Decreased Systemic Polymorphonuclear Neutrophil (PMN) Rolling Without Increased PMN Adhesion in Peritonitis at Remote Sites

Daniel E. Swartz, MD; Andrew J. E. Seely, MD; Lorenzo Ferri, MD; Betty Giannias, BSc; Nicolas V. Christou, MD, PhD, FRCSC

**Background:** Previous in vitro studies have demonstrated that the host response to intra-abdominal infection produces increased generalized polymorphonuclear neutrophil (PMN) adherence to vascular endothelial cells (ECs), which may lead to subsequent endothelial damage, leaky capillaries, and organ dysfunction. There are scant data to demonstrate this enhanced systemic PMN adherence in vivo or the influence of PMN rolling on PMN endothelial adherence.

**Hypothesis:** Systemic PMN adherence in the animal with sepsis is increased.

**Design:** In vivo murine model of a 2-front infection using intravital microscopy of the cremasteric muscle to quantify PMN-EC adherence in a septic response.

**Setting:** Basic science laboratory and animal surgical facility.

**Patients or Other Participants:** One hundred CD1 male mice.

**Interventions:** Animals underwent cecal ligation and puncture peritonitis, cremasteric muscle *Escherichia coli* infection, both infections, or neither (controls). Eighteen hours later, the mice underwent exteriorization of the cremasteric muscle under an intravital microscope for measurement of PMN-EC interactions. Blood was then drawn for calculation of circulating PMN counts.

**Main Outcome Measures:** Adherence of PMNs, PMN rolling flux, PMN rolling velocity, and circulating PMN counts.

**Results:** Circulatory mechanics did not differ between the groups. Unlike static in vitro systems, we could not detect an increase in PMN adherence after peritonitis with this dynamic in vivo model. A local (cremasteric) infection was associated with marked PMN adherence. Peritonitis was associated with reduced PMN adherence at a local infection site as well as reduced rolling adhesion and PMN rolling velocity.

**Conclusions:** The data suggest that intra-abdominal infection does not increase remote PMN adherence, and may actually result in reduction of systemic adherence via modulation of PMN rolling.

**Secondary Peritonitis** is associated with a significant mortality rate ranging from 0% to 70%, with survival correlating to a successful host response rather than the cause, severity, or anatomic location. Patients with peritonitis are at an increased risk for development of infectious complications at remote sites such as pneumonia and wound, urinary tract, and vascular-catheter–related infections. In a recent multicenter evaluation of more than 10000 critically ill patients, there was a 45% rate of intensive care unit (ICU)–acquired infections; risk factors for mortality were identified as pneumonia, bacteremia, and sepsis. For these patients to survive with multiple sites of infections, an appropriate immune response must successfully distribute the limited resources to competing stimuli. The mechanism of this immune-mediated triage is presently unclear.

The polymorphonuclear neutrophil (PMN) is the predominant granulocyte at the site of acute infection in the initial 48 hours. The PMNs are recruited to the site of injury by a series of specific interactions with endothelial cells (ECs) via cell-surface adhesion molecules. After transendothelial migration, the PMN becomes an effector cell capable of tracking to the site of injury and of phagocytosing and then destroying the foreign microorganisms. Under normal circumstances after the successful elimination of microbial invaders, the PMN undergoes apoptosis, or programmed cell death. Although all aspects

From the L. D. MacLean Surgical Research Laboratories, Division of General Surgery, McGill University Health Centre, Montreal, Quebec.
MATERIALS AND METHODS

The protocols for these studies were approved by the Animal Care Committee of McGill University, Montreal, Quebec. The CD1 male mice (25-35 g; Charles River, St Constant, Quebec) were allowed to acclimate to the animal facility for 3 to 5 days before entry into the study. Animals that did not survive the perioperative period were excluded from the study, and subsequent animals were added to the study until 20 survived in each group. A total of 107 animals were used, of which 15 were killed humanely or died before the conclusion (overall mortality rate, 14%), to permit 60 subjects in 3 experimental groups, 20 controls, and 12 subjects for determination of optimal E.coli concentration.

DETERMINATION OF OPTIMAL E.coli CONCENTRATION

To determine the optimal concentration of E.coli to create a local inflammatory response in the cremaster muscle sheath for intravital microscopy, 10^9, 10^8, 10^7, and 10^6 E.coli NCTC9001 organisms in 150 µl of sterile 0.9% isotonic sodium chloride solution were injected into the cremaster muscle. These organisms were provided graciously by the Department of Pathology at McGill University. Each concentration was administered to 3 mice via intracremasteric injection, and the mice were observed for 18 hours. Marked systemic toxic effects were observed in animals receiving 10^6 and 10^7 organisms, and they succumbed on induction of anesthesia. Animals receiving 10^7 and 10^6 organisms appeared moderately ill, but tolerated the anesthesia well. On cremasteric dissection, animals that had received 10^5 organisms demonstrated gross and microscopic inflammatory changes, whereas those with 10^6 organisms had no visible evidence of such changes. Thus a dose of 10^7 organisms was selected for use in this study.

STUDY DESIGN

Animals were assigned to 1 of 4 groups: CLP, ORC, ORC-CLP, or no infection (controls). Eighteen to 24 hours later, each animal was anesthetized and underwent cremasteric muscle dissection and mounting on an intravital microscope. Straight, nonbranching postcapillary venules 30 to 60 µm in diameter and segments 150 µm in length were selected for study. After a 20-minute period to allow postdissection hemodynamic changes in the vessels to stabilize, centerline red blood cell velocity (Vrbc) and vessel diameter were measured, allowing calculation of mean red blood cell velocity (Vmean), mean blood flow, mean shear rate, and mean venular wall shear stress. Video recording techniques allowed the measurement of PMN rolling adhesion, rolling velocity, and stationary adhesion. After data acquisition, blood was drawn for PMN isolation and determination of circulating PMN concentration.

CLP-INDUCED PERITONITIS

Cecal ligation and puncture–induced peritonitis was performed according to the technique of Chaudry et al.10 After intraperitoneal anesthesia with ketamine hydrochloride (200 mg/kg) and xylazine hydrochloride (10 mg/kg), the abdomen was shaved with electric clippers, and the skin was prepared with 1% povidone-iodine solution. A 1-cm midline laparotomy incision was made, and the cecum was delivered carefully into the operative field. The stool was gently milked into the cecum before ligation with 3-0 silk proximal to the iliocaval valve so as not to cause intestinal obstruction. Two punctures with a 21-gauge needle were made 1 cm apart, and manual compression was used to extrude feces from the puncture sites. The cecum was replaced into the peritoneum, and the abdomen was closed in 2 layers with 3-0 nylon. The animals were allowed to awaken under heating lamps and received subcutaneous buprenorphine hydrochloride analgesia (0.05-0.1 mg/kg) immediately after operation and then every 8 to 12 hours as needed.

E.coli-INDUCED CREMATERIC MUSCLE INFECTION

Mice assigned to the ORC group underwent intraperitoneal anesthesia as described, followed by a 3-mm transverse skin snip of the scrotum. The cremaster muscle was exteriorized and injected with 10^7 E.coli cells in 150 µl of 0.9% isotonic sodium chloride solution. The muscle was replaced in the scrotum, and the skin was closed with a single 3-0 nylon suture. Animals in the ORC-CLP group underwent both procedures at the same time.

INTRAVITAL MICROSCOPY

Eighteen to 24 hours later, all animals underwent intraperitoneal anesthesia with ketamine hydrochloride (200 mg/kg) and xylazine hydrochloride (10 mg/kg). Under a heat lamp, a longitudinal midline neck incision was made, and the right internal jugular vein was cannulated. During the course of the experiment, both drugs were administered intravenously in 50- to 100-µL boluses titrated to anesthesia. Cremasteric muscle dissection for intravital microscopy was performed according to the technique of Granger and Kubec.11 With the animals in the supine position and with the assistance of a dissection light microscope (Nikon SMZ-1B Stereoscopic Dissecting Microscope; Nikon of these events are important for a successful immune response, the recruitment of PMNs between competing sites of infection occurs at the level of initial PMN-EC interaction.

A sustained immune response giving rise to a widespread inflammatory state after a significant injury or insult can lead to dire systemic events such as multiple-organ dysfunction syndrome, a significant cause of morbidity and mortality in patients in the ICU. This syndrome is defined as a persistent maladaptive and dysregulated state of the inflammatory and immune systems.4 The pathophysiology of this disease state involves an uncontrolled immunomediator response of the PMN, leading to systemic organ destruction.11 Despite the widespread belief that organ destruction in multiple-organ dysfunction syndrome is mediated by PMNs, in vivo broad evidence of this effect has been scarce. Although some adherence studies performed in vitro support increased
The entire cremaster was scanned visually under light microscopy (Nikon Eclipse TE 2000 Inverted Microscope with Epifluorescence; Nikon Canada Inc) for adequate visualization of blood flow in postcapillary venules. A straight, unbranched segment approximately 150 µm in length and 30 to 50 µm in diameter was located and centered. The animal was observed via video microscopy (Cohu 4915-2010 CCD Monochrome Video Camera; Scion Corporation, Frederick, Md) for 20 minutes to allow PMN kinetics to return to baseline values before measurement. The animal was excluded if centerline Vrbc, measured continuously using a velocimeter (Optical Doppler Velocimeter; Microcirculation Research Institute, College Station, Tex), fell below 3 mm/s. Vessels were also excluded if Vmean was greater than 6 mm/s, at which shear stresses would have a significant impact on PMN-EC interactions. Vessel blood flow was then recorded for 10 minutes by means of video cassette recorder (RCA Video Cassette Recorder VR4564; Thomson Consumer Electronics, Indianapolis, Ind) with a time-date generator (WJ-810; Panasonic, Secaucus, NJ). One to 3 venules per animal were recorded depending on adequate vessel quality. At the end of the data acquisition, the mice were killed humanely in a carbon dioxide chamber before undergoing percutaneous cardiac puncture. The blood was stored in a heparinized tube and placed onto the microscope under a heat lamp. The blood was splayed open using an anterior longitudinal incision with electrocautery and secured with a 5-point suture fixation at the periphery. The tissue was kept moist with warmed bicarbonate buffer perfusion (Peristaltic Microperfusion Pump; Instech Laboratories Inc, Plymouth Meeting, Pa). The tests, epididymis, and vas deferens were removed gently into the abdominal cavity. The animal was then turned prone, resecured onto the intravital microscopy specimen board, and placed onto the microscope under a heat lamp.

MEASUREMENT OF VENUS KINETICS

The recorded PMN-EC interactions were analyzed by means of off-line video playback and using a stage micrometer to calibrate on-screen measurements of vessel diameter and length. Vessel kinetics were determined using the following formulas and calculations. Centerline RBC velocity was determined as previously mentioned, using the velocimeter with the sensors placed in the center of the vessel. Mean RBC velocity was determined by the formula Vrbc/1.6; venular blood flow (VBF), Vmean × cross-sectional area, assuming cylindrical geometry. Venular shear rate (VSR) was calculated by the formula 8 (Vmean/Dv), where Dv indicates vessel diameter, and venular wall shear stress, VSR × η, where η is 0.25 poise, the viscosity of blood.

PMN PURITY

The PMNs were isolated from the blood using the standardized technique described. Purity of the PMNs was assessed in 4 mice (2 controls and 2 CLP animals) using isolation, smear, and Giemsa staining. The differential was counted in 3 high-power fields per slide, and PMNs were reported as raw number and percentage of total leukocytes. Figure 1 demonstrates a differential smear of CLP animals, and Figure 2 depicts that of controls. The percentage of purity of PMNs was 94% for controls and 68% for CLP animals.

STATISTICAL ANALYSIS

All data were analyzed using commercially available software (Systat 8.0; SPSS, Chicago, Ill). The differences among groups were analyzed for statistical significance by means of analysis of variance. Between-group comparisons were analyzed using Bonferroni-corrected t tests. P < .05 was considered statistically significant.
herence was measured in animals with sepsis undergoing a cecal ligation and puncture–induced peritonitis (CLP group), with an E coli–induced cremasteric muscle infection (orchitis; ORC group), and with both competing sites of injury (ORC-CLP group). Intravital microscopy of the cremaster muscle allowed quantification of several PMN-EC interactions before transendothelial migration. In addition to stationary adhesion, we measured rolling adhesion and its velocity.

RESULTS

The numbers of circulating PMNs among the intravital microscopy study groups are demonstrated in Figure 3. Within the blood, there was no significant difference in the numbers of circulating PMNs between the controls and ORC mice. In contrast, when compared with the controls, there was a significant reduction in the numbers of circulating PMNs in the CLP animals. These results are corroborated in the photomicrographs. In ORC-CLP mice, there was no further reduction in the numbers of circulating PMNs compared with CLP alone.

The circulatory variables for the groups of mice in the intravital microscopy study are shown in the Table. Mean vessel diameter and VBF did not differ significantly among the 4 groups of animals. However, Vmean was significantly reduced from 2.9 to 2.3 mm/s in CLP mice, with and without ORC. Furthermore, venular wall shear stress was reduced significantly in the ORC animals compared with controls, and this was observed in the presence and absence of CLP.

To determine the extent of any relationship between Vmean and venular wall shear stress and the kinetics of PMN-EC interactions, we performed correlational analyses. These analyses revealed that there were significant relationships between the Vmean and vessel shear stress and between the Vmean and PMN rolling ($r^2=0.39$ and $r^2=0.56$, respectively; $P<.001$). No other significant correlations between baseline hemodynamic variables and kinetics of PMN-EC interactions were observed. Since alterations in Vmean occurred systemically in CLP animals, changes in PMN delivery in the circulation could result in different adherence rates (rolling and stationary) among different groups. To account for these differences, the effect of plasma PMN concentration was adjusted for evaluating PMN adherence. For completeness, a similar adjustment was performed for rolling flux.

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The results for PMN rolling flux, which was defined as the number of PMNs rolling past a stationary point per minute, are represented in Figure 4, left. Although the greatest PMN rolling flux was observed in the controls, these values did not differ significantly from those of the ORC group. In contrast, the CLP animals showed a significant reduction in PMN rolling flux compared with controls and ORC animals.

Since circulating PMN concentrations differed among the groups and thus could account for differences in rolling adherence, the data was replotted after adjusting for the effect of PMN concentration in the blood (Figure 4, B). An adjustment factor for each data point was created by dividing the circulating PMN concentration for each animal by the mean PMN concentration of the controls. The adjusted rolling adherence was determined by multiplying the number of rolling PMNs by the adjustment factor. To account for any effect of PMN concentration on stationary adherence, the same adjustment factor was used to calculate the adjusted stationary adherence (Figure 5, right). Adjusting the rolling adherence data for the effect of circulating PMN concentration did not significantly alter the results.

Mean PMN rolling velocities for the 4 groups of mice are represented in Figure 6. The highest rolling velocity was observed in the controls, although this did not differ significantly from that of the CLP animals. There was, however, a significant reduction in rolling velocity in the ORC mice. The addition of CLP to the ORC animals did not significantly further reduce the PMN rolling velocity.

The results of PMN adherence in the 4 groups of mice, defined as the number of PMNs that become adherent to a 100-µm section of the postcapillary venule endothelium during a 5-minute period, are demonstrated in Figure 5. The data on the left side of the Figure represent only the newly adherent PMNs, which remained stationary for at least 30 seconds during the survey period, while excluding the previously adherent PMNs.
This was believed to represent a more temporally concise sampling of stationary adherence pertaining to factors present only during the 5-minute survey period. When compared with overall adherence (cells already adherent plus those that became adherent), the proportion of cells adherent between groups were equivalent (data not shown). In the ORC group, there was a significant increase in the numbers of adherent PMNs, compared with the other groups. There was no difference in the numbers of PMNs adhering to the venular endothelium in CLP animals with or without ORC or in controls ($P > .05$). The ORC-CLP mice had significantly fewer adherent PMNs than the ORC group ($P < .001$).

Adjusting the stationary adherence of PMNs for the effect of circulating PMN concentration demonstrated a more than 50% reduction of stationary adherence in the CLP group (Figure 5, right).

**COMMENT**

Firm adherence of PMNs to the endothelium requires the sequential involvement of selectins, chemotactants, and integrins under physiologic conditions of blood flow. It is commonly accepted that sepsis is associated with systemic PMN adherence as a prerequisite to PMN-mediated organ injury. In vitro studies of PMN-EC interaction have supported this hypothesis, however, confirmation with in vivo data is lacking. Using intravital microscopy in a rat model of cecal ligation and puncture, Piper et al examined PMN adherence in vivo in postcapillary venules of extensor digitorum longus muscles. The authors demonstrated a reduction in PMN adherence in animals with sepsis that was abolished when data were adjusted for circulating PMN count. Using a murine cremasteric muscle model, our data similarly demonstrated a lack of increased systemic adherence in sepsis. When adjusted for circulating PMN concentration, our results failed to show any increase in adherence between CLP and control animals. Instead, the stationary adherence in the CLP and CLP-ORC groups fell by more than 50%.

Potential causes of this decrease in adherence include technical errors in measurement of adherence or measurement of circulating PMN concentration as well as the effect of differences in circulatory hemodynamics between groups.

Whether or not the data were adjusted for circulating PMN concentration, the ORC group showed a significant increase of PMN adherence above that of CLP animals, demonstrating that our method of detection of PMN adherence works because PMN adherence is greater at the site of infection. In ORC-CLP animals, we observed a dramatic reduction in PMN adherence within the cremaster muscle. This suggests a picture of competing sites of injury in which PMNs are preferentially delivered to the more significant peritoneal injury.

Hemodynamic variables were measured to examine their contribution to the profile of cellular responses seen. We demonstrated significant differences in $V_{\text{mean}}$ and mean venular wall shear stress among some of the groups, despite equivalent values for vessel diameters and VBF among all 4 groups of animals. The results of correlational analyses performed demonstrated that only differences in the numbers of rolling PMNs between groups could be attributed to differences in $V_{\text{mean}}$ and mean venular wall shear stress. Since the reduced RBC velocity was seen only in CLP animals from the reference point of the cremaster muscle, such changes manifest as a systemic effect. On the other hand, the reduced venular wall shear stress was seen only in the ORC groups and not in the CLP group, thereby implying a local rather than systemic effect. Thus, although it is unlikely that changes in venular wall shear stress occur systemically in the face of injury, we cannot exclude a reduction in $V_{\text{mean}}$ as a cause of differences found in numbers of rolling PMNs. Factors responsible for the reduction in $V_{\text{mean}}$ in our experimental groups remain to be established.

To adjust for the effect of circulating PMN concentration, we determined an adjustment factor relating to the mean circulating PMN count of the controls. When adjusted values were plotted against each group, no alteration in statistical significance for each group was noted when compared with unadjusted data. Therefore, any results that occurred could not be attributed to differences in PMN concentrations in the circulation. Thus, the differences in hemodynamic parameters seen did not significantly alter the rolling or stationary adherence of the PMNs.

It is possible that our technique of purifying circulatory PMNs before counting them with a hemocytometer underestimated the PMN concentration in the plasma. Ficoll-Paque separation by density gradient has been used extensively to purify human PMNs for counting in individuals with sepsis and controls. To our knowledge, this technique has not been validated in mice, and it has been proposed that sepsis alters the density of PMNs in the circulation, leading to their loss during density separation. We subsequently compared our technique with a Coulter counter and found approximately 50% fewer PMNs per unit volume in CLP mice. However, given the more than 50% reduction of adherence in controls to CLP after the adjustment for circulatory PMN concentration.
(from 6.4±1.0 to 2.1±1.1 cells per 5 minutes along 100 μm), error in PMN separation could not account completely for these differences.

All CLP animals, with or without ORC, demonstrated a marked reduction in numbers of rolling PMNs compared with controls. These differences correspond to similar results reported elsewhere. One explanation for reduced stationary adherence in CLP animals could be the significantly fewer PMNs undergoing rolling adhesion compared with controls, since rolling is a prerequisite for stationary adherence. This could not fully explain the reduced systemic adherence in CLP animals, since significantly less rolling adhesion was demonstrated in the ORC group as well. Other explanations for a lack of increased stationary adherence in CLP animals could be the animal model itself. The mouse model has been widely used in sepsis and inflammatory response research, but there may be inherent differences in inflammation from humans.

Rolling velocity of PMNs reflects the presence of adhesive interactions between selectins and their ligands on the PMN and the underlying ECs. The time course of our experiments, 18 to 24 hours after intracremasteric injection of the E coli organisms, suggested that selectins would have been the predominant selectin expressed. The predominance of this adhesion molecule may account for the reduced rolling velocity of the PMNs within the cremasteric vasculature, since in vitro studies of selectin have demonstrated slower rolling than P- or L-selectin. Further studies to address this hypothesis using specific anti-E-selectin antibodies in this model are necessary. Differences in PMN rolling velocity could also explain the lack of increased stationary adherence in CLP animals. The PMNs rolled at greater velocity through the cremasteric postcapillary venules in CLP animals compared with ORC animals. Lower PMN velocity implies an enhanced PMN-EC interaction through greater exposure of surface adhesion molecules. Specific selectin molecule expression has been associated with changes in rolling adherence, velocity, or both, and this may account for increased PMN recruitment to infected cremasteric tissue. Other investigators have demonstrated elevated systemic CD11b (integrin) expression in CLP animals compared with controls in a similarly designed study.

One explanation for the reduction in PMN adherence remote from the peritoneum in peritonitis is the action of soluble L-selectin. Shedding of L-selectin is a concomitant step in transition from rolling to firm adhesion and is associated with up-regulation of the β2 integrin molecules on the PMN. When PMNs are pretreated with interleukin 8 (IL-8) and shed their L-selectin before interaction with the endothelium, adhesion does not occur. Human in vivo data has demonstrated that patients with sepsis have increased soluble L-selectin levels compared with controls. Little is known of the fate or the subsequent role played by these soluble L-selectin molecules on PMN-EC interactions downstream. It is possible that once shed, the soluble L-selectin may bind to its receptors peripherally and competitively block quiescent PMN rolling on the endothelium. This would lead to a reduced PMN flux at sites remote from injury and increased numbers at sites of injury where the soluble L-selectin would less effectively compete with up-regulated L-selectin receptors.

Secondary peritonitis is a significant cause of morbidity and mortality in the ICU, and patients in the ICU as a group have the highest rate of nosocomial infections. Once recruited to the site of injury, the PMN interacts with ECs via rolling adhesion, firm adhesion, and transendothelial migration. Using a murine cecal ligation and puncture peritonitis model and skin or cremaster injury as the secondary site in a 2-front injury model, we examined the role of injury severity on the triage of PMNs to competing sites of injury. With the use of intravital microscopy, we demonstrated that numbers of PMNs involved in rolling adhesion, rolling velocity, and stationary adhesion varied depending on the presence of a minor local or a major remote injury, both, or the absence of injury. Most importantly, we have demonstrated for the first time that peritonitis in the mouse model is associated with reduced systemic PMN adherence.

In summary, we used intravital microscopy of the cremasteric muscle postcapillary venules to assess PMN-EC interactions in vivo in a murine 2-front infection model consisting of CLP and E coli–induced cremaster muscle infection. Our data do not support a systemic increase in stationary adherence of PMNs to ECs in sepsis, contrary to current hypotheses of the pathophysiologic features of multiple-organ dysfunction syndrome and some in vitro investigations.


Corresponding author: Nicolas V. Christou, MD, PhD, FRCSC, Division of General Surgery, McGill University Health Center, Royal Victoria Hospital, 687 Pine Ave W, Montreal, Quebec, Canada H3A 1A1 (e-mail: nicholas.christou@muhc.mcgill.ca).

REFERENCES


**Quotation**

T here are still among us “brilliant” operators from whom I pray to be spared when my hour has come. For them it is the mere quality of effort that counts.

*Sir Berkeley Moynihan*  
1865-1936