor burden. To compare adduct levels, liver DNA was isolated from mice euthanized 24 h after administration of either treatment regimen (Figure 1) and analyzed for amounts of both AFB_1-N^7-guanine and AFB_1-FAPY adducts (Figure 3). A single dose of 6 mg/kg AFB_1 administered on day 4 produced similar levels of AFB_1-N^7-guanine in males and females as observed at 24 h in the kinetic study described above (Fig. 3A); male 2.7 ± 1; female 2.6 ± 0.8 adducts/10^6 nucleotides. Levels of the highly mutagenic AFB_1-FAPY were twice as high as those of AFB_1-N^7-guanine in both sexes; male 5.5 ± 3 and female 5.8 ± 2 adducts/10^6 nucleotides.

The multiple dose regimen of 3 x 2 mg/kg AFB_1 given on days 4, 7 and 10, resulted in lower levels of adducts measured 24 h after the final dose, compared to the single 6 mg/kg dose. As shown in Fig. 3B, the levels of AFB_1-N^7-guanine were 6-7 fold lower; male 0.36 ± 0.2 adducts/10^6 nucleotides, female 0.40 ± 0.2 adducts/10^6 nucleotides. Levels of AFB_1-FAPY adducts were also lower in the multiple dose animals, but only by 1.5-2 fold compared to the single dose; male 3.6 ± 2 adducts/10^6 nucleotides, female 2.5 ± 1 adducts/10^6 nucleotides. This pattern is consistent with the persistence and accumulation of FAPY adducts during chronic administration of AFB_1 that has been observed in other animal models (Croy and Wogan 1981). Importantly, however, levels of AFB_1-N^7-guanine or AFB_1-FAPY induced by either treatment protocol showed no evidence of gender-related differences.

***Mutation frequencies in the gpt gene of AFB_1-treated neonatal mice*.** The early stage genotoxicity and mutagenesis of AFB_1 adducts in liver DNA were assessed in the *gpt* transgene following selection for 6-TG resistance. A minimum of 4 mice of each sex were treated with the single or multiple dose regimens as described above. Based on previous studies (Thybaud *et al.* 2003), for mutation analysis, livers were collected from animals euthanized 21 days after final dosing. Figure 4 shows significant increases in mutation frequency in livers of mice treated with AFB_1 administered by either the single or multiple dose regimens. In DMSO-treated mice the average mutation frequency was 3 x 10^{-6} , which is approximately half the spontaneous mutation frequency reported for *gpt* delta C57BL/6J mice (Masumura *et al.* 1999). The mutation frequency induced by the single dose AFB_1 regimen in males and females combined was 92 ± 14 x 10^{-6}. A lower mutation frequency was observed in mice treated with the multiple dose regimen; 66 ± 19 x 10^{-6} (P = 0.01, single vs multiple dose). This is consonant with the lower amounts of mutagenic AFB_1 adducts found at 24 h following the multiple dose protocol. There was no significant difference in the mutation frequencies observed in males as compared to females treated with either regimen, indicating that liver cells of both sexes were similarly susceptible to mutagenesis by AFB_1.

***Mutation types in the gpt gene*.** DNA sequence analyses were performed on 6-TG resistant mutants isolated from AFB_1- and DMSO-treated mice. Although fewer 6-TG resistant mutants from the DMSO-treated controls than from AFB_1-treated mice were available for sequencing, the types of mutations present in both were very similar. Tables 1 and 2 summarize the types of mutations in livers of males and females treated with the two regimens. A total of 264 6-TG resistant colonies from 17 AFB_1-treated mice were analyzed by DNA sequencing to identify mutations present in the 459 bp *gpt* gene. Mutations found included base substitutions (248/264, 94%), insertions (4/264, 2%) and small deletions (12/264, 5%). Both transitions and transversions were present at G:C and A:T base pairs. The most frequent type of mutation seen in all the AFB_1-treated groups was the G:C to T:A transversion (169/264, 64%) followed by G:C to A:T transition (44/264, 17%). Single base pair deletions occurred primarily at G:C base pairs (9/11, 81%). Overall, the types of mutations were similar in male and female mice treated with either a single dose (6 mg/kg) or multiple doses (3 x 2 mg/kg) of AFB_1.

Sequence analysis of 62 mutants obtained from 16 DMSO-treated mice (combined data from both the single- and multiple dose groups) revealed that G:C to A:T transitions were the predominant mutation (22/62, 35%) followed by G:C to T:A transversions (13/62, 21%). Single base pair deletions at G:C base pairs were found at the same frequency as in AFB_1-treated animals (7/9, 78%). As described below, distribution of these mutations within the *gpt* gene differs significantly from that of AFB_1-treated mice.

Comparison of mutational spectra. Differences in the distribution of mutations with regard to base number in the *gpt* gene among treatment groups were tested using Monte Carlo analysis (Adams and Skopek 1987). Mutation spectra were significantly different between AFB_1-treated and DMSO control groups (multiple dose vs DMSO, $P < 0.01$; single dose vs DMSO, $P < 0.01$). For illustration, Figure 5 shows the mutation spectrum in the *gpt* gene in liver of mice treated with a single (6 mg/kg) AFB_1 dose. (See supplemental data for mutant spectra from the multiple dose and control animals.) Several mutational hot spots for base substitution mutations were observed in AFB_1-treated mice. G:C to T:A transversions occurred as independent mutations in $\geq$ 5 mice

at nucleotides 101 (5'-GCC-3'), 108 (5'-AGC-3'), 115 (5'-GGT-3'), 140 (5'-GCG-3') and 208 (5'-GAG-3'). In DMSO-treated mice, G:C to A:T transitions occurred most frequently at nucleotides 64 (5'-CGA-3'), 110 (5'-CGT-3'), and 115 (5'-GGT-3'). These CpG sites have previously been identified as *gpt* mutational hot spots in untreated animals (Masumura *et al.* 2000).

When the distributions of mutations within the *gpt* gene were compared between AFB_1-treated groups there was no significant difference in the types or positions of mutations (multiple dose vs single dose, P = 0.214; 95% C.L 0.199 – 0.229). Thus, the AFB_1 mutational spectra in the *gpt* sequence were not dependent on the exposure conditions in this experimental model. Further comparisons of mutational spectra between male and female animals showed that they were also indistinguishable.

DISCUSSION

This study examined relationships among levels of liver AFB_1-DNA adducts and mutagenesis in liver cells of newborn transgenic *gpt* delta B6C3F1 mice treated with dosing protocols previously shown to be highly effective in inducing HCC in male, but not female, B6C3F1 mice (Vesselinovitch *et al.* 1972). We found that dosing with AFB_1 created very similar levels of adducts in liver DNA of both sexes, and induced nearly identical mutation frequencies and types of mutations in the *gpt* transgene in liver cells. These results demonstrate that during infancy male and female B6C3F1 mice are at similar risks of genotoxic damage and mutagenesis resulting from AFB_1 exposure.

Monitoring of AFB_1-DNA adducts revealed that administration of a single 6 mg/kg dose produced a higher level of DNA adducts at 24 h, compared to the multiple dose regimen. Levels of AFB_1-N^7-guanine decreased rapidly during the 24 h after treatment, which has been shown to be a result of its removal by DNA repair processes together with its chemical transformation into secondary lesions, including apurinic sites and two imidazole ring-opened AFB_1-FAPY forms that are believed to be rotamers (Brown *et al.* 2006). The lower level of adducts in liver DNA induced by treatment with the multiple

dose regimen was reflected in a lower *gpt* mutation frequency observed in animals treated with multiple doses of AFB_1.

Our results identified G:C to T:A transversions in the *gpt* gene as the most prevalent mutation induced by AFB_1 in the liver of mice. Studies of similar design by other investigators produced similar findings, with AFB_1 inducing primarily G:C to T:A (76%) mutations in the *cII* gene in the neonatal liver of Big Blue mice (Chen *et al.* 2010). Overall, the types of mutations found in the *gpt* gene of AFB_1-treated mice were characteristic of those induced by it in other experimental systems. The G:C to T:A transversion is the predominant mutation induced by either the AFB_1-N^7-guanine or AFB_1-FAPY adduct in *in vitro* experimental systems, (Bailey *et al.* 1996; Foster *et al.* 1983; Smela *et al.* 2002). In *E. coli* the AFB_1-FAPY minor rotamer was found to be the most potent mutagen, producing G:C to T:A transversions about 6 times more frequently than AFB_1-N^7-guanine (Smela *et al.* 2002). The high mutagenic potency of the FAPY adduct together with its persistence in DNA suggests that it could be responsible for a major fraction of the mutations induced by AFB_1 in the rapidly growing neonatal liver.

Several mutational hot spots for G:C to T:A transversions in the *gpt* gene were observed in livers of AFB_1-treated mice, including nucleotide positions 101, 108, 115, 140, 208, 244 and 320. Such hot spots may result from structural features of the DNA sequence that increase the reactivity of guanine with the AFB_1-8,9-oxide (Benasutti *et al.* 1988), actions of the repair and replication processes, or properties of the *gpt* gene product used to select 6-TG-resistant mutants. The selection procedure requires nearly complete inactivation of the *E. coli gpt* enzyme, since toxic levels of 6-TG remain even in the presence of very low enzyme activity (Thilly *et al.* 1978). In this regard it is noteworthy that the most frequently encountered hot spots, including positions 108, 115, 140 and 208 are located in codons for amino acids involved in the binding sites of guanine or phosphoribosylpyrophosphate, the enzyme's two substrates (Vos *et al.* 1998). This observation provides a plausible explanation of the partial overlap observed between AFB_1-induced mutational spectra and those of other agents. Furthermore it

suggests the possibility that unrecognized mutational hot spots may exist at nucleotides in the *gpt* sequence that result in amino acid changes that do not strongly affect *gpt* enzyme activity. If this were the case, mutation frequency values calculated from our data would represent minimal estimates.

In both experimental animals and humans, the incidence of HCC is much higher in males than in females. Our findings of similar levels of AFB_1-adducts and mutation frequencies in infants of both sexes suggests the possibility that biochemical or other factors may be responsible for the observed sex difference in HCC incidence occurring in adult mice treated with AFB_1 as infants. Current conceptual models of HCC pathogenesis are based on initiating mutations produced by genotoxic compounds followed by enhanced tumor development driven by factors such as liver cell regeneration and chronic inflammation. In humans, aflatoxin synergizes with hepatitis B virus (HBV) to greatly increase the risk for HCC (Wild and Montesano 2009). Host responses to HBV infection include activation of the NF-κB signaling pathway that contributes to chronic inflammation and promotion of HCC (Sun and Karin 2008). One explanation offered for the resistance of female mice to HCC is the observation that estrogenic hormones can suppress inflammation and reduce cancer risk during the promotion phase of hepatocarcinogenesis in mice (Nakatani *et al.* 2001b; Naugler *et al.* 2007a). While mechanisms responsible for different sex-based incidence of HCC remain to be defined, our findings support the suggestion that post-initiation host responses are important modulators of HCC development.

Aflatoxin-DNA adducts are validated biomarkers of AFB_1 exposure and have enabled estimation of risk for HCC in exposed human populations (Groopman *et al.* 2008). In experimental animals, the chemoprotective agent oltapraz has been shown to be effective in inhibiting formation of AFB_1-DNA adducts and reducing the frequency of preneoplastic lesions as well as tumor formation in the livers of AFB_1-treated rats (Roebuck *et al.* 2003a). Insights gained from these experimental studies laid the foundation for clinical trials of chemopreventive strategies that are effective in reducing AFB_1-DNA adducts in exposed populations (Kensler *et al.* 2005). Our results suggest

that aflatoxin-induced genetic changes are important contributors to tumor initiation, but additional factors yet to be defined are also determinants of the ultimate development of HCC in mice. The nine-fold higher incidence of HCC in men compared to women highlights the importance of elucidating the additional hormonal and environmental factors that regulate HCC development.

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Figure Legends

Figure 1. Experimental scheme for treatment of newborn *gpt* delta B6C3F1 mice with AFB_1, analysis of AFB_1-DNA adducts and mutations in the *gpt* gene in liver.

Figure 2. Amounts of AFB_1-N^7-guanine in liver DNA of 4-day old male (-▲-) and female (-■-) *gpt* delta B6C3F1 mice after administration of 6 mg/kg AFB_1. Points = mean ± SD.

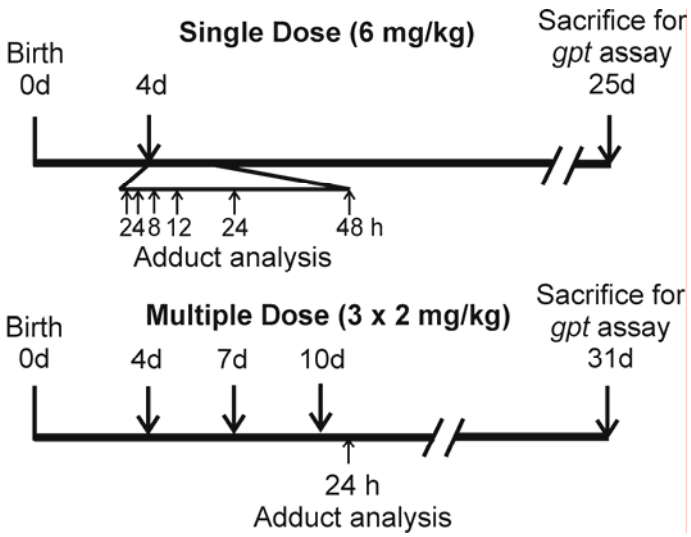
Figure 3. AFB_1 DNA adducts in liver of *gpt* delta B6C3F1 mice 24 h after a single 6 mg/kg dose (**A**) or 3 x 2 mg/kg administered every third day (**B**). Plotted data = mean ± SD.

Figure 4. Mutation frequency in neonatal *gpt* delta B6C3F1 mice treated with AFB_1 or DMSO vehicle. Mice were administered a single 6 mg/kg dose of AFB_1 (single) or treated with 3 x 2 mg/kg doses of AFB_1 (multiple). At 25 (single) or 31 (multiple) days of age DNA was isolated from liver and the number of 6-TG resistant colonies determined. Plotted data = mean ± SD.

Figure 5. Nucleotide sequence of the *gpt* gene indicating the position and type of base substitutions, deletions (□) and insertions (V) induced by a single 6 mg/kg AFB_1 dose administered to 4-day old *gpt* delta B6C3F1 mice. Mutations were assayed 21 days after dosing.

Figure S1. Nucleotide sequence of the *gpt* gene indicating the position and type of base substitutions, deletions (□) and insertions (V) present in *gpt* delta B6C3F1 mice administered DMSO vehicle. Mutations were assayed 21 days after dosing.

Figure S2. Nucleotide sequence of the *gpt* gene indicating the position and type of base substitutions, deletions (□) and insertions (V) induced by 3 x 2 mg/kg AFB_1 administered on days 4, 7 and 10 after birth to *gpt* delta B6C3F1 mice. Mutations were assayed 21 days after dosing.


Single Dose (6 mg/kg)
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4d
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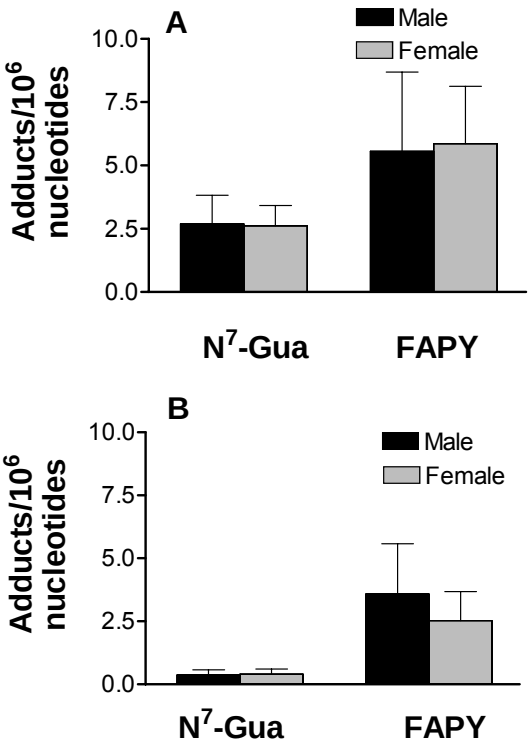
gpt assay
25d
2 4 8 12 24 48 h
Adduct analysis
Multiple Dose (3 x 2 mg/kg)
Birth
0d
4d
7d
10d
Sacrifice for
gpt assay
31d
24 h
Adduct analysis

Figure 1.


A
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Adducts/10^6 nucleotides
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FAPY
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Female
10.0
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Adducts/10^6 nucleotides
N7-Gua
FAPY

Figure 3

Table 1. Summary of mutations in the *gpt* gene of male and female *gpt* delta B6C3F1 neonates 21 days after treatment with a single 6 mg/kg dose of AFB_1. The percentage of each type of mutation is shown in parentheses.

Mutation Types	**Control**		**AFB_1**	
	Male	Female	Male	Female
Transition:				
G:C to A:T	4 (29)	6 (32)	11 (15)	12 (18)
A:T to G:C	0 (0)	1 (5)	2 (3)	0 (0)
Transversion:				
G:C to T:A	4 (29)	4 (21)	48 (65)	45 (68)
G:C to C:G	1 (7)	1 (5)	6 (8)	5 (8)
A:T to T:A	1 (7)	0 (0)	3 (4)	0 (0)
A:T to C:G	0 (0)	0 (0)	1 (1)	0 (0)
Deletion				
1 bp	1 (7)	5 (26)	1 (1)	3 (5)
>1 bp	1 (7)	0 (0)	0 (0)	0 (0)
Insertion	2 (14)	2 (11)	2 (3)	1 (2)
Total number of mutants	14 (100)	19 (100)	74 (100)	66 (100)

Table 2. Summary of mutations in the *gpt* gene of male and female B6C3F1 neonates 21 days after treatment with multiple doses (3 x 2 mg/kg) of AFB_1. The percentage of each type of mutation is shown in parentheses.

Mutation Types	**Control**		**AFB_1**	
	Male	Female	Male	Female
Transition:				
G:C to A:T	9 (38)	3 (60)	8 (17)	13 (17)
A:T to G:C	1 (4)	0 (0)	0 (0)	1 (1)
Transversion:				
G:C to T:A	4 (17)	1 (20)	31 (66)	45 (58)
G:C to C:G	1 (4)	0 (0)	1 (2)	13 (17)
A:T to T:A	1 (4)	0 (0)	0 (0)	2 (3)
A:T to C:G	0 (0)	0 (0)	1 (2)	0 (0)
Deletion				
1 bp	4 (17)	0 (0)	6 (13)	2 (3)
>1 bp	0 (0)	0 (0)	0 (0)	0 (0)
Insertion	4 (17)	1 (20)	0 (0)	1(1)
Total number of mutants	24 (100)	5 (100)	47 (100)	77 (100)

Reference List

Adams, W. T., and Skopek, T. R. (1987). Statistical test for the comparison of samples from mutational spectra. *J. Mol. Biol.* **194**(3), 391-396.

Anderson, L. M., Diwan, B. A., Fear, N. T., and Roman, E. (2000). Critical windows of exposure for children's health: cancer in human epidemiological studies and neoplasms in experimental animal models. *Environ. Health Perspect.* **108 Suppl 3**, 573-594.

Bailey, E. A., Iyer, R. S., Stone, M. P., Harris, T. M., and Essigmann, J. M. (1996). Mutational properties of the primary aflatoxin B1-DNA adduct. *Proc. Natl. Acad. Sci. U. S. A* **93**(4), 1535-1539.

Benasutti, M., Ejadi, S., Whitlow, M. D., and Loechler, E. L. (1988). Mapping the binding site of aflatoxin B1 in DNA: systematic analysis of the reactivity of aflatoxin B1 with guanines in different DNA sequences. *Biochemistry* **27**(1), 472-481.

Brown, K. L., Deng, J. Z., Iyer, R. S., Iyer, L. G., Voehler, M. W., Stone, M. P., Harris, C. M., and Harris, T. M. (2006). Unraveling the aflatoxin-FAPY conundrum: structural basis for differential replicative processing of isomeric forms of the formamidopyrimidine-type DNA adduct of aflatoxin B1. *J. Am. Chem. Soc.* **128**(47), 15188-15199.

Cariello, N. F., Piegorsch, W. W., Adams, W. T., and Skopek, T. R. (1994). Computer program for the analysis of mutational spectra: application to p53 mutations. *Carcinogenesis* **15**(10), 2281-2285.

Chen, T., Heflich, R. H., Moore, M. M., and Mei, N. (2010). Differential mutagenicity of aflatoxin B1 in the liver of neonatal and adult mice. *Environ. Mol. Mutagen.* **51**(2), 156-163.

Croy, R. G., and Wogan, G. N. (1981). Temporal patterns of covalent DNA adducts in rat liver after single and multiple doses of aflatoxin B1. *Cancer Res.* **41**(1), 197-203.

Eaton, D. L., and Gallagher, E. P. (1994). Mechanisms of aflatoxin carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* **34**, 135-172.

Egner, P. A., Groopman, J. D., Wang, J. S., Kensler, T. W., and Friesen, M. D. (2006). Quantification of aflatoxin-B1-N7-Guanine in human urine by high-performance liquid chromatography and isotope dilution tandem mass spectrometry. *Chem. Res. Toxicol.* **19**(9), 1191-1195.

Foster, P. L., Eisenstadt, E., and Miller, J. H. (1983). Base substitution mutations induced by metabolically activated aflatoxin B1. *Proc. Natl. Acad. Sci. U. S. A* **80**(9), 2695-2698.

Groopman, J. D., Busby, W. F., Jr., and Wogan, G. N. (1980). Nuclear distribution of aflatoxin B1 and its interaction with histones in rat liver in vivo. *Cancer Res.* **40**(12), 4343-4351.

Groopman, J. D., Croy, R. G., and Wogan, G. N. (1981). In vitro reactions of aflatoxin B1-adducted DNA. *Proc. Natl. Acad. Sci. U. S. A* **78**(9), 5445-5449.

Groopman, J. D., Jackson, P. E., Turner, P., Wild, C. P., and Kensler, T. W. (2002). Validation of exposure and risk biomarkers: Aflatoxin as a case study. *Progress in Nucleic Acid Research and Molecular Biology*, 307-318.

Groopman, J. D., Kensler, T. W., and Wild, C. P. (2008). Protective interventions to prevent aflatoxin-induced carcinogenesis in developing countries. *Annu. Rev. Public Health* **29**, 187-203.

Hayes, J. D., Judah, D. J., Neal, G. E., and Nguyen, T. (1992). Molecular cloning and heterologous expression of a cDNA encoding a mouse glutathione S-transferase Yc subunit possessing high catalytic activity for aflatoxin B1-8,9-epoxide. *Biochem. J.* **285 (Pt 1)**, 173-180.

Hussain, S. P., Schwank, J., Staib, F., Wang, X. W., and Harris, C. C. (2007). TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. *Oncogene* **26**(15), 2166-2176.

Kensler, T. W., Chen, J. G., Egner, P. A., Fahey, J. W., Jacobson, L. P., Stephenson, K. K., Ye, L., Coady, J. L., Wang, J. B., Wu, Y., Sun, Y., Zhang, Q. N., Zhang, B. C., Zhu, Y. R., Qian, G. S., Carmella, S. G., Hecht, S. S., Benning, L., Gange, S. J., Groopman, J. D., and Talalay, P. (2005). Effects of glucosinolate-rich broccoli sprouts on urinary levels of aflatoxin-DNA adducts and phenanthrene tetraols in a randomized clinical trial in He Zuo township, Qidong, People's Republic of China. *Cancer Epidemiol. Biomarkers Prev.* **14**(11 Pt 1), 2605-2613.

Kensler, T. W., Egner, P. A., Davidson, N. E., Roebuck, B. D., Pikul, A., and Groopman, J. D. (1986). Modulation of aflatoxin metabolism, aflatoxin-N7-guanine formation, and hepatic tumorigenesis in rats fed ethoxyquin: role of induction of glutathione S-transferases. *Cancer Res.* **46**(8), 3924-3931.

Masumura, K., Matsui, K., Yamada, M., Horiguchi, M., Ishida, K., Watanabe, M., Ueda, O., Suzuki, H., Kanke, Y., Tindall, K. R., Wakabayashi, K., Sofuni, T., and Nohmi, T. (1999). Mutagenicity of 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) in the new gpt delta transgenic mouse. *Cancer Lett.* **143**(2), 241-244.

Masumura, K., Matsui, K., Yamada, M., Horiguchi, M., Ishida, K., Watanabe, M., Wakabayashi, K., and Nohmi, T. (2000). Characterization of mutations induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in the colon of gpt delta transgenic mouse: novel G:C deletions beside runs of identical bases. *Carcinogenesis* **21**(11), 2049-2056.

Nakatani, T., Roy, G., Fujimoto, N., Asahara, T., and Ito, A. (2001b). Sex hormone dependency of diethylnitrosamine-induced liver tumors in mice and chemoprevention by leuprorelin. *Jpn. J. Cancer Res.* **92**(3), 249-256.

Nakatani, T., Roy, G., Fujimoto, N., Asahara, T., and Ito, A. (2001a). Sex hormone dependency of diethylnitrosamine-induced liver tumors in mice and chemoprevention by leuprorelin. *Jpn. J. Cancer Res.* **92**(3), 249-256.

Naugler, W. E., Sakurai, T., Kim, S., Maeda, S., Kim, K., Elsharkawy, A. M., and Karin, M. (2007b). Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. *Science* **317**(5834), 121-124.

Naugler, W. E., Sakurai, T., Kim, S., Maeda, S., Kim, K., Elsharkawy, A. M., and Karin, M. (2007a). Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. *Science* **317**(5834), 121-124.

Nohmi, T., Katoh, M., Suzuki, H., Matsui, M., Yamada, M., Watanabe, M., Suzuki, M., Horiya, N., Ueda, O., Shibuya, T., Ikeda, H., and Sofuni, T. (1996). A new transgenic mouse mutagenesis test system using Spi- and 6-thioguanine selections. *Environ. Mol. Mutagen.* **28**(4), 465-470.

Rice, J. M. (1981). Prenatal susceptibility to carcinogenesis by xenobiotic substances including vinyl chloride. *Environ. Health Perspect.* **41**, 179-188.

Roebuck, B. D., Curphey, T. J., Li, Y., Baumgartner, K. J., Bodreddigari, S., Yan, J., Gange, S. J., Kensler, T. W., and Sutter, T. R. (2003a). Evaluation of the cancer chemopreventive potency of dithiolethione analogs of oltipraz. *Carcinogenesis* **24**(12), 1919-1928.

Roebuck, B. D., Curphey, T. J., Li, Y., Baumgartner, K. J., Bodreddigari, S., Yan, J., Gange, S. J., Kensler, T. W., and Sutter, T. R. (2003b). Evaluation of the cancer chemopreventive potency of dithiolethione analogs of oltipraz. *Carcinogenesis* **24**(12), 1919-1928.

Shupe, T., and Sell, S. (2004a). Low hepatic glutathione S-transferase and increased hepatic DNA adduction contribute to increased tumorigenicity of aflatoxin B1 in newborn and partially hepatectomized mice. *Toxicol. Lett.* **148**(1-2), 1-9.

Shupe, T., and Sell, S. (2004b). Low hepatic glutathione S-transferase and increased hepatic DNA adduction contribute to increased tumorigenicity of aflatoxin B1 in newborn and partially hepatectomized mice. *Toxicol. Lett.* **148**(1-2), 1-9.

Smela, M. E., Hamm, M. L., Henderson, P. T., Harris, C. M., Harris, T. M., and Essigmann, J. M. (2002). The aflatoxin B(1) formamidopyrimidine adduct plays a major role in causing the types of mutations observed in human hepatocellular carcinoma. *Proc. Natl. Acad. Sci. U. S. A* **99**(10), 6655-6660.

Sun, B., and Karin, M. (2008). NF-kappaB signaling, liver disease and hepatoprotective agents. *Oncogene* **27**(48), 6228-6244.

Thilly, W. G., Deluca, J. G., Hoppe, H., and Penman, B. W. (1978). Phenotypic lag and mutation to 6-thioguanine resistance in diploid human lymphoblasts. *Mutat. Res.* **50**(1), 137-144.

Thybaud, V., Dean, S., Nohmi, T., de, B. J., Douglas, G. R., Glickman, B. W., Gorelick, N. J., Heddle, J. A., Heflich, R. H., Lambert, I., Martus, H. J., Mirsalis, J. C., Suzuki, T., and Yajima, N. (2003). In vivo transgenic mutation assays. *Mutat. Res.* **540**(2), 141-151.

Vesselinovitch, S. D. (1990). Perinatal mouse liver carcinogenesis as a sensitive carcinogenesis model and the role of the sex hormonal environment in tumor development. *Prog. Clin. Biol. Res.* **331**, 53-68.

Vesselinovitch, S. D., Mihailovich, N., Wogan, G. N., Lombard, L. S., and Rao, K. V. (1972). Aflatoxin B 1 , a hepatocarcinogen in the infant mouse. *Cancer Res.* **32**(11), 2289-2291.

Vos, S., Parry, R. J., Burns, M. R., de, J. J., and Martin, J. L. (1998). Structures of free and complexed forms of Escherichia coli xanthine-guanine phosphoribosyltransferase. *J. Mol. Biol.* **282**(4), 875-889.

Wild, C. P., and Montesano, R. (2009). A model of interaction: aflatoxins and hepatitis viruses in liver cancer aetiology and prevention. *Cancer Lett.* **286**(1), 22-28.

SUPPLEMENTARY FIGURES

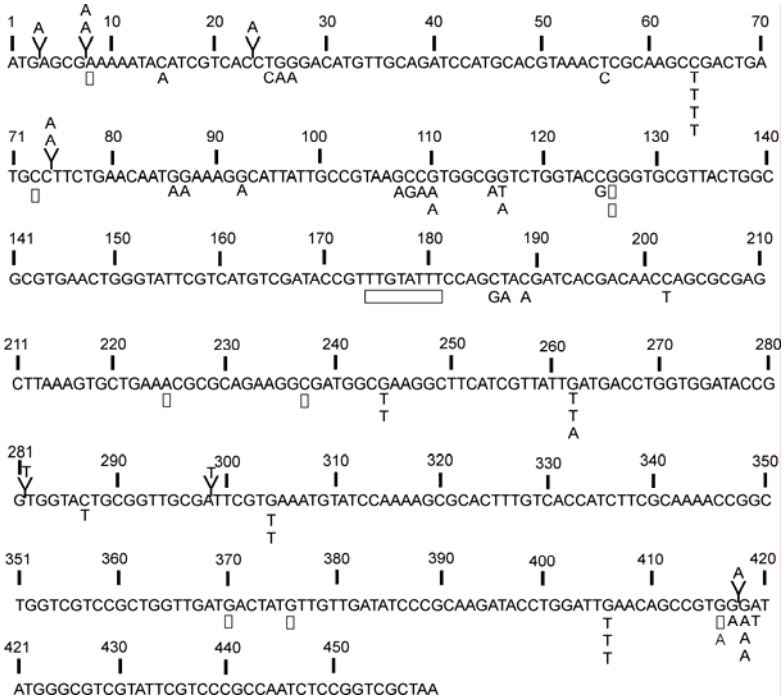

Figure S1. Base pair substitutions, deletions (□) and insertions (Y) in the *gpt* gene from liver of B6C3HF1 mice (male and female) treated with DMSO vehicle. Mutations assayed 21 days after dosing.

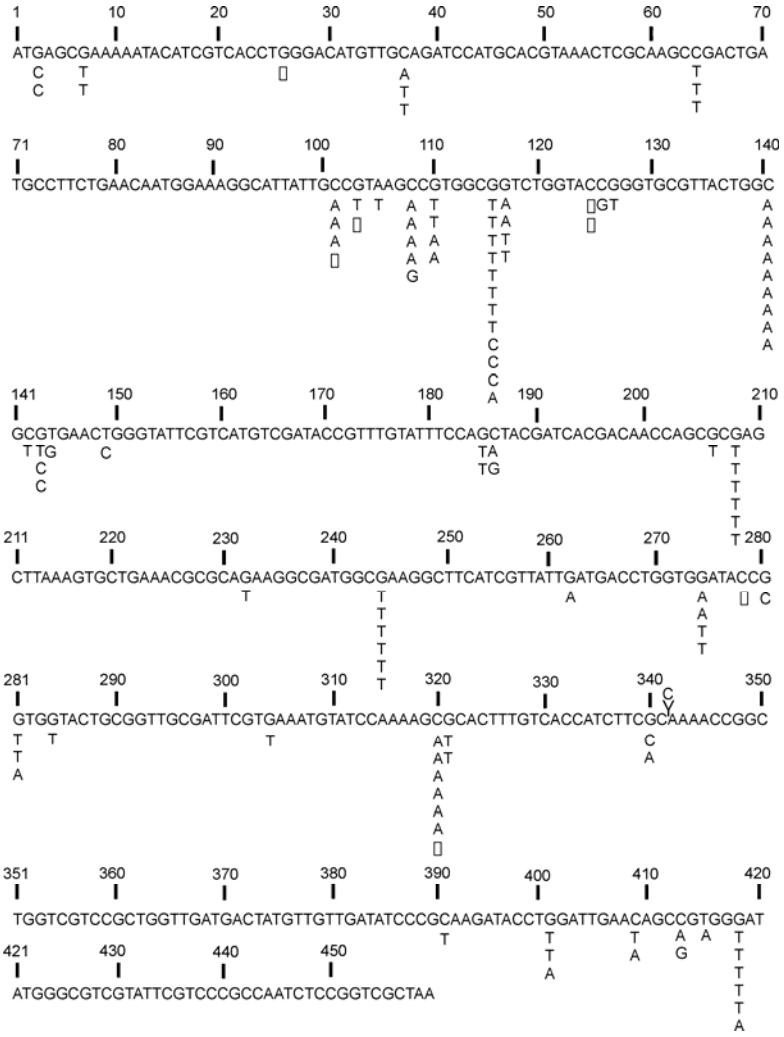

Figure S2. Base pair substitutions, deletions (□) and insertions (Y) in the *gpt* gene from liver of B6C3F1 mice (male and female) treated with 3 x 2 mg/kg AFB1 on neonatal days 4, 7 and 10.