Microvascular perfusion deficits are not a prerequisite for mucosal injury in septic rats


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Neviere, R. R., M. L. Pitt-Hyde, R. D. Piper, W. J. Sibbald, and R. F. Potter. Microvascular perfusion deficits are not a prerequisite for mucosal injury in septic rats. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G933–G940, 1999.—Our major objective was to investigate whether injury to the mucosa of the small intestine occurred in a normotensive model of sepsis and whether such injury was associated with microvascular perfusion deficits. Using fluorescence intravital microscopy, we show direct evidence of cell injury within the mucosa (pneumonia 12.4 ± 2.6 cells/field, sham 2.2 ± 0.7 cells/field), whereas use of $^{51}$Cr-labeled EDTA showed evidence of increased mucosal permeability (pneumonia 1.90 ± 0.67 ml·min$^{-1}$·100 g$^{-1}$; sham 0.24 ± 0.04 ml·min$^{-1}$·100 g$^{-1}$), 48 h following induction of pneumonia. Despite such injury the capillary density in the ileal mucosa and submucosa of pneumonic rats (1,027 ± 77 and 1,717 ± 86 mm$^{-2}$) was not significantly different compared with sham (998 ± 63 and 1,812 ± 101 mm$^{-2}$). However, a modest albeit significant decrease in capillary perfusion was measured in the muscularis layer of pneumonia (11.0 ± 1.3 mm) compared with sham (13.9 ± 0.63 mm) and appeared to be associated with leukocyte entrapment. Pretreatment using low doses of endotoxin to induce endotoxin tolerance not only increased muscularis capillary density but reduced the number of leukocytes trapped within the microvasculature, decreased myeloperoxidase activity within the ileum in pneumonic rats, and prevented mucosal injury. In conclusion, we have shown that pneumonia results in remote injury to the mucosa of the ileum and that such injury was not associated with concurrent microvascular perfusion deficits.

Changes in microcirculatory perfusion, including excessive increases in spatial and temporal flow heterogeneity, have been demonstrated in skeletal muscle (20, 23) and intestinal mucosa (9, 11, 12, 25, 30, 33) in various animal models of sepsis. The animal models used to study such circulatory derangements in sepsis have induced the syndrome either by injecting endotoxin or inducing peritonitis by cecal ligation and perforation (CLP). Whereas these models have provided important insights regarding intestinal injury, they may be inappropriate if the goal is to test the hypothesis that a distant focus of infection establishes a sequence of events that causes microcirculatory flow deficits, which lead to cellular injury in the intestine. This is a potentially important weakness because mechanisms describing remote tissue injury cannot be adequately examined if the model is complicated by either circulatory shock (as may complicate endotoxin infusion) or has the potential for direct intestinal injury (as might occur in peritonitis following CLP). Therefore, the first objective of the present study was to test the hypothesis that microvascular flow deficits occur within the small intestine in a normotensive model of sepsis, induced by a nonabdominal source of infection, and that such flow deficits either precede or occur concurrently with cell injury. We evaluated this hypothesis with particular emphasis on the mucosa of the intestine, because injury to this layer is generally believed to be critical during sepsis. We produced sepsis by inducing bacterial pneumonia in rats and used sufficient fluid resuscitation to prevent hypotension. This approach eliminated potentially important confounding variables such as circulatory shock or direct manipulation of the intestine.

Mechanisms leading to microvascular flow deficits have been investigated extensively in conditions such as ischemia-reperfusion and include microvascular compression due to tissue edema (21), endothelial cell swelling (17), or plugging of capillaries by leukocytes (7, 17). In contrast, mechanisms leading to altered microvascular perfusion in remote organs in the context of bacterial sepsis have not been as thoroughly studied. Although endotoxic shock causes leukocyte activation and accumulation within the microvasculature of the lung (34) and liver (36), concurrent hypotension in such models raises concern as to whether altered leukocyte behavior was due to the low flow state or to the effects of a remote inflammatory focus. Thus our second objective was to test the hypothesis that altered microvascular flow within the intestine during sepsis, following bacterial pneumonia, was due to leukocyte plugging of the capillary bed. To address this hypothesis, it was necessary to use an approach that

SEPSIS IS CONSIDERED a whole body inflammatory response to a focus of infection and may be characterized by multiple-organ injury (5, 6), a condition referred to as multiple-organ dysfunction syndrome (MODS). Although MODS accounts for a substantial mortality rate in intensive care units, mechanisms leading to sepsis-associated remote organ injury have not been fully elucidated. Critically ill patients are susceptible to intestinal injury, which may include changes in gut permeability and failure of intestinal defence mechanisms. It may be possible that intestinal dysfunction complicating sepsis is the consequence of mucosal injury, which follows reduced microcirculatory perfusion in this syndrome.

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reduced the potential for leukocyte accumulation within the intestine. To accomplish this, we employed endotoxin-induced tolerance because this phenomenon not only reduces the activation of circulating leukocytes (1, 2) but also dramatically reduced their accumulation within the mesenteric microcirculation (1). We found that a distant focus of infection produced by bacterial pneumonia resulted in significant cellular injury within the mucosa and that such injury was not associated with loss of perfused capillaries or leukocyte accumulation. In the muscularis, however, a significant, albeit minor, reduction in the density of perfused capillaries was measured and appeared to be a result of leukocyte plugging.

**METHODS**

**General Methods**

The protocol was approved by the Council on Animal Care at the University of Western Ontario. Male Lewis rats, weighing 325–375 g, were housed in groups of six in standard cages and supplied ad libitum with laboratory chow and water. After a 1-wk acclimatization period, the rats were entered into the study.

Experimental pneumonia and sham procedure. A bacterial culture (Pseudomonas aeruginosa) was prepared fresh daily and suspended in PBS solution [1.5 x 10^6 colony-forming units (CFU)/ml]. General anesthesia was induced by halothane inhalation, after which the right external jugular vein and the right carotid artery were cannulated with 0.25 mm Silastic tubing (Dow Corning, Midland, MI) and PE-50 tubing (Intramedic), respectively. The lines were tunneled to the back of the neck and attached to a swivel device. In the pneumonia group, the trachea was incised and animals were injected intratracheally with 0.15 ml of a solution containing 1.5 x 10^8 CFU/ml. Animals in the sham group were prepared in the same way but had intratracheal injection of 0.15 ml saline. In addition, naive rats were prepared as described but did not have either tracheal incision or injection. Postoperatively, the rats were housed separately and allowed free access to standard rat chow and water. Fluid was administered to all animals (experimental, sham, naive) by a continuous infusion of saline at 400 ml·kg⁻¹·day⁻¹ and fentanyl at 400 mg·kg⁻¹·day⁻¹. Forty-eight hours after surgery, mean systemic arterial blood pressure and respiratory rate were recorded. Arterial blood samples for blood gases and lactate analysis and complete blood count were obtained.

**Endotoxin Pretreatment Schedule.** Pretreatment using incremental low doses of endotoxin has been shown to reduce the activation of circulating leukocytes (1, 2) and leukocyte accumulation within the mesenteric microcirculation (1). Such pretreatment was used in the present study to determine if leukocytes, rendered tolerant to endotoxin, altered microvascular perfusion within the wall of the intestine following induction of bacterial pneumonia. Endotoxin-pretreated animals received daily intraperitoneal injections of increasing doses of endotoxin (Escherichia coli 026:B6, Sigma, Mississauga, ON, Canada) for 3 consecutive days. The schedule of endotoxin injection was 0.15 mg/kg on day 1, 0.30 and 0.45 mg/kg (at least 8 h apart) on day 2, and 0.60 mg/kg on day 3 (2).

**Biochemical Evaluation of Lung Injury.** At the end of each study, lungs from each animal were cannulated via the trachea, and bronchoalveolar lavage was undertaken with 15 ml of saline. Returned fluid was pooled and centrifuged (200 g for 10 min), and the cellular pellet was washed in saline. A cell count was directly performed by hemocytometer, and the total number of cells was calculated. Cellular monolayers were prepared with a cytocentrifuge and stained with Wright-Giemsa stain. Cellular morphotype differential was obtained by counting 200 cells per sample and expressing each type of cell as a percentage of the total number counted. A sample of lavage fluid supernatant from each animal was stored at -70°C, and total protein assay was performed with the Bradford protein microassay utilizing an albumin standard curve (8).

**Preparation of Intestine and Intravital Microscopy**

Forty-eight hours after surgery, rats were reanesthetized with pentobarbital sodium (20 mg/kg iv) and the animal was mechanically ventilated with an O₂-N₂ mixture (FIO₂ = 0.3). A laparotomy was performed to exteriorize the ileum, and the animal was then placed onto the microscope stage. Blood gases were analyzed to ensure sufficient oxygenation (Sao₂ >95%) and to adjust ventilation to maintain a Paco₂ of 35–45 torr. Throughout the study, anesthesia was maintained by bolus pentobarbital doses (10 mg/kg iv) and body temperature was kept at 36.5–37.5°C by use of a heat lamp.

The preparation of the intestine followed that previously described by Bohlen and Gore (4) with slight modifications. After right lateral placement of the animal onto the microscope stage, a 3- to 4-cm portion of distal ileum, 5 cm proximal to the ileocecal valve, was exteriorized using cotton-tip applicators wetted with warmed Krebs solution. The intestinal loop was placed gently into a bath containing Krebs solution, which was prepared on the surface of a transparent window on the stage of an inverted intravital microscope (Nikon Diaphot 300). The temperature of the intestine was monitored using a thermocouple catheter placed in contact with the preparation and was maintained constant at 37°C using heat lamps. Any exposed tissues were intermittently irrigated with warmed Krebs solution and were maintained at 37°C throughout the study period. Fiberoptic light guides were used to illuminate the field of view.

With use of unipolar diathermy, the bowel was opened along a segment of 3 cm at the antimesenteric border of the gut. Any residual bleeding was carefully controlled with unipolar diathermy. With the mucosa facing upward, bowel contents and mucus were gently removed from the surface of the mucosa using Krebs-wetted cotton swabs. The ileum was then inverted so that the mucosal surface was facing down on the stage and maintained flat using a balloon applicator device. The mucosa was studied in rats separate from those used for the study of the submucosa and muscularis layers. In rats used for the submucosa and muscularis the ileum was prepared in the same way as for the mucosal study, but the muscularis layer was placed onto the surface of the stage, not the mucosa. By focusing through the muscularis layer, views of the submucosa were obtained. Differentiation of the muscularis and submucosa was easily made due to the ordered arrangement of the microvasculature within the muscularis compared with the random arrangement within the submucosa. For all preparations the balloon applicator, applied on the surface of the ileum, was used to establish a flat field of view for intravital microscopy.

Leukocytes were labeled by intravenous injection of carboxyfluorescein diacetate succinimidyl ester (CFDASE, Molecular Probes, Eugene, OR) (29). CFDASE was dissolved in 0.25 ml DMSO immediately before the experiment, diluted in 2 ml of sterile water, and injected into the jugular vein over 3 min (3 mg/kg). CFDASE is a nonfluorescent precursor that diffuses into cells and forms a stable fluorochrome (carboxyfluorescein succinimidyl ester) after being catalyzed by ester-
ases, which occurs predominantly in leukocytes and platelets. After CF DASE injection, circulating leukocytes were recognized within the microvasculature by fluorogenic activity and could be distinguished from the smaller pin-point-sized platelets (29). All the preparations were allowed to stabilize for 30 min to exclude the influence of hyperemia induced by preparatory methods. Images from the inverted microscope were visualized using a camera (VE-1000CCD, Dage-MTI) connected in series to a time-date generator (WJ -810, Panasonic) and a videotape recorder (AG-1970, Panasonic). During the course of the experiment, images were recorded onto super VHS videotape for later analysis.

Microvascular flow events in the mucosa (6 villi examined for a period of 60 s in each animal), the submucosa (10 fields examined for a period of 60 s in each animal), and the muscularis (10 fields examined for a period of 60 s in each animal) were recorded at three magnifications (× 370, × 700, × 1,000). After these recordings, fluorescence microscopy was undertaken to visualize labeled leukocytes or tissue injury.

Image analysis of the recorded video images was undertaken by an investigator blinded to the experimental group. Microvascular networks in each layer of the ileum were traced onto transparent sheets and digitized for the analysis of intercapillary area (ICA, inversely related to capillary density) using Mocha software (Jandel, San Rafael, CA). To obtain absolute values of ICA in square millimeter, measurements were calibrated to a standard image of a taped microscope graticule. The density of capillaries having flowing red blood cells in the muscularis was assessed by the number of capillaries crossing three equally spaced vertical lines drawn on the video monitor. The total number of capillaries encountered with flowing red blood cells was divided by the total length of the sampling lines and expressed as the number of capillaries per millimeter. Leukocyte accumulation within the capillary bed was determined as the number of stationary leukocytes within each field of view, and the average of the total fields was taken as the estimate of leukocyte accumulation. High magnification was used to determine their location within the microvascular network.

Methods Used to Determine Intestinal Injury

Small intestine myeloperoxidase and wet-to-dry weight ratio. Tissue myeloperoxidase (MPO) activity was determined as previously described (26). Briefly, tissue from ileum was placed in PBS and homogenized. All homogenates were then centrifuged at 6,000 g for 20 min at 4°C. The pellet was homogenized and sonicated with an equivalent volume of 50 mM acetic acid (pH 6.0) containing 0.5% hexadecyltrimethylammonium hydroxide (HETOH) detergent. MPO activity was determined by measuring the hydrogen peroxide-dependent oxidation of 3,5-tetramethylbenzidine and expressed as units of optical density per gram of tissue. A portion of distal ileum was harvested and then weighed. After drying under an infrared heating lamp for 48 h, the ileum was reweighed and the wet-to-dry weight ratio was calculated.

Cell injury. Propidium iodide (0.36 mg/ml) was administered as a bolus injection (0.5 ml) via the arterial catheter and was used to estimate the number of injured cells using fluorescence intravital microscopy. The number of fluorescently labeled nuclei was counted per field of view and thus expressed as the number of injured cells per field. The use of propidium iodide has been generally accepted as a reliable method of identifying severely injured cells. It is a fluorescent nuclear dye (excitation 520 nm, emission 610 nm), which will gain access to and label the nuclei of lethally injured cells only (15).

Functional injury: ileal epithelial cell permeability. The blood-to-lumen clearance of 51Cr-labeled EDTA was measured in the distal portion of the ileum 48 h following induction of pneumonia (n = 5), in sham rats (no pneumonia, n = 4), and in endotoxin-pretreated pneumonia rats (n = 4). Briefly, a laparotomy was performed, and a loop of the distal ileum (10 cm) was isolated with blood vessels intact. In all animals, both renal pedicles were ligated to prevent excretion of 51Cr-EDTA. Inflow and outflow tubes were secured on each end of the ileal segment, and warm buffered Krebs-Henseleit solution (pH 7.4) was perfused through the segment at a rate of 1 ml/min. The intestinal segment and abdominal contents were kept moist with Krebs-Henseleit-soaked gauze and covered with a clear plastic sheet to minimize evaporation and tissue dehydration. The rat then received 100 µCi of 51Cr-EDTA intravenously. Thirty minutes were allowed for tissue equilibration of 51Cr-EDTA before samples of lumen perfusate were collected (every 10 min). Plasma samples (0.5 ml) were obtained immediately before collection of the lumen perfusate and each 30 min for 60 min. Samples were analyzed for 51Cr-EDTA using a gamma counter. At the end of the experiment, the distal ileal loop was removed, rinsed, and weighed. The plasma-to-lumen clearance of 51Cr-EDTA was calculated as clearance (ml·min⁻¹·100 g⁻¹) = cpm perfusate × perfusion rate × 100/cpm plasma × weight of intestinal segment, where cpm is counts per minute per milliliter. The value of 51Cr-EDTA clearance in each animal was based on the mean value of the luminal perfusate samples collected during the 1-h study period.

Experimental Protocol

A total of 64 rats was used in this study and assigned to either sham (n = 9), naive (n = 20), pneumonia (n = 22), or endotoxin-pretreated pneumonia (n = 13). Because results obtained from sham and naive rats were not statistically different, these groups were combined and are referred to as sham. For intravital microscopy a total of 51 rats was used to measure capillary density and leukocyte accumulation in the mucosa (sham 14, pneumonia 9, endotoxin pretreated 3), and muscularis (sham 11, pneumonia 8, endotoxin pretreated 6) of the small intestine (ileum). In seven rats used for the muscularis we also measured perfusion in the submucosa. Of the total rats used for intravital microscopy of the mucosa, 11 rats were also used for direct estimation of cell injury. A total of 13 rats was used to measure mucosal permeability, using 51Cr-EDTA (sham 4, pneumonia 5, endotoxin pretreatment 4). Although a sample size of three and four appears small for the effect of endotoxin pretreatment, the power of the statistical tests was greater than 80% and thus did not justify the use of additional animals.

Statistics

Data are expressed as means ± SE. The data were analyzed using standard statistical procedures, i.e., ANOVA with Scheffé’s post hoc test for data sets that were normally distributed, and the Kruskal-Wallis test was used with a secondary multiple comparison procedure for data sets that were not normally distributed (Sigma Stat 1.0, Jandel). Statistical significance was set at P < 0.05.

RESULTS

Pneumonia Model

Forty-eight hours following the induction of pneumonia, all of the pneumatic rats demonstrated reduced activity, piloerection, and exudation around the eyes...
and nose. In contrast, sham animals demonstrated full recovery from anesthesia and surgery with no detectable sign of disease. Pneumonia, macroscopically estimated at postmortem, involved 36% of the lung in the pneumonia group, whereas sham rats showed no signs of this lesion. Neutrophil differential count and protein concentration in bronchoalveolar lavage were increased in pneumonia animals compared with sham animals, confirming a local inflammatory response (Table 1). Blood cultures were positive (Pseudomonas aeruginosa) in 64% of pneumonia animals. Blood cultures were negative in all but one sham animal, which grew a gram-positive cocci. Physiological and hematological results from sham and pneumonia animals are summarized in Table 2.

Effects of Endotoxin Pretreatment

Compared with pneumonia, endotoxin pretreatment had no effect on the behavior of the rats, the severity of pneumonia (estimated macroscopically), or the percentage of positive blood cultures. Bronchoalveolar lavage results from endotoxin-pretreated and pneumonia rats are summarized in Tables 1 and 2.

Mucosal Injury

A significant increase in the number of propidium iodide labeled nuclei was measured in the mucosa of pneumonia (12.4 ± 2.6 cells/field) compared with sham (2.2 ± 0.7 cells/field) rats. However, after endotoxin pretreatment the number of injured cells in pneumonic rats was indistinguishable from the sham rats (Fig. 1A).

Table 1. Bronchoalveolar lavage results in sham, pneumonia, and endotoxin-pretreated pneumonia animals

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Pneumonia</th>
<th>LPS Pneumonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleated cell count, 10⁶/l</td>
<td>5.2 ± 1.5</td>
<td>16.6 ± 2.3*</td>
<td>16.6 ± 2.3*</td>
</tr>
<tr>
<td>Neutrophils, 10⁶/l</td>
<td>1.2 ± 0.5</td>
<td>10.7 ± 5.3*</td>
<td>10.5 ± 3.9*</td>
</tr>
<tr>
<td>Protein, mg/l</td>
<td>333 ± 52</td>
<td>543 ± 104*</td>
<td>475 ± 108*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 14 rats (sham), 17 rats (pneumonia), 9 rats (LPS pneumonia). Lipopolysaccharide (LPS) pneumonia, endotoxin-pretreated pneumonia. *P < 0.05 compared with sham.

Table 2. Physiological and hematological results in sham, pneumonia, and endotoxin-pretreated pneumonia animals

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Pneumonia</th>
<th>LPS Pneumonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mmHg</td>
<td>120 ± 4</td>
<td>116 ± 4</td>
<td>101 ± 8</td>
</tr>
<tr>
<td>RR, breaths/min</td>
<td>75 ± 5</td>
<td>75 ± 3</td>
<td>77 ± 4</td>
</tr>
<tr>
<td>Leukocytes, 10⁶/l</td>
<td>5.65 ± 0.59</td>
<td>3.25 ± 0.40*</td>
<td>2.77 ± 0.72*</td>
</tr>
<tr>
<td>Neutrophils, 10⁶/l</td>
<td>2.02 ± 0.29</td>
<td>0.79 ± 0.14*</td>
<td>1.13 ± 0.48*</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>0.67 ± 0.28</td>
<td>0.84 ± 0.22</td>
<td>1.55 ± 1.25</td>
</tr>
<tr>
<td>pH</td>
<td>7.44 ± 0.01</td>
<td>7.45 ± 0.02</td>
<td>7.42 ± 0.03</td>
</tr>
<tr>
<td>Pao₂, Torr</td>
<td>81 ± 3</td>
<td>75 ± 4</td>
<td>65 ± 5</td>
</tr>
<tr>
<td>Pac₂, Torr</td>
<td>37 ± 2</td>
<td>37 ± 2</td>
<td>40 ± 5</td>
</tr>
<tr>
<td>HCO₃, mmol/l</td>
<td>25 ± 1</td>
<td>25 ± 1</td>
<td>25 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 14 rats (sham), 17 rats (pneumonia), 9 rats (LPS pneumonia). MAP, mean arterial pressure; RR, respiratory rate; Pao₂, oxygen arterial partial pressure; Pac₂, carbon dioxide partial pressure; HCO₃, arterial bicarbonate. *P < 0.05 compared with sham.

Fig. 1. Average number of injured (propidium iodide labeled) cells within mucosa (A) and permeability of mucosa to ^⁵¹Cr-EDTA (B) show that mucosal injury occurs as a result of pneumonia, whereas endotoxin (lipopolysaccharide, LPS) pretreatment prevented such injury. Values are expressed as means ± SE. *P < 0.05 compared with sham. #P < 0.05 compared with pneumonia.

The plasma-to-lumen clearance of ^⁵¹Cr-EDTA was significantly increased (P = 0.02) in pneumonia rats (1.90 ± 0.67 ml·min⁻¹·100 g⁻¹) compared with sham (0.24 ± 0.04 ml·min⁻¹·100 g⁻¹). However, after endotoxin pretreatment, the plasma-to-lumen clearance was significantly reduced in pneumonia rats (0.50 ± 0.17 ml·min⁻¹·100 g⁻¹, P = 0.04) compared with the untreated group (Fig. 1B) but failed to return to sham levels (0.50 ± 0.17 vs. 0.24 ± 0.04 ml·min⁻¹·100 g⁻¹, P = 0.01).

Microvascular Perfusion and Leukocyte Accumulation

No significant differences were measured between the sham and naive rats when comparing either the...
ileal mucosa or muscularis, nor was there any differences in any of the physiological parameters measured. Thus these groups were combined and used as the shams for comparison to pneumonia and endotoxin-pretreated pneumonia groups.

The densities of capillaries with flowing red blood cells in the ileal mucosa and submucosa of sham (998 ± 63 and 1,812 ± 101 µm², respectively) and pneumonia rats (1,027 ± 77 and 1,717 ± 86 µm², respectively) were not significantly different (Fig. 2, A and B). However, a small (15–20%) but significant decrease in the number of perfused capillaries was measured in the muscularis layer of pneumonia (11.0 ± 1.3 mm) compared with sham (13.9 ± 0.63 mm) rats (Fig. 2C). Endotoxin pretreatment prevented the small but significant decline in the number of perfused capillaries within the muscularis, while having no effect on perfusion in the mucosa (Fig. 2, A and C). The effect of endotoxin tolerance was not studied in the submucosa.

MPO activity was greater in the ileum of pneumonia animals compared with sham (Fig. 3). However, no difference was measured in ileum wet-to-dry weight ratio between sham (3.24 ± 0.22) and pneumonia rats (2.82 ± 0.16). Intravital microscopy showed increased accumulation of stationary leukocytes in the microvasculature of the muscularis layer in pneumonia rats compared with sham (Fig. 4A), whereas a decrease in leukocyte accumulation occurred in the mucosal layer (Fig. 4B). Leukocytes in the muscularis layer were located within capillaries (Fig. 5) and were always associated with the absence of red blood cell flow. Endotoxin pretreatment decreased MPO activity within the ileum compared with pneumonia rats (Fig. 3) and prevented the accumulation of leukocytes within the muscularis layer (Fig. 4A).

**DISCUSSION**

Using intravital video microscopy, we provided evidence of a significant increase in the number of injured cells within the mucosa, 48 h following the induction of bacterial pneumonia. In addition to such direct measures, we showed evidence of functional mucosal injury in the form of significantly increased permeability.
However, such injury occurred in the absence of concurrent microvascular perfusion deficits. In fact, our results showed that the capillary density in the mucosa and submucosa of sham and pneumonia animals was similar. The only evidence of microvascular perfusion abnormalities occurred in the muscularis layer, which showed evidence of a minor, albeit significant, decrease in the number of perfused capillaries compared with sham. Pretreatment with low doses of endotoxin, to induce endotoxin tolerance, not only preserved the functional capillary density (number of capillaries perfused with red blood cells) within the muscularis layer but reduced leukocyte accumulation and completely prevented cell injury within the mucosa.

**Experimental Model and Rationale**

Based on the physiological parameters measured in this and our previous study (12), the degree of sepsis induced by bacterial pneumonia, or CLP, are comparable. The only significant difference between models appears to be the temporal expression of such symptoms (i.e., 24 h following CLP compared with 48 h following induction of pneumonia). Thus the severe derangements in microcirculatory flow we had previously noted in the CLP model (12) may, in part, be the result of direct injury induced by the infected peritoneal fluid and/or manipulation of the bowel during CLP.

It is often argued that microcirculatory flow deficits leading to hypoxic stress represent one of the initiating events leading to tissue injury during sepsis. In the case of the intestine, such injury is believed to result in increased mucosal permeability (13, 16, 30, 31, 33). In this context, the hypothesis that mucosal injury is followed by abnormally excessive translocation of bacteria and/or endotoxin during sepsis was not tested in this study. However, our study does provide direct evidence of significant cell injury, which was accompanied by significantly increased permeability of the mucosa. Because we found no change in the number of perfused capillaries and significantly reduced leukocytes within the microvasculature of the mucosa following induction of pneumonia, it seems reasonable to suggest that such mucosal injury would not likely be a consequence of either the vascularly delivered leukocytes or oxygen deprivation. However, we cannot exclude the possibility that oxidant injury may occur either from the resident (i.e., extravascular) leukocytes within the mucosa or as a direct consequence of the maintained microvascular perfusion. Such possibilities await future study.

The mucosal and muscularis layers of the intestine constitute two parallel vascular compartments that
may respond differently to various stimuli, including endotoxin infusion (24, 27, 28). Although not directly tested, the possibility remains that the minor flow deficits within the muscularis layer may have resulted in a redistribution of blood flow to the mucosa. The possibility of such altered distribution of blood flow between the layers of the small intestine has been reported in septic models (24).

Our study showed evidence of increased leukocyte accumulation within the intestine (MPO assay) following pneumonia, and the use of intravital microscopy suggested that such accumulation was likely restricted to the muscularis layer. To determine the role of leukocytes in the altered microvascular perfusion within the muscularis layer, it was necessary to either reduce leukocyte-endothelial cell interactions, reduce their activation, or remove leukocytes from the circulation. To accomplish the latter, we undertook pilot experiments with anti-neutrophil serum but found that use of such an intervention in combination with bacterial pneumonia resulted in unacceptably high mortality rates (90–95%). Such results might be expected because anti-neutrophil serum lowers the circulating levels by increasing the sequestration of such cells in the lung, liver, and spleen. Pneumonia is a pathological process, which results in the extensive accumulation of leukocytes within the lung, and thus the addition of anti-neutrophil serum likely exacerbates the already compromised lung.

Previous studies had shown that pretreatment with low doses of endotoxin reduced leukocyte activation (1, 2, 35), adhesion to the microvascular endothelium of the mesentery (1, 2), and entrapment within capillaries in experimental myocardial infarct (2, 35). Thus we employed this technique to depress leukocyte accumulation within the intestine in our model. Endotoxin pretreatment not only preserved the functional capillary density within the muscularis layer but reduced leukocyte sticking and accumulation. Although such evidence suggests that leukocytes may play an active (direct) role in the microvascular perfusion anomalies within the muscularis layer during sepsis, it is also possible that their entrapment reflects low-flow conditions induced by other factors such as swollen endothelial cells.

The role of leukocytes in the loss of capillary perfusion in models of ischemia and reperfusion remains controversial. Studies have reported leukocyte entrapment within capillaries of skeletal muscle following ischemia and reperfusion (14). However, more recent studies suggested that leukocyte entrapment within capillaries was not statistically correlated to the loss of capillary perfusion following ischemia (29). Our results suggest a cause-and-effect relationship between leukocyte entrapment and loss of capillary perfusion within the muscularis layer of the small intestine during sepsis. However, the benefits afforded endotoxin tolerance are not limited to reduced leukocyte activation and accumulation within the intestine, and thus use of more selective interventions await further study.

In addition to reducing the microvascular perfusion deficits and accumulation of leukocytes within the muscularis layer, endotoxin pretreatment also prevented the incidence of mucosal injury (both direct cellular injury and permeability). Such injury was not mediated by an increase in vascularly delivered leukocytes because no increase in leukocyte accumulation was measured within the mucosa. However, endotoxin tolerance has been shown to provide protection not only against the injuries induced by administration of high doses of endotoxin (10) but to reactive oxygen metabolites (3). The mechanisms leading to mucosal injury and the protection afforded by preadministration of endotoxin following a distant focus of infection await further study.

In conclusion, we have provided direct evidence of significant mucosal injury 48 h following induction of bacterial pneumonia and the fact that such injury occurred in the absence of concurrent microvascular perfusion deficits. Such results suggest that microvascular perfusion deficits cannot be considered a general prerequisite for remote organ injury during sepsis. In addition, we showed that induction of endotoxin tolerance not only reduced leukocyte accumulation within the intestine but was an effective means of preventing mucosal injury during sepsis.

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