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Single-Dose Tumor Necrosis Factor Protection against Endotoxin-Induced Shock and Tissue Injury in Rats

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Tumor necrosis factor (TNF), a macrophage product released in response to endotoxin and other stimuli, has been shown to be a central mediator of endotoxin or septic shock. However, its highly conserved and wide-ranging physiological effects suggest that it may also be an essential cytokine in the host defense against acute bacterial infection or sepsis. A single nontoxic dose of human recombinant TNF administered intravenously 24 h prior to a lethal infusion of *Escherichia coli* lipopolysaccharide (LPS) completely prevented acute LPS-induced hypotension, ameliorated tissue injury in the lungs and liver, and improved survival in male Fisher 344 rats. The protective effects of TNF were dose dependent and required a 24-h pretreatment interval. After the infusion of LPS, animals in both groups (TNF-treated animals and saline-pretreated controls) initially appeared acutely ill and had a similar severe metabolic acidosis, indicating that TNF did not inactivate or prevent the toxic effects of LPS. Twelve hours after the administration of TNF, the gene for manganous superoxide dismutase, a mitochondrial enzyme which scavenges toxic reactive oxygen species and is induced during conditions which generate a free radical stress, was expressed in liver tissue, suggesting that the induction of manganous superoxide dismutase may be an important in vivo protective mechanism against cellular injury during lethal endotoxemia.

In rats the administration of tumor necrosis factor (TNF) induces many of the pathophysiological changes observed during lethal endotoxin shock (15). Prior immunization against TNF improves survival following lethal endotoxemia in mice and also following *Escherichia coli* septic shock in baboons (4, 16). However, TNF is a highly conserved, pluripotent molecule, and exposure to TNF has also been shown to induce beneficial effects and will confer resistance to TNF cytotoxicity in vitro and TNF lethality in vivo (2, 13, 20). In addition, TNF pretreatment appears to ameliorate pulmonary oxygen toxicity (17), prolong the survival of tumor-bearing animals without affecting tumor growth (14), and prevent the lethal effects of gram-negative sepsis induced by cecal ligation and puncture (CLP) (12). The conditions under which exogenous TNF exerts primarily beneficial or deleterious effects are complex and may be dependent on the dose, route of administration, and a number of physiological conditions present in the experimental model being used. The current experiments were performed to characterize the protective effects of a single intravenous (i.v.) dose of TNF against lipopolysaccharide (LPS)-induced shock, tissue injury, and lethality in rats. In addition, we sought to determine whether a single dose of TNF which was protective against a lethal endotoxin challenge was associated with the induction of the gene for manganous superoxide dismutase (MnSOD), as this has been implicated as an important protective mechanism of TNF in vitro (20, 21).

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MATERIALS AND METHODS

Animals. Male Fisher 344 rats weighing 200 to 225 g (n = 380) were group housed in a controlled environment and fed rat chow and water ad libitum. All experiments were conducted in compliance with the Animal Care and Use Committee, National Institutes of Health.

Reagents. Human recombinant TNFα was generously supplied by the Cetus Corp., Emeryville, Calif., and had a specific activity of 1×10^6 to 2.5×10^6 U/mg as measured by the L929 bioassay and an endotoxin level of 30 to 50 pg/6.5×10^6 U as measured by the *Limulus* assay (19). TNF was reconstituted in sterile water and brought to final concentration in normal saline with 0.5% fatty acid-poor endotoxin-free bovine serum albumin (<6 EU/mg) purchased from Calbiochem, La Jolla, Calif. Control animals received an equal volume of protein carrier solution in normal saline (NS). *Escherichia coli* endotoxin (LPS, serotype O127:B8) was purchased from Sigma, St. Louis, Mo., and brought to final concentration in normal saline.

***Limulus* endotoxin assay.** Endotoxin levels were determined by using a standard *Limulus* test (Sigma). Briefly, endotoxin-free glassware was prepared by autoclaving at 121°C for 1 h followed by heating in an oven at 175°C for 3 h. Then 100 μl of previously obtained serum was coincubated with 100 μl of *Limulus* amebocyte lysate at 37.5°C for 1 h in a closed water bath. The presence of a hard gel, which was not disrupted on inversion, was taken as evidence of a positive test. Samples that tested positive were then serially diluted in endotoxin-free water and incubated as above. The endotoxin level was determined by multiplying the inverse of the highest dilution found positive by the lowest concentration of endotoxin standard found positive. The lower limits of endotoxin detection in serum were 40 to 60 pg/ml.

Survival studies. All survival experiments followed the

same general format and were performed in a blinded fashion. In the initial experiments, i.v. injections were made into the dorsal vein of the penis with either TNF at 10, 50, or 100 μg/kg or NS after animals had been anesthetized by intraperitoneal (i.p.) administration of pentobarbital at 20 mg/kg. LPS at 10, 15, or 20 mg/kg was administered 24 h later via the i.p. or i.v. route. In the subsequent experiments injections of TNF at 50 μg/kg or NS were staggered at 6, 12, or 24 h prior to i.p. administration of LPS (20 mg/kg). In all experiments survival was assessed at 24 h, at which time surviving animals appeared stable or recovering. Late deaths were exceedingly rare in this animal model.

Physiology studies. Animals were anesthetized as above and given either i.v. TNF at 50 μg/kg ($n = 6$) or i.v. NS ($n = 5$). Indwelling carotid artery and superior vena cava (SVC) catheters were then placed through a ventral cervical incision, tunneled subcutaneously, and brought out through a dorsal interscapular incision. Catheters were fashioned by melding PE-10 to PE-90 polyethylene tubing (Clay-Adams, Parsippany, N.Y.) and were protected by a metal coiled-spring tube anchored to the scapular masculature and connected to a swivel apparatus atop a metabolic cage. The arterial line was connected to a pressure transducer, and the SVC catheter was brought out through the top of the cage, flushed with 0.2 ml of heparinized saline, and capped. This allowed animals to recover from anesthesia and move about the metabolic cage freely. Administration of substances and measurement of parameters could be performed without disturbing the animal and without using anesthesia. After 24 h, LPS at 10 mg/kg was infused over 5 min into the SVC of the awake unrestrained rats. Heart rate, respirations, and mean arterial blood pressure were recorded at frequent intervals for 6 h. Prior to and then 1.5 and 4 h after LPS administration, 0.5 ml of arterial blood was withdrawn for determination of arterial blood gas and endotoxin levels in serum (only at 1.5 h) and replaced with the same volume of NS.

Histology studies. Rats were anesthetized as described above, and TNF at 50 μg/kg ($n = 6$) or an equal volume of NS ($n = 6$) was administered i.v. LPS at 20 mg/kg was given i.p. 24 h later. At 12 h after LPS administration, animals were sacrificed by cervical dislocation and tissue was harvested and preserved in 10% formalin, stained with hematoxylin and eosin, and read in a blinded fashion by a pathologist (P.J.K.) for assessment of injury. Sections of liver examined for hepatocellular necrosis were graded as having either no necrosis, scattered foci of single-cell necrosis, confluent necrosis of two to six hepatocytes, or zonal hepatocellular necrosis.

Northern blot hybridization. Fifteen animals were treated with TNF or NS as described above. At baseline (no TNF) and at 12 and 24 h later, the liver was harvested ($n = 2$ or 3 per group at each time point) and frozen in liquid nitrogen. LPS at 20 mg/kg was administered i.p. to the remaining animals, and liver tissue was harvested 4 and 8 h later. The tissue was pulverized with a precooled mortar and pestle and homogenized in guanidine isothiocyanate, and total cellular RNA was separated over a cesium chloride gradient as described previously (5). RNA was quantitated (DU-65 spectrophotometer; Beckman Instruments, Fullerton, Calif.), and 25 μg per lane was loaded into a 10% formaldehyde agarose gel, separated electrophoretically overnight, and transferred to a nylon filter. Sequential hybridization was performed with ^{32}P-labeled human cDNA probes for MnSOD and CuZnSOD, a noninducible cytosolic enzyme which is constitutively expressed (generously supplied by G. W. Wong and A. Singh, Genentech, Inc., South San Francisco, Calif.). Optical densitometry was performed on each autoradiogram to quantitate relative gene expression (Beckman DU-8 spectrophotometer).

TABLE 1. Dose-dependent effect of 1-day i.v. pretreatment with TNF against endotoxin-induced lethality in rats

Dose of endotoxin	Actual endotoxin dose (mg/kg)	Route of administration	% Survival (no. surviving) following TNF or NS administration:		
			NS	TNF (10 μg/kg)	TNF (50 μg/kg)
LD_{25}	10	i.p.	75 (46)	97 (32)[a]	ND[b]
LD_{60}	10	i.v.	40 (68)	75 (28)[a]	92 (13)[c]
LD_{75}	15	i.p.	25 (15)	ND	94 (18)[c]
LD_{95}	20	i.p.	5 (25)	20 (20)	80 (20)[c,d]

[a] $P < 0.01$ versus NS.
[b] ND, not done.
[c] $P < 0.004$ versus NS.
[d] $P < 0.004$ versus TNF at 10 μg/kg.

Statistics. Data are presented as mean ± standard error of the mean. Survival proportions were compared by using Fisher's exact test, and other parametric data were analyzed by using Student's t test with unknown but assumed equal variance. Physiology data were analyzed by analysis of variance.

RESULTS AND DISCUSSION

The protective effects of various doses of TNF administered 24 h prior to increasingly lethal doses of LPS are summarized in Table 1. Administration of TNF at 10 μg/kg significantly improved survival against 25 and 60% lethal doses (LD_{25} and LD_{60}) of LPS ($P < 0.01$). However, the protective effects were overcome when an LD_{95} of LPS was used, since there was no difference in survival compared with NS-treated controls. After i.v. pretreatment with TNF at 50 μg/kg, there was a significant improvement in survival over that of NS-treated animals at all doses of LPS administered 24 h later ($P < 0.004$), and after an LD_{95} of LPS, survival was significantly better than after pretreatment with TNF at 10 μg/kg ($P < 0.004$). The protective effects of i.v. TNF at 100 μg/kg, administered 24 h prior to an LD_{95} of LPS, were comparable to the protective effects of TNF at 50 μg/kg (data not shown). TNF doses of 200 μg/kg or higher are toxic and occasionally lethal in this animal model, and therefore 50 μg/kg appeared to be the most efficacious dose. In the subsequent experiments TNF was not protective when administered 6 or 12 h prior to LPS (data not shown). This lack of protection of TNF at 6 or 12 h prior to LPS was unexpected because Galanos et al. have seen protection in mice pretreated with TNF at only 4 h prior to lethal challenge. However, they challenged the mice with a combination of LPS and D-galactosamine instead of LPS alone (8). The differences in the challenge agent may account for differences in the time to onset of protection.

One day after the insertion of indwelling carotid artery and SVC catheters, animals appeared to have recovered from the procedure as evidenced by an alert state, spontaneous activity, and grooming behavior. Initial vital signs and arterial blood gas determinations were not different in TNF- and NS-pretreated animals. After a 5-min infusion of LPS at 10 mg/kg into the SVC, all animals in these studies (and in the initial survival experiments), whether pretreated with TNF or NS, initially appeared very ill as manifested by piloerec-

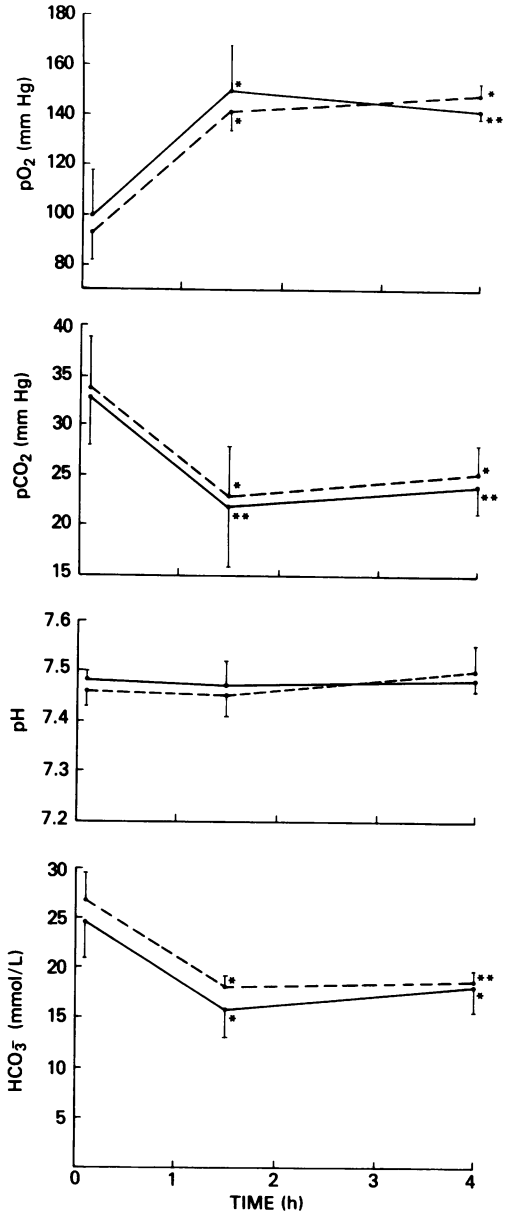


FIG. 1. Effect of LPS on pO_2, pCO_2, pH, and arterial HCO_3^- after TNF (dashed lines) and NS (solid lines) pretreatment. Animals were prepared as described in the text. Then 0.5 ml of arterial blood was withdrawn at baseline and at 1.5 and 4 h after LPS administration; it was replaced with the same volume of NS. Prior to LPS administration there was no difference in pO_2, pCO_2, pH, or HCO_3^- in TNF- or NS-pretreated animals, and the pH did not change within each group or between groups after LPS administration. At 1.5 h after LPS administration there was a marked metabolic acidosis in both groups as evidenced by a similar significant decrease in HCO_3^- compared with baseline levels (*, $P < 0.01$; **, $P < 0.05$), which was compensated by alveolar hyperventilation with a decrease in pCO_2 and increase in pO_2. The severe metabolic acidosis was still present in both groups at 4 h after LPS.

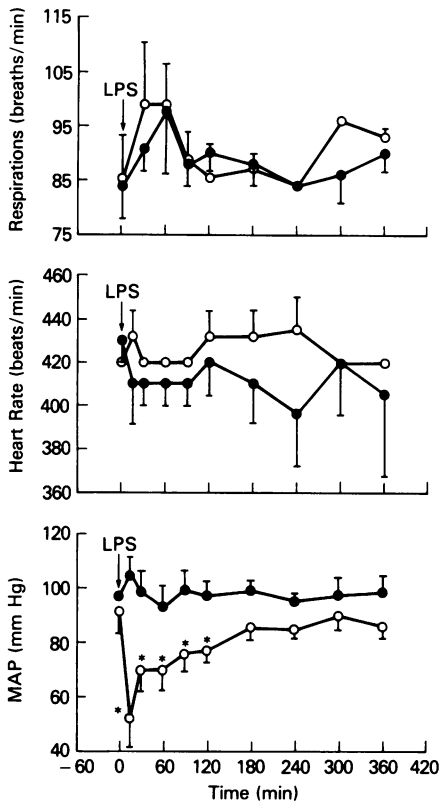
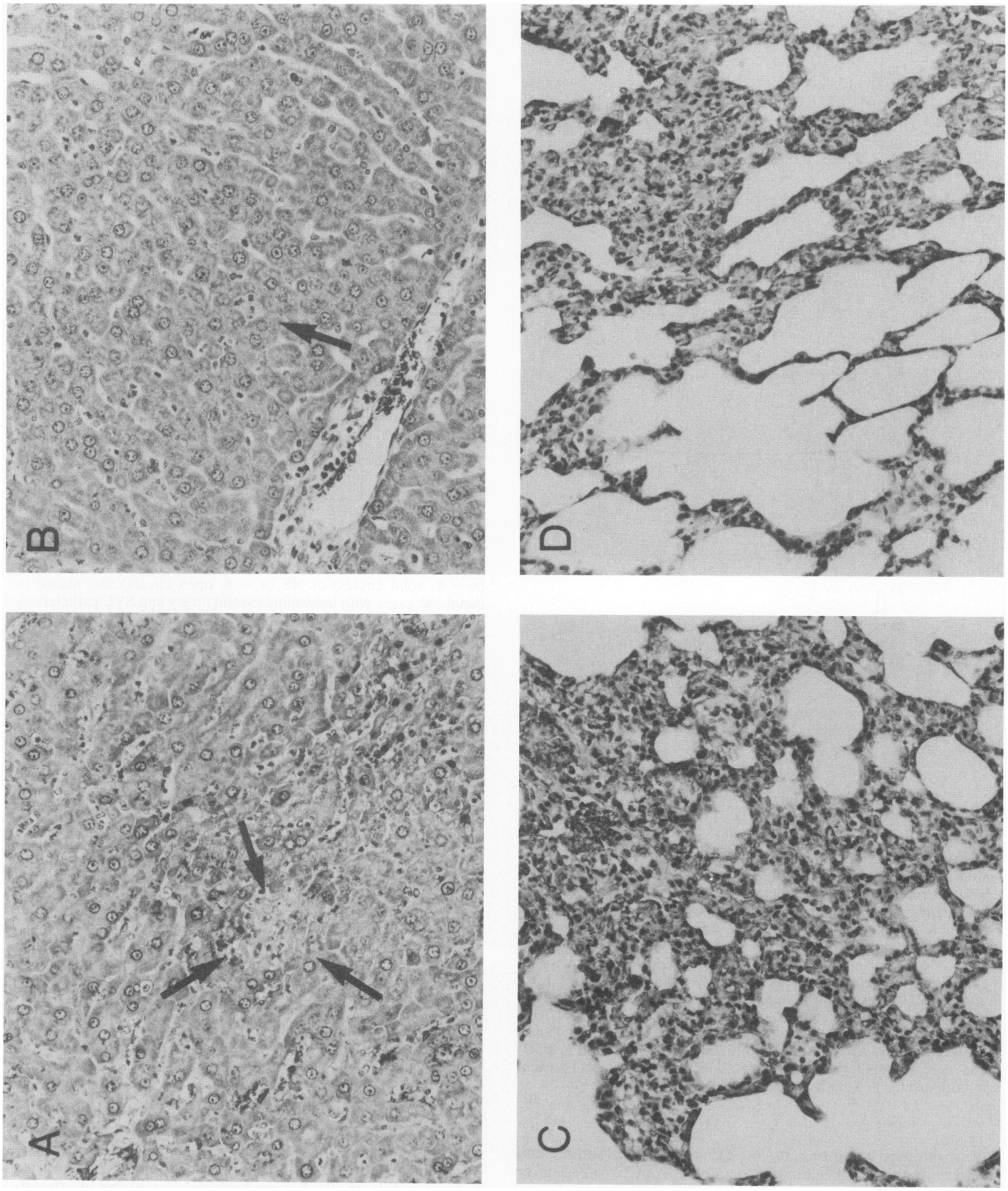


FIG. 2. Effect of LPS on respiratory rate, heart rate, and MAP after TNF and after NS pretreatment. Rats were treated with TNF ($n = 6$, solid circles) or NS ($n = 5$, open circles) and placed in metabolic cages with indwelling carotid artery and SVC catheters as described in the text. After 24 h, LPS at 10 mg/kg was infused into the SVC over 5 min. Respiratory rate, heart rate, and MAP were recorded at frequent intervals for 6 h. Prior to LPS administration there was no difference in respiratory rate, heart rate, or MAP between groups. After LPS administration there was no significant difference within each group or between groups in respiratory rate or heart rate (top and middle panels). However, in NS-pretreated rats, LPS caused an immediate decrease in MAP compared with baseline levels, which remained significantly lower than in TNF-pretreated animals for 3 h ($P < 0.01$). MAP did not change after LPS administration in TNF-pretreated rats.

tion, huddled posture, crusted eyes, hyperventilation, and severe diarrhea. LPS lethality occurred from 12 to 24 h after LPS administration, at which time surviving animals appeared stable or recovering. At 1.5 and 4 h after LPS administration there was a marked metabolic acidosis in both groups of animals, manifested by a significant and similar decrease in the level of arterial plasma bicarbonate compared with baseline levels ($P < 0.05$; Fig. 1). This was compensated by alveolar hyperventilation in both groups, with a significant decrease in pCO_2 and increase in pO_2 compared with baseline levels ($P < 0.05$). On the basis of appearance and the comparable severe metabolic stress as reflected by a marked acidemia in both groups of animals, TNF pretreatment did not appear to exert its protective effects by inactivating LPS or preventing its toxic actions. At 1.5 h, circulating levels of endotoxin in serum were the same in TNF- and NS-pretreated animals (TNF, 1,433 ± 1,250 U/ml; NS, 1,679 ± 1,154 U/ml).

Infusion of LPS has been shown to initiate a number of cardiovascular and metabolic derangements mediated by neural, humoral, and vasoactive substances, resulting in hypotension, decreased tissue perfusion, multiple organ failure, and death (9). Severe hypotension during life-threatening sepsis is a significant clinical problem associated with a very poor prognosis (10), and strategies directed towards


B
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A
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preserving blood pressure during septicemia may improve the outcome. In these experiments there were no significant differences in vital signs between groups prior to the administration of LPS, and after LPS administration there was no difference in heart rate or respiratory rate within or between groups of animals treated with TNF or NS (Fig. 2). However, LPS infusion in NS-pretreated animals produced a prompt and marked drop in mean arterial blood pressure, which recovered partly but remained significantly lower than in TNF-treated animals for almost 3 h ($P < 0.01$). The mean arterial blood pressure did not change after LPS infusion in animals pretreated with TNF.

Microscopically, confluent hepatocellular necrosis was present in all tissue samples examined from NS-pretreated rats. However, TNF pretreatment uniformly prevented this degree of LPS-induced tissue injury in the liver (Fig. 3A and B). Sections of lung showed interstitial pneumonitis in all NS-pretreated rats, which was present but partially ameliorated in three of five TNF-pretreated rats (Fig. 3A and B). No differences were observed microscopically in sections of gastrointestinal tract or kidney from either group of animals. Our laboratory has previously shown that repetitive sublethal doses of TNF given i.p. twice daily for 6 days will also protect rats from the tissue injury and lethality of an LPS challenge given 1 day later (7). The histological protection in the current experiments is not as dramatic as that seen with a longer TNF pretreatment interval. However, a single i.v. dose of TNF confers comparable protection against the lethal effects of LPS and is considerably less toxic. In addition, the time course and degree of tissue injury are variable after LPS administration, and this study has evaluated only a single time point in an evolving process.

In vitro, exposure to TNF induces the gene for MnSOD, a mitochondrially based metalloenzyme which is induced during periods of oxygen-mediated free radical stress, but not other antioxidant or mitochondrial enzymes such as catalase, glutathione peroxidase, or cytochrome *c* oxidase (21). MnSOD is ubiquitous in mammalian tissues and is thought to be an important cellular defense mechanism against toxic reactive superoxide radicals (3). In vitro, gene induction of MnSOD has been shown to be important for resistance to subsequent TNF cytotoxicity (2, 20). In the current experiments, a single nontoxic dose of TNF which protected against endotoxin lethality induced the gene for MnSOD (Fig. 4). Gene expression was transient, being maximal at 12 h after TNF administration; a greater than threefold increase in gene expression was produced than in NS-treated rats. In vitro and in vivo, the induction of MnSOD gene expression by TNF has been shown to correlate with increased MnSOD protein production and enzyme activity (18, 20). In rats, a single intratracheal dose of TNF will induce MnSOD gene expression and increase protein production and will result in a significant increase in enzyme activity (18). Cells exposed to TNF will also increase their enzyme activity in association with MnSOD gene expression (20). In this study we have shown that the i.v. administration of a protective dose of TNF induces hepatic MnSOD gene expression, which is likely to be associated with protein production and enzyme activity.

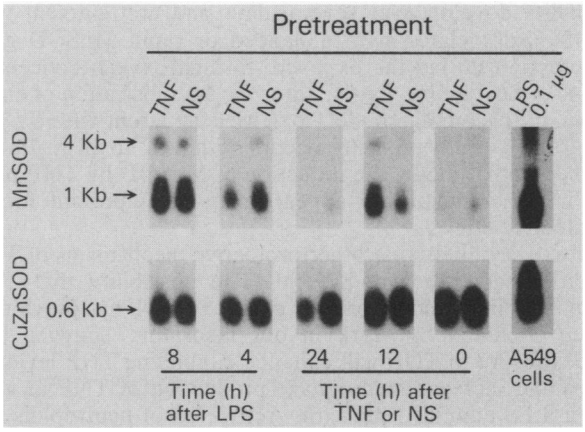

FIG. 4. Induction of the gene for MnSOD in liver after TNF or NS pretreatment. Animals were treated with TNF or NS as described in the text and sacrificed at time 0 (no TNF), 12, and 24 h. At 24 h, the remaining animals were given LPS at 20 mg/kg i.p.; they were sacrificed 4 and 8 h later ($n = 2$ to 3 per group per time point). Liver RNA was extracted. Then 25 μg of RNA was loaded into each lane and run on an agarose gel. The activity of the gene for MnSOD and CuZnSOD was determined by autoradiography with specific ^{32}P-labeled cDNA probes for human MnSOD and CuZnSOD. As expected, CuZnSOD was constitutively expressed in all animals at all time points and expression was not affected by TNF pretreatment or LPS. MnSOD was induced at 12 h after a single nontoxic dose of TNF at 50 μg/kg i.v. Four hours after LPS, the mRNA for MnSOD appeared lower in TNF-pretreated animals, suggesting that the LPS-induced free radical stress had been ameliorated by TNF pretreatment.

Because MnSOD gene expression is induced in eukaryotic cells under conditions which generate toxic reactive oxygen species, 4 h after LPS administration MnSOD gene expression was also increased in both groups. Interestingly, it was higher in NS- than TNF-pretreated animals. It may be that TNF induction of the gene for MnSOD resulted in enough increased enzyme activity to scavenge reactive oxygen species during the first hours of endotoxemia. Researchers in our laboratory are currently investigating the relationship of TNF pretreatment with MnSOD gene expression, enzyme activity, and survival in a murine model of endotoxin shock. The gene expression of CuZnSOD was constitutive, did not change after TNF or LPS treatment, and ensured equal loading of RNA in all lanes.

Researchers in our laboratory have previously shown that chronic exposure to sublethal doses of TNF (given twice daily i.p. for 6 days) will improve survival following lethal gram-negative sepsis induced by CLP (1). The chronic exposure to TNF results in a slight increase in gene expression of MnSOD prior to CLP but facilitates its expression soon after CLP. This observation is similar to the augmented induction of pulmonary MnSOD gene expression by intratracheal instillation of TNF in rats subsequently exposed to hyperoxia (18). In a hyperoxia or CLP model, toxicity and

FIG. 3. Effect of TNF pretreatment on amelioration of tissue injury 12 h after LPS administration. Animals were pretreated with TNF ($n = 5$) or NS ($n = 4$) and given LPS (20 mg/kg i.p.) 24 h later. At 12 h after LPS administration animals were sacrificed and tissue was preserved and evaluated for the degree of tissue injury, as described in the text. Areas of confluent hepatocellular necrosis were present in all NS-pretreated animals after LPS administration (arrows in panel A). However, liver tissue from TNF-pretreated animals showed very minimal changes, with only occasional single-cell necrosis (arrow in panel B). Interstitial pneumonitis was present in all NS-pretreated animals (C) and, to a lesser degree, in three of five TNF-pretreated animals (D), whereas two TNF-pretreated rats had microscopically normal lung parenchyma. (Hematoxylin and eosin; original magnification, ×250.)

lethality develop over several days and pretreatment with TNF is associated with enhanced or rapid MnSOD gene expression during the exposure to high oxygen concentrations or developing sepsis. After the administration of endotoxin in this animal model, toxicity is apparent within a few hours and death typically occurs within 12 to 18 h. In this rapidly lethal model the induction of MnSOD by TNF prior to LPS administration appears may be important for its protective effect.

It is very likely that other protective mechanisms of TNF pretreatment are also present. The possibility that TNF release is inhibited by prior exposure to TNF has previously been excluded by work in our laboratory showing that preexposure to TNF will enhance circulating TNF levels in vivo and increase macrophage production of TNF in vitro after LPS administration (6). Activation of neutrophils has been shown to initiate the shedding of TNF receptors from cells (11). Although the physiological importance of this phenomenon is not known, it is possible that TNF exposure reduces the subsequent responsiveness of cells to TNF or induces a large number of circulating receptors which act to clear the subsequent exuberant production of TNF during endotoxemia.

In summary, a single i.v. dose of TNF given 24 h prior to a lethal LPS challenge improved survival over NS-pretreated controls. LPS-induced toxic effects were observed early in both groups of animals, as manifested by their ill appearance and severe acidosis during endotoxemia. However, LPS-induced hypotension was completely prevented and tissue injury was substantially ameliorated by TNF pretreatment. In addition, TNF induced the gene for MnSOD, suggesting that this may be an important early in vivo mechanism of action against LPS-induced shock and tissue injury. TNF treatment may have important clinical application for patients at high risk for the development of life-threatening sepsis or in conditions which generate free radical stress such as hyperoxia or ischemia-reperfusion injury.

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