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Peritoneal fluid: A potential mechanism of systemic neutrophil priming in experimental intra-abdominal sepsis

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Abstract

Background—Recent studies suggest that peritoneal fluid (PF) may be an important mediator of inflammation. We hypothesize that PF may drive systemic inflammation in intra-abdominal sepsis by representing a priming agent for neutrophils.

Methods—PF was collected 12 hours after initiation of intra-abdominal sepsis in swine. Naïve human neutrophils were primed with PF prior to treatment with *N*-formyl-met-leu-phe or phorbol 12-myristate 13-acetate to elucidate receptor dependent and independent mechanisms of neutrophil activation. Flow cytometry was used to quantify neutrophil surface adhesion marker expression of integrins and selectins and superoxide anion production. Additionally, pro-inflammatory cytokines were quantified in PF.

Results—PF primed neutrophils via receptor dependent and independent mechanisms. There were significant increases in the pro-inflammatory cytokines IL-6 and TNF-alpha in PF correlating with the development of intra-abdominal sepsis.

Conclusions—PF represents a priming agent for naïve PMN's in intra-abdominal sepsis. This may be secondary to increased levels of pro-inflammatory cytokines. Strategies to reduce the amount of PF may decrease the systemic inflammatory response by reducing a priming agent for neutrophils.

MeSH Keywords

Trauma; resuscitation; abdomen; white blood cells; neutrophil activation; intra-abdominal sepsis

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Introduction

Care of the trauma patient with multiple injuries is complicated by a variety of pathological processes including hemorrhagic shock, crystalloid resuscitation, and regional ischemia/reperfusion. Intra-abdominal sepsis from intestinal injury may add to this picture. Translocation of fluid into the peritoneal cavity is often a consequence of these factors and is compounded by decreased plasma protein (hemodilution) and factors affecting capillary permeability (i.e., hemorrhagic shock/resuscitation leading to global ischemia/reperfusion injury).

The biological activity of peritoneal fluid is not well characterized. Previous research has indicated that peritoneal fluid collected from burn patients who were undergoing resuscitation to contain increased levels of pro-inflammatory cytokines¹ and that trans-serosally derived peritoneal fluid accumulated from the small intestine during repair of abdominal aortic aneurysms increased polymorphonuclear (PMN) cell expression of CD11b.² More recently, our group has demonstrated that peritoneal fluid from a non-infectious model of abdominal compartment syndrome may contribute to the inflammatory response by serving as a primer of naïve neutrophils.³

Peritoneal fluid can be reabsorbed into the circulation predominantly via lymphatic conduits allowing for a plausible explanation of systemic effects of this fluid.⁴ This pathway becomes even more important in cases of significant intra-abdominal inflammation (as in intra-abdominal sepsis) where the inflammatory response may accentuate the reabsorption of peritoneal fluid.

In this study, we hypothesized that peritoneal fluid from an intra-abdominal sepsis model may contribute to systemic inflammation by serving as a priming agent for naïve neutrophils. To that end, we interrogated the PMN cell response to peritoneal fluid from a two hit model of intra-abdominal sepsis, and tested the ability of this fluid to prime naïve neutrophils as determined by surface marker expression of integrins and selectins and superoxide production.

Materials and Methods

All procedures were approved by the University of Texas Health Science Center at Houston and Surpass, Inc. (Osceola, WI) Institutional Animal Care and Use Committee and were consistent with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

Intra-abdominal Sepsis Model

A two hit model of intra-abdominal sepsis as developed by Steinberg et al. was utilized for all experiments.⁵ Female swine (30-40 kilograms, n=10) were fasted overnight (12-16 hours) with free access to water. General anesthesia was induced by ketamine (20 mg/kg intramuscular injection), atropine (0.04 mg/kg intramuscular injection) and xylazine (2 to 3 mg/kg intramuscular injection). The animals were intubated and ventilated with a fraction of inspired oxygen (FiO₂) of 21%, positive end expiratory pressure (PEEP) of 5 mmHg, and a tidal volume of 10 cc/kg. Catheters were placed into the left common carotid artery and right internal jugular vein for hemodynamic monitoring and fluid infusion, respectively. A Swan Ganz pulmonary artery thermodilution catheter was placed into the right internal jugular vein and positioned into the pulmonary artery using flow direction. Additional instrumentation consisted of placing a suprapubic bladder catheter.

A midline laparotomy was then performed. The superior mesenteric artery was identified and dissected free of mesenteric attachments. A clamp was placed on the superior mesenteric artery for 30 minutes to induce intestinal ischemia. During the ischemia period, the cecum was identified and exteriorized. An enterotomy was made and 0.5 cc/kg of feces was collected. Additionally, 2 cc/kg of blood was obtained from the animal. The collected feces and blood were combined to form a fecal blood clot. The enterotomy was closed and the cecum was returned to the abdominal cavity. The fecal blood clot was implanted in the right lower quadrant. A catheter was placed into the left paracolic gutter and exteriorized for the collection of peritoneal fluid. The abdomen was then closed and the animals were allowed to recover. During the surgical period, the animals received one liter of 0.9% normal saline. Additionally, at 6 and 12 hours post-injury, the animals received intravenous injection of ampicillin (2 grams) and metronidazole (500 milligrams). To confirm development of organ dysfunction following the experimental protocol, various physiological and laboratory parameters were monitored, including mean arterial pressure (MAP), cardiac output (CO), cardiac index (CI), change in FiO₂ settings, base excess, blood urea nitrogen (BUN), and urine output.

Peritoneal fluid was collected at 12 hours post surgical instrumentation per the following protocol. The peritoneal fluid catheter was flushed with forty milliliters of warm sterile saline prior to collection. After one minute, the fluid was withdrawn and collected. The fluid was centrifuged at 3500 rpm (15°C) for 10 minutes and the supernatant was flash frozen and subsequently stored at -70°C or below until time of experimentation.

An additional 6 animals were used as negative controls and served as baseline. These animals underwent a laparotomy and peritoneal fluid was collected immediately upon opening of the peritoneal cavity. The fluid was flash frozen and stored at -70°C or below until time of experimentation.

Materials

N-formyl-met-leu-phe (fMLP) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (St. Louis, MO). Phosphate buffered saline (PBS) was obtained from Mediatech, Inc (Manassas, VA). Dihydroethidium (HE) was obtained from Invitrogen Corporation (Carlsbad, CA). Allophycocyanin (APC) conjugated CD11b, phycoerythrin (PE) conjugated CD18, and fluorescein (FITC) conjugated CD62L antibodies (and corresponding isotype controls) optimized for flow cytometry were obtained from Biolegend (San Diego, CA). Human neutrophils were isolated as previously described.^{6, 7}

Experimental Design

Peritoneal fluid was evaluated as a potential priming agent for PMNs via receptor dependent and receptor independent pathways of neutrophil activation. To accomplish this objective, we evaluated the response of naïve human neutrophils to fMLP (receptor dependent) and PMA (receptor independent) after priming with peritoneal fluid. Activation was determined by superoxide anion production as well as surface marker expression of integrins (CD11b and CD18) and selectins (CD62L). In addition, we determined whether the concentration of pro-inflammatory cytokines (IL-6 and TNF- α) changed relative to baseline.

Neutrophil Activation Assay

Stock solutions of HE (10 mM), fMLP (10 mM) and PMA (10 mg/ml) were prepared in dimethyl sulfoxide and were aliquotted and stored at -20°C until time of use.

Superoxide Production

Superoxide anion production was determined as the conversion of HE to ethidium bromide. All reactions were done in a 96-well plate with a total reaction volume of 200 μ l. Each well (reaction) contained approximately one million cells. HE (final concentration 10 μ M) was added to all wells except for blank wells (true negative control) and allowed to incubate for 15 minutes in the dark at 37°C. After the incubation period, peritoneal fluid (10 μ l, end concentration 5% total assay volume) was added to wells to test the priming capabilities of peritoneal fluid. The samples were allowed to incubate for 5 minutes in the dark at 37°C. Subsequently, fMLP (final concentration 1 μ M) or PMA (final concentration 200 ng/ml) were added to wells incubated only with HE and peritoneal fluid. The samples were allowed to incubate for 20 minutes in the dark at 37°C. At the conclusion of the incubation period, the plate was immediately removed and placed on ice for 10 minutes in the dark to stop the reaction prior to analysis by flow cytometry. A sample size (n) of 10 (i.e., peritoneal fluid from 10 different animals) was used for all intra-abdominal sepsis animals and an n of 6 was utilized for all control animals (referred to as baseline). Of note, the doses for fMLP and PMA are based on previous, published literature.^{6, 8, 9}

Surface Marker expression of CD62L, CD11b, and CD18

All reactions were done in a 96-well plate with a total reaction volume of 200 μ l. Each well (reaction) contained approximately 1×10^6 cells. Peritoneal fluid (10 μ l, end concentration 5% total assay volume) was added to wells to test the priming capabilities of peritoneal fluid. The samples were allowed to incubate for 5 minutes in the dark at 37°C. Subsequently, fMLP (final concentration 1 μ M) or PMA (final concentration 200 ng/ml) were added to the experimental wells. The samples were allowed to incubate for 20 minutes in the dark at 37°C. At the conclusion of the incubation period, the plate was immediately removed and placed on ice for 10 minutes in the dark to stop the reaction. Subsequently, APC conjugated CD11b, PE conjugated CD18, and FITC conjugated CD62L were added to all experimental wells as per manufacturers suggested protocol. APC, PE, and FITC isotype controls were added to designated isotype control wells. The samples were then incubated for 30 minutes protected from light prior to analysis by flow cytometry. An n of 10 (i.e., peritoneal fluid from 10 different animals) was used for all intra-abdominal sepsis animals and an n of 6 was utilized for all control animals (i.e., baseline). Of note, the doses for fMLP and PMA are based on previous, published literature.^{6, 8, 9}

Flow Cytometry Analysis

All flow cytometry was performed on an LSR II flow cytometer (BD Biosciences, San Jose, CA) using the high throughput sampler (BD Biosciences, San Jose, CA). The PMN population was gated using the forward scatter and side scatter plot and the mean fluorescent intensity (MFI) for each sample was recorded. For each sample, 5,000 events were collected for analysis. Analysis was performed using FACSDiva software (BD Biosciences, San Jose, CA). All values were normalized to peritoneal fluid protein concentration as determined by the bicinchoninic acid (BCA) protein assay.

IL-6/TNF- α ELISA

Levels of IL-6 and TNF- α were measured in peritoneal fluid at baseline and at time of decompression using a commercially available ELISA kit as per manufacturers suggested protocol (R&D Systems, Minneapolis, MN).

Statistical Analysis

Unless otherwise mentioned, all values are represented as mean $\pm$ SEM. Statistical comparisons were made using an un-paired two tailed t-test. A p-value of <0.05 was used to denote statistical significance.

Results

Model Related Organ Injury

Model related organ injury in instrumented swine was manifested by several parameters. All values are presented at baseline followed by the value 12 hours after initiation of the intra-abdominal sepsis protocol. Cardiovascular dysfunction was evidenced by significantly decreased MAP (101 ± 6 to 53 ± 2 mmHg, $p<0.0001$), CO (3.09 ± 0.20 to 1.73 ± 0.14 L/min, $p<0.0001$), and CI (3.02 ± 0.20 to 1.71 ± 0.15 , $p<0.0001$). There was a significant increase in FiO_2 from baseline ($21 \pm 0\%$) to 12 hours after protocol completion ($27 \pm 1\%$, $p<0.0001$). Renal dysfunction was manifested by a significant decrease in urine output when comparing the first three hours post protocol completion (7.17 ± 1.28 cc/kg/hr) as compared to the three hours immediately prior to peritoneal fluid collection (1.16 ± 0.12 cc/kg/hr, $p=0.0006$). In addition, there were significant decreases in base excess (7.8 ± 1.0 to 2.3 ± 1.6 mmol/L, $p=0.002$; also an indicator of non-respiratory acid-base balance) and significant increases in BUN (8.3 ± 0.6 to 20.3 ± 1.7 mg/dL, $p<0.0001$). Based on these parameters, at 12 hours, the instrumentation induced organ injury consistent with developing gut ischemia/reperfusion and intra-abdominal sepsis. We therefore utilized peritoneal fluid collected at this timepoint for all downstream analyses as described below.

Receptor Dependent Neutrophil Activation

Superoxide production

When peritoneal fluid from the intra-abdominal sepsis animals was utilized as a primer for fMLP stimulated activation, there was a significant increase in neutrophil activation as measured by superoxide anion production. Superoxide anion production was significantly increased ($p=0.007$) when peritoneal fluid from the intra-abdominal sepsis group was used as a primer (29.32 ± 1.55) as compared to peritoneal fluid collected at baseline (3.90 ± 0.32) (Figure 1). Values are presented as mean fluorescent intensity per mg protein.

CD62L, CD11b, and CD18 surface marker expression

When peritoneal fluid from the intra-abdominal sepsis animals was utilized as a primer for fMLP stimulated activation of PMNs, there was a significant increase in neutrophil activation as measured by cell surface marker expression of CD11b and CD18. Expression of CD11b significantly increased (0.15 ± 0.01 versus 1.35 ± 0.24 , $p=0.001$, baseline versus intra-abdominal sepsis sample) (Figure 2). CD18 expression was also significantly increased (0.42 ± 0.03 versus 3.17 ± 0.58 , baseline versus intra-abdominal sepsis sample, $p=0.002$) (Figure 2). Expression of CD62L was not decreased (0.06 ± 0.00 versus 0.15 ± 0.02 , baseline versus intra-abdominal sepsis sample). Values are presented as mean fluorescent intensity per mg protein.

Receptor Independent Neutrophil Activation

Superoxide production

When peritoneal fluid from the intra-abdominal sepsis animals was utilized as a primer for PMA stimulated activation of neutrophils, there was a significant increase in activation as measured by superoxide anion production. Superoxide anion production was significantly increased ($p=0.004$) when peritoneal fluid from the intra-abdominal sepsis group was used

as a primer (292.41 ± 54.72) as compared to peritoneal fluid collected at baseline (28.05 ± 6.11) (Figure 1). Values are presented as mean fluorescent intensity per mg protein.

CD62L, CD11b, and CD18 surface marker expression

We next examined the effect of peritoneal fluid as a primer for PMA induced neutrophil activation. There were significant increases in CD11b (0.29 ± 0.01 versus 2.72 ± 0.47 , $p=0.001$, baseline versus intra-abdominal sepsis sample) and CD18 (0.84 ± 0.05 versus 7.05 ± 1.2 , $p=0.002$, baseline versus intra-abdominal sepsis sample) (Figure 3). Similar to fMLP, expression of CD62L was not decreased when peritoneal fluid from the intra-abdominal sepsis was used as a primer for PMA induced neutrophil activation (0.04 ± 0.00 versus 0.16 ± 0.03 , baseline versus intra-abdominal sepsis sample). Values are presented as mean fluorescent intensity per mg protein.

Quantification of IL-6 and TNF- α in Peritoneal Fluid

To examine a potential mechanism for neutrophils priming, we evaluated whether there was a change in concentration of pro-inflammatory cytokines (IL-6 and TNF- α) from baseline peritoneal fluid to that collected from the intra-abdominal sepsis animals. Significant increases in IL-6 (3.16 ± 0.27 to 21.89 ± 7.34 ng/mg, $p=0.01$) and TNF- α (1.89 ± 0.16 to 3.62 ± 0.30 ng/mg, $p<0.0001$) were noted in peritoneal fluid from septic swine (Figure 4).

Discussion

Our data demonstrate that peritoneal fluid from a two-hit model of intra-abdominal sepsis serves as a priming agent for receptor dependent and independent pathways for activation in naïve PMN's as measured by increases in CD11b and 18 and superoxide production. Although speculative, one mechanism for the peritoneal fluid-induced priming of PMNs observed may be the increased levels of pro-inflammatory cytokines (IL-6 and TNF- α) found in the peritoneal fluid from intra-abdominal sepsis animals. Cytokines, including IL-6 and TNF- α , have previously been shown to prime inflammatory cells and mediate subsequent tissue injury.¹⁰

We utilized derivatives of two species for the neutrophil activation assay, i.e., we tested the effect of peritoneal fluid derived from a swine intra-abdominal sepsis model for ability to prime naïve human PMN's. Our decision for the use of human neutrophils in the assay is based on several factors. Sarin et. al. demonstrated in previous work that PMN's derived from swine or humans behave similarly when exposed to potential stimulants (i.e., mesenteric lymph and isolated components) from swine.⁹ Furthermore, peritoneal fluid and mesenteric lymph are similar in makeup, i.e., the major content is gut-derived interstitial fluid. Assays for the evaluation of superoxide production and changes in surface marker expression in human neutrophils have been extensively studied and characterized. However, as an internal experimental control for testing porcine biological fluids following injury, we utilized peritoneal fluid collected at baseline to exclude any baseline effects of porcine derived fluid on human neutrophils.

The exact component(s) of peritoneal fluid that lead to neutrophil priming is currently unknown. Increased levels of pro-inflammatory cytokines have been shown to be present with injury in human and animal studies, leading to our hypothesis that these cytokines may account for the observed effect of peritoneal fluid on neutrophil priming. Additionally, increased peritoneal fluid levels of TNF- α and IL-6 were associated with death in an animal model of peritonitis.¹¹ Although the concentration of IL-6 and TNF- α were small (ng) in our model, other investigators have demonstrated alterations in neutrophil biology at similar

concentrations.¹² Further mechanistic experiments are necessary, however, to determine if these (or other) cytokines are responsible for neutrophil priming.

Inflammation is an important stimulant to the reabsorption of peritoneal fluid into the systemic circulation.⁴ The major pathway for peritoneal fluid uptake into the systemic circulation is via lymphatic conduits in the peritoneal wall and diaphragm.¹³ In this study, we present evidence that peritoneal fluid from an intra-abdominal sepsis model may serve as a pro-inflammatory stimulus by acting as a primer for naïve neutrophils. In the setting of intra-abdominal inflammation and especially when the initiating focus is of an intra-abdominal nature, peritoneal fluid may serve as an initiator and/or propagator of a subsequent systemic inflammatory response. Recent work supports the notion that peritoneal fluid may serve as a propagator of neutrophil priming even after acute treatment. Decompressive laparotomy after development of abdominal compartment syndrome in pigs has been shown not to decrease the ability of peritoneal fluid to prime naïve neutrophils when measured at up to 12 hours post decompression.³ More detailed time course studies are required to determine the exact role of peritoneal fluid in propagation of the inflammatory response.

Gut-derived lymph has been shown to result in distant lung injury, and lymphatic diversion (at the level of the thoracic duct) has been shown to ameliorate this injury, presumably by preventing the systemic release of pro-inflammatory mediators.¹⁴ Our results suggest that peritoneal fluid may serve to increase the pro-inflammatory characteristics of abdominal cavity-derived lymph secondary to its major route of uptake via diaphragmatic and peritoneal cavity lymphatics. Serving to corroborate this hypothesis is preliminary data from Kubiak et al. Utilizing the animal model as described, abdominal negative pressure therapy was shown to prevent the development of lung injury, as demonstrated by decreased atelectasis, alveolar fibrin, alveolar red and white blood cells, and alveolar thickness. In addition, negative pressure therapy was associated with decreases in leukocytes in kidney and liver.^{15, 16} The results presented here suggest that one possible explanation for these observations is that augmented removal of peritoneal fluid decreases the pro-inflammatory characteristics of abdominal-activity derived lymph resulting in decreased lung injury. Additionally, it suggests the importance of leukocytes in abdominal sepsis induced organ injury.

We chose to use the described model of intra-abdominal sepsis for several reasons. It is a validated model of intra-abdominal sepsis that provides a platform for translational large animal pre-clinical research. The major endpoint produced by the model is lung injury. One of our major research interests is in neutrophil priming/distant organ injury. Given the multitude of data suggesting a role of gut derived lymph in lung injury and our hypothesis that peritoneal fluid may be a component of intra-abdominal derived lymph reaching the lungs, this model provided us with the opportunity to see if peritoneal fluid derived from this model led *in vitro* to neutrophil priming/activation as has been shown by gut derived lymph. Also suggesting to corroborate our results and providing further evidence of this link is data described above by Kubiak et al demonstrating that use of negative pressure therapy in this model leads to amelioration of lung injury.

Primed neutrophils may make patients more susceptible to a second hit injury, including, for example, an exaggerated response to a subsequent operation.^{17, 18} Multiple investigators have demonstrated that post-shock mesenteric lymph and its components serve as priming agents for neutrophils.^{9, 19} We have demonstrated that peritoneal fluid may additionally serve as a continued priming and activating stimulus for neutrophils. Whether this finding translates into the clinical scenario where early removal of inflammatory ascites

downregulates the local and/or systemic inflammatory response or alters the pro-inflammatory characteristics of mesenteric lymph is the subject of future investigation.

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Figure 1.

Peritoneal fluid from an intra-abdominal sepsis model primes neutrophils in both receptor dependent (fMLP) and receptor independent (PMA) manners as evidenced by increased superoxide production. Results are demonstrated $\pm$ standard error of the mean. (* $p < 0.05$ versus baseline; $n = 6$ (baseline) or $n = 10$ (intra-abdominal sepsis)/group; MFI=mean fluorescence intensity)

Figure 2.

When examining the receptor dependent pathway, peritoneal fluid from an intra-abdominal sepsis animal model primes neutrophils as evidenced by increased CD11b and CD18 surface marker expression. Results are demonstrated $\pm$ standard error of the mean. (* $p < 0.05$ versus baseline; $n = 6$ (baseline) or $n = 10$ (intra-abdominal sepsis)/group; MFI=mean fluorescence intensity)

Figure 3.

When examining the receptor independent pathway, peritoneal fluid from an intra-abdominal sepsis animal model primes neutrophils as evidenced by increased CD11b and CD18 surface marker expression. Results are demonstrated $\pm$ standard error of the mean. (* $p < 0.05$ versus baseline; $n = 6$ (baseline) or $n = 10$ (intra-abdominal sepsis)/group; MFI=mean fluorescence intensity)

Figure 4.

A significant increase in the concentration of the pro-inflammatory cytokines IL-6 and TNF- α in peritoneal fluid is noted with the development of intra-abdominal sepsis. (* $p < 0.05$ versus baseline; $n = 6$ (baseline) or $n = 10$ (intra-abdominal sepsis)/group)