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Tumor Necrosis Factor-α-Null Mice Are Not Resistant to Cadmium Chloride-Induced Hepatotoxicity[1]

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Acute administration of cadmium results in hepatotoxicity. Recent reports indicate that Kupffer cells, the resident macrophages of the liver, participate in the manifestation of chemical-induced hepatotoxicity. Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that is a major product of Kupffer cells and mediates the hepatotoxic effects of lipopolysaccharide (LPS). It has been speculated that cadmium also may exert its hepatotoxicity via the production of TNF-α by the Kupffer cells. Therefore, this study was undertaken to determine whether mice deficient in TNF-α are resistant to Cd-induced hepatotoxicity. TNF-α-null (TNF-KO) and wild-type (WT) mice were dosed ip with saline, LPS (0.1 mg/kg)/Gln (D-galactosamine, 700 mg/kg), or $CdCl_2$ (2.2, 2.8, 3.4, and 3.9 mg Cd/kg). Serum alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) activities were quantified to assess liver injury. Caspase-3 activity was quantified to assess hepatocellular apoptosis. LPS/Gln treatment increased ALT (17-fold) and SDH (21-fold) in WT mice. In contrast, LPS/Gln-treatment did not significantly increase ALT or SDH in TNF-KO mice. LPS/Gln-treatment caused a 7.8-fold increase in caspase-3 activity in WT mice but did not increase caspase-3 in TNF-KO mice. Cadmium caused a dose-dependent increase in liver injury in both WT and TNF-KO mice. However, the liver injury produced by Cd in the TNF-KO mice was not different from that in WT at any dose. No significant increase in caspase-3 activity was detected in any of the Cd-treated mice. These data indicate that, in contrast to LPS/Gln-induced hepatotoxicity, TNF-α does not appear to mediate Cd-induced hepatotoxicity.

Key Words: cadmium; tumor necrosis factor; TNF; liver; hepatotoxicity; necrosis; apoptosis.

The heavy metal cadmium (Cd) is an industrial and environmental pollutant. It is toxic to several tissues, most notably causing hepatotoxicity with acute exposure and nephrotoxicity with chronic exposure. Histological evaluation of liver injury reveals that acute Cd exposure causes swelling, congestion, pyknosis, karyorrhexis, apoptosis, and necrosis in the liver (Dudley *et al.*, 1982). However, few reports have definitively addressed the mechanism of toxicity at the molecular level. Cadmium has been shown to cause inhibition of electron transport (Diamond and Kench, 1974), DNA damage (Bagchi *et al.*, 1996), and lipid peroxidation (Stacey *et al.*, 1980; Harvey and Klaassen, 1983). These data describe some of the toxic effects of acute Cd, yet do not identify the mechanism of toxicity.

Several current reports implicate endogenous mediators in the pathogenesis of chemical-induced hepatic injury. The prevailing theory is that Kupffer cells, the resident macrophages of the liver, produce proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), and that this endogenous mediator contributes to chemical-induced hepatotoxicity. Normally, TNF-α participates in the regulation of inflammation and immunity to pathogens. However, unusually high amounts of TNF-α can result in host cell damage through its proinflammatory or cytotoxic properties. TNF-α can be directly cytotoxic to hepatocytes by inducing apoptosis and necrosis (Adamson and Billings, 1992; Tartaglia *et al.*, 1993). In addition, TNF-α initiates signal transduction pathways responsible for the production of cell adhesion molecules and chemotactic factors affecting hepatocytes and neutrophils (Witthaut *et al.*, 1994; Essani *et al.*, 1995). As a result, neutrophils adhere to damaged cells and release a host of cytotoxic intermediates, such as cytokines, proteases, and reactive oxygen species (Jaeschke *et al.*, 1996).

It is widely accepted that TNF-α is the endogenous mediator of toxicity for bacterial lipopolysaccharide (LPS) (Taniguchi *et al.*, 1997; Bopst *et al.*, 1998). The development of mice deficient in TNF-α (TNF-KO) has provided an animal model that can most accurately define specific functions of TNF-α (Marino *et al.*, 1997). Recent studies have shown TNF-KO mice to be resistant to LPS as well as LPS/D-galactosamine (LPS/Gln)-induced hepatotoxicity (Marino *et al.*, 1997; Taniguchi *et al.*, 1997; Bopst *et al.*, 1998).

TNF-α may also be involved in the manifestation of hepatotoxicity caused by nonbacterial hepatotoxins, such as carbon

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tetrachloride (Czaja *et al.*, 1989; Laskin, 1996) and acetaminophen (Blazka *et al.*, 1995). Experiments involving suppression of Kupffer cell function with gadolinium chloride ($GdCl_3$) have shown that Kupffer cell activation appears to be involved in producing the hepatotoxicity caused by these chemicals (Edwards *et al.*, 1993; Laskin *et al.*, 1995; Michael *et al.*, 1999). TNF-KO mice are less sensitive to the hepatotoxic effects of carbon tetrachloride, demonstrating a role for TNF-α in the mechanism of chemical-induced liver injury (Morio *et al.*, 2001). In contrast, TNF-KO mice are not less sensitive to the hepatotoxic effects of acetaminophen (Boess *et al.*, 1998). These studies demonstrate that TNF-α can be involved in the mechanism of some chemical-induced as well as bacterial-induced hepatotoxicity.

Limited data indicate that TNF-α is also involved in Cd-induced hepatotoxicity. Suppression of Kupffer cells with $GdCl_3$ has been shown to decrease Cd-induced hepatotoxicity (Sauer *et al.*, 1997; Yamano *et al.*, 1998b). This observed decrease in sensitivity to Cd may be related to decreased TNF-α levels; however, $GdCl_3$ may have other protective actions in the liver. For example, $GdCl_3$ has been shown to suppress superoxide production, interfere with inducible nitric oxide synthase (iNOS) expression, and weakly induce metallothionein (Iimuro *et al.*, 1994; Roland *et al.*, 1996). A more specific method of elucidating the role of TNF-α in Cd-induced hepatotoxicity was to treat mice with anti-TNF-α antibodies prior to Cd administration. However, pretreatment with anti-TNF-α antibodies resulted in only slight protection against Cd-induced hepatotoxicity (Kayama *et al.*, 1995). Most recently, Yamano *et al.* (2000) reported that hepatic TNF-α protein concentrations were not increased after Cd administration. This evidence cannot be interpreted as conclusive proof that TNF-α is involved in Cd-induced hepatotoxicity. Consequently, the current study was designed to specifically examine the role of TNF-α in Cd-induced hepatotoxicity utilizing TNF-KO mice, which is the most conclusive model currently available. This study therefore tested the hypothesis that TNF-KO mice are resistant to the hepatotoxic effects of Cd.

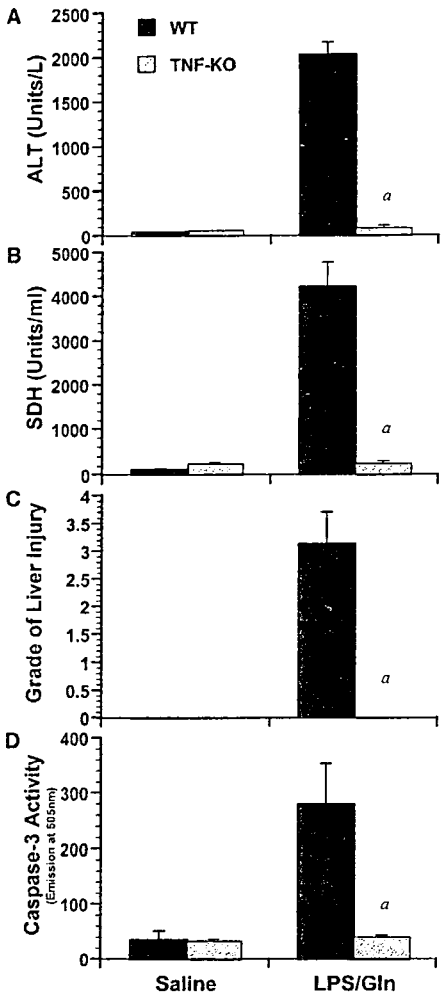

FIG. 1. Hepatotoxicity following LPS/Gln administration in WT and TNF-KO mice. WT (solid bars) and TNF-KO mice (hatched bars) were administered LPS/Gln (100 μg LPS and 700 mg D-galactosamine ip). Blood and livers were collected 9 h later. Hepatotoxicity was assessed by measuring serum indices of hepatotoxicity (A) ALT and (B) SDH. (C) Liver sections were examined histologically and scored semiquantitatively for liver injury. (D) Apoptosis was assayed by quantifying caspase-3 activity. Data are presented as means ± SE (n = 12). *TNF-KO mice exhibited significantly less toxicity than WT mice ($p < 0.05$).

MATERIALS AND METHODS

Chemicals. Cadmium chloride was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Lipopolysaccharide from *Escherichia coli* 0127:B8 (TCA extract), D-galactosamine, and serum enzyme activity kits were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade.

Animals. Wild-type (WT) and homozygous TNF-α-null (TNF-KO) mice (background C57BL/6 × 129) were used throughout the study. Both strains of mice were a generous gift from Dr. Marino (Marino *et al.*, 1997) at Memorial Sloan-Kettering Cancer Center. Mice were housed in an AAALAC certified facility at 70 ± 2°F with a 12-h light/dark cycle and were fed laboratory mouse chow (Purina, St. Louis, MO) and water *ad libitum*. Male mice (8 weeks old, approximately 25–30 g) were dosed ip with saline or a combination of 100 μg/kg lipopolysaccharide and 700 mg/kg LPS/Gln and hepatotoxicity was assessed 9 h later (n = 12). For the cadmium dose–response experiments, hepatotoxicity was assessed at two different time points, 9 h after doses of 2.2, 2.8, 3.4, or 3.9 mg Cd/kg ip (n = 12) and 16 h after doses of 2.0, 2.4, 2.8, 3.2, 3.6, or 4.0 mg Cd/kg ip (n = 8). Mice were decapitated and blood was collected and processed for serum enzyme activity analysis. Livers were removed and a portion of the left lobe of the each liver was placed in 10%

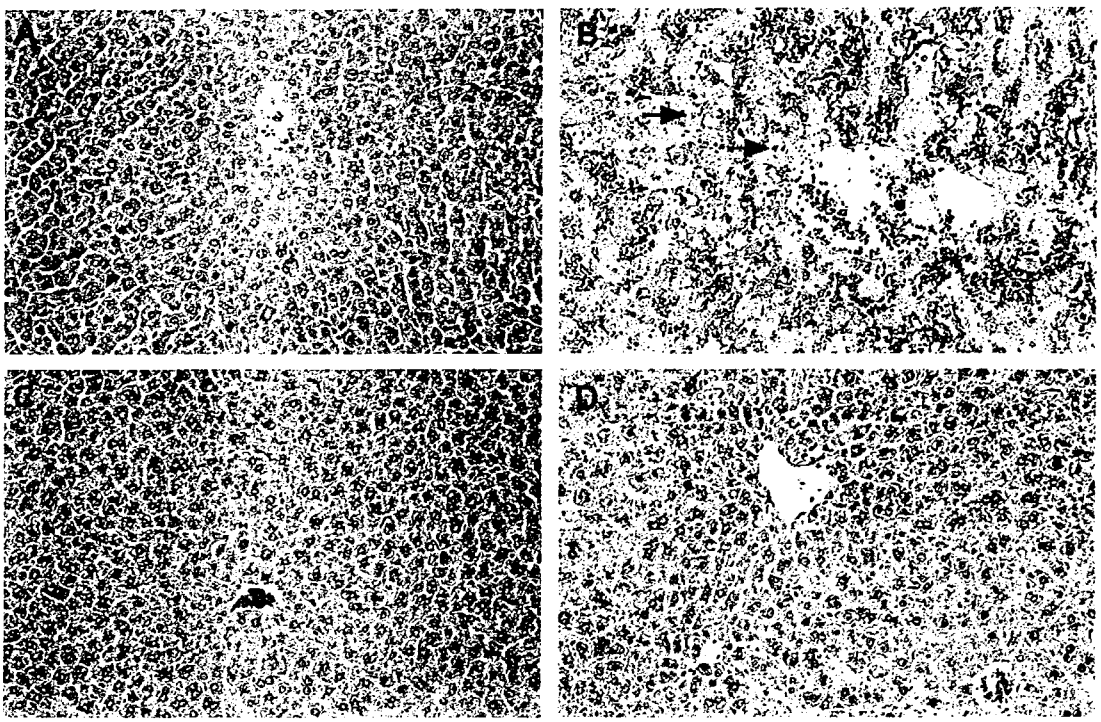

FIG. 2. Photomicrographs of liver sections after administration of saline or LPS/Gln in WT and TNF-KO mice. Wild-type and TNF-KO mice were administered saline or LPS/Gln (100 μg LPS and 700 mg D-galactosamine ip). Livers were collected 9 h later, processed routinely, and stained with hematoxylin and eosin. Photomicrographs (200×) are representative of mouse liver from wild-type (A and B) and TNF-KO (C and D) mice 9 h after administration of saline (A and C) or LPS/Gln (B and D). Note that apoptotic bodies (arrows) are present after LPS/Gln administration.

neutral buffered formalin. The remainder of the liver was stored at −80°C until assayed for caspase-3 activity.

Serum enzyme activity assays. Biochemical evaluation of liver injury was performed by quantifying serum activities of alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) spectrophotometrically, using Sigma test kits (Sigma Chemical Co.) according to the manufacturer's instructions.

Histopathology. Samples were taken consistently as cross-sections of the largest lobe of the liver. Liver sections, approximately 5 μm thick, were processed, stained with hematoxylin and eosin, and analyzed by light microscopy for liver injury. Grade of liver injury was analyzed semiquantitatively with six scores of severity: 0, none; 1, minimal (>2 foci of single cell necrosis per section); 2, mild (at least 5 areas of focal necrosis per section); 3, moderate (at least five foci of zonal necrosis per section); 4, severe (lobular damage, with many viable lobules per section); and 5, global (severe lobular damage, few areas of viability per section).

Caspase-3 activity assay. Liver samples were homogenized (1:5) w/v in lysis buffer (Tris-buffered saline with 1% Tween-20) using a Teflon pestle and a mortar. Homogenates were then centrifuged at 12,000*g* for 10 min. Supernatants were retained and stored at −80°C until assayed for caspase-3 activity. This assay was modified from Gillardon *et al.* (1997) by Harstad *et al.* (1999). Final assay mixture contained 150 μg protein, 5 mM DTT, 15 mM *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (P-409; Biomol, Plymouth Meeting, PA), 1 μl of the protease inhibitor mixture (P-8340; Sigma Chemical Co.), 25 mM Hepes, and 2 mM $MgCl_2$ in a total volume of 100 μl per reaction. After 1 h incubation at 37°C, fluorescence of the liberated fluorophore was quantified at 505 nm using a 96-well luminescence spectrometer (LS 50B; Perkin—Elmer, Norwalk, CT).

Statistics. Data from LPS/Gln-treated groups were analyzed using Student's *t* test. Data from Cd-treated groups were analyzed by two-way ANOVA.

RESULTS

LPS/Gln-induced hepatotoxicity in WT and TNF-KO mice. LPS/Gln-treatment produced a marked increase in serum ALT activity (43-fold) and SDH activity (33-fold) in WT mice, indicating extensive liver injury (Figs. 1A and 1B). Histological grade of liver injury was increased only in WT mice after LPS/Gln administration (Fig. 1C). LPS/Gln-treatment also increased caspase-3 activity in WT mice demonstrating hepatocyte apoptosis (Fig. 1D). Apoptosis and necrosis were confirmed by histolology and the samples were scored semiquantitatively for liver damage. The biochemical measures of liver injury were confirmed by histological examination (Fig. 2). LPS/Gln

treatment of WT mice caused extensive liver injury, consisting of sinusoidal congestion, apoptosis, and necrosis (Fig. 2B). Endothelial cells were indiscernible, while parenchymal cells demonstrated morphology characteristic of both necrosis and apoptosis. Hepatic sinusoids were congested with erythrocytes and leukocyte infiltration was prominent. This morphology was evenly distributed throughout the liver. LPS/Gln administration to TNF-KO mice caused no observable change in histology compared to saline-treated controls (Fig. 2D). These results demonstrate that LPS/Gln treatment causes both apoptosis and necrosis in WT mice. In contrast, administration of LPS/Gln to TNF-KO mice did not result in liver injury by any of the four indices measured. Saline treatment had no deleterious effect in either WT or TNF-KO mice.

Cd-induced hepatotoxicity in WT and TNF-KO mice. Cadmium caused a dose-dependent increase in serum ALT (Fig. 3A) and SDH (Fig. 3B) activities in WT mice (Fig. 3, solid squares), indicating liver damage. Administration of 2.2 mg Cd/kg in WT mice resulted in minor increases (2-fold) in serum ALT and SDH, but no injury was apparent histologically. Dosages of 2.8, 3.4, and 3.9 mg Cd/kg increased serum ALT (16-, 19-, and 26-fold, respectively) as well as SDH (9-, 17-, and 25-fold, respectively) compared to saline control. The dose-related increase in serum enzyme activity paralleled increasing grades of liver damage (Fig. 3C). Cadmium treatment did not increase caspase-3 activity in WT mice at any dose, indicating a minimal amount of apoptosis (Fig. 3D). Histologically, Cd-induced hepatotoxicity in both WT and TNF-KO mice consisted of necrosis with no apoptosis observed histologically (not shown).

Cadmium also produced significant liver injury in TNF-KO mice (Fig. 3; open circles) 9 h after exposure. As in WT mice, administration of 2.2 mg Cd/kg did not result in hepatotoxicity. Dosages of 2.8, 3.4, and 3.9 mg Cd/kg increased serum ALT (4-, 17-, and 18-fold, respectively) as well as SDH (17-, 11-, and 17-fold, respectively) compared to saline control TNF-KO mice. Increases in ALT and SDH in TNF-KO mice were not significantly different from WT mice at any dose of Cd. The grade of liver injury increased with dose of Cd and paralleled the increases in serum enzyme activities. Cadmium treatment did not increase caspase-3 activity in TNF-KO mice. Histologically, it was not possible to discriminate between WT and TNF-KO mice after Cd administration. These data indicated that Cd-induced hepatotoxicity in TNF-KO mice was not significantly different from WT mice at any dose.

Nine hours after Cd administration, WT mice could not be histologically or biochemically discriminated from TNF-KO mice. However, it was determined that more extensive hepatotoxicity may demonstrate differential toxicity in the two mouse types. Thus, a similar experiment was conducted and liver injury was assessed at a later time (16 h after Cd administration) to determine whether the lack of difference was due to a short exposure (Fig. 4). Cadmium produced more liver injury at 16 than at 9 h after Cd administration in WT mice. Serum ALT activity was increased (10-fold) at the dosage of 2.4 mg Cd/kg. Serum SDH activity was increased (9- and 7-fold) at dosages of 2.0 and 2.4 mg Cd/kg, respectively, compared to saline control in WT mice. Dosages of 2.8 and 3.2 mg Cd/kg in WT mice caused extensive liver damage, increasing serum ALT activity (36- and 29-fold, respectively) and SDH activity (19- and 16-fold, respectively). At dosages of 3.6 and 4.0 mg Cd/kg, the increases in serum ALT activity (36- and 29-fold, respectively) and SDH activity (20- and 16-fold, respectively) were different from saline control. The histological grade of liver injury increased in parallel to serum enzyme activities. Again, the dose-related increase in serum enzyme activity paralleled increasing grades of liver damage. Cadmium treatment did not cause an increase in caspase-3 activity at any dose, indicating that the absence of apoptosis at 9 h was not related to elapsed time.

TNF-KO mice were also sensitive to Cd-induced liver injury 16 h after Cd administration (Fig. 4). Dosages of 2.0 and 2.4 mg Cd/kg caused only minor injury, resulting in insignificant increases in ALT (2- and 2-fold, respectively) and SDH (2- and 2-fold, respectively) activities. Doses of 2.8 to 4.0 mg Cd/kg caused significantly more hepatotoxicity in TNF-KO mice. Serum ALT activity was increased 45- to 55-fold and serum SDH activity was increased 22- to 23-fold. As in WT mice, grade of liver injury in TNF-KO mice increased with dose and correlated with the increases in serum enzyme activities. Again, Cd treatment did not increase caspase-3 activity in TNF-KO mice. Histological examination confirmed the biochemical measures of liver damage in both WT and TNF-KO mice 16 h after Cd administration (Fig. 5). Dosages of 2.0 and 2.4 mg Cd/kg caused little apparent damage, consisting of only mild congestion and hydropic degeneration. Cadmium doses of 2.8 and 3.2 mg Cd/kg caused moderate liver damage. Cd-induced liver injury consisted primarily of focal necrosis as well as isolated single cell necrosis. Foci of necrosis were numerous but small, consisting of approximately 10–20 cells each. Congestion was more extensive and the presence of leukocytes was noted, although the leukocytes were primarily contained within the sinusoids. At dosages of 3.6 and 4.0 mg Cd/kg, the necrotic foci were larger and often spanned several lobules. Congestion was more severe and extended into the parenchyma as peliosis hepatis. Thus, after 16 h, Cd caused more significant liver damage in both WT and TNF-KO mice. However, the longer time point did not demonstrate that the liver injury produced by Cd in the TNF-KO mice was different from that in WT mice.

DISCUSSION

The aim of this study was to determine whether TNF-α is involved in cadmium-induced liver damage. Several indepen-

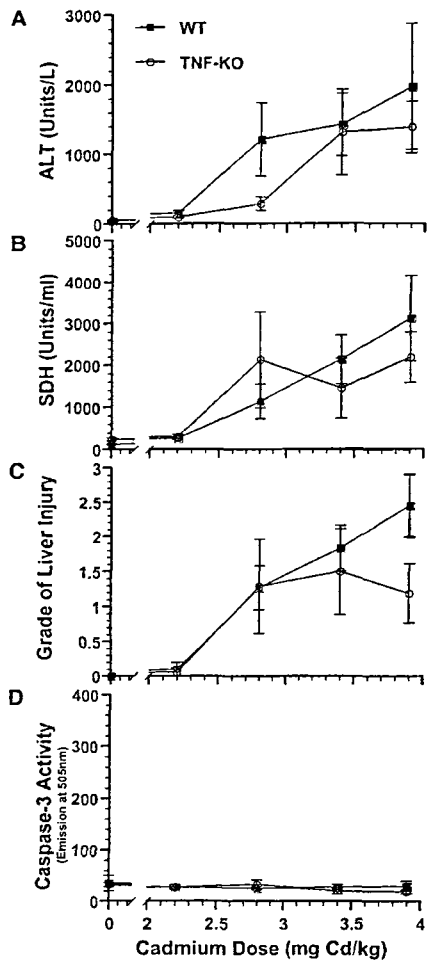

FIG. 3. Hepatotoxicity 9 h after administration of various dosages of Cd in WT and TNF-KO mice. WT (solid squares) and TNF-KO mice (open circles) were administered various doses of Cd (2.2, 2.8, 3.4, or 3.9 mg Cd/kg ip, n = 12). Blood and livers were collected 9 h later. Hepatotoxicity was assessed by measuring serum indices of hepatotoxicity (A) ALT and (B) SDH. (C) Liver sections were examined histologically and scored semiquantitatively for liver injury. (D) Apoptosis was assayed by quantifying caspase-3 activity. Data are presented as means ± SE (n = 12).

dent researchers have provided strong evidence for involvement of TNF-α in liver damage produced by bacterial endotoxin (Marino *et al.*, 1997; Taniguchi *et al.*, 1997; Bopst *et al.*, 1998) and some evidence that TNF-α plays a role in acetaminophen- (Blazka *et al.*, 1995, 1996) and carbon tetrachloride-induced hepatotoxicity (Czaja *et al.*, 1989; DeCicco *et al.*, 1998; Rikans *et al.*, 1999; Morio *et al.*, 2001). These studies have included models of Kupffer cell suppression, anti-TNF-α-antibody pretreatment, and TNF-KO mice. All of these data clearly support a role of TNF-α in hepatotoxicity produced by these chemicals. The role of TNF-α in Cd-induced hepatotoxicity is alluded to, but insufficiently defined.

TNF-α is widely accepted as the primary mediator of LPS/

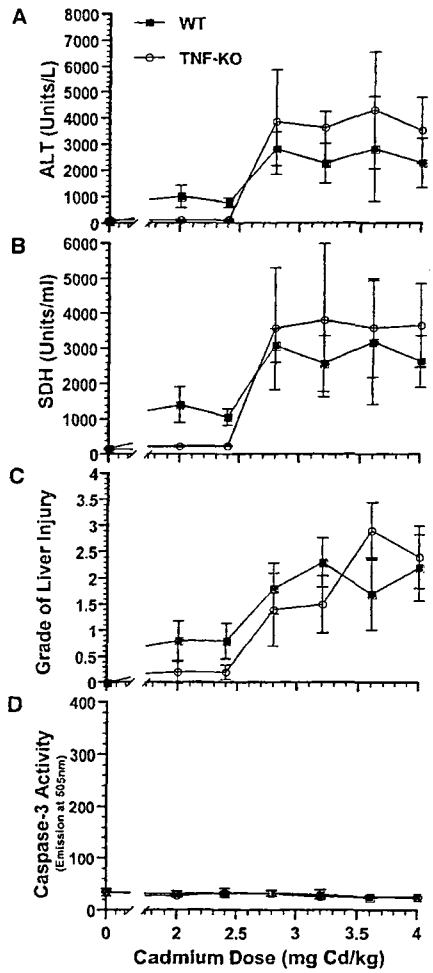

FIG. 4. Hepatotoxicity 16 h following administration of various dosages of cadmium in WT and TNF-KO mice. WT (solid squares) and TNF-KO mice (open circles) were administered various doses of Cd (2.0, 2.4, 2.8, 3.2, 3.6, or 4.0 mg Cd/kg ip, n = 8). Blood and livers were collected 16 h later. Hepatotoxicity was assessed by measuring serum indices of hepatotoxicity (A) ALT and (B) SDH. (C) Liver sections were examined histologically and scored semiquantitatively for liver injury. (D) Apoptosis was assayed by quantifying caspase-3 activity. Data are presented as means ± SE (n = 8).

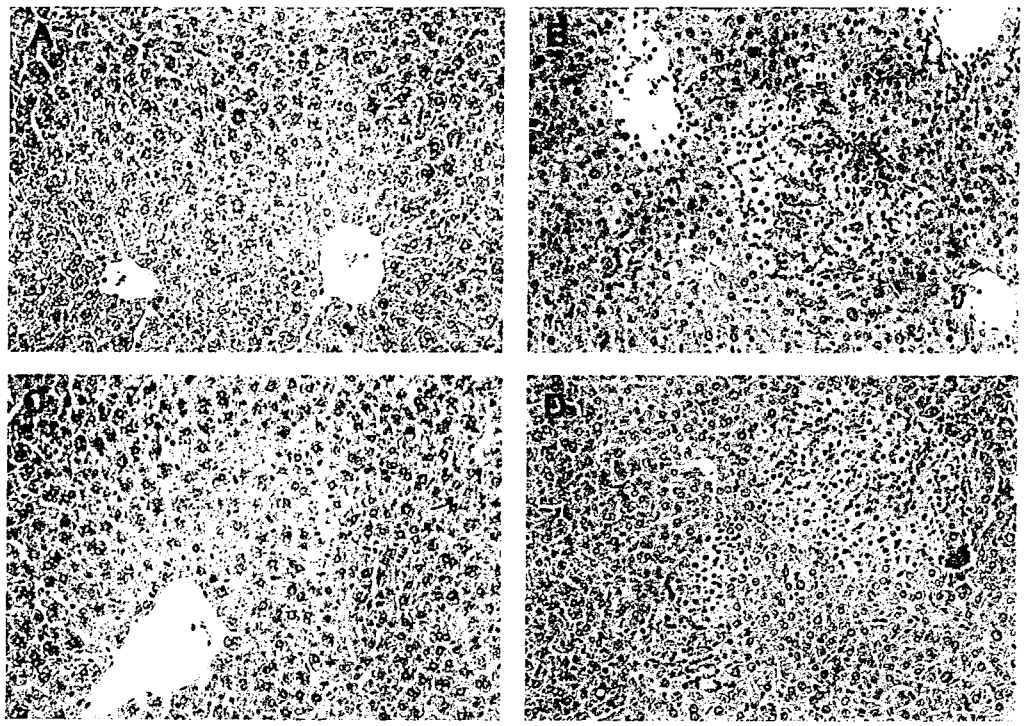

FIG. 5. Photomicrographs of liver sections after administration of saline or 4.0 mg Cd/kg in WT and TNF-KO mice. Wild-type and TNF-KO mice were administered saline or 4.0 mg Cd/kg. Livers were collected 16 h after Cd administration, processed routinely, and stained with hematoxylin and eosin. Photomicrographs (200×) are representative of mouse liver from wild-type (A and B) and TNF-KO (C and D) mice 16 h after administration of saline (A and C) or 4.0 mg Cd/kg (B and D).

Gln-induced hepatotoxicity in WT mice. Therefore, LPS/Gln was chosen as a positive control for these experiments. LPS/Gln administration caused extensive liver damage, including both necrosis and apoptosis, in all areas of the livers of WT mice. However, necrosis and apoptosis were absent in TNF-KO mice after LPS/Gln treatment. These data are consistent with published data (Taniguchi *et al.*, 1997) and clearly confirm that TNF-α is a required component in the production of LPS/Gln-induced liver damage. In addition, this experiment demonstrates the validity of the TNF-KO mouse as an animal model that can define a role for TNF-α in the manifestation of chemical-induced hepatotoxicity.

Several studies have attempted to address the role of TNF-α in Cd-induced hepatotoxicity. Suppression of Kupffer cells prior to Cd administration protects rats against Cd-induced liver injury (Sauer *et al.*, 1997; Yamano *et al.*, 1998a), most likely in the absence of MT induction. While these data do not directly point to TNF-α, it strongly supports a role for Kupffer cells and, thus, suggests proinflammatory cytokines as mediators. More specifically, pretreatment of rats with anti-TNF-α antibodies offers mild protection against Cd toxicity (Kayama *et al.*, 1995). Yamano *et al.* (2000) recently published a report that contrasted these data in demonstrating no induction of TNF-α upon Cd treatment. Rather, they showed that chemokines may be more important in the manifestation of Cd-induced hepatotoxicity.

In this study, we utilized the TNF-KO mouse to more conclusively determine whether TNF-α is involved in Cd-induced hepatotoxicity. Two separate time points were chosen to examine Cd-induced hepatic injury. Initially, the 9-h time point was chosen to correlate with earlier studies from this lab (Habeebu *et al.*, 1998). However, in this study, only moderate liver injury was observed 9 h after Cd treatment. This is most likely due to the relative resistance of the background strain (C57BL/6) for the TNF-KO mice to Cd-induced liver injury. Previous studies in this lab used a relatively more sensitive strain of mice (129Sv/Ola). Therefore, hepatotoxicity was also examined at a later time point (16 h) in order to cause more extensive toxicity and to possibly reveal whether there is a difference between WT and TNF-KO mice in susceptibility to

Cd-induced hepatotoxicity. Cadmium caused more hepatotoxicity after 16 than 9 h but still did not result in a significant shift in the dose response in TNF-KO in comparison to the WT mice. Therefore, it can be concluded from these data that TNF-α does not appear to be required for Cd-induced hepatotoxicity.

Previous experiments indicate that apoptosis may contribute to the hepatotoxicity of Cd (Habeebu *et al.*, 1998). In that study, apoptosis was observed maximally from 9 to 14 h in 129Sv/Ola mice. In the current study, Cd treatment did not cause an increase in apoptosis in either WT or TNF-KO mice (background C57BL/6) at any dose or time. These results may be interpreted as indicative of a strain difference in apoptosis in Cd-induced hepatotoxicity and require further investigation.

In summary, the present data demonstrate that, in contrast to LPS/Gln-induced hepatotoxicity, TNF-α does not appear to play a role in Cd-induced hepatotoxicity. These data do not exclude the involvement of other inducible proinflammatory cytokines and chemokines from the mechanism of cadmium-induced hepatotoxicity nor do they exclude a compensatory role of other proinflammatory cytokines in the TNF-KO mice after Cd administration. However, from these data, it can be concluded that, while TNF-α is clearly involved in LPS/Gln-induced hepatotoxicity, TNF-α does not appear to be important in Cd-induced hepatotoxicity.

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