Bacterial Lipopolysaccharide Exposure Alters Aflatoxin B₁ Hepatotoxicity: Benchmark Dose Analysis for Markers of Liver Injury

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Received November 11, 2001; accepted February 14, 2002

Aflatoxin B₁ (AFB₁) is a fungal toxin that causes both acute hepatotoxicity and hepatocellular carcinoma in humans and experimental animals. Previous studies demonstrated that a small, noninjurious dose of bacterial lipopolysaccharide (LPS) augments the hepatotoxicity of AFB₁ through activation of inflammatory cells and production of soluble inflammatory mediators (Barton et al., 2000b, 2001). This study was conducted to examine the effect of LPS on the dose-response relationship for AFB₁-induced liver injury. Male Sprague-Dawley rats (250–350g) were treated with AFB₁ (0.1 mg/kg–6.3 mg/kg, ip) and 4 h later with a noninjurious dose of E. coli LPS (7.4 × 10⁵ EU/kg, iv). Twenty-four h after AFB₁ administration, hepatic parenchymal cell injury was estimated from elevations in serum alanine aminotransferase and g-glutamyl transferase activities. Based on benchmark dose (BMD) analysis, the AFB₁ BMD for parenchymal cell injury was decreased 10-fold by LPS cotreatment, whereas AFB₁ BMDs for bile duct injury were decreased nearly 20-fold. The data suggest that concurrent inflammation renders the liver considerably more sensitive to the hepatotoxic effects of AFB₁.

Key Words: lipopolysaccharide; aflatoxin B₁; liver injury; risk assessment; benchmark dose.

Aflatoxin B₁ (AFB₁) is a mycotoxin produced by Aspergillus flavus and Aspergillus parvisicus. It is a common contaminant of grain foods for both human and animal consumption. Human exposure to AFB₁ is greatly influenced by quality of grain storage, climate, and culinary customs (Hall and Wild, 1994; Wilson and Payne, 1994). Indeed, in contrast to the United States, human exposure to AFB₁ in developing countries can be quite large. Consumption of contaminated corn is probably the most important mode of exposure (Wood, 1989). In the Guangxi province of the People’s Republic of China, where corn is a dietary staple, AFB₁ contamination of corn has been measured at 460 mg/kg (Li et al., 2001). Human exposure in this region has been estimated to be between 50 to 75 mg/day (Groopman et al., 1992).

AFB₁ causes acute hepatotoxicity and liver carcinomas in people and laboratory animals (Roebuck and Maxuutenko, 1994). It is metabolized to a highly reactive 8,9-epoxide that binds to cellular macromolecules, primarily in the periporal region of the liver. AFB₁-induced liver injury manifests itself as periporal parenchymal cell necrosis, hemorrhage, and injury to intrahepatic bile ducts. Clinical manifestations of acute AFB₁ exposure in humans include abdominal pain, pulmonary edema, and liver necrosis, and these are collectively referred to as aflatoxicosis (Cullen and Newberne, 1994).

Identification of populations susceptible to chemical toxicity is an integral component of risk assessment. Epidemiological studies of AFB₁ exposure have proved to be crucial in identification of “at risk” populations for hepatotoxicity and liver carcinoma. In regions where AFB₁ exposure is commonplace, there is a strong correlation between hepatocellular carcinoma incidence and hepatitis B infection, a defining feature of which is inflammation of the liver (Groopman et al., 1993). Strong association can be seen between expression of hepatitis B viral proteins and an inflammatory response, and it has been suggested that this may enhance the action of certain hepatocarcinogens by increasing rates of hepatocellular injury and proliferation (Jin et al., 2001; Sell et al., 1991). Moreover, strong positive correlations have been found in rats between AFB₁-induced acute liver injury and preneoplastic lesions (Maxuutenko et al., 1996).

Supporting the correlations identified in people are studies in experimental animals, which suggest that modest inflammation increases the hepatotoxic response to AFB₁ (Barton et al., 2000a). Endotoxic lipopolysaccharide (LPS) is an outer cell-wall component of gram-negative bacteria. It is a potent inflammmagen and contributes significantly to the pathogenesis of gram-negative bacterial infections by activating toll-like receptors on inflammatory cells, which in turn precipitate the expression of numerous soluble inflammatory mediators. Exposure to large amounts of LPS during conditions such as sepsis is associated with fever, circulatory shock, disseminated intra-vascular coagulation, and injury to several organs, including the liver (Ghosh et al., 1993). In contrast, small doses of LPS do not cause overt tissue injury but can nevertheless lead to

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tissue accumulation of inflammatory cells and release of inflammatory mediators. Episodes of modest inflammation, although benign on their own, are probably commonplace in people and have the ability to augment the toxicities of several xenobiotic agents (Ganey and Roth, 2001; Roth et al., 1997).

The aim of this study was to quantify the ability of LPS to shift the dose-response relationship for AFB<sub>1</sub>-induced liver injury. AFB<sub>1</sub> was given at various doses in the presence or absence of a small, noninjurious dose of LPS, and liver injury was determined via analysis of serum enzyme markers. Benchmark dose (BMD) analysis was used to estimate the magnitude of LPS-induced shifts in sensitivity to AFB<sub>1</sub> hepatotoxicity.

**MATERIALS AND METHODS**

**Animals and materials.** Male Sprague-Dawley rats (CD-Crl: CD-(SD)BR VAF/Plus; Charles River, Portage, MI) weighing 250–350 grams were used for these studies. Reagent kits used to measure serum markers of liver injury (Infinity-ALT, Infinity-AST, ALP, GGT) were purchased from Sigma Chemical Co. (St. Louis, MO), as was lipopolysaccharide derived from E. coli serotype 0128:B12 with an activity of 1.7×10<sup>6</sup> EU (endotoxin units)/mg. A colorometric, kinetic limulus amebocyte lysate (LAL) assay was used to estimate LPS-specific activity using a kit (#50-650U) purchased from Bio-whittaker (Walkersville, MD). Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co.

**Treatment protocol.** In preliminary studies, 24-h fasting had minimal effect on the magnitude of hepatotoxicity but decreased variability in response among animals. Rats fasted for 24 h were given a dose of AFB<sub>1</sub>, ranging from 0.1 mg/kg to 6.3 mg/kg intraperitoneally in a vehicle comprising 8% DMSO in sterile water. Four h later they were given 7.4×10<sup>6</sup> EU/kg LPS or sterile saline via the tail vein. This dose of LPS was not overtly hepatotoxic when administered alone (Barton et al., 2000a). Twenty-four h after AFB<sub>1</sub> administration, rats were anesthetized with sodium pentobarbital (50 mg/kg, ip), and blood was drawn from the dorsal aorta, allowed to clot, and centrifuged to separate serum.

**Serum markers of liver injury.** Commercial reagent kits (see above) were used to measure serum enzyme activities. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured spectrophotometrically by the methods of Wroblewski and LaDue (1956) and Karmen (1955), respectively. Serum γ-glutamyl transferase (GGT) and alkaline phosphatase (ALP) activities were measured by the methods of Szasz (1974) and Bowers and McComb (1966), respectively.

**Benchmark dose (BMD) analysis.** Background and technical information on BMD analysis were obtained from the most recent United States Environmental Protection Agency guidance publication (U.S. EPA, 1995). Dose-response curves were analyzed using the EPA’s Benchmark Dose Response software (Version 1.3), which was developed by the National Center for Environmental Assessment to aid in computer analysis of dose-response data (information and software available at http://cfpub.epa.gov/ncea/cfm/ nceahome.cfm). A continuous Hill model was employed to calculate benchmark doses for individual dose-response curves. This model was utilized because it provided an adequate fit to data for each of the toxicity endpoints measured. The benchmark response was defined as the response corresponding to one control standard deviation from the control mean. Assuming serum ALT activities above the 99th percentile of the control mean (control mean + 2.33 standard deviations) are considered adverse, this benchmark response identifies an AFB<sub>1</sub> dose at which 10% of treated animals would have serum ALT activities above the 99th percentile (Crump, 1995). Assumption of equal slopes was confirmed via calculation of the Hill slope from a best-fit, four-parameter logistic model for each curve. Hill slopes for individual curves were compared statistically using Student’s t-test.

**Statistical analysis.** Results are expressed as mean ± SE. N for treatment groups was 4–11; vehicle-treated rats were included on each experimental day such that n for combined animals was 24. Data were analyzed using a one-way ANOVA, with group comparisons made with Tukey’s test. The Kolmogorov-Smirnov test was applied to test homogeneity of variance. Data with non-homogeneous variance were analyzed using Kruskal-Wallis one-way ANOVA on ranks, with Dunn’s post hoc test for multiple comparisons. The criterion for significance was p < 0.05 for all studies.

**RESULTS**

**Hepatic Parenchymal Cell Injury**

Animals received either intravenous saline or 7.4×10<sup>6</sup> EU/kg LPS 4 h after AFB<sub>1</sub> administration, and hepatic parenchymal cell injury was assessed 24 h after AFB<sub>1</sub> treatment. Doses for the AFB<sub>1</sub>/Veh curve ranged from 0.63 mg/kg to 6.3 mg/kg AFB<sub>1</sub>, whereas doses for the AFB<sub>1</sub>/LPS curve ranged from 0.1 mg/kg to 1.0 mg/kg AFB<sub>1</sub>. In the AFB<sub>1</sub>/saline and AFB<sub>1</sub>/LPS groups given the largest dose of AFB<sub>1</sub>, mortality was approximately 30% and 50%, respectively, whereas survival at the other AFB<sub>1</sub> doses was between 90–100%. Blood samples were taken from surviving rats for determination of biomarkers of liver injury. Increases in ALT (Fig. 1A) and AST (Fig. 1B) activities in AFB<sub>1</sub>/Veh-treated animals were dose-dependent, with a sharp increase in activity near 4.0 mg/kg AFB<sub>1</sub> for both markers. No observed adverse-effect levels (NOAELs) for ALT and AST were 4.0 mg/kg and 2.0 mg/kg AFB<sub>1</sub>, respectively. Significant increases in both markers were observed at markedly smaller AFB<sub>1</sub> doses in animals cotreated with LPS. The NOAEL for both ALT and AST in AFB<sub>1</sub>/LPS-cotreated animals was 0.25 mg/kg. Thus, using the NOAEL as a marker of hepatic parenchymal cell injury, LPS cotreatment resulted in an 8–16-fold increase in AFB<sub>1</sub> toxicity.

**Bile Duct Injury**

Consistent with markers of parenchymal cell injury, appreciably larger AFB<sub>1</sub> doses were required to cause significant increases in ALP (Fig. 2A) and GGT (Fig. 2B) activities in the AFB<sub>1</sub>/Veh group than in the AFB<sub>1</sub>/LPS cotreated group. NOAEL values for increases in ALP activity were 4.0 mg/kg and 0.25 mg/kg AFB<sub>1</sub> for AFB<sub>1</sub>/Veh- and AFB<sub>1</sub>/LPS-treated animals, respectively. The NOAEL value for GGT in AFB<sub>1</sub>/LPS cotreated animals was 0.4 mg/kg, whereas no significant increases in GGT were seen for animals treated with AFB<sub>1</sub> alone (i.e., NOAEL ≥ 6.3 mg/kg). Therefore, the NOAEL dose for markers of bile duct injury in AFB<sub>1</sub>/LPS cotreated animals was about 16-fold less than the NOAEL for animals treated with AFB<sub>1</sub> alone.

**Benchmark Analysis of AFB<sub>1</sub> Dose Response**

Due to limitations of NOAEL as an estimation of threshold response, benchmark dose analysis was performed (U.S. EPA, 1995). Table 1 illustrates the BMD calculated for a continuous benchmark response defined as the response
corresponding to one control standard deviation above the control mean. AFB₁ BMD values for ALT and AST in animals treated with AFB₁ alone were 3.97 mg/kg and 3.71 mg/kg, respectively. AFB₁ BMD values for LPS-cotreated animals were markedly decreased to 0.36 mg/kg and 0.38 mg/kg, respectively. Thus, the BMD calculation indicated a 10-fold leftward shift in the BMD for enzyme markers of hepatic parenchymal cell injury. AFB₁ BMD values for ALP were 4.61 mg/kg and 0.18 mg/kg for AFB₁/Veh- and AFB₁/LPS-treated animals, respectively. The benchmark response for GGT activity was not achieved within the dose range for animals treated with AFB₁ alone. However, cotreated animals displayed an AFB₁ BMD of 0.22 mg/kg, signifying a pronounced leftward shift in BMD for GGT activity. Hence, AFB₁ BMDs for markers of bile duct injury were decreased ≥ 20-fold.

DISCUSSION

Liver lesions associated with LPS-enhanced AFB₁ hepatotoxicity mimic those occurring with a large dose of AFB₁ and are characterized by peribiliary necrosis and bile duct epithelial cell injury (Barton et al., 2000a; Kalengayi and Desmet, 1975).

FIG. 1. AFB₁ dose response for serum markers of hepatic parenchymal cell injury. Rats were treated with various doses of AFB₁, then 4 h later with 7.4 × 10⁶ EU/kg LPS or saline. AFB₁ doses used for saline-treated animals were 0.63, 1.0, 2.0, 4.0, 6.0, and 6.3 mg/kg. AFB₁ doses used for LPS-treated animals were 0.1, 0.25, 0.4, 0.63, and 1.0 mg/kg. Hepatic parenchymal cell injury was estimated by measuring the serum activities of (A) alanine aminotransferase (ALT) and (B) aspartate aminotransferase (AST). Data are expressed as means ± SEM; n = 4–24 animals; *significantly different from respective control group not treated with AFB₁ (p < 0.05).

FIG. 2. AFB₁ dose response for serum markers of bile-duct injury. Rats were treated with various doses of AFB₁, then 4 h later with 7.4 × 10⁶ EU/kg LPS or saline. AFB₁ doses used for saline-treated animals were 0.63, 1.0, 2.0, 4.0, 6.0, and 6.3 mg/kg. AFB₁ doses used for LPS-treated animals were 0.1, 0.25, 0.4, 0.63, and 1.0 mg/kg. Bile duct injury was estimated by measuring the serum activities of (A) alkaline phosphatase (ALP) and (B) γ-glutamyltransferase (GGT). Data are expressed as means ± SEM; n = 4–24 animals; *significantly different from respective control group not treated with AFB₁ (p < 0.05).
Thus, based on the nature of the lesions, LPS appears to enhance AFB$_1$ hepatotoxicity (Barton et al., 2000a). Dose-response curves for markers of AFB$_1$-induced hepatic parenchymal cell and bile duct injury showed a marked leftward shift in animals coexposed to a nontoxic dose of LPS. We used both NOAEL and BMD analysis to estimate shifts in thresholds for toxicity. Application of the traditional NOAEL analysis to dose-response data for non-cancer health effects comes with several disadvantages, and NOAEL values often differ markedly from derived BMDs (Allen et al., 1994; Gaylor et al., 1998). For example, the assignment of a NOAEL relies critically on data from only one dose, whereas BMD analysis takes all of the dose-response relationship into account, including slope, in determining the BMD. Moreover, in contrast to BMD analysis, NOAEL values are highly dependent on sample size and tend to be larger in studies with a smaller sample size (U.S. EPA, 1995). In an effort to overcome the shortcomings of NOAEL analysis, numerous investigators have used BMD analysis to analyze dose-response toxicity data for diverse effects including neurotoxicity, developmental toxicity, and effects on the endocrine system (Mantovani et al., 1998; Rabovsky et al., 2001; Zhou et al., 2001). In this study, benchmark doses determined for each curve indicated that the NOAEL values often differ markedly from derived BMDs (Allen et al., 1994; Gaylor et al., 1998). For example, the assignment of a NOAEL relies critically on data from only one dose, whereas BMD analysis takes all of the dose-response relationship into account, including slope, in determining the BMD. Moreover, in contrast to BMD analysis, NOAEL values are highly dependent on sample size and tend to be larger in studies with a smaller sample size (U.S. EPA, 1995). In an effort to overcome the shortcomings of NOAEL analysis, numerous investigators have used BMD analysis to analyze dose-response toxicity data for diverse effects including neurotoxicity, developmental toxicity, and effects on the endocrine system (Mantovani et al., 1998; Rabovsky et al., 2001; Zhou et al., 2001). In this study, benchmark doses determined for each curve indicated that the NOAEL and BMD analyses showed similar estimates of the increase in AFB$_1$ toxicity in response to LPS co-treatment. Thus, as measures of toxicity threshold, both the NOAEL and BMD analyses showed similar estimates of the increase in AFB$_1$ toxicity in response to LPS co-treatment.

Inflammatory events initiated by LPS are responsible for its ability to augment AFB$_1$ toxicity. Elevations in plasma tumor necrosis factor-α (TNF-α) are seen before the onset of AFB$_1$/LPS-induced liver injury, and neutralization of TNF-α protects against the augmentation of both parenchymal cell injury and bile duct injury (Barton et al., 2001). Similarly, neutrophils (PMNs) accumulate early in the livers of AFB$_1$/LPS-treated rats, and PMN depletion prior to AFB$_1$/LPS treatment causes a significant reduction in hepatocellular injury. By contrast, PMN depletion does not alter bile duct epithelial cell (BDEC) injury in this model (Barton et al., 2000b). This result suggests that two different mechanisms are operative, one for hepatocellular injury that depends on PMNs and another for bile-duct damage that is independent of PMNs. In the present dose-response analysis, LPS produced a greater leftward shift in biliary injury markers than in markers of hepatocellular damage. This result is consistent with different mechanisms underlying injury to parenchymal cells vs. BDECs. Another possibility is that the same mechanism contributes to injury in both cell types but that parenchymal cells are less easily damaged than BDECs and require an additional, PMN-dependent insult for the expression of overt injury.

AFB$_1$ hepatotoxicity requires metabolic activation of AFB$_1$ to its toxic 8,9-epoxide (Eaton et al., 1994). Formation of the AFB$_1$ 8,9-epoxide is catalyzed by cytochrome (CYP) P450 family members including CYP1A2 and CYP3A4 (Eaton et al., 1994). One possible explanation for the enhancing effects of LPS on AFB$_1$ toxicity is that LPS might increase production of reactive 8,9-epoxide. However, LPS treatment has been shown to cause a decrease in hepatic CYP450 levels (Liu et al., 2000). Additionally, inflammatory cytokines, including TNF-α and IL-1, decrease expression of CYP450 isoforms responsible for AFB$_1$ metabolism, both in vitro and in vivo (Muntane-Relat et al., 1995; Pous et al., 1990). Accordingly, it seems unlikely that LPS enhanced hepatotoxicity in this model via increased production of reactive AFB$_1$ metabolites. Nevertheless, in some human studies, evidence suggests that chronic inflammation of the liver caused by disease states (e.g., hepatitis) causes upregulation of the CYP1A2 and CYP3A4 isoforms of CYP450 (Kirby et al., 1996).

One important route of AFB$_1$, 8,9-epoxide detoxification is via conjugation to glutathione (GSH) (Degen and Neumann, 1978; Eaton et al., 1994). It is conceivable that LPS might decrease liver GSH concentration, thereby decreasing capacity...
to detoxify the AFB$_1$ 8,9-epoxide, and resulting in enhanced hepatotoxicity. However, LPS given to rats at a hepatotoxic dose nearly 13-fold greater than that used in this study did not cause significant reduction in hepatic GSH concentration (Sneed et al., 1996). Therefore, it is unlikely that the small dose of LPS used in this study enhances AFB$_1$ toxicity via alteration of hepatic GSH levels. Nevertheless, to minimize variation in responses, we used rats that had been fasted for 24 h, a procedure shown to decrease liver GSH content (Maruyama et al., 1968). The possibility of a synergistic action of LPS and fasting on hepatic GSH levels cannot be ruled out, but preliminary studies indicated that fasting decreased variability among animal responses, with little effect on the magnitude of hepatotoxicity produced after AFB$_1$/LPS cotreatment (data not shown).

People with hepatitis B who are exposed to AFB$_1$ are at greater risk for hepatocellular carcinoma (Qian et al., 1994). Localized expression of hepatitis viral proteins in perportal regions of the liver strongly correlates with perportal inflammation, and Jin et al. (2001) have suggested that inflammation induced by these proteins during hepatitis may contribute to multistage carcinogenesis by increasing rates of cellular damage and proliferation. Indeed, partial hepatectomy or pretreatment with carbon tetrachloride in AFB$_1$-treated rats increases the quantity and size of placential glutathione S-transferase (GST-P)-positive, preneoplastic foci (Hiruma et al., 1996). Additionally, in AFB$_1$-treated rats, the incidence of GST-P-positive preneoplastic lesions in livers strongly correlates with periportal inflammation and Jin et al. (2001) have suggested that inflammation induced by these proteins during hepatitis may contribute to multistage carcinogenesis by increasing rates of cellular damage and proliferation. Indeed, partial hepatectomy or pretreatment with carbon tetrachloride in AFB$_1$-treated rats increases the quantity and size of placential glutathione S-transferase (GST-P)-positive, preneoplastic foci (Hiruma et al., 1996). Additionally, in AFB$_1$-treated rats, the incidence of GST-P-positive preneoplastic lesions in livers strongly correlates with increases in biomarkers of acute AFB$_1$ hepatotoxicity (Max et al., 1997). Therefore, it is unlikely that the small dose of LPS used in this study enhances AFB$_1$ toxicity via alteration of hepatic GSH levels. Nevertheless, to minimize variation in responses, we used rats that had been fasted for 24 h, a procedure shown to decrease liver GSH content (Maruyama et al., 1968). The possibility of a synergistic action of LPS and fasting on hepatic GSH levels cannot be ruled out, but preliminary studies indicated that fasting decreased variability among animal responses, with little effect on the magnitude of hepatotoxicity produced after AFB$_1$/LPS cotreatment (data not shown).

In conclusion, modest inflammation induced by LPS exposure causes a pronounced leftward shift in dose-response curves for AFB$_1$-induced liver injury. Considering the commonplace nature of exposure of humans to LPS and other inflammagens, concurrent inflammation should be considered as a potentially important risk factor for hepatotoxic effects of AFB$_1$ and other xenobiotics agents. Concurrent inflammation is only one of many determinants of individual sensitivity to chemical toxicity. Others include age, gender, metabolic polymorphisms, diet, and coexposure to other xenobiotics. The present observation that a single one of these has the potential to increase sensitivity by 10–20-fold is remarkable, especially when one considers the potential for additivity or synergy among such factors. Inflammation should be considered along with other determinants of sensitivity in the setting of safety factors or estimations of risk in risk assessment paradigms.

ACKNOWLEDGMENT

This research was supported by NIH grant ES04139.

REFERENCES


INFLAMMATION AND AFB1, DOSE RESPONSE


