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Lipopolysaccharide Detoxification by Endotoxin Neutralizing Protein

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Endotoxin neutralizing protein (ENP), a recombinant form of the anti-lipopolysaccharide factor that was isolated from amebocytes of the American horseshoe crab, *Limulus polyphemus*, detoxifies lipopolysaccharide (LPS) both *in vitro* and *in vivo*. Using the *Limulus* amebocyte lysate assay, LPS was detoxified by ENP at a 1 to 1 weight ratio (1:1). When isolated rat aortic rings were preincubated for 16 hr with either LPS or LPS/ENP (1:5), only aortas in the LPS/ENP group contracted normally under norepinephrine stimulation. To show that detoxification of a lethal amount of LPS (18 mg/kg, LD_{50} at 48 hr) persists *in vivo*, LPS/albumin (1:1) or LPS/ENP (1:1) mixtures were preincubated (30 min, 37°C) and then injected intravenously into rats. In the 8 hr after injection, LPS/ENP challenged rats, in contrast to their LPS/albumin injected counterparts, had significantly fewer physical signs of acute LPS toxicity ($P < 0.001$). At 48 hr after challenge, *all* LPS/ENP treated rats survived ($P < 0.01$ vs LPS/albumin), and with significantly less weight loss ($P < 0.001$ vs LPS/albumin challenged survivors). At necropsy, the LPS/ENP group was free of typical LPS-induced gross organ lesions, notably in the liver, spleen, gut-associated lymphoid tissue (GALT), and small intestine. By microscopic examination, lymphocytic necrosis in the spleen and GALT of the LPS/ENP treated survivors was significantly milder than that in the LPS/albumin challenged survivors, although the degree of hepatocellular necrosis and small intestinal enteritis was similar. LPS-neutralizing proteins such as ENP may be useful in treating LPS toxicity. © 1993 Academic Press, Inc.

INTRODUCTION

Lipopolysaccharide (LPS), a toxic constituent of the outer cell wall of gram-negative bacteria, plays a central role in the pathophysiology of sepsis [1]. Many physiologic changes have been associated with LPS or the cytokines and prostaglandins it induces. These include cachexia, fever, loss of vascular contractile responsiveness to pressors, and increased adhesiveness of the endothelial lining of blood vessels for leukocytes [2]. Other effects of LPS exposure are diffuse intravascular coagulation and hemorrhagic necrosis of liver and small intestine [3]. The derangements that this bacterial toxin can induce often end in cardiovascular system collapse or multiple organ system failure [2, 3].

Mechanisms of protection from LPS have evolved in many species, including the American horseshoe crab, *Limulus polyphemus*, and the Japanese horseshoe crab, *Tachypleus tridentatus*. When horseshoe crab hemolymph encounters LPS, amebocytes rapidly aggregate and then degranulate, and a coagulin gel surrounds the LPS or bacteria [4]. Besides the zymogens responsible for this gelation, which is the basis of the *Limulus* amebocyte lysate agglutination assay (LAL) for LPS, other amebocyte proteins also bind this toxin. One of these is anti-lipopolysaccharide factor (ALF), a protein of molecular weight 11,800 [5, 6]. This protein competes directly for LPS with the coagulation zymogens, thus acting as a neutralizer of LPS and a hemolymph anticoagulant [6, 7].

In contrast to the horseshoe crab's hemolymph, in mammalian serum, antibodies are one of the major protein species to bind, and perhaps neutralize, toxic antigens like LPS [8]. With human antiserum raised against the J5 antigen, which represents the lipid A and core regions of LPS, Ziegler *et al.* [9] passively immunized septic patients and showed that the severity of septic shock could be greatly reduced by this treatment. In a further development, monoclonal anti-LPS antibodies have now undergone clinical trials [10].

Just as anti-LPS antibodies are now being regarded as a possible therapy against sepsis, other smaller, less complicated LPS-neutralizing proteins, like ALF, may eventually be useful against LPS-induced toxicity [11]. There are some precedents in the literature for this. In 1975, Fumarola *et al.* protected rats against endotoxin lethality [12] by simultaneously injecting into the peritoneum both *Limulus* amebocyte lysate and a lethal dose of LPS. In the rabbit pyrogen assay studies of Niwa *et al.*, preincubation of LPS with *Tachypleus* ALF significantly attenuated the characteristic LPS fever [13], and

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Alpert *et al.* recently protected rabbits from meningococcal lipooligosaccharide (LOS) shock by either premixing the LOS with ALF or treating the animals *in vivo* a half hour after LOS challenge [14]. These reports suggest that the detoxifying activity of ALF known to occur *in vitro* may persist *in vivo*.

With a recombinant form of ALF called the endotoxin neutralizing protein (ENP), produced by Associates of Cape Cod, Inc. (Woods Hole, MA), Alpert *et al.* recently protected rabbits in their model of LOS shock [15]. The aim of our study was to delineate the neutralization of *Escherichia coli* LPS by ENP, as measured both *in vitro* and in a rat model of acute LPS toxicity. Our results suggest that LPS-neutralizing proteins such as ENP might eventually be used to treat bacteria-induced shock.

METHODS

Endotoxin Neutralizing Protein

A synthetic gene coding for the amino acid sequence of *Limulus* ALF, as reported by Muta *et al.* [16], was constructed and modified for expression in the yeast *Saccharomyces cerevisiae*. Yeast preferred codons were chosen and the alpha factor promoter and signal sequence was inserted for gene expression by Collaborative Research, Inc. (Bedford, MA). The crude protein, having a final sequence

EAEADGIWTQLIFTLVNNLATLWQSGDF

QFLDHECHYRIKPTFRRLKWKYKGK

FWCPSWTSITGRATKSSRSGAVEHSV

RNFVGQAKSSGLITQRQAEQFISQYN

was secreted into the yeast broth.

The fermentation was stopped and harvested at late log phase growth. The broth reached an optical density of 150–200 measured at 620 nm. This corresponds to 18–24 g of cells per liter dry weight or 4- to 8×10^9 cells per ml. Samples were collected at various times throughout the fermentation, and rapid HPLC analysis of the clarified broth was performed with a Poros S column (Perceptive Biosystems, Cambridge, MA). Clarified broth, 50 ml, was applied and eluted with a NaCl gradient (0–2 *M* over 30 min). The HPLC peak heights, coupled with the biological assay of the broth, enabled harvest time to be optimized. The fermentation broth was collected into autoclaved 20-liter polypropylene carboys. A hollow fiber unit (Sepracor, Marlboro, MA) with a 0.3-μm cutoff was used to remove cells and clarify the broth.

The filtrate (20 liters) was then applied to a 500-ml radial flow model column (Sepragen, Hercules, CA) packed with a sulfopropyl cation exchange resin (IBF, Savage, MD) in equilibration buffer consisting of 3 *M* urea, 50 m*M* ammonium acetate, and 10 m*M* EDTA, at pH 7.0. The column was then washed with equilibration buffer until the absorbance at 280 nm approached baseline. Step elution was performed with 100 m*M* and 2 *M* NaCl in equilibration buffer. The 2 *M* NaCl fraction contained active ENP, as measured by endotoxin neutralization bioassay, and was next purified by reversed phase chromatography. The 2 *M* NaCl peak (750 ml) was applied to a radial flow column (Sepragen) packed with 500 ml of C-4 silica resin (Vydac) equilibrated in 0.2% trifluoroacetic acid (TFA). The column was then step eluted with 25, 35, and 50% isopropanol (IPA) in 0.2% TFA. The 50% IPA fraction containing active ENP was then dialyzed against distilled water using an 8000 molecular weight cutoff hollow fiber (AG Technologies, Needham, MA) and lyophilized for storage.

Carbohydrate Analysis of ENP

Aliquots of ENP were submitted for monosaccharide compositional analysis (Oxford Glycosystems Limited, England). Samples (ca. 3 mg) were exposed to anhydrous methanolic HCl to liberate monosaccharides as 1-*O*-methyl derivatives. N-acetylation of available primary amino groups was performed, and samples were reacted with per-*O*-trimethylsilyl (TMS) to form the TMS-methylglycosides. The TMS-methylglycosides were separated on capillary gas liquid chromatography using a CP-SIL8 column with detection by mass spectroscopy. Identification of methylglycosides was by retention time and mass spectrum, through comparison to TMS-methylglycoside reference standards.

Amino Acid Analysis of ENP

Protein samples were hydrolyzed in 6 *N* HCl with trace amounts of phenol for 24 hr at 110°C using a Waters (Milford, MA) workstation. Dried aliquots were derivitized with phenylisothiocyanate (Pierce) to yield phenylthiocarbamyl amino acids. Separation was achieved using a Waters Amino Acid Analyzer with WISP Injector. Data was processed using Maxima 820 (Waters) data collection and analysis software.

In Vitro Limulus Amebocyte Lysate Assay

The neutralizing activity of ENP was assayed *in vitro* in 96-well microtiter plates [16]. Protein fractions were serially diluted across the top row and mixed with a 1-μg standard *E. coli* LPS solution. The mixture was then diluted down the plate and LAL assays (Associates of Cape Cod, Inc.) were performed. Absorbance was measured at 340 nm. The dilution of protein necessary to get 90% reduction of the LPS was a measure of the specific activity.

Animals

Male Sprague–Dawley rats (Taconic Farms, Germantown, NY), 280 to 330 g each, were placed in isolettes

upon their arrival from the vendor. They were maintained in an environment controlled for both light (12 hr on, 12 hr off) and temperature (constant 23°C). They also were given tap water and standard rat chow (RMH-2000) *ad libitum* until the day of the experiment.

In Vitro Rat Aortic Ring Contraction Studies

Following decapitation of the rats, the thoracic aortas were excised and sectioned into rings (3.5-mm segments). They were incubated at 37°C for 16 hr under a 95% O_2–5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium containing 1% fetal calf serum, 100 U penicillin, and 100 μg streptomycin. During this period, the rings ($N = 8$ for each group) were subjected to one of four experimental incubation regimes. They were exposed to either 10 ng/ml LPS (055:B5, Difco, Detroit, MI), 50 ng/ml ENP, a mixture of 10 ng/ml LPS and 50 ng/ml ENP, or medium only. After the 16-hr incubation, contractile performance of rings was assessed as described previously [18]. Generated tension was measured as a response to cumulative NE concentration (1 n*M* to 30 μM).

The parameters E_{max} (maximum contraction, mg tension/mg tissue) and EC_{50} (molar NE concentration responsible for half-maximum contraction) were obtained for each ring by fitting expression (1) to the NE dose–response curve:

$$\text{Tension} = \frac{(E_{max})\,([\text{NE}])}{EC_{50} + [\text{NE}]}. \qquad (1)$$

The E_{max} or EC_{50} values for different treatments were compared by random blocks analysis of variance followed by Student–Newman–Keuls a posteriori tests. Tests for differences between EC_{50} values were based on log EC_{50} values [17]. Probabilities of 0.05 or less were taken as significant in this and all later tests.

In Vivo Rat Model of Acute LPS Toxicity

LPS prepared by the hot phenol–water extraction method (*E. coli* 0111:B4, Sigma Chemical Company, St. Louis, MO) was suspended by vortexing to a concentration of 10 mg/ml in 0.15 *M* PBS (0.15 *M* NaCl, 0.020 *M* sodium phosphate, pyrogen-free water, pH 7.4). The control protein, human serum albumin (Plasbumin-25, USP, Cutter Biological, Berkeley, CA), was dissolved in PBS to a concentration of 10 mg/ml. The ENP was diluted with PBS to a 10 mg/ml concentration. After preparation of each solution, multiple aliquots were frozen at −20°C.

Rats were randomized into one of six groups. The groups included a buffer control group (PBS/PBS), three groups of LPS-challenged rats (LPS/PBS, LPS/albumin, and LPS/ENP), and two protein control groups (PBS/albumin, and PBS/ENP). On the day of the experiment, the appropriate injection mixtures were prepared by thawing and then mixing equal volumes of the component solutions. After incubation (37°C for 30 min), each injection mixture was gently vortexed just before injection. Under anesthesia by halothane inhalation (2-bromo-2-chloro-1,1,1-trifluoroethane, Halocarbon Laboratories, North Augusta, SC), rats were injected intravenously (iv) via the dorsal vein of the penis (in a fluid load of 3.0 ml/kg). The *in vivo* challenge to the rats was 18 mg/kg of the LPS and 18 mg/kg of ENP. Halothane was then removed, and the animals quickly awoke. They were then maintained on water and standard rat chow.

Rats were weighed before injection and then weighed 24 and 48 hr after injection. Statistical comparisons of the percentage of weight change among survivors were by Mann–Whitney *U* test. Rats were evaluated hourly (by the principal author and the veterinary pathologist) for the first 8 hr for the presence of each of the 11 signs of LPS toxicity defined in Table 1. After that, survival was noted at 24 and 48 hr after injection. The experimental group identity of each rat was unknown to the evaluator. The hourly LPS toxicity scores for each rat were totaled, with differences between group scores evaluated by the Mann–Whitney *U* test. Survival was measured in the first 8 hr, at 24 hr, and 48 hr after iv injection. Survival statistics were determined by 2×2 χ^2.

Rats were necropsied immediately after death (nonsurvivors) or immediately following sacrifice by halothane inhalation at 48 hr (survivors). (The nature of survival studies is such that animals can only be necropsied at the time of death or at the end of the experiment. Therefore, any comparisons of pathologic state made between survivors and nonsurvivors represent discontinuous time points, and these comparisons emphasize persistent organ and tissue changes in the surviving rats.) The livers, spleens, small intestines, gut-associated lymphoid tissues (Peyer's patches; GALT), large intestines, and kidneys were examined. Gross changes in tissue color and composition, signs of necrosis, congestion, and frank or petechial hemorrhage were noted at necropsy (by the principal author and the veterinary pathologist.) The normalized organ weight (organ weight at necropsy divided by preinjection body weight) for liver, spleen, and kidney of each animal was calculated. Statistical comparisons of normalized organ weights were by Mann–Whitney *U* test. Tissue samples were fixed for at least 24 hr in 10% buffered formalin, cut at 6 μm, stained with hematoxylin and eosin, and then examined microscopically. Samples included liver, spleen, small intestine, GALT, large intestine, and kidney. Tissue damage was quantitated on a six-level scale (normal, minimal, mild, moderate, marked, and severe). Comparisons were made by contingency tables between tissues from animals treated with combinations of PBS, albumin, LPS, or ENP.

TABLE 1

Carbohydrate and Amino Acid Analysis of ENP

Monosaccharide	*Limulus* ALF molar content[a]	ENP molar content[a]
	Carbohydrate analysis	
Fucose	8.6	ND[b]
Galactose	3.2	0.8
Glucose	ND	4.1
Mannose	10.1	132.7
Galactosamine	2.6	ND
Glucosamine	5.8	4.9
Xylose	2.3	3.2

Amino acid	*Limulus* ALF molar content[c]	ENP molar content[c]
	Amino acid analysis[d]	
Asp	6.1 (7)	7.2 (7)
Glu	9.8 (10)	11.8 (12)
Ser	8.2 (10)	9.5 (10)
Gly	8.0 (7)	7.0 (7)
His	2.6 (3)	2.9 (3)
Arg	8.2 (7)	6.3 (7)
Thr	6.2 (8)	8.5 (8)
Ala	8.4 (5)	7.9 (7)
Pro	3.4 (2)	2.2 (2)
Tyr	3.8 (3)	2.8 (3)
Val	5.5 (4)	4.9 (4)
Met	0.0 (0)	0.0 (0)
Cys	0.0 (2)	0.0 (2)
Ile	7.2 (6)	5.9 (6)
Leu	6.5 (7)	7.3 (7)
Phe	3.9 (7)	7.0 (7)
Trp	0.0 (5)	0.0 (5)
Lys	6.0 (8)	6.9 (8)

[a] nmol saccharide/mg protein.
[b] Not detected.
[c] Amino acid residues/mole; found (predicted).
[d] Predicted values were derived from amino acid sequences deduced from *Limulus* ALF or ENP gene sequences.

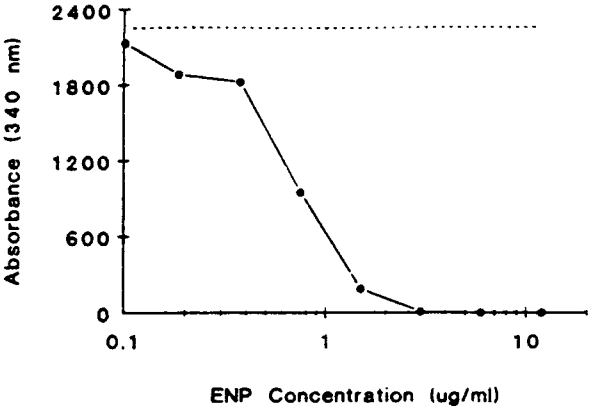

FIG. 1. Neutralization of LPS by ENP, as assayed by the LAL. ENP is diluted serially across a microtiter plate (solid line) and challenged with a constant 1 μg/ml *E. coli* LPS (dotted line). ENP/LPS mixtures are measured for available LPS by the kinetic turbidimetric LAL assay. Neutralization of 90% of the added LPS occurs at 1.2 μg of ENP, suggesting an ENP/LPS ratio ca. 1.

RESULTS

Analysis of Limulus ALF and ENP

The ENP, expressed in recombinant form in yeast, was found to have an abundance of mannose (Table 1). In *Limulus* ALF the mannose concentration (nmole mannose/mg protein) was 10.1, whereas in ENP it was 132.7. The results of the amino acid analysis (Table 1), on the other hand, were consistent with expected values [18].

In Vitro Limulus Amebocyte Lysate Assay

A 1 to 1 ratio by weight of ENP to LPS was sufficient, as assessed by inhibition of the LAL assay, to reduce the LPS activity by 90% (Fig. 1). This ratio was consistent with our previous determination for select batches of *Limulus* ALF using this *in vitro* neutralization assay [5].

In Vitro Rat Aortic Ring Contraction Studies

When aortas have been incubated in only medium for 18 hrs, they respond to NE with a contractile strength, E_{max}, of 453 ± 34 and a sensitivity, $-\log(EC_{50})$, of 7.49 ± 0.10. (All values are expressed as means ± SEM, for eight rings in each group.) The *in vitro* incubation of rat aorta with LPS causes a reduced contractile responsiveness to NE [19]. The changes in tissue response are time dependent, with some effects being seen as soon as 4 hr after either *in vitro* or *in vivo* exposures, and they are complete by 18 hr [20]. In our experiments (Fig. 2), a 16-hr incubation of isolated rat aortic rings with LPS

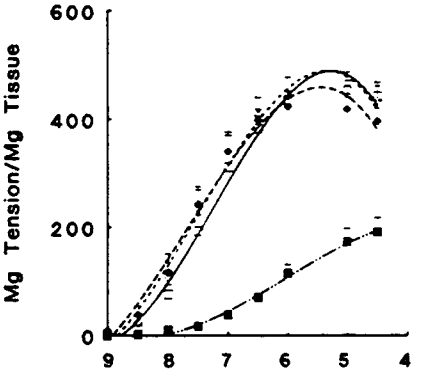

FIG. 2. Contractile response to NE of aortic rings isolated from rats and incubated with medium only (open diamonds), LPS (closed squares), ENP (closed diamonds), or LPS ENP (open squares). N = 8, symbols denote means ± SEM. Curves are best fits of Eq. (1) to the data.

TABLE 2

Physical Signs of LPS Toxicity in the Rat

Mild (1 point)	Moderate (2 points)	Severe (3 points)
Piloerection of fur	—	—
Hunched posture	Irritable when handled	Convulsive
No exploring behavior	Not easily arousable	—
Red foot pads	—	Cyanotic paws
Soft stools	Diarrhea	Bloody diarrhea
Deep, slow breaths	Alternating rapid/ slow breaths	Gasping breaths

Note. Each rat was evaluated hourly, in the first 8 hr after injection, for the presence of each of these physical signs.

significantly reduced maximal vascular contractility, E_{max} = 187 ± 29, and sensitivity, $-\log(EC_{50})$ = 6.39 ± 0.13, to NE stimulation. Incubation of aortic tissue with only ENP did not change tissue responses to NE: E_{max} = 424 ± 20, $-\log(EC_{50})$ = 7.60 ± 0.12. The contractile response of LPS/ENP (1:5) treated vascular tissue was indistinguishable from that of control tissue: E_{max} = 453 ± 17, $-\log(EC_{50})$ = 7.32 ± 0.09. So, a ratio of 1:5 LPS/ENP was found to provide significant protection in our vascular tissue assay. In preliminary experiments, however, smaller doses of ENP provided less, but still important, protection against LPS (data not shown).

In Vivo Rat Model of Acute LPS Toxicity

The *in vivo* rat model was first characterized by injecting a 20 mg/kg iv bolus dose of LPS/PBS and monitoring serum concentrations of LPS with the *Limulus* lysate chromogenic assay. Most of the LPS was cleared from the blood within 10 to 20 min after injection, and residual serum levels at 60 min were less than 5% of the estimated initial peak concentration (data not shown). The lethal threshold for LPS/PBS injection was about 3 mg/kg, and at LPS doses above 25 mg/kg, lethality approached 100%. The toxic effects of LPS injection, as used in this study, were consistent with previous reports [21, 22].

Signs of LPS toxicity (Table 2) appeared within 15 min of LPS/albumin injection and, at first, were mostly mild. By 2 hr, about half the group had soft stools or diarrhea. By 4 hr, the more ill rats exhibited many moderate signs of LPS toxicity. Eventual nonsurvivors precipitously displayed severe signs of LPS toxicity within an hour of death. By 24 hr, no physical signs of LPS toxicity were present in the survivors. Group LPS toxicity scores for the LPS/albumin challenged rats, calculated as detailed in Table 2, rose rapidly in the first 3 hr. They continued to be high for the remaining 5 hr of the observation period (Fig. 3). In the LPS/ENP group, physical signs of LPS toxicity were nearly absent

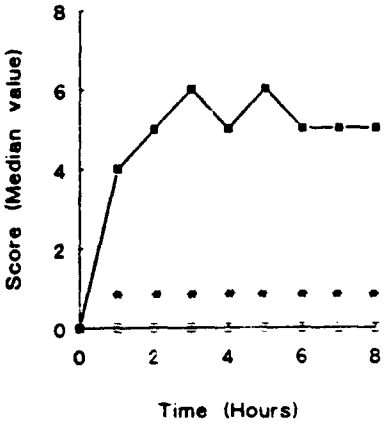

FIG. 3. Median LPS shock score for treated rats at each hour. LPS/albumin (1 to 1) treatment, (closed squares) (N = 10). LPS/ENP (1 to 1) treatment, open squares (N = 10). (•) denotes significant differences in the LPS shock score of rats that received LPS/ENP compared with rats that received LPS/albumin.

throughout the experiment, which represented a significant lowering of the hourly LPS toxicity scores (Fig. 3).

The time until death ranged from 3 to 48 hr, with most (over 80%) dying 3 to 6 hr after injection. Median survival times for the LPS/PBS and LPS/albumin injected groups were, respectively, 4 and 5 hr. No mortalities were observed in the groups challenged with PBS/PBS or PBS/albumin. All animals given PBS/ENP survived (10/10, 100%). The lethality in the LPS/PBS group was 3/10 (30%). After preincubation with albumin (1:1), survival in the LPS/albumin group was 5/10 (50%), which was not significantly different. All rats receiving LPS/ENP survived (10/10, 100%). Compared with the 50% survival of the concomitant LPS/albumin challenged group, this was a significant improvement in survival.

Weights of survivors were assessed 48 hr after injection, and the results are summarized in Fig. 4. The LPS/

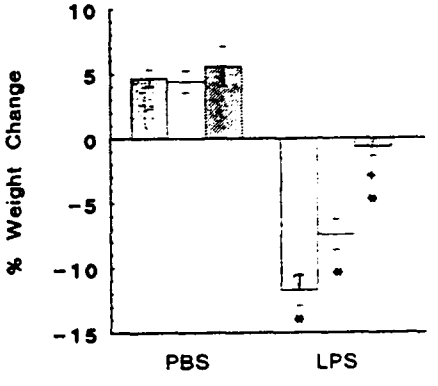

FIG. 4. Percentage change from initial weight 48 hr after injection by rats treated with: PBS/PBS (dotted bar; N = 10), PBS/albumin (open bar; N = 8), PBS/ENP (striated bar; N = 6), LPS/PBS (dotted bar; N = 4), LPS/albumin (1 to 1; open bar; N = 5), and LPS/ENP (1 to 1; striated bar; N = 5). (•) Denotes significant difference as compared with PBS/albumin treated animals. (+) Denotes significance from LPS/albumin treated rats.

TABLE 3

Gross Pathology Organs

	LPS/albumin nonsurvivors	LPS/albumin survivors	LPS/ENP survivors
Liver			
Color	Darkened	Yellow-white	Normal
Composition	Normal	Friable	Normal
Necrosis	Absent	Diffuse	Absent
Spleen			
Color	Darkened	Normal	Normal
Surface	Normal	Multinodular	Normal
Small intestine			
Serosa color	Purple-red	Brown-yellow	Normal
Mucosa surface	Congested	Normal	Normal
Hyperemia	Diffuse	Absent	Absent
Large intestine	Normal	Normal	Normal
GALT	Hemorrhagic	Normal	Normal
Kidney	Normal	Normal	Normal
Number of rats	18	10	10

PBS and LPS/albumin challenged groups lost much more weight than the PBS/PBS and PBS/albumin control groups. Preincubation of LPS with ENP significantly mitigated this LPS-induced weight loss. These rats lost, on average, only 0.7% of their initial body weight. Regardless, their weight gain was still impaired, in contrast to the 5% weight gain of the PBS/albumin group (Fig. 4).

In our model, liver, spleen, and small intestine were most affected by LPS injection. Necropsy of the nonsurvivors of the LPS/albumin or LPS/PBS groups revealed marked damage to these organs, as summarized in Table 3. A different pattern of lesions was detected in the livers, spleens, and small intestines of survivors. In contrast to these stark effects, it is noteworthy that injection of PBS/ENP or LPS/ENP caused *no* gross signs of damage at the 48 hr necropsy. Grossly, the livers, spleens, and small intestines of these groups appeared to be normal.

About organ weight changes, injection of LPS (in PBS or albumin) caused the spleen to enlarge significantly to nearly twice normal size and weight. Nonsurvivors (median time of death, 4 to 5 hr) and survivors (sacrificed at 48 hr) showed the same degree of splenomegaly. Incubation of LPS with ENP did not modulate the increase in weight. Of the other organs weighed in the LPS/PBS or LPS/albumin groups, neither liver nor kidney were enlarged or heavier.

At the microscopic level, injection of LPS/albumin caused, in 60% of nonsurvivors, prominent tissue hemorrhage in the liver, intestine, and GALT; for the survivors, by contrast, tissue hemorrhage was absent. As with the marked hemorrhage found in the nonsurvivors, enteritis and hepatocellular necrosis also were significantly more severe (Table 4). Whether nonsurvivors or survivors, the hepatocellular necrosis seen in all LPS/PBS or LPS/albumin injected rats lacked an inflammatory cell component, suggesting a peracute nature to the damage. In both of these groups, the large intestines and kidneys were unremarkable.

When ENP alone was injected, PBS/ENP group, multifocal areas of peracute hepatocellular necrosis, of minimal to mild severity, were seen (Table 4). ENP, itself, had caused some mild histologic changes of an immediate nature, but only in the liver.

Microscopic examination of the LPS/ENP group revealed that lymphocytic necrosis of the spleen and GALT was significantly attenuated, as Table 4 shows. For the liver and small intestine, on the other hand, premixture with ENP did not lessen the LPS-induced damage visible under the microscope. Considering the liver, in particular, a normal appearance by gross examination concealed significant microscopic lesions.

DISCUSSION

In vitro, ALF, like other LPS-binding peptides found in nature, exhibits antimicrobial activity [11, 23]. *Limulus* ALF has an 83% sequence homology with *Tachypleus* ALF, and both are structurally similar to the α-lactalbumin/lysozyme family of mammalian proteins [24, 25]. At low concentrations, ALF is bacteriostatic against rough gram-negative bacteria; while at higher titers, ALF is reported to kill such bacteria [13]. In addition, ALF has been shown to neutralize LPS directly [18].

ENP is a single-chain, basic protein that can bind to and neutralize LPS. This amphipathic protein has a rich clustering of hydrophobic amino acids at the amine terminal region and an array of basic amino acids in the central disulfide-bonded loop region [23, 24]. When LPS and ENP are mixed, they aggregate. We found that as ENP was added dropwise to the colorless solution of LPS (1:1), a white turbid suspension initially formed around the settling drop. This was followed by the appearance of a flocculent white precipitate after incubation (37°C for 30 mins). The exact mode of binding of ENP to LPS is uncertain, although it is likely that the hydrophobic and cationic amino acids of ENP interact, respectively, with the fatty acid chains and the phosphate groups of the toxic lipid A region of LPS.

Different ratios of LPS to neutralizing protein are reported to detoxify LPS. For example, the lysis of LPS-treated erythrocytes by *Tachypleus* ALF is inhibited after LPS preincubation with ALF at a ratio of at least 1 to 1.6 [26]. In the endothelial cell activation studies of Desch *et al.*, LPS to *Tachypleus* ALF ratios of 1 to 10 up to 1 to 100 are needed to block LPS-induced activation [27]. In our vascular tissue study with recombinant ENP, a 1:5 ratio of LPS/ENP was required to retain optimal contraction under NE stimulation.

In our *in vivo* rat model, LPS, after 37°C incubation with ENP (1:1), is effectively transformed from a solution of 50% lethality (18 mg/kg) to one of 0% lethality (less than 3 mg/kg). ENP also reduces or eliminates

TABLE 4

Microscopic Pathology of Organs

	Liver (hepatocellular necrosis)						Spleen (lymphocytic necrosis)						Small intestine (Enteritis)						GALT (lymphocytic necrosis)					
Severity:	0	1	2	3	4	5	0	1	2	3	4	5	0	1	2	3	4	5	0	1	2	3	4	5
PBS/																								
Albumin	10						1	7	2				2	6	2				3	3	4			
ENP	1	2	6			*	3	2	4				2	5	1	1			2	3	4			
LPS/																								
Albumin ns		1	1	4	9	**			2	9	3	1		1	3	5	6	**	4	9	2			
Albumin s		1	1	8				1	3	5	1			5	5				1	1	5	3		
ENP s	1	2	2	3	2		1	4	5			**	3	3	2	1		1	3	5	2			**

Note. Number of rats in each experimental group showing liver, spleen, intestine, and GALT lesions of differing severity. ns, nonsurvivors; s, survivors. Severity: 0, normal; 1, minimal; 2, mild; 3, moderate; 4, marked; 5, severe.

* $P < 0.05$ in comparison with PBS/albumin.

** $P < 0.05$ in comparison with LPS/albumin s.

LPS-induced tissue damage. Comparable *in vivo* LPS neutralization has been reported for LPS-shocked sheep treated with polymyxin B [28].

Disordered vascular function may be an important contributor to the lethality associated with LPS shock [29]. The ability of ENP to shield isolated vascular tissue from LPS was documented in our *in vitro* study. Our vascular protection study also showed that the neutralization of LPS by ENP can be maintained for an extended period of time, suggesting that a decreased effective concentration of LPS resulting from persistent ENP activity also contributed to the enhanced survival in our *in vivo* model.

The LPS–ENP complex seems to persist *in vivo* in a form not as toxic as free LPS. For example, the LPS toxicity scores in our rat model were significantly attenuated over the 8 hr observation period in the LPS/ENP group. Less desirable *in vivo* effects of this aggregation, such as prolonging LPS clearance or encouraging tissue localization, are hinted at by the condition of the LPS/ENP survivors at necropsy. These include the lingering histopathology (hepatocellular necrosis and small intestine enteritis) and the persistence of the LPS-induced splenomegaly.

Although much of the *in vivo* toxicity of LPS can be lessened by preincubation with ALF or ENP, administration of the proteins themselves had some untoward effects. For example, in our initial experiments with *Limulus* ALF, a 15 mg/kg dose of PBS/ALF killed 2 of 15 animals, one 6 hr after, and the other 22 hr after injection. In addition, the kidneys of some of these PBS/ALF injected rats, nonsurvivors and survivors, were yellow and shrunken. Administration of PBS/ENP, on the other hand, is not as toxic as *Limulus* ALF, but it is associated with a significant degree of hepatocellular necrosis. We speculate that the more harmful effects particular to *Limulus* ALF may have been caused by a combination of lower solubility, greater protein degradation, and extraneous proteins in the ALF extraction mixture (data not shown).

The mild hepatocellular necrosis caused by injection of ENP was multifocal and marked by an absence of inflammatory cells. Yet only the liver showed such necrosis. Together, this suggests that ENP may be hepatotoxic. Many different agents can cause hepatocellular necrosis, including the cyclic octapeptide amatoxins of the toxic mushroom genus *Amanita* [30]. Amatoxins can selectively enter the hepatocyte, after uptake by the Kupffer cells, and then bind to and inhibit RNA polymerase [31]. We speculate that ENP, or some fragment of the peptide, also can be taken up by the hepatic reticuloendothelial system. We can only guess the cause of the hepatocellular necrosis associated with ENP, although the histology suggests that it is secondary to a cytotoxic effect of the protein on either the Kuppfer cells or the hepatocytes.

In summary, ENP neutralized LPS *in vitro* in both the LAL and the aortic contractility assays. By the *in vivo* rat model, LPS/ENP was not lethal. ENP also tempered weight loss, physical signs of LPS toxicity, and much of the pathologic damage to vital organs. The recent cloning and expression of ENP has made it more available. This availability, together with the detoxifying capability of ENP we have shown in our study, suggests that recombinant forms of proteins that can bind to and neutralize LPS, or even synthetic analogues of such peptides, may be used in the future to treat sepsis and septic shock.

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