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LPS Pretreatment Protects from Hepatic Ischemia/Reperfusion[1]

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In vivo **administration of nonlethal doses of lipopolysaccharide (LPS) to rodents can result in protection from subsequent lethal doses of endotoxin or LPS. We have previously demonstrated that hepatic ischemia/reperfusion (I/R) results in a TNF-dependent lung and liver injury and we postulated that pretreatment with sublethal concentrations of LPS prior to hepatic I/R could be protective from this injury. To test this hypothesis, five groups of rats were studied. LPS-I/R received 25 μg of LPS iv 24 hr prior to I/R, VEH-I/R received an equivalent volume of vehicle iv 24 hr prior to I/R, LPS-LPS received 25 μg of LPS iv 24 hr prior to sham laparotomy at which time an additional 25 μg of LPS was given iv, VEH-LPS received an equivalent volume of vehicle 24 hr prior to sham laparotomy and 25 μg of LPS iv immediately prior to sham laparotomy, and SHAM consisted of sham-operated control animals. Peak plasma tumor necrosis factor-α (TNF) levels occurred between 30 and 150 min of reperfusion: LPS-I/R = 778 ± 150 pg/ml ($n = 5$), VEH-I/R = 145 ± 46 pg/ml ($n = 5$), LPS-LPS = 970 ± 716 pg/ml ($n = 4$), VEH-LPS = 15,949 ± 10,937 ($n = 5$), and SHAM = 3 ± 1 ($n = 5$). As previously demonstrated by other investigators, pretreatment with LPS decreases TNF release in response to a second dose of LPS; however, TNF release was increased following hepatic I/R in those animals pretreated with LPS (LPS-I/R vs VEH-I/R, $P = 0.014$). Pulmonary injury was assessed by total protein and cell counts in bronchoalveolar lavage (BAL) fluid. Total protein concentration in BAL fluid from LPS-I/R = 59.48 ± 14.87 μg/ml ($n = 11$) and from VEH-I/R = 239.41 ± 60.12 μg/ml ($n = 16$) ($P < 0.05$). BAL fluid protein contents from LPS-LPS, VEH-LPS, and SHAM were 63.49 ± 8.10 μg/ml ($n = 7$), 81.71 ± 10.14 μg/ml ($n = 7$), and 33.02 ± 12.22 μg/ml ($n = 8$), respectively, and were not significantly different from each other. BAL fluid cell counts paralleled protein levels in each group. These data suggest that pretreatment with LPS increases TNF release in response to I/R, but protects from the subsequent pulmonary injury. In addition, liver injury in this model was assessed by quantitation of serum AST. There was no evidence of a difference in the liver injury which followed hepatic ischemia/reperfusion in animals which were pretreated with LPS before hepatic I/R.** © 1994 Academic Press, Inc.

INTRODUCTION

Tumor necrosis factor-α (TNF)[2] has been shown to be intimately involved in the pathophysiologic alterations observed in several disease states. A major working hypothesis has been that different stimuli will induce production of TNF, and that the organ injury associated with a disease was actually due to the induced TNF and not the stimulus itself. This hypothesis has been most rigorously tested in septic shock, where (i) injection of live bacteria [1, 2] or lipopolysaccharide (LPS) [3] rapidly induces production of circulating TNF, (ii) injection of purified, recombinant TNF can reproduce many of the alterations observed in septic shock [4, 5], and (iii) neutralization of TNF with antibodies will prevent lethality [1, 2, 6]. However, this rigid hypothesis is changing in light of new evidence that the production of TNF is not necessarily harmful [7] and in fact may be required to orchestrate an effective immune response. Despite these more recent studies, multiple investigations which have inhibited TNF activity with specific antibodies have demonstrated that too much TNF can damage organs.

One interesting aspect of TNF regulation is the induction of tolerance. This phenomena is observed when a small dose of LPS induces TNF, yet a second much larger dose of LPS fails to induce detectable TNF [8, 9]. The failure to respond to the second challenge is not due to a global inhibition of cellular function, since a second non-LPS challenge will still induce TNF [10]. This failure to produce significant amounts of TNF following the second larger LPS challenge has been postulated to be responsible for the development of tolerance and the prevention of organ injury [8, 9].

We have recently described a model of partial hepatic

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[2] Abbreviations used: LPS, lipopolysaccharide; TNF, tumor necrosis factor-α; I/R, ischemia/reperfusion; BAL, bronchoalveolar lavage.

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ischemia/reperfusion (I/R) which induces TNF production and also results in pulmonary and hepatic injury [11, 12]. We sought to determine if animals would become tolerant to the effects of I/R if they were pretreated with LPS. Our data show that LPS pretreatment will result in more TNF production after hepatic I/R, yet less organ injury.

MATERIALS AND METHODS

Animal Model

A rat model of lobar, rather than total, hepatic ischemia was used in order to produce a severe ischemic insult without inducing mesenteric venous hypertension [13]. All experiments utilized 200- to 300-g adult male pathogen-free Sprague–Dawley rats (Charles River, Portage, MI). Anesthesia for laparotomy was induced with intramuscular ketamine hydrochloride (100 mg/kg) and an indwelling intravenous line was placed via the right external jugular vein for serial blood sampling and administration of intravenous fluids and medications. By positioning the catheter tip in the suprahepatic vena cava just above the dome of the liver, immediately posthepatic blood samples were obtained; correct catheter position was confirmed at laparotomy. Following intravenous heparinization with 100 units/100 g body wt, midline laparotomy was performed. Hepatic ischemia was initiated by application of an atraumatic microaneurysm (Heifitz) clip across the portal venous and hepatic arterial blood supply to the cephalad three lobes of the liver; the three caudal lobes retained an intact portal inflow and venous outflow, preventing intestinal venous congestion. Lobar ischemia was maintained for 90 min and the Heifitz clip was then removed at a second laparotomy. Intravenous lactated Ringer's solution in a dose of 0.75 ml was administered to replace operative fluid and blood losses. Sham-operated control animals were treated in an identical fashion with the omission of vascular occlusion.

Experimental Design

Five experimental groups were constructed. Animals in LPS-I/R received 25 μg of LPS (*Escherichia coli* type 0111:B4; Sigma, St. Louis, MO) in 0.5 cc of RPMI intravenously (iv) via penile vein injection 24 hr prior to induction of hepatic ischemia/reperfusion; anesthesia was induaed with 50 mg/kg of intramuscular ketamine for all penile vein injections in all groups. VEH-I/R rats received an equivalent volume of RPMI (0.5 cc) without LPS iv 24 hr prior to hepatic ischemia/reperfusion. LPS-LPS animals received 25 μg of LPS in 0.5 cc of RPMI iv 24 hr prior to sham laparotomy at which time an additional 25 μg of LPS in 0.5 cc of RPMI was given intravenously. Animals in the VEH-LPS group received 0.5 cc of RPMI iv 24 hr prior to sham laparotomy and 25 μg of LPS in 0.5 cc of RPMI iv immediately prior to sham laparotomy, and SHAM rats underwent sham laparotomy alone. Plasma specimens were analyzed for TNF at 30, 60, 90, 120, 150, 180, and 240 min following reperfusion or conclusion of sham laparotomy by cytolytic bioassay. Three hundred-microliter aliquots of blood were sampled from the indwelling suprahepatic catheter at each time point. Intravenous lactated Ringer's solution was administered as blood volume replacement in a ratio of 3 cc of Ringer's solution for each 1 cc of blood lost. Plasma was separated from the samples and stored at −70°C. Of note, separate groups of animals were used to obtain TNF levels and assess lung and liver injury in order to avoid problems with excessive blood loss due to blood sampling.

TNF Assay [14]

TNF activity in the plasma specimens was measured using a sensitive bioassay with the WEHI 164, subclone 13 cell line as previously described [14]. Briefly, plasma samples were serially diluted directly into 96-well flat-bottom cell culture trays and 100 μl of WEHI cells (5×10^4 cells) with 0.5 μg/ml actinomycin D (Calbiochem, LaJolla, CA) was added to each well. The plates were incubated for 20 hr at 37°C. Following the addition of 20 μl of 3-(4,5-dimethylthiiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) at a concentration of 5 mg/ml in phosphate-buffered saline to each well, the plates were again incubated at 37°C for 4 hr. Next, 150 μl of medium was removed and 100 μl of isopropanol with 0.04 *N* hydrochloric acid was added to each well to dissolve the purple formazan crystals and the plates were read on a Bio-Tek ELISA reader (Winooski, VT) at an absorbance of 550 nm. Standard curves utilizing human recombinant TNF were generated with each assay; a medium only well served as a negative control. The standard curve was used to calculate TNF values in the experimental samples. TNF values are expressed in pg/ml. The assay was repeated after the positive samples were neutralized with a polyclonal rabbit anti-murine TNF antiserum which cross-reacts with rat TNF [15]. A negative assay following this treatment confirmed that the cytotoxic activity wao due to TNF.

Assessment of Lung Injury

Lung injury in this model was assessed by measuring protein concentration and cell counts in bronchoalveolar lavage (BAL) fluid. Previous studies in our laboratory have demonstrated that the time of peak increase in pulmonary microvascular permeability occurs following 12 hr of reperfusion [12]. Animals in these experiments were therefore sacrificed following 12 hr of liver reperfusion and lung injury assessed by bronchoalveolar lavage. Immediately following sacrifice, the animal's lungs were excised *in toto* and bronchoalveolar lavage was performed via the transected trachea. Each set of lungs was lavaged five times with 5 cc of normal saline, resulting in a

total lavage volume of approximately 25 cc. This lavage fluid was then analyzed for protein content and cell counts. The lavage fluid was centrifuged and the cells were resuspended in RPMI and counted. The protein content of the fluid was determined using albumin as a standard and the Coomassie protein assay reagent (Pierce, Rockford, IL). Cells were counted with a Coulter counter (Hialeh, FL).

Measurement of Serum Liver Enzymes.

Blood samples for measurement of serum AST were obtained at sacrifice. AST was quantitated using standard clinical automated analysis. Again, previous studies from our laboratory have demonstrated several peaks in AST release following hepatic ischemia/reperfusion; statistically significant increases, compared to sham-operated control animals, occur following 3, 12, and 24 hr of hepatic reperfusion [12]. In addition, these previous studies demonstrated that serum AST is a sensitive indicator of liver injury in this model and elevations is serum AST occurred well in advance of any histopathologically demonstrable abnormalities within the liver parenchyma [12]. AST levels in these experiments were obtained following 12 hr of hepatic reperfusion.

Statistical Analysis.

TNF levels are expressed in pg/ml as the mean ± the standard error of the mean. Lung lavage protein levels are expressed in μg/ml as the mean ± the standard error of the mean. Cell counts are the mean number of cells × 10^5 ± the standard error of the mean. AST levels are expressed in international units (IU) and are expressed as the mean ± the standard error of the mean. Statistical analysis was performed using an unpaired Students' t test unless otherwise stated.

RESULTS

TNF Release Following Hepatic Ischemia/Reperfusion

Peak TNF levels occurred between 30 and 150 min of reperfusion in all animals. Very low or absent levels of TNF were seen in the sham-operated control animals (n = 5); the mean peak TNF level in this group was 3 ± 1 pg/ml. As previously demonstrated by other investigators [8, 9], pretreatment with nonlethal doses of LPS results in a decrease in TNF release following rechallenge with LPS. Administration of vehicle 24 hr prior to sham laparotomy and subsequent administration of 25 μg of LPS immediately prior to sham laparotomy (VEH-LPS, n = 5) resulted in a mean peak TNF level of 15,949 ± 10,937 pg/ml. Pretreatment with 25 μg of LPS 24 hr prior to rechallenge with the same dose of LPS immediately prior to sham laparotomy (LPS-LPS, n = 4) resulted in a lower mean peak level of TNF (970 ± 716 pg/ml) (Fig. 1). The mean peak TNF level following pretreatment with vehicle 24 hr prior to hepatic ischemia/reperfusion (VEH-I/R, n = 5) was 145 ± 46 pg/ml, which is consistent with our previous studies which demonstrated TNF release following hepatic ischemia/reperfusion [11, 12]. Pretreatment with 25 μg of LPS 24 hr prior to hepatic ischemia/reperfusion (LPS-I/R, n = 5) resulted in a significantly higher mean peak level of TNF (778 ± 150 pg/ml, P = 0.014; Fig. 2).

Lung Injury

Lung injury in this model was evaluated by measuring protein concentration and cell counts in bronchoalveolar lavage fluid. Previous experiments in our laboratory have determined that the time of maximal increase in pulmonary microvascular permeability following hepatic ischemia/reperfusion follows 12 hr of hepatic reperfusion. Therefore, animals in these experiments were sacrificed 12 hr following revascularization and lung lavage was performed. LPS treatment prior to hepatic ischemia/reperfusion incurred a significant ($P < 0.05$) protective effect against the pulmonary injury which occurs after liver revascularization. Mean protein concentration in LPS-I/R (n = 11) was 59.48 ± 14.87 μg/ml, compared to the protein concentration in the lung lavage from VEH-I/R (n = 16), which was 239.41 ± 60.12 (Fig. 3). The differences in mean cell counts (×10^5) were also significantly different between these two groups: the lavage fluid cell counts in LPS-I/R (n = 11) were 6.76 ± 0.80, as compared to the cell counts in VEH-I/R (n = 16), which were 18.03 ± 3.87 ($P < 0.05$, Fig. 4). Of note, there was no statistically significant difference in either mean protein concentration or cell counts in the lavage fluid between animals treated with LPS prior to hepatic ischemia/reperfusion and sham-operated control animals. Mean protein concentrations in LPS-LPS (n = 7), VEH-LPS (n = 7), and SHAM (n = 8) were 63.49 ± 8.10, 81.71 ± 10.14, and 33.02 ± 12.22 and were also not significantly different from each other. Similarly, the mean cell counts in the lavage fluid from LPS-LPS (n = 7), VEH-LPS (n = 7), and SHAM (n = 8) were 5.19 ± 0.61, 6.40 ± 0.87, and 2.8 ± 1.33 and also were not significantly different from each other.

Liver Injury

Serum levels of AST were used to assess liver injury in these experiments. Previous studies in our laboratory have demonstrated significant increases in AST following 3, 12, and 24 hr of hepatic reperfusion. We have previously demonstrated that AST is a sensitive indicator of liver injury in this model and that AST levels become significantly increased prior to the development of any histopathologically demonstrable hepatic parenchymal abnormalities. In this particular study we chose to measure AST at 12 hr of reperfusion. In this case, there was no protective or deleterious effect of pretreatment with LPS prior to hepatic ischemia/reperfusion. Mean AST

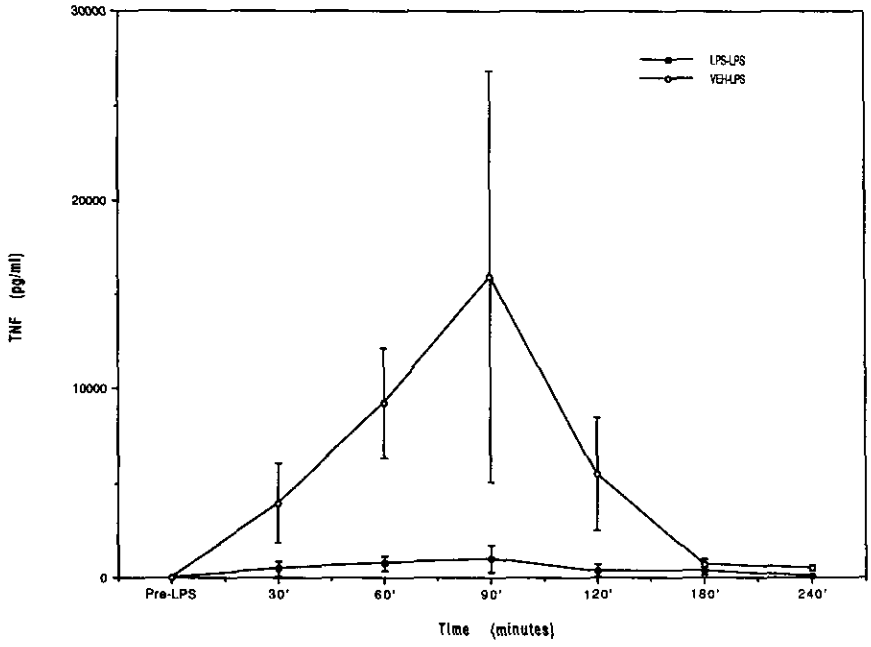

FIG. 1. TNF release following administration of 25 μg of LPS immediately prior to sham laparotomy in rats pretreated with 25 μg of LPS or an equivalent amount of vehicle 24 hr prior to sham laparotomy. This confirms observations made previously by other investigators, demonstrating that pretreatment with small nonlethal doses of LPS decreases subsequent TNF release in response to a second challenge with LPS.

in animals pretreated with vehicle prior to hepatic ischemia/reperfusion (VEH-I/R, $n = 16$) was 13,072 ± 2736, compared to animals pretreated with LPS 24 hr prior to initiation of hepatic ischemia/reperfusion (LPS-I/R, $n = 8$), where mean AST levels were 12,376 ± 4679 (P = NS, Fig. 5). Mean AST in LPS-LPS ($n = 7$), VEH-LPS ($n = 7$), and SHAM ($n = 8$) were 134 ± 12,244 ± 70, and 83 ± 10, respectively.

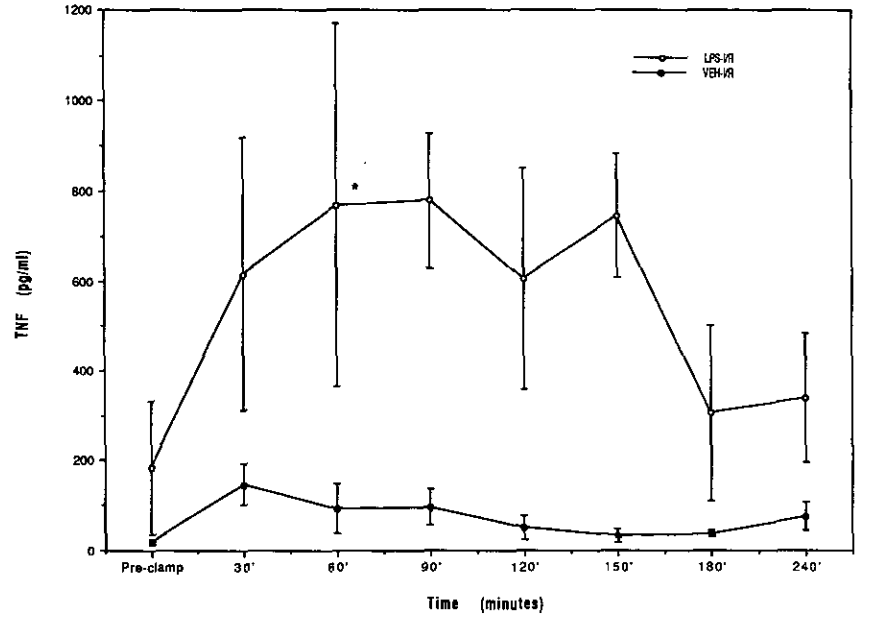

FIG. 2. TNF release following hepatic ischemia/reperfusion in rats pretreated with LPS or vehicle 24 hr prior to hepatic ischemia/reperfusion. Peak TNF levels occurred between 30 and 150 min of reperfusion in all animals. Very low or absent levels of TNF were seen in the sham-operated control animals ($n = 5$); the mean peak TNF level in this group was 3 ± 1 pg/ml. Mean peak TNF levels in animals pretreated with LPS are significantly higher (*$P = 0.014$) than mean peak TNF levels in animals pretreated with vehicle 24 hr prior to hepatic ischemia/reperfusion.

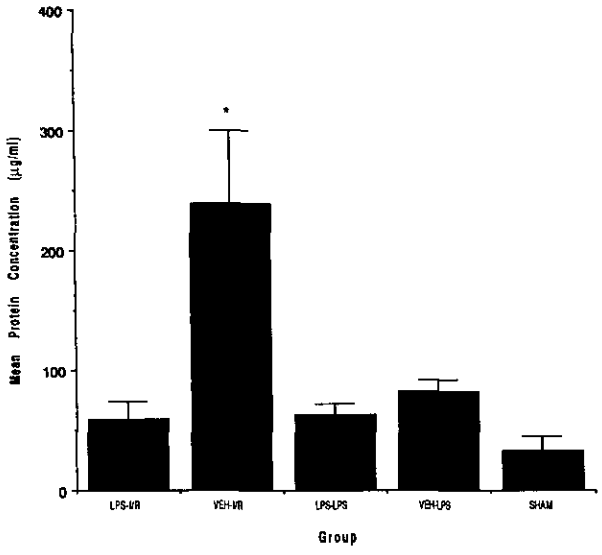

FIG. 3. Bronchoalveolar lavage fluid protein content (µg/ml) following pretreatment with LPS or vehicle 24 hr prior to induction of hepatic ischemia/reperfusion. There is a statistically significant decrease ($P < 0.05$) in BAL fluid protein content in those animals pretreated with LPS 24 hr prior to hepatic ischemia/reperfusion compared to animals receiving vehicle 24 hr prior to hepatic ischemia/reperfusion. Of note, there is no statistically significant difference between animals pretreated with LPS prior to I/R and sham-operated control animals.

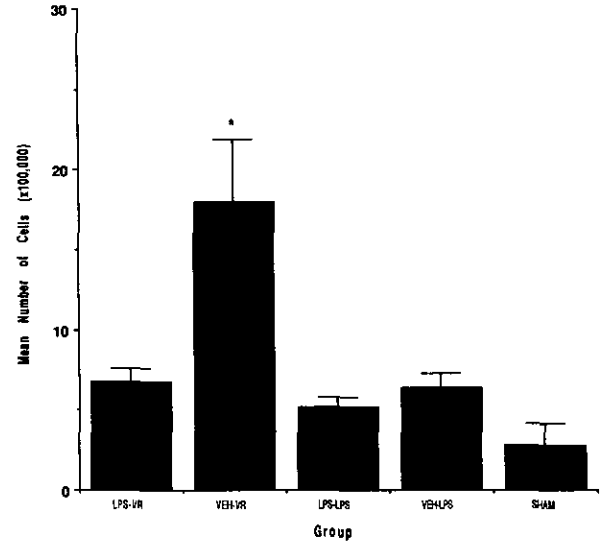

FIG. 4. Cell counts in bronchoalveolar lavage fluid following pretreatment with LPS or vehicle 24 hr prior to induction of hepatic ischemia/reperfusion. There is a statistically significant decrease ($P < 0.05$) in the cell counts in the lavage fluid in those animals pretreated with LPS 24 hr prior to hepatic ischemia/reperfusion compared to animals receiving vehicle 24 hr prior to hepatic ischemia/reperfusion. Of note, there is no statistically significant difference between animals pretreated with LPS prior to I/R and sham-operated control animals.

DISCUSSION

Our data demonstrate a discrepancy between the production of TNF and the injurious effects of this peptide mediator. These results are somewhat surprising in view of the current doctrine that overproduction of TNF is responsible for organ injury. However, newer studies have begun to illustrate that the simple dogma of TNF equals injury and death does not adequately describe the complex behavior of this molecule during *in vivo* inflammatory events. In many instances, TNF exerts a beneficial effect and may actually provide protection against later, organ-damaging effects.

In our model, TNF is probably not exerting a protective effect since we have already demonstrated that inhibition of TNF activity with anti-TNF antibodies will provide protection against the liver and lung injury observed after hepatic ischemia and reperfusion. The most likely hypothesis for the organ protection induced by prior nonlethal LPS administration is prior, transient induction of TNF. This early TNF will then provide protection against the subsequent organ damaging effects of TNF [16–18]. This concept that prior exposure to TNF will protect against subsequent TNF is supported by a series of articles showing that injection of recombinant TNF will prevent death in models of sepsis. Further work has shown that prior exposure to a nontoxic form of LPS will induce tolerance to toxic forms of LPS [19]. It is possible that these experiments could have been repeated using exposure to a nontoxic form of LPS and achieved similar results.

The mechanism of protection following induction of tolerance is not well understood. Even low-dose LPS re-

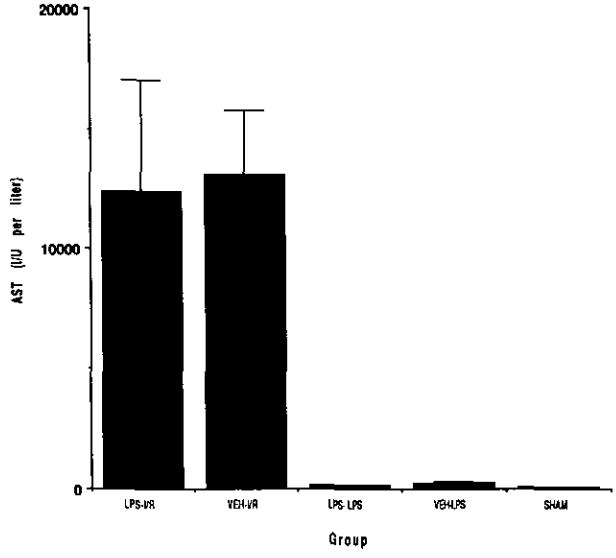

FIG. 5. SGPT levels following hepatic ischemia/reperfusion in animals pretreated with LPS or vehicle 24 hr prior to hepatic ischemia/reperfusion. There is no statistically significant difference between animals pretreated with LPS or vehicle prior to hepatic ischemia/reperfusion.

sults in the release of a number of mediators [20], including IL-1, TNF, GM-CSF, and G-CSF [21]. These induced cytokines most probably account for the induced organ protection. This has been most elegantly shown by Neta's group looking at the prevention of lethality due to radiation, where prior administration of cytokines will prevent death [22, 23]. The exogenous cytokines, and their induced products such as IL-6 and acute-phase proteins, probably account for the majority of the radioprotection.

The current experiments demonstrated a paradoxical rise in TNF following hepatic ischemia/reperfusion in animals pretreated with LPS. In addition, despite the increase in TNF levels, there is a decrease in the lung injury which follows hepatic reperfusion and what appears to be a stable liver injury, which one could interpret as a protective effect, since there is no increase in liver injury concurrent with the increase levels of TNF produced. Since other studies have demonstrated distinct mechanisms for the development of tolerance to ultraviolet radiation (UVR) and LPS [24], one can postulate that there are also distinct mechanisms for release of TNF in response to hepatic ischemia/reperfusion. These studies have demonstrated that the desensitization to the inflammatory properties of UVR is mediated at the site of interaction between the exogenous inflammatory stimulus and the cells having the potential to produce the cytokines [24]. *In vivo* studies demonstrated that animals desensitized to UVR remain fully responsive to the inflammatory effects of LPS and animals desensitized to LPS undergo an acute inflammatory reaction following a single exposure to UVR [24]. Furthermore, UVR-desensitized mice respond normally to the inflammatory effects of uv irradiation at a previously unexposed skin site [24], suggesting that the target cells within both UVR- and LPS-desensitized mice remain responsive to the endogenous mediators of inflammation. This was further substantiated by the fact that both LPS- and UVR-desensitized mice underwent a normal acute-phase response after stimulation with an exogenous source of IL-1 [24].

Since the inflammatory response *in vivo* is a complex cascade of events involving both soluble mediators and inflammatory cells, one could also postulate that pretreatment with LPS in the context of hepatic ischemia/reperfusion results in down-regulation of one of the more distal mediators or inflammatory cells involved in the development of organ injury following hepatic ischemia/reperfusion. The activated neutrophil appears to be the primary effector cell mediating the development of tissue injury, regardless of the precipitating event [25–28]. Activated neutrophils release a variety of inflammatory mediators including proteolytic enzymes, arachadonic acid metabolites, reactive oxygen species, and cytokines, all of which can impact directly on the pulmonary and hepatic microvasculature, leading to increased microvascular permeability, hemorrhage, and increased leukocyte migration into the tissue parenchyma [25, 26, 28–30]. We have previously demonstrated that TNF is intimately involved in initiating the events leading to acute pulmonary microvascular inflammation following hepatic ischemia/reperfusion [11, 12]. The lung and liver injuries in this model of lobar hepatic ischemia/reperfusion are characterized by neutrophil influx and increased microvascular permeability [lung injury: 11, 12; liver injury: Colletti *et al.*, unpublished data]. Hepatic ischemia/reperfusion triggers a cascade of inflammatory events which precipitate an influx of activated neutrophils into the hepatic and pulmonary interstitium, resulting in neutrophil-mediated injury, manifested as an increase in microvascular permeability, tissue injury, and organ dysfunction. The complete details and chronology of the series of events which ultimately culminate in the arrival of activated neutrophils into the interstitium with the subsequent development of microvascular leak, tissue injury, and end-organ dysfunction is as yet incompletely understood; however, the steps involved in the recruitment of activated neutrophils into the pulmonary interstitium may include endothelial cell activation and expression of endothelial cell-derived neutrophil adhesion molecules, neutrophil activation and expression of neutrophil-derived adhesion molecules, neutrophil–endothelial cell adhesion, neutrophil diapedesis, and neutrophil migration beyond the vascular barrier via established chemotactic gradients. Thus, given the potential complexity of the cascade precipitating neutrophil recruitment into the tissues, one could postulate that LPS pretreatment in the context of hepatic ischemia/reperfusion may down-regulate any or all of the steps precipitating neutrophil migration into the tissues.

The mechanism for the enhanced production of TNF in those rats treated with LPS and then subjected to hepatic I/R is difficult to explain. It is possible that the small nonlethal LPS immunologically primed the animal such that it would hyperrespond to a second challenge. This has been demonstrated to occur in mice where a small challenge of IL-1 12 hr prior to a challenge of TNF results in augmented TNF and IL-6 release [31]. Alternatively, the LPS may have down-regulated the endogenous inhibitory mechanisms that are normally used to control production and release of TNF, such that the second challenge was allowed to proceed more vigorously, a previously proposed hypothesis [32]. Further studies are needed to decipher the exact causes of the increased TNF with decreased organ injury.

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