



Published in final edited form as:

*Environ Mol Mutagen.* 2010 March ; 51(2): 156–163. doi:10.1002/em.20518.

## Differential Mutagenicity of Aflatoxin B<sub>1</sub> in the Liver of Neonatal and Adult Mice

Tao Chen<sup>\*</sup>, Robert H. Heflich, Martha M. Moore, and Nan Mei

Division of Genetic and Reproductive Toxicology, National Center for Toxicological Research, US Food and Drug Administration, Jefferson, Arkansas

### Abstract

Children are generally more sensitive to toxicants than adults, including an increased sensitivity to genotoxic carcinogens. We previously demonstrated that neonatal mice are also more sensitive to the mutagenic effects of the direct alkylating agents *N*-ethyl-*N*-nitrosoamine and the arylamine 4-aminobiphenyl than adult mice. In this study, we have evaluated the effect of age on the mutagenicity of the fungal toxin and liver carcinogen aflatoxin B<sub>1</sub> (AFB<sub>1</sub>). Neonatal Big Blue transgenic mice were treated with 6 mg/kg AFB<sub>1</sub>, a treatment that produces liver tumors, while adult mice were treated with 6 and 60 mg/kg AFB<sub>1</sub>, treatments that do not result in tumors. The *CII* liver mutant frequency (MF) in mice treated with AFB<sub>1</sub> as neonates was 22-fold higher than in control neonatal mice, whereas the treatment of adult mice with either dose of AFB<sub>1</sub> did not significantly increase the liver MF over the controls. In AFB<sub>1</sub>-treated neonatal mice, the frequency of G:C → T:A transversion, a major type of mutation induced by AFB<sub>1</sub>, was about 82-fold higher than for the control and 31-fold higher than for adult mice treated with 60 mg/kg AFB<sub>1</sub>. Our mutagenicity findings parallel the relative carcinogenicity of AFB<sub>1</sub> in neonatal and adult mice, and are consistent with previous observations of the lower level of hepatic glutathione S-transferase and higher level of hepatic AFB<sub>1</sub>-DNA adduction in neonatal mice compared to adult mice. *Environ. Mol. Mutagen.* 51:156–163, 2010. Published 2009 by Wiley-Liss, Inc.<sup>†</sup>

### Keywords

aflatoxin B<sub>1</sub>; neonate; mutagenicity; mutation; glutathione S-transferase

### INTRODUCTION

Cancer is the second leading cause of death among 1- to 14-year-old children in the United States, following only accidents as a cause of death in this age group. Although there have been significant improvements in the relative survival rate for many childhood cancers, the overall cancer incidence in children continues to increase slowly [Jemal et al., 2006]. The reason for this trend is not clear, although many possibilities exist. Infants and children may be more vulnerable to the toxicity of carcinogenic chemicals than adults because of unique

<sup>†</sup>This article is a US Government work and, as such, is in the public domain in the United States of America.

<sup>\*</sup>Correspondence to: Tao Chen, HFT-130, 3900 NCTR Road, Jefferson, AR 72079. tao.chen@fda.hhs.gov.

routes and types of exposure, unique susceptibilities, physiological and metabolic factors, pharmacokinetics, diet, and behavior.

Our previous studies with mice demonstrated one possible factor promoting the higher incidence of childhood cancer that DNA damage induced by carcinogens is more efficiently converted into mutations in neonates than in adults [Slikker et al., 2004; Chen et al., 2005; Mei et al., 2005]. These results were attributed to faster cell turnover rates in neonates than adults. In addition, it has been recognized that, for some agents, neonatal mice may have lower activity than adults for the metabolic enzymes that convert carcinogenic metabolites into inactive conjugates [Gorrod et al., 1968]. The resulting increased levels of carcinogenic metabolites, coupled with rapid cell division, could induce more mutations in neonates than adults. Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is an example of an agent that may be conjugated poorly by neonates.

AFB<sub>1</sub>, a metabolite of the grain mold *Aspergillus flavus*, is a potent human hepatocarcinogen and widespread contaminant of human food supplies. AFB<sub>1</sub> also induces tumors or preneoplastic lesions in experimental animals [IARC, 1990, 1993]. AFB<sub>1</sub>, however, displays striking interspecies variation in carcinogenic potency, with rats being the most sensitive species and mice being refractory to dietary levels three orders of magnitude higher than those carcinogenic in rats. An efficient conjugation with glutathione, catalyzed by glutathione S-transferase (GST), confers AFB<sub>1</sub> resistance in mice [Hengstler et al., 1999]. Species-specific differences in hepatic mutations among adult lambda/*lacI* transgenic rats and mice following exposure to AFB<sub>1</sub> also have been reported [Dycaico et al., 1996]. A single dose of 2.5 mg/kg AFB<sub>1</sub> did not increase liver mutant frequency (MF) relative to vehicle-treated controls in adult mice, while rats subjected to one-tenth of this dose responded with an ~20-fold induction in liver MF over background. The mutants were sequenced and the mutational spectra were different between AFB<sub>1</sub>-treated mice and rats. A large increase in G:C → T:A transversions was observed among the mutations isolated from the AFB<sub>1</sub>-treated rats while the major mutation type for AFB<sub>1</sub>-treated mice was G:C → A:T transition. In contrast to the resistance of adult mice to AFB<sub>1</sub> carcinogenesis, AFB<sub>1</sub> is a strong carcinogen in the neonatal mouse tumorigenicity assay. The liver of neonatal mice is known to be relatively deficient in its ability to conjugate AFB<sub>1</sub>, perhaps accounting for the sensitivity of neonatal mice to AFB<sub>1</sub> tumorigenicity [Vesselinovitch et al., 1972; Flammang et al., 1997]. Therefore, AFB<sub>1</sub> should be a good chemical for testing the hypothesis that a lower conjugating activity in neonatal mice might be a factor responsible for the greater sensitivity of neonates than adults to the carcinogenicity of certain agents.

The Big Blue transgenic mouse assay provides a unique opportunity for studying the induction of tissue-specific mutation. In this in vivo model, reporter genes are located on shuttle vectors that are derivatives of the bacteriophage lambda (λ). Multiple genomic copies of the phage are contained within the genome of each transgenic animal cell as stably integrated concatamers. After exposure of transgenic animals to a test substance, DNA can be isolated from individual organs, such as liver, and single copies of the phage genome can be excised from high molecular weight DNA and packaged into infectious particles with the help of a packaging mix [Kohler et al., 1990]. If appropriate *E. coli* host cells are infected, plated, and incubated, plaques become visible on the plates within hours. Mutant phage can

be isolated from the plates and mutations identified in the target gene, most often the *cII* gene. The *cII* gene encodes a protein that activates transcriptional promoters in  $\lambda$  that are essential for lysogenization. Mutations in the *cII* region that lower the levels of the *cII* protein result in a decreased ability of  $\lambda$  to lysogenize. When grown under conditions that favor lysogeny,  $\lambda$  prophages carrying *cII* mutations ( $\lambda cII^-$ ) survive only by entering the lytic pathway of development, forming plaques. Prophages that are wild-type for the *cII* region ( $\lambda cII^+$ ) integrate into the host genome and become part of the developing bacterial lawn [Banuett et al., 1986; Jakubczak et al., 1996]. In this study, we have continued our examination of the sensitivity of adult and neonatal mice to the mutagenicity of genotoxic carcinogens by measuring the mutagenicity of AFB<sub>1</sub> in neonatal and adult Big Blue transgenic mice.

## MATERIALS AND METHODS

### Animals and AFB<sub>1</sub> Treatment

Big Blue B6C3F<sub>1</sub> transgenic mice were obtained from Taconic Laboratories (Germantown, NY). We followed the recommendations of our Institutional Animal Care and Use Committee for the handling, maintenance, treatment, and sacrifice of the animals. AFB<sub>1</sub> was purchased from Sigma (St. Louis, MO). Male neonatal and adult Big Blue B6C3F<sub>1</sub> mice were divided into five groups, with each group containing five mice. Table I shows the dose levels of AFB<sub>1</sub> and treatment schedule for the various experimental groups. The mice in the two neonatal groups were injected i.p. with total doses of 0 or 6 mg/kg body weight AFB<sub>1</sub> at the age of 4, 7, and 10 days. This three-dose, every third-day treatment schedule has also been used for AFB<sub>1</sub> liver tumor induction in adult male mice [Wogan, 1969]. Thus, mice in the three adult groups in our study were injected i.p. with total doses of 0, 6, or 60 mg/kg body weight AFB<sub>1</sub> in three split-doses at 120, 123, and 126 days of age. AFB<sub>1</sub> was dissolved in DMSO for treatment and the volume of the chemical administration was 2  $\mu$ l/g body weight. The animals were sacrificed 6 weeks after the treatment. The choice of a 6-week mutant manifestation time was based on the recommendations for adult mice by Thybaud et al. [2003] and our observations on mutant manifestation following transplacental exposure [Mei et al., 2005]. The livers were isolated, frozen quickly by using liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ .

### Isolation of DNA from Liver Tissue

Liver DNA was isolated with a RecoverEase DNA isolation kit (Stratagene, La Jolla, CA). Each DNA sample was isolated from  $\sim 100$  mg of mouse liver tissue. Liver tissue was gently homogenized in 5 ml of cold lysis buffer to disaggregate the cells, then sent through a sterile cell strainer into a 50 ml conical tube and centrifuged at 1,100g for 12 min at  $4^{\circ}\text{C}$ . The supernatant fluid was discarded and any residual droplets were removed from the tube with a sterile applicator. One milliliter of digestion solution from the DNA isolation kit was supplemented with 20  $\mu$ l RNase-It ribonuclease; 70  $\mu$ l of this digestion solution and 70  $\mu$ l of warmed proteinase K solution were mixed and added to each liver cell pellet. The conical tube was then placed in a  $50^{\circ}\text{C}$  water bath for 45 min. The mixture was transferred to a dialysis cup floating on TE buffer, and left to dialyze at room temperature for  $\sim 42$  hr. Fully hydrated genomic DNA was then removed from the dialysis cup and stored at  $4^{\circ}\text{C}$  until use.

### ***cII* Mutation Assay**

The packaging of  $\lambda$  phage, plating of the packaged DNA samples, and determination of MF were conducted following Stratagene's procedure for the  $\lambda$  select-*cII* mutation detection system for Big Blue rodents. The  $\lambda$  shuttle vector containing the *cII* target gene was rescued from total genomic DNA with  $\lambda$  phage packaging extract (Transpack; Stratagene). Plating was performed with the *Escherichia coli* host strain G1250. The bacteria were grown in TB1 liquid medium with 1% maltose-MgSO<sub>4</sub> (1 M) overnight at 30°C in preparation for the experiment. To determine the total titer of packaged phages, G1250 bacteria were mixed with a 1:3,000 dilution of packaged phage, plated on TB1 plates, and incubated overnight at 37°C (nonselective conditions). For mutant selection, the packaged phages were mixed with G1250 cells, plated on TB1 plates, and incubated at 24°C for about 42 hr (conditions for  $\lambda$  *cII* selection). The *cII*MF was calculated as the ratio of the total number of mutant plaques (as determined at 24°C) to the total number of plaques screened (as determined at 37°C). Statistical analyses of MFs were performed using SigmaStat (SPSS Science, Chicago, IL). All MF data are expressed as the mean  $\pm$  standard deviation (SD) from five animals. Statistical significance was determined by one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test for comparison of multiple treatment groups.

### **Sequence Analysis of Mutation Types in the *cII* Gene**

Mutants for sequencing were selected from mutant plaques recovered from mice in the 6 mg/kg neonate and 60 mg/kg adult AFB<sub>1</sub> treatment groups. The sequencing protocol was adapted from a previous procedure [Chen et al., 2002], with minor modifications. The *cII* mutant plaques were selected at random from selection plates assaying liver DNA from different animals and replated at low density to verify the mutant phenotype. Single, well-isolated plaques were selected from these plates and transferred to microcentrifuge tubes containing 100  $\mu$ l of autoclaved distilled water. The tubes were placed in a thermocycler at 99.9°C for 10 min and centrifuged at 1,500g for 5 min immediately after the heating. The *cII* target DNA was amplified by PCR with primers 5'-AAAAA GGGCATCAAATTAACC-3' and 5'-CCGAAGTTGAGTATTTTTGCTG-3'. For PCR amplification, 10  $\mu$ l of the supernatant and 0.03  $\mu$ l of the each primer (148  $\mu$ M) were added to 10  $\mu$ l of 2 $\times$  PCR Master Mix (Promega, Madison, WI). The PCR reaction was carried out with the following cycling parameters: a 3 min denaturation at 95°C; followed by 30 cycles of 30 sec at 95°C, 1 min at 60°C, and 1 min at 72°C; with a final extension of 10 min at 72°C. The PCR products were purified using QiaQuick PCR purification kits (Qiagen, Chatsworth, CA). The *cII* mutant DNA was sequenced and analyzed with a CEQ DTCS-Quick Start Kit and a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA), according to the manufacturer's instructions. The primers for *cII* mutation sequencing were the same as those used for the PCR. The differences in mutational types detected in different groups were tested statistically using Monte Carlo analysis [Adams and Skopek, 1987].

### **Mutation Frequency Calculation**

MF was defined as the frequency of plaques that were phenotypically mutants in the *cII* mutant assay, while mutation frequency was the frequency of mutant plaques carrying independent mutations and therefore requires correction for clonal expansion. In this

calculation, mutations found more than once in the mutants isolated from a single animal were assumed to be siblings and were considered to represent a single independent mutation. The total mutation frequency was calculated as MF  $\times$  the ratio of independent mutations to the total number of analyzed mutations. The mutation frequency for each type of mutation was determined as the total mutation frequency  $\times$  the percentage of the mutation type among all independent mutations.

## RESULTS

### Mutant Frequency

Groups of five neonatal and adult male Big Blue mice were administered AFB<sub>1</sub> and sacrificed 6 weeks later along with corresponding controls. The MFs in the liver *cII* genes from the different treatment groups and their concurrent controls are compared in Figure 1. The mice treated as neonates with 6 mg/kg AFB<sub>1</sub> had a significant, ~22-fold increase in liver MF ( $P < 0.001$ ;  $914 \pm 311 \times 10^{-6}$  in mice treated with AFB<sub>1</sub> vs.  $41 \pm 13 \times 10^{-6}$  in the neonatal control). In addition, the liver *cII*MF in mice treated with AFB<sub>1</sub> as neonates was significantly greater than the MFs in mice treated with AFB<sub>1</sub> as adults (neonate vs. 6 mg/kg AFB<sub>1</sub> adult,  $P < 0.001$  and neonate vs. 60 mg/kg AFB<sub>1</sub> adult,  $P < 0.001$ ). Although AFB<sub>1</sub> treatment of adults produced a dose-related increase in MF, the MFs in the treated mice were not significantly increased compared to the concurrent control group ( $46 \pm 14 \times 10^{-6}$  in the adult control vs.  $69 \pm 22 \times 10^{-6}$  in 6 mg/kg AFB<sub>1</sub> adult group,  $P = 0.800$ ;  $76 \pm 30 \times 10^{-6}$  in 60 mg/kg AFB<sub>1</sub> adult group vs. the adult control,  $P = 0.941$ ). Also, there was no significant difference between the liver MFs in the two adult AFB<sub>1</sub>-treatment groups ( $P = 0.941$ ).

### Mutation Types

AFB<sub>1</sub>-induced liver *cII* mutations were evaluated by DNA sequence analysis of 93 and 63 mutants isolated from the neonatal and adult AFB<sub>1</sub>-treated sample groups, respectively (Table II). Fewer mutants were sequenced in the adult group because the low mutant frequency provided a relatively small number of mutants for analysis. A total of 70 independent mutations from neonatal and 55 from adult mice were identified (Table III). The mutation spectrum for control mice was obtained from our previous study [Chen et al., 2005] because the ages of the control neonates and adults, as well as the type of vehicle used, were the same in the previous study and in this study. Table III summarizes the types of mutations in the liver *cII* gene from mice treated with AFB<sub>1</sub> as neonates and adults, as well as the control mice. The most common type of mutation detected in AFB<sub>1</sub>-treated neonates was G:C  $\rightarrow$  T:A transversion (76%), while G:C  $\rightarrow$  T:A transversion (51%) and G:C  $\rightarrow$  A:T transition (32%) were the predominant mutations found in AFB<sub>1</sub>-treated adults. The main type of mutation for the control groups was G:C  $\rightarrow$  A:T transition. The mutation spectra in the neonatal and adult AFB<sub>1</sub>-treatment groups were significantly different from their respective controls ( $P < 0.0001$ ). Also, there was a significant difference between the AFB<sub>1</sub>-treated neonatal and adult mouse spectra ( $P < 0.01$ ) while there was no significant difference between the spectra for neonatal and adult control mice.

## Mutation Frequency

Table IV shows the mutation frequencies that were detected for each of the AFB<sub>1</sub>-treated and control groups of mice. Mutation frequencies are given that include all types of mutations, along with mutation frequencies for each of the major types of mutation that were detected, G:C → T:A, G:C → A:T, and frameshifts. The total mutation frequencies in the different groups were very similar to the corresponding MFs for these groups, indicating that clonal expansion was not a factor in producing the high MFs induced by AFB<sub>1</sub> in the neonatal mice. The mutation frequencies for G:C → A:T and frameshifts showed relatively small differences among the treatment groups. In contrast, the mutation frequencies for G:C → T:A differed greatly between the treatment and control groups, 82-fold and up to fivefold changes for neonatal and adult mice, respectively, indicating that AFB<sub>1</sub> mainly induced a G:C → T:A transversion in mouse liver.

## DISCUSSION

### Mutagenicity of AFB<sub>1</sub> Correlates With the Carcinogenicity of AFB<sub>1</sub> in Neonatal and Adult Mice

AFB<sub>1</sub> is an extremely potent hepatocarcinogen in neonatal mice, but not in adult mice. The same protocol used for treating neonatal mice with 6 mg/kg AFB<sub>1</sub> in this study resulted in liver tumor incidences of 70% at 52 weeks of age, and 100% at 82 weeks of age, whereas treatment of adult mice with 60 mg/kg AFB<sub>1</sub> did not produce significant toxic or carcinogenic responses [Wogan, 1969]. Carcinogenesis is commonly broken down into three main stages: initiation, promotion, and progression. Initiation can result from carcinogen-induced mutations in critical genes involved in carcinogenesis. If initiation is the main reason for the sensitivity of neonates to AFB<sub>1</sub> carcinogenicity, a high MF in liver would be expected in the neonatal, but not in the adult, AFB<sub>1</sub>-treatment group. In this study, the *cII* liver MF in neonatal mice treated with 6 mg/kg AFB<sub>1</sub> was 22-fold higher than that of the concurrent control, whereas treatment of adult mice with 6 and 60 mg/kg AFB<sub>1</sub> did not significantly increase the MFs over the control (Fig. 1, about 1.5-fold and 1.7-fold more than the MF of the control, respectively). A previous study also showed that a single dose of 2.5 mg/kg AFB<sub>1</sub> did not increase liver *lacI*MF in adult Big Blue mice (1.3-fold increase,  $P=0.25$ ) [Dycaico et al., 1996]. These results suggest that neonatal mice are much more sensitive to the mutagenicity of AFB<sub>1</sub> than adult mice. This correlation between the mutagenicity and carcinogenicity of AFB<sub>1</sub> in neonatal and adult mice is consistent with mutagenicity being an important factor for AFB<sub>1</sub> carcinogenesis.

### The Mutational Spectra in Neonatal and Adult Mice Reflect the Mutational Specificity of AFB<sub>1</sub>-DNA Adducts

The spectra of *cII* gene mutations in liver from AFB<sub>1</sub>-treated neonatal and adult mice were significantly different from their respective controls. Also, there was a significant difference between the spectra in AFB<sub>1</sub>-treated neonatal and adult mice (Table III). Relative to the mutational spectrum in control mice, AFB<sub>1</sub>-treated neonatal mice had a dramatic increase in the proportion of G:C → T:A transversions, to 76% of all mutations, and a large reduction in the proportion of G:C → A:T transitions, the major mutation in control mice, to only 9%. In the AFB<sub>1</sub>-treated adult mice, the changes in the proportions of G:C → T:A transversions

and G:C → A:T transitions (to 51% and 32%, respectively) were not as great, even though the overall spectrum was significantly different from the control. These more modest changes indicate that AFB<sub>1</sub> did induce mutations in adult mice, but that the induction was relatively low, and not sufficient to alter the MF significantly. The difference between the mutational spectra in the AFB<sub>1</sub>-treated neonatal and adult mice resulted from the different percentages of G:C → T:A transversions and G:C → A:T transitions, and reflected a lower impact of AFB<sub>1</sub>-specific mutation in the adult mice.

AFB<sub>1</sub> is bioactivated by epoxidation of the terminal furan ring double bond to form AFB<sub>1</sub> exo-8,9-epoxide, which reacts covalently with DNA [Miller, 1978; Essigmann et al., 1983]. The major DNA adduct induced by AFB<sub>1</sub> is 8,9-dihydro-8-(N7-guanyl)29-hydroxyafatoxin B<sub>1</sub> (AFB<sub>1</sub>-N7-Gua) [Essigmann et al., 1977; Lin et al., 1977; Martin and Garner, 1977; Swenson et al., 1977; Croy et al., 1978; Croy and Wogan, 1981a,b; Groopman et al., 1981]. This DNA lesion induces mainly G:C → T:A transversions in a variety of biological systems. AFB<sub>1</sub> is known to produce G:C → T:A transversion in bacteria [Foster et al., 1983; Bailey et al., 1996], in Big Blue mice treated with a glutathione depleting agent [Autrup et al., 1996], and in Big Blue rats [Dycaico et al., 1996]. This mutation is also the principal mutation detected in human liver tumors that are believed to be induced by aflatoxin. A high proportion of liver cancers in populations exposed to high levels of dietary aflatoxin have p53 codon 249 G:C → T:A transversions [Bressac et al., 1991; Hsu et al., 1991; Li et al., 1993]. Analysis of mutation frequencies in mice from our study showed large AFB<sub>1</sub>-induced increases in G:C → T:A transversion in both neonatal and adult mice. Therefore, the mutational spectra induced by AFB<sub>1</sub> in our study are largely consistent with the reported mutational specificity of AFB<sub>1</sub> in other systems. The spectra reflect a common biological activity of AFB<sub>1</sub> in neonatal and adult mice, with a much higher sensitivity to mutation induction by AFB<sub>1</sub> in neonatal mice.

The high MFs in AFB<sub>1</sub>-treated neonatal mice could, in theory, result from clonal expansion due to rapid division of a relatively few cells containing *cH* mutations. In that case, a low induced mutation frequency would be found in the AFB<sub>1</sub>-treated neonatal mice. Our analysis of mutation frequencies showed that the total mutation frequencies in the different treatment groups, including the AFB<sub>1</sub>-treated neonatal mouse group (Table IV), were similar to the MFs for these groups (Fig. 1). This observation indicates that clonal expansion was not a factor in producing the elevated MFs detected in AFB<sub>1</sub>-treated neonatal mice.

### **The Mutagenicity of AFB<sub>1</sub> Is Consistent With GST Enzyme Activity in Neonatal and Adult Mice**

In mammalian cells, the primary pathway for AFB<sub>1</sub> detoxification is through GST-mediated conjugation of the exo-8,9-epoxide with reduced glutathione (GSH), with the level of GST conjugation for a species being inversely related to the susceptibility of the species to AFB<sub>1</sub>-induced hepatocarcinogenesis [Hengstler et al., 1999]. Adult mice are comparatively resistant to AFB<sub>1</sub>-induced toxicity and carcinogenicity and this resistance is thought to be mainly due to their relatively high levels of GST [Eaton et al., 1993].

A previous study [Autrup et al., 1996] investigated the mutagenic activity of AFB<sub>1</sub> in the liver of adult mice co-treated with phorone, a GSH depleting agent. While AFB<sub>1</sub> alone did

not result in a significant increase in liver *lacIMF*, co-treatment of AFB<sub>1</sub> and phorone resulted in a significant increase in MF (four-fold over the control). These results suggest that the GST levels in liver could be a major factor for the differential susceptibility of neonatal and adult mice for mutation induction by AFB<sub>1</sub>. It is theoretically possible that DNA repair in neonatal mice is not as efficient as in adult mice, and may account for some of the sensitivity of neonatal mice to AFB<sub>1</sub>. Some studies indicate that differences in DNA repair are an important determinant in tissue- and species-related differences in susceptibility to AFB<sub>1</sub> [Bedard et al., 2005]. However, there are no reports indicating that neonatal mice have a lower DNA repair capacity for AFB<sub>1</sub>-induced DNA damage than adults.

Shupe and Sell [2004] measured AFB<sub>1</sub>-DNA adduct formation in relation to GST levels in murine liver. Based on their results, it is expected that during the first 10 days of age, when we treated neonatal mice with AFB<sub>1</sub> in this study, GST levels in murine liver were about 0.05 mmol/(mg protein min), while at 120 days of age, when we treated our adult mice, liver GST levels were 0.26 mmol/(mg protein min). Shupe and Sell [2004] also reported that AFB<sub>1</sub>-DNA adduct formation was inversely correlated with GST levels at the different ages. DNA adducts were 13-fold higher in the 10-day-old neonatal mice than the 120-day-old adult mice. The results of our study are consistent with these data. The MF induced by AFB<sub>1</sub> in the neonatal mice was about 13-fold higher than that in the treated adults.

In summary, AFB<sub>1</sub> treatment of neonatal and adult mice resulted in very different liver MFs and mutation frequencies. Neonatal treatment with AFB<sub>1</sub> resulted in a significant induction of liver *cII*MF, whereas AFB<sub>1</sub> treatment of adults had no significant effect on MF. The differential mutagenicities of AFB<sub>1</sub> in mouse liver correlate well with the carcinogenicities resulting from neonatal and adult exposure to AFB<sub>1</sub>. A dramatic increase in G:C → T:A transversion frequency in mice treated with AFB<sub>1</sub> as neonates is consistent with the reported mutational specificity of AFB<sub>1</sub> in other systems. These observations indicate that the sensitivity of neonatal mice to AFB<sub>1</sub>-induced G:C → T:A transversions plays a critical role in their sensitivity to AFB<sub>1</sub> carcinogenicity. Our results suggest that young children might be more sensitive to AFB<sub>1</sub> mutagenicity and have a higher risk to AFB<sub>1</sub> exposure.

## ACKNOWLEDGMENTS

The views presented in this article do not necessarily reflect those of the Food and Drug Administration.

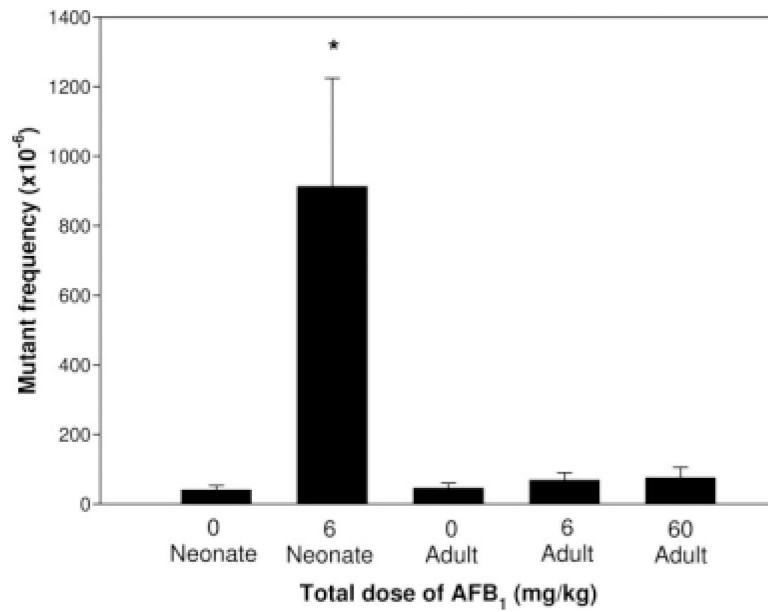
## REFERENCES

- Adams WT, Skopek TR. 1987 Statistical test for the comparison of samples from mutational spectra. *J Mol Biol* 194:391–396. [PubMed: 3305960]
- Autrup H, Jorgensen EC, Jensen O. 1996 Aflatoxin B1 induced *lacI* mutation in liver and kidney of transgenic mice C57BL/6N: Effect of phorone. *Mutagen* 11:69–73.
- Bailey EA, Iyer RS, Stone MP, Harris TM, Essigmann JM. 1996 Mutational properties of the primary aflatoxin B1-DNA adduct. *Proc Natl Acad Sci USA* 93:1535–1539. [PubMed: 8643667]
- Banuett F, Hoyt MA, McFarlane L, Echols H, Herskowitz I. 1986 *hflB*, a new *Escherichia coli* locus regulating lysogeny and the level of bacteriophage lambda *cII* protein. *J Mol Biol* 187:213–224. [PubMed: 2939254]



- Bedard LL, Alessi M, Davey S, Massey TE. 2005 Susceptibility to aflatoxin B1-induced carcinogenesis correlates with tissue-specific differences in DNA repair activity in mouse and in rat. *Cancer Res* 65:1265–1270. [PubMed: 15735011]
- Bressac B, Kew M, Wands J, Ozturk M. 1991 Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature* 350:429–431. [PubMed: 1672732]
- Chen T, Gamboa da Costa G, Marques MM, Shelton SD, Beland FA, Manjanatha MG. 2002 Mutations induced by alpha-hydroxytamoxifen in the lacI and cII genes of Big Blue transgenic rats. *Carcinogenesis* 23:1751–1757. [PubMed: 12376486]
- Chen T, Mittelstaedt RA, Beland FA, Heflich RH, Moore MM, Parsons BL. 2005 4-Aminobiphenyl induces liver DNA adducts in both neonatal and adult mice but induces liver mutations only in neonatal mice. *Int J Cancer* 117:182–187. [PubMed: 15880493]
- Croy RG, Wogan GN. 1981a Quantitative comparison of covalent aflatoxin-DNA adducts formed in rat and mouse livers and kidneys. *J Natl Cancer Inst* 66:761–768. [PubMed: 6939922]
- Croy RG, Wogan GN. 1981b Temporal patterns of covalent DNA adducts in rat liver after single and multiple doses of aflatoxin B1. *Cancer Res* 41:197–203. [PubMed: 7448760]
- Croy RG, Essigmann JM, Reinhold VN, Wogan GN. 1978 Identification of the principal aflatoxin B1-DNA adduct formed in vivo in rat liver. *Proc Natl Acad Sci USA* 75:1745–1749. [PubMed: 273905]
- Dycaico MJ, Stuart GR, Tobal GM, de Boer JG, Glickman BW, Provost GS. 1996 Species-specific differences in hepatic mutant frequency and mutational spectrum among lambda/lacI transgenic rats and mice following exposure to aflatoxin B1. *Carcinogenesis* 17:2347–2356. [PubMed: 8968048]
- Eaton D, Van Ness K, Beutler T, editors. 1993 GST Mediated Protection Against Carcinogens: Aflatoxin B1 as an Example. Boca Raton: CRC Press pp 187–198.
- Essigmann JM, Croy RG, Nadzan AM, Busby WF, Jr, Reinhold VN, Buchi G, Wogan GN. 1977 Structural identification of the major DNA adduct formed by aflatoxin B1 in vitro. *Proc Natl Acad Sci USA* 74:1870–1874. [PubMed: 266709]
- Essigmann JM, Green CL, Croy RG, Fowler KW, Buchi GH, Wogan GN. 1983 Interactions of aflatoxin B1 and alkylating agents with DNA: Structural and functional studies. *Cold Spring Harb Symp Quant Biol* 47 (Part 1):327–337. [PubMed: 6407800]
- Flammang TJ, Tungeln LS, Kadlubar FF, Fu PP. 1997 Neonatal mouse assay for tumorigenicity: Alternative to the chronic rodent bioassay. *Regul Toxicol Pharmacol* 26:230–240. [PubMed: 9356286]
- Foster PL, Eisenstadt E, Miller JH. 1983 Base substitution mutations induced by metabolically activated aflatoxin B1. *Proc Natl Acad Sci USA* 80:2695–2698. [PubMed: 6405385]
- Gorrod JW, Carter RL, Roe FJ. 1968 Induction of hepatomas by 4-aminobiphenyl and three of its hydroxylated derivatives administered to newborn mice. *J Natl Cancer Inst* 41:403–410. [PubMed: 4299538]
- Groopman JD, Croy RG, Wogan GN. 1981 In vitro reactions of aflatoxin B1-adducted DNA. *Proc Natl Acad Sci USA* 78:5445–5449. [PubMed: 6795633]
- Hengstler JG, Van der Burg B, Steinberg P, Oesch F. 1999 Interspecies differences in cancer susceptibility and toxicity. *Drug Metab Rev* 31:917–970. [PubMed: 10575555]
- Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. 1991 Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature* 350:427–428. [PubMed: 1849234]
- IARC. 1990 IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Pharmaceutical Drugs. Lyon: IARC pp 123–141.
- IARC. 1993 IARC Monographs on the Evaluation of Carcinogenic Risks to Humans Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. Lyon: IARC pp 197–572.
- Jakubczak JL, Merlino G, French JE, Muller WJ, Paul B, Adhya S, Garges S. 1996 Analysis of genetic instability during mammary tumor progression using a novel selection-based assay for in vivo mutations in a bacteriophage lambda transgene target. *Proc Natl Acad Sci USA* 93:9073–9078. [PubMed: 8799156]

- Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ. 2006 Cancer statistics, 2006. *CA Cancer J Clin* 56:106–130. [PubMed: 16514137]
- Kohler SW, Provost GS, Kretz PL, Fieck A, Sorge JA, Short JM. 1990 The use of transgenic mice for short-term, in vivo mutagenicity testing. *Genet Anal Tech Appl* 7:212–218. [PubMed: 2151115]
- Li D, Cao Y, He L, Wang NJ, Gu JR. 1993 Aberrations of p53 gene in human hepatocellular carcinoma from China. *Carcinogenesis* 14:169–173. [PubMed: 8382111]
- Lin JK, Miller JA, Miller EC. 1977 2,3-Dihydro-2-(guan-7-yl)-3-hydroxy-aflatoxin B1, a major acid hydrolysis product of aflatoxin B1-DNA or -ribosomal RNA adducts formed in hepatic microsome-mediated reactions and in rat liver in vivo. *Cancer Res* 37:4430–4438. [PubMed: 922734]
- Martin CN, Garner RC. 1977 Aflatoxin B-oxide generated by chemical or enzymic oxidation of aflatoxin B1 causes guanine substitution in nucleic acids. *Nature* 267:863–865. [PubMed: 895848]
- Mei N, Heflich RH, Moore MM, Chen T. 2005 Age-dependent sensitivity of Big Blue transgenic mice to the mutagenicity of N-ethyl-N-nitrosourea (ENU) in liver. *Mutat Res* 572:14–26. [PubMed: 15790487]
- Miller EC. 1978 Some current perspectives on chemical carcinogenesis in humans and experimental animals: Presidential Address. *Cancer Res* 38:1479–1496. [PubMed: 348302]
- Shupe T, Sell S. 2004 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* 148:1–9. [PubMed: 15019083]
- Slikker W III, Mei N, Chen T. 2004 N-ethyl-N-nitrosourea (ENU) increased brain mutations in prenatal and neonatal mice but not in the adults. *Toxicol Sci* 81:112–120. [PubMed: 15159527]
- Swenson DH, Lin JK, Miller EC, Miller JA. 1977 Aflatoxin B1–2,3-oxide as a probable intermediate in the covalent binding of aflatoxins B1 and B2 to rat liver DNA, ribosomal RNA in vivo. *Cancer Res* 37:172–181. [PubMed: 187331]
- Thybaud V, Dean S, Nohmi T, de Boer J, Douglas GR, Glickman BW, Gorelick NJ, Heddle JA, Heflich RH, Lambert I, Martus H-J, Mirsalis JC, Suzuki T, Yajima N. 2003 In vivo transgenic mutation assays. *Mutat Res* 540:141–151. [PubMed: 14550498]
- Vesselinovitch SD, Mihailovich N, Wogan GN, Lombard LS, Rao KV. 1972 Aflatoxin B 1, a hepatocarcinogen in the infant mouse. *Cancer Res* 32:2289–2291. [PubMed: 4343225]
- Wogan GN. 1969 Metabolism and biochemical effects of aflatoxins In: Goldblatt LA, editor. *Aflatoxin-Scientific Background Control and Implications*. New York: Wiley pp 321–350.



**Fig. 1.**

Comparison of MFs in the liver *cII* gene of mice treated with AFB<sub>1</sub> as neonates and adults. Data are the mean  $\pm$  SD for five mice per group. The asterisk indicates a significant difference in MF between the neonatal AFB<sub>1</sub>-treatment group and other groups. There was a significant difference between the MFs induced by AFB<sub>1</sub> in the neonatal and adult treatment groups ( $P < 0.001$ ). While the MF in the neonatal treatment group was about 22-fold higher than that in the concurrent control, the MFs in the adult AFB<sub>1</sub>-treatment groups were only marginally higher than that in their concurrent control.

**Table I.**Schedule for AFB<sub>1</sub> Treatment and Sacrifice of Big Blue B6C3F<sub>1</sub> Mice

Group <sup>a</sup>	Age at dosing (days)	Dose at each treatment time (mg/kg bw)	Total dose (mg/kg bw)	Age at sacrifice (weeks)
I	4, 7, and 10	0	0	8
II	4, 7, and 10	2	6	8
III	120, 123, and 126	0	0	24
IV	120, 123, and 126	2	6	24
V	120, 123, and 126	20	60	24

bw, body weight.

<sup>a</sup>Each group contained five male mice.

Table II.

Mutations in the Liver *cII* Gene of Big Blue Mice Treated With AFB<sub>1</sub> as Neonates and Adults

Position <sup>a</sup>	Mutation <sup>b</sup>	Amino acid change	Sequence context 5' → 3' <sup>c</sup>	Number of mutationsn (number of independent mutations when different from mutants)	
				Neonate	Adult
-14	G → T	N/A	ctaAGGaaa	1	1
-13	G → T	N/A	ctaAGGaaa	2	
3	G → T	Met → Ile	catATGgtt	1	
4	G → T	Val → Phe	atgGTTcgt	1	
24/25	CG → AA	AsnGlu → LysLys	cgcAACGAGgct		1
25	G → A	Glu → Lys	aacGAGgct		1
	G → T	Glu → Stop	aacGAGgct	2	
28	G → T	Ala → Ser	gagGCTcta		2
29	C → A	Ala → Asp	gagGCTcta		2(1)
34	C → T	Arg → Stop	ctaCGAatc		2
40	G → T	Glu → Stop	atcGAGagt	1	1
47	C → A	Ala → Glu	agtGCCgtg		1
	C → G	Ala → Gly	agtGCCgtg	1	
51	G → T	Leu → Phe	gcgTTGctt	2(1)	1
64	G → A	Ala → Thr	atcGCAatg		3
73	G → T	Gly → Stop	cttGGAact	1	1
74	G → T	Gly → Val	cttGGAact		2
89	C → G	Ala → Gly	acaGCGgaa	1	
	C → A	Ala → Glu	acaGCGgaa	8 (5)	3 (2)
	C → T	Ala → Val	acaGCGgaa	1	2
91	G → T	Glu → Stop	gcgGAAgct		1
94	G → C	Ala → Pro	gaaGCTgtg	1	
95	C → A	Ala → Asp	gaaGCTgtg	1	
101	G → T	Gly → Val	gtgGGCgtt		1
103	G → A	Val → Ile	ggcGTTgat		1
	G → T	Val → Phe	ggcGTTgat	5 (3)	4 (2)
108	T → A	Asp → Glu	gttGATaag		1
113	C → A	Ser → Stop	aagTCGcag		1
122	G → T	Ser → Ile	atcAGCagg	4 (3)	
123	C → A	Ser → Arg	atcAGCagg	2	2
125	G → T	Arg → Met	agcAGGtgg	2	
128	G → T	Trp → Leu	aggTGGaag	1	
129	G → A	Trp → Stop	aggTGGaag	1	
131	A → T	Lys → Met	tggAAGagg	1	
134	G → T	Arg → Met	aagAGGgac	1	
	G → C	Arg → Thr	aagAGGgac	1	
135	G → T	Arg → Ser	aagAGGgac	1	1

Position <sup>a</sup>	Mutation <sup>b</sup>	Amino acid change	Sequence context 5' → 3' <sup>c</sup>	Number of mutationsn (number of independent mutations when different from mutants)	
				Neonate	Adult
135–136	GG → TT	ArgAsp → SerTry	aagAGGGACTgg	1	
140	G → C	Trp → Ser	gacTGGatt		1
141	G → T	Trp → Cys	gacTGGatt	2(1)	
145	C → A	Pro → Thr	attCCAaag	1	1
145–146	CC → AA	Pro → Lys	attCCAaag		1
145/146	–C	Frameshift	attCCAaag	1	
150	G → T	Lys → Asn	ccaAAGttc	1	
155	C → T	Ser → Leu	ttcTCAatg		1
158	–T	Frameshift	tcaATGctg		1
159	G → A	Met → Iie	tcaATGctg		1
159–160	GC → TG	MetLeu → IieVal	tcaATGCTGctt	1	
160	C → A	Leu → Met	atgCTGctt	2	1
161	T → A	Leu → Gln	atgCTGctt		1
163	C → A	Leu → Ile	ctgCTTgct	2(1)	
	C → T	Leu → Phe	ctgCTTgct	1	
166	G → T	Ala → Ser	cttGCTggt	2	
167	C → A	Ala → Asp	cttGCTggt	2	
173/174	+GTTC	Frameshift	gttCTTgaa		1
178/185	+G	Frameshift	gaaTGGGGGGTCggt	1	
179	G → T	Trp → Leu	gaaTGGggg	1	
179–184	–G	Frameshift	gaaTGGGGGGTCggt		1
181	G → T	Gly → Trp	tggGGGgtc	2(1)	
190	G → T	Asp → Tyr	gttGACgac	1	
193	G → T	Asp → Tyr	gacGACgac	1	1
196	G → A	Asp → Asn	gacGACatg	1	1
	G → C	Asp → His	gacGACatg	1	
	G → T	Asp → Tyr	gacGACatg		1
212	C → A	Ala → Glu	ttgGCGcga	18(6)	7 (4)
	C → T	Ala → Val	ttgGCGcga	3 (2)	5 (4)
214	C → T	Arg → Stop	gcgCGAcaa		2
217	C → A	Gln → Lys	cgaCAAgtt	1	
224	C → A	Ala → Asp	gttGCTgcg	3	
232	C → A	Leu → Ile	attCTCacc		1
256	–G	Frameshift	gcgGCAacc	1	
285/286	–G	Frameshift	cagATGGAGttc		1
Total				93 (70)	63 (55)

–, deletion; +, insertion.

<sup>a</sup>Position 1 is the first base of the start codon in the *cII* coding sequence.

<sup>b</sup>Presented in term of sequence change on nontranscribed DNA strand.

<sup>c</sup>Uppercase indicates target codon and target bases are underlined.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

**Table III.**

Summary of the Independent Mutations in the Liver *cII* Gene of Big Blue Mice Treated With AFB<sub>1</sub> as Neonates and Adults

Type of mutation	AFB <sub>1</sub> -treated <sup>a</sup>				Control <sup>b</sup>			
	Neonatal		Adult		Neonatal		Adult	
	Number	%	Number	%	Number	%	Number	%
G:C → C:G	5	7	1	2	3	5	3	9
G:C → A:T	6	9	18	32	28	50	13	41
G:C → T:A	53	76	28	51	12	21	2	6
A:T → T:A	1	1	2	4	0	0	0	0
A:T → C:G	0	0	0	0	5	9	3	9
A:T → G:C	0	0	0	0	1	2	3	9
Frameshift	3	4	4	7	7	13	8	25
Tandem	2	3	2	4	0	0	0	0
Total mutants screened	70	100	55	100	56	100	32	100

<sup>a</sup>The mutational spectra for AFB<sub>1</sub>-treated neonatal and adult mice were significantly different from their respective concurrent controls ( $P < 0.0001$ ). Also, there was a significant difference between the AFB<sub>1</sub>-treated neonatal and adult mice ( $P < 0.01$ ). There was no significant difference between the two control groups.

<sup>b</sup>Data for controls were obtained from our previous study [Chen et al., 2005]. The control animals were treated with DMSO at 2  $\mu$ l/g body weight.



**Table IV.**

Comparison of the Mutation Frequencies for All Mutation Types and for Three Major Types of Mutations in the Liver *cII* Gene of Mice Treated With AFB<sub>1</sub> as

Treatment group	Total dose	Mutation frequencies (X10 <sup>-6</sup> )			Frameshift
		All types	G:C → A:T	G:C → T:A	
Neonate	0	28	14	6	4
	6	688	62	489	28
Adult	0	42	17	3	10
	60	66	21	16	5

The unit for total dose is mg AFB<sub>1</sub>/kg body weight.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript