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Remote Thermal Injury Increases LPS-Induced Intestinal IL-6 Production[1]

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Background. **Patients suffering from burn injury are at high risk for subsequent infection. Thermal injury followed by endotoxemia may result in a "second hit," causing an exaggerated inflammatory response with increased morbidity and mortality. The role of the intestine in this "second hit" response is unknown. We hypothesized that remote thermal injury increases the inflammatory response of intestinal mucosa to subsequent treatment with lipopolysaccharide (LPS).**

Methods. **Mice underwent sham or scald injury. Seven days after injury, mice were treated with LPS. Blood and bowel specimens were obtained. Serum and intestinal inflammatory cytokines were measured by enzyme-linked immunosorbent assay (ELISA). Changes in TLR-4 pathway components in intestine were measured by reverse transcription-polymerase chain reaction (RT-PCR), Western blot, and electrophoretic mobility shift assay (EMSA). Intestinal leukocyte infiltration was analyzed by myeloperoxidase assay.**

Results. **A "second hit" of injected LPS resulted in increased IL-6 in intestine of burned mice compared with sham. Similarly, jejunal IL-6 mRNA levels increased in mice with prior thermal injury, suggesting a transcriptional mechanism. Of transcription factors known to drive IL-6 expression, only AP-1 activation was significantly elevated by a "second hit" of LPS.**

Conclusion. **Prior thermal injury potentiates LPS-induced IL-6 cytokine production in intestine. These results indicate a heightened inflammatory response to a second hit by intestine after burn injury.**

Key Words: **IL-6; intestinal mucosa; burn; TLR-4; AP-1.**

INTRODUCTION

The inflammatory response to thermal injury is associated with significant morbidity and mortality. Despite improvements in critical care, the systemic inflammatory response syndrome and sepsis remain the leading cause of death in intensive care units [1–3]. Infection complicates the hospital course of as many as 50% of patients admitted with thermal injuries, and may be responsible for as many as 75% of burn related deaths [4]. In these patients, the acute inflammatory insult of thermal injury is followed by infection, which can represent a "second hit" to the patient [5]. Under these circumstances, a normally well-tolerated insult, such as pneumonia, may lead to an exaggerated inflammatory response and progression to multiple organ failure with associated high morbidity and mortality. Burn patients appear to be especially susceptible to a "second hit." The development of pneumonia is associated with increased mortality, and sepsis is associated with at least 50% mortality in this patient population [6, 7]. Thus, attempts to decrease morbidity and mortality following thermal injury require understanding the effect of the "first hit" on the "second hit." Despite intensive study, the etiology of susceptibility to the "second hit" remains incompletely understood.

Following systemic injury, the intestinal mucosa produces multiple pro-inflammatory cytokines. Prior studies investigating the role of the intestine in the inflammatory response to thermal injury have shown increased gut bacterial translocation and elevation of plasma endotoxin and IL-6 after burn [8, 9]. Another

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study utilizing *in situ* hybridization in mouse intestinal lamina propria demonstrated increased IL-6 expression after burn [10]. Preliminary studies suggest a role for the intestinal mucosa in the second hit. *Ex vivo* experiments have shown that enterocytes harvested from previously burned animals produced more IL-6 in the presence of LPS compared with enterocytes from control animals [11]. The mechanisms of this altered responsiveness are unknown.

In order to examine the role of the intestine in the second hit after thermal injury, we investigated the effect of remote thermal injury on the inflammatory response to subsequent endotoxemia. We hypothesized that remote thermal injury increases the inflammatory response of intestinal mucosa to subsequent treatment with lipopolysaccharide (LPS).

METHODS

Experimental Conditions

Male C57/BL6 mice weighing 22–28 g were purchased from The Jackson Laboratory (Bar Harbor, ME), fed standard laboratory diet and water *ad libitum*, and acclimated for 1 wk in a climate-controlled room with a 12-hlight-dark cycle. Experiments were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati.

Thermal injury was induced as described previously with minor modifications [12]. Briefly, mice were anesthetized with pentobarbital 60 mg/kg intraperitoneal (i.p.) injection and inhaled isoflurane (1.5%). Dorsal fur was removed by clipping, and mice were exposed to 90 °C water for nine s to create a 25% total body surface area full thickness burn. Sham mice were exposed to room temperature water. All mice were resuscitated immediately with 1 mL i.p. normal saline, allowed to recover in a warmed oxygen tent, and then housed individually. In initial experiments, we examined the effect of sham or thermal injury on serum cytokine levels in mice sacrificed at 1, 4, or 7 d after injury.

In subsequent experiments, a "second hit" of inflammation was induced by i.p. injection of 10 mg/kg LPS (*E. coli* 0111:B4; Calbiochem La Jolla, CA) 7 d after sham or burn injury. Additionally, we tested the effect of LPS injection in otherwise untreated mice. Mice were sacrificed at 1 or 4 h after LPS injection. Blood was obtained by cardiac puncture, and serum separated by centrifugation, and stored at –80 °C until analysis. Small and large bowel were removed and irrigated with ice-cold normal saline. Large bowel was procured whole. Small bowel mucosa was harvested by scraping as described previously [13]. Specimens were frozen in liquid nitrogen and stored at –80 °C until analysis.

Tissue Extraction

Nuclear and cytoplasmic fractions were prepared as described previously [13]. All steps were carried out on ice. Tissue samples were homogenized in 0.5 mL of buffer A (10-mmol/L HEPES [pH 7.9], 1.5-mmol/L $MgCl_2$, 10-mmol/L KCl, 1-mmol/L DTT, and 1-mmol/L PMSF), incubated for 10 min, and then centrifuged at 850 *g* for 10 min at 4 °C. The pellets were resuspended in 1.5× cell volume of buffer A with 0.1% Triton X-100, incubated for 10 min, and centrifuged as above. The supernatant was removed and saved as the cytoplasmic fraction. The pellet was resuspended in 300 μL of buffer A, centrifuged as above, and resuspended in 1 cell volume of a buffer of 20-mmol/L HEPES (pH 7.9), 25% glycerol (vol/vol), 420-mmol/L NaCl, 1.5-mmol/L $MgCl_2$, and 0.2-mmol/L EDTA. After incubation for 30 min, the nuclear fraction was recovered by centrifugation at 20,000 *g* for 15 min at 4 °C. Fractions were assayed for protein concentration (BCA Protein Assay Kit; Pierce, Rockford, IL) and stored at –80 °C until analysis.

Cytokine analysis was performed as previously described [14]. Jejunal scrapings were sonicated for two 10 s periods in 1 mL phosphate-buffered saline (PBS) containing complete protease inhibitor cocktail tablets (Roche, Indianapolis, IN) and 2 mM PMSF (Sigma, St. Louis, MO). Colon whole bowel specimens were first homogenized, then sonicated for one 10 s period in this same solution. Samples were centrifuged at 12,000 *g* at 4 °C for 45 min. Supernatant density was determined using BCA Protein Assay Kit (Pierce). IL-6 was measured using commercially available ELISA kits (R and D Systems, Minneapolis, MN) per the manufacturer's instructions. Serum IL-6 is expressed as pg/mL and intestinal IL-6 is expressed as ng/g protein.

Electrophoretic Mobility Shift Assay

Nuclear extracts of intestinal tissue were analyzed by electrophoretic mobility shift assay (EMSA) as described previously [13]. Double-stranded consensus oligonucleotides of nuclear factor-kappa B (NF-κB) or activator protein 1 (AP-1) (Promega, Madison, WI) or consensus and mutant oligonucleotides of CCAAT/enhancer binding protein (C/EBP) (Santa Cruz Biotechnology, Santa Cruz, CA) were end labeled with (^{32}P) gamma-adenosine triphosphate (γATP) (Perkin Elmer, Boston, MA) using polynucleotide kinase T4 (Promega).

End-labeled probe was purified from unincorporated (^{32}P) γATP using a purification column (Bio-Rad Laboratories, Hercules, CA). Binding reactions (total volume 15 μL) with equal amounts of nuclear extracts (20 μg) and oligonucleotide and buffer containing 20% glycerol (vol/vol), 50 mM Tris-HCl, pH 7.9, 2.5 mM EDTA, 2.5 mM DTT, 5 mM $MgCl_2$, 250 mM NaCl, and 0.25 μg/μL poly[d(I-C)] (USB Corp., Cleveland, OH) were incubated at room temperature for 30 min. Samples were subjected to electrophoretic separation on a nondenaturing 5% polyacrylamide gel at 100 V. Blots were dried at 53 °C for 3 h and analyzed by exposure to PhosphorImager screen (GE Healthcare, Piscataway, NJ) or autoradiography film.

Western Blot Analysis

Equal concentrations of protein were boiled in equal concentrations of loading buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol) for 5 min, then separated by electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). For TLR-4 analysis, membranes were blocked with 10% nonfat dried milk in Tris-buffered saline (TBS, pH 7.6) containing 0.05% Tween-20 (TTBS), for 2.5 h, incubated overnight with anti-TLR4 antibody (H-80; Santa Cruz Biotechnology) at 1:500 dilutions in 5% milk, washed three times in TTBS, and incubated with a peroxidase-conjugated goat anti-rabbit IgG secondary for 60 min. The blots were washed in TTBS three times, incubated in Western Blotting Luminol reagent (Santa Cruz Biotechnology) and exposed on radiographic film (X-Omat AR; Eastman-Kodak, Rochester, NY). Western blot for MD-2 (Abcam, Cambridge, MA) was performed similarly except that 10% milk was used.

Quantitative RT-PCR

RNA was isolated from jejunal mucosal samples by a commercially available kit (RNeasy and Qiashredder; Qiagen, Valencia, CA), the concentration was determined spectrophotometrically, and the purity verified by electrophoresis on 1% agarose gel. cDNA was synthesized, and quantitative RT- PCR performed using a commercially available kit (RT^2 PCR Array First Strand Kit, Mouse Toll-Like Receptor Signaling Pathway Microarray RT^2 Profiler PCR Array; SuperArray Bioscience, Frederick, MD) and analyzed following the manufacturer's instructions. Data analysis was performed utilizing manufacturer's