Functional α_4-integrin: A newly identified pathway of neutrophil recruitment in critically ill septic patients

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Using a novel flow chamber assay system and whole blood, we show that leukocytes from septic individuals have a four-fold elevation of adhesion, but not rolling, on a P-selectin/β_2-integrin substrate. Most leukocytes from septic patients (but not healthy controls) that bound vascular cell adhesion molecule 1 (VCAM-1) were neutrophils. All adhesion was inhibited with an antibody specific for the VCAM-1 ligand α_4-integrin. The α_4-integrin was present on neutrophils from septic patients but not on neutrophils from patients with localized bacterial infections. The plasma milieu of septic patients was sufficient to induce neutrophils from healthy subjects to bind VCAM-1 under flow conditions. This is the first description of α_4-integrin/VCAM-1 pathway of neutrophil recruitment in human disease. This pathway may provide a new therapeutic target to reduce inappropriate neutrophil adhesion without altering the normal yet critical β_2-integrin–mediated adhesive function of neutrophils.

Severe sepsis syndrome/septic shock is a clinically defined syndrome which continues to be associated with a mortality rate of 20–50%, and is the leading cause of death in adult intensive care units[1,2] (ICU). Despite advances in the physiologic support of these patients there is still no definitive treatment for this condition, in part due to our lack of understanding of the mechanisms that underlie the pathophysiology of sepsis. For example, the leukocyte recruitment hypothesis, developed using animal models, suggests that sepsis is a multi-factor process involving a cascade of inflammatory responses culminating in the inappropriate recruitment of leukocytes from the circulation[3,4]. Studies examining human sepsis however do not necessarily mirror the animal models and have shown both decreased and increased adhesivity of leukocytes for various substrata[5–10]. These differences may be due in part to the animal models poorly representing the human condition. Another crucial limitation is that most *in vitro* experiments with human cells have used non-biologic substrata (nylon wool, glass) and non-physiologic conditions (neutrophils in buffer in static systems), and therefore do not reflect the dynamic behaviour of leukocytes in the septic milieu of the microvasculature.

Due to the high shear forces in blood vessels, leukocyte recruitment is absolutely dependent upon the ability of the leukocytes to roll on the endothelium before they can adhere[11,12]. The rolling and adherence of neutrophils is mediated by the selectins, whereas the α_4-integrin/VCAM-1 pathway mediates eosinophil, monocyte and lymphocyte rolling and adherence[13,14]. Studies of adhesion of leukocytes from septic patients to date has been under static conditions in which selectin and VCAM-1-mediated rolling does not occur[6,8,9]. Moreover, most studies have used leukocytes isolated from the whole blood of septic patients, which removes the cells from the septic environment and the pro-inflammatory mediators found in septic blood. These approaches have yielded conflicting results. For example, neutrophils from septic patients have been reported to have decreased adhesion to nylon fiber[15], increased adhesion to glass cover slips (assessed as leuko-aggregation)[9] and similar adhesion to albumin-coated latex beads when compared with healthy controls[6]. Others have reported different results for the same non-physiologic substrata[8].

We have previously described a novel way of studying leukocyte adhesion which mimics the shear conditions found in the human microvasculature while maintaining the blood milieu[16]. Human whole blood is perfused through a laminar flow chamber in which soluble adhesion molecules are immobilized to a glass cover slip. With this system we can visualize leukocyte rolling and adhesion, and at the end of each experiment determine the leukocyte differentials on the cover slip. This approach has revealed selectivity of various leukocytes for specific adhesion molecules. For example, neutrophils have a propensity to adhere to selectins but not VCAM-1, whereas mononuclear cells adhere to VCAM-1 but not selectins[16]. Here we have applied this technique for the first time to a clinical disease state, specifically sepsis, to directly examine the behavior of leukocytes in their natural septic milieu under flow conditions. We describe a novel pathway (α_4-integrin/VCAM-1) of neutrophil recruitment (rolling and adhesion) present in septic individuals but not in post-operative patients or patients with acute bacterial pneumonia. We postulate that the induction of this pathway may lead to inappropriate neutrophil recruitment in the pathogenesis of sepsis, and ultimately sequelae associated with multiple organ dysfunction syndrome.

Demographic data

For the study, we included 28 patients with sepsis syndrome/septic shock. The ages of the septic patients ranged between 24 and 86 years (mean: 59.3). The male:female ratio was 18:10. Overall mortality was 17.9%. Acute physiologic and

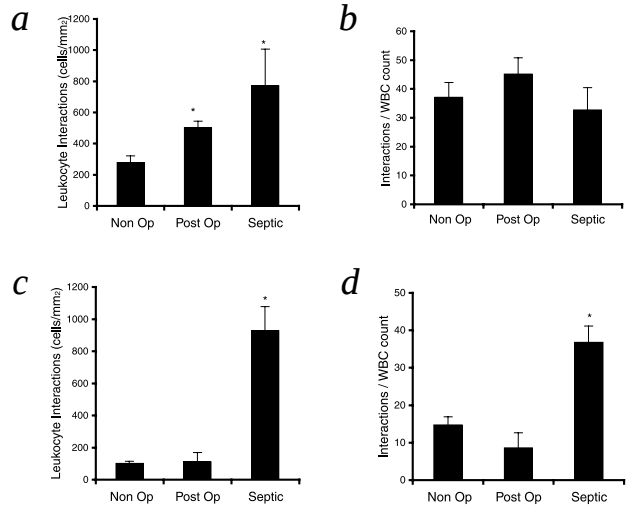

Fig. 1 The number of rolling and adhering leukocytes on P-selectin/CD18 ligand substratum. ***a***, Leukocytes rolling in nonoperative controls (Non Op, *n* = 13), healthy postoperative controls (Post Op, *n* = 5) and septic individuals (Septic, *n* = 9). ***b***, data normalized for the circulating leukocytes (WBC counts). ***c*** and ***d***, Number of adherent leukocytes in each group (*c*) and normalized for circulating leukocytes (*d*). *, $P < 0.05$ relative to control group unless specified otherwise in the text.

chronic health evaluation II (APACHE II) scores were recorded on the day that blood was drawn for the study (mean: 23.7 ± 3.0). The white blood cell (WBC) counts for the septic patients were 12.2–40.5 × 10^9/l (normal range: 4.0–11.0) with a mean of 24.1 ± 3.4. Of the 28 patients, 16 had pneumonia (8 community-acquired, 5 ventilator-associated and 3 nosocomial), 10 had intra-abdominal sepsis and 2 had necrotizing fasciitis. Gram-positive organisms were responsible for 11 of 16 pneumonias, and 8 subjects with pneumonia had concurrent bacteremia. Group A streptococcus was responsible for both cases of fasciitis. All 10 intra-abdominal infections were characterized by polymicrobial infections, and 4 had concurrent bacteremia with Gram-negative organisms. Of 28 patients, 6 (21.4%) had severe sepsis syndrome, whereas the remainder met the criteria for septic shock.

The nonoperative control group had WBC counts within the normal range with a mean of 7.8 ± 0.7 × 10^9/l and ages ranging from 22 to 52 years with a male:female ratio of 7:5. There were 5 patients in the postoperative control group with an average age of 59 years. This group had WBC counts ranging from 7.5 to 16.1 × 10^9/l with a mean of 11.8 ± 1.6 × 10^9/L

Leukocyte interactions on P-selectin/CD18 ligand

We investigated leukocyte rolling and adhesion to platelet monolayers in blood from critically ill septic patients and healthy nonoperative and postoperative controls. In the nonoperative controls, approximately 300 cells rolled on the platelet monolayer (Fig. 1*a*) and less than 100 cells firmly adhered (Fig. 1*c*). Rolling was significantly decreased by a P-selectin antibody, and the adhesion was markedly reduced by an antibody specific for CD18 (data not shown), which supports the view that this substratum functions as a surrogate P-selectin and CD18 ligand[17–19]. The numbers of rolling leukocytes from the postoperative control group and the septic group were significantly elevated over the nonoperative control group ($P < 0.05$; Fig. 1*a*). However, this, was entirely due to elevated numbers of circulating cells as no differences were observed when the number of rolling cells was normalized for the number of circulating leukocytes (Fig. 1*b*). Clearly the increase in circulating cells increases the number of rolling leukocytes.

The number of adherent cells was similar in the control and postoperative group but was greatly enhanced in the septic patients ($P = 0.001$; Fig. 1*c*). This increase did not depend on the number of circulating leukocytes, because the number of adherent cells per WBC count ($P < 0.01$) remained significantly elevated in the septic patients relative to either the nonoperative or postoperative group (Fig. 1*d*). Rolling leukocytes from septic patients therefore have a greater propensity to adhere when compared with leukocytes from healthy controls. This is best exemplified by the adhesion/rolling ratio, which is four-fold greater ($P < 0.01$) in leukocytes from septic patients than those from controls (Fig. 2). Moreover, despite the increased number of rolling leukocytes in the postoperative control group, the adhesion/rolling ratio was significantly lower, again indicating that although there was increased rolling, leukocytes from the postoperative control group did not show increased adhesion (unlike the septic leukocytes).

Staining of the cover slips at the end of the experiments revealed that the majority of cells interacting with the P-selectin and CD18 ligands were neutrophils (~90%). Moreover, the enhanced number of adherent cells within the septic group could be entirely accounted for by increased numbers of neutrophils (data not shown).

Leukocyte recruitment on VCAM-1

To test whether leukocytes other than neutrophils also had increased adhesion, we performed similar experiments with VCAM-1, which predominately recruits mononuclear leukocytes but not neutrophils[16]. We used VCAM-1 at concentrations that induced numbers of rolling and adherent cells similar to those observed with optimized TNF-α treatment of endothelia (Fig. 3*a*). TNF-α is the most potent cytokine for leukocyte recruitment in our model system. We observed approximately 100 cells/field-of-view roll and adhere on both substrata. Leukocytes from control, postoperative and septic patients rolled and adhered on VCAM-1 cover slips with similar magnitude (Fig. 3*b*). This is surprising, as normalizing for circulating numbers decreased the total number of leukocytes on VCAM-1 from septic

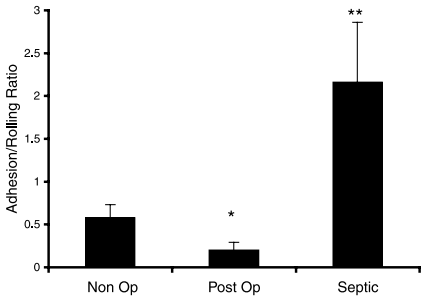

Fig. 2 Number of rolling cells that adhere (adhesion/rolling) for healthy controls (Non Op, *n* = 13; Post Op, *n* = 5) and septic individuals (Septic, *n* = 9) . *, $P < 0.05$ relative to the nonoperative group; **, $P < 0.01$ relative to both control groups.

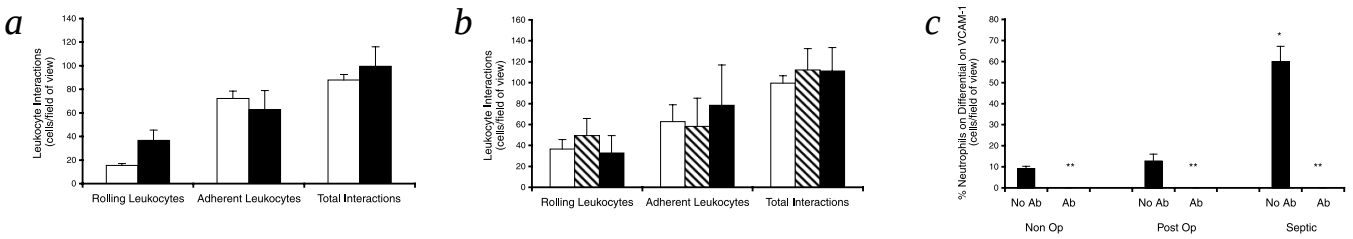

Fig. 3 Neutrophils from septic patients adhere to VCAM-1 via α_4-integration. ***a***, Number of rolling and adhering leukocytes as well as the total number of cells interacting with either VCAM-1 (■, $n = 4$) or TNF-α–treated endothelium (□, $n = 3$). ***b***, Number of rolling and adhering leukocytes as well as total interactions for nonoperative controls (□, $n = 10$), postoperative controls (▨, $n = 5$) and septic patients (■, $n = 11$) on VCAM-1. ***c***, Percent of neutrophils adhering to VCAM-1 immobilized protein under flow conditions. *, $P = 0.001$ relative to both control groups. Also shown is the complete inhibition of adhesion to VCAM-1 by HP1/2 antibody (Ab) directed against the VCAM-1 ligand, α_4-integrin. **, $P < 0.05$ relative to respective group without antibody.

patients. In both control groups, most cells were mononuclear leukocytes with less than 10% neutrophils binding to VCAM-1 (Fig. 3*c*). However, more than 60% of the septic leukocytes adherent to VCAM-1 were neutrophils (Fig. 3*c*; $P = 0.001$ relative to control groups). Moreover, the 2–3-fold increase in circulating neutrophil counts could not account for the 7-fold increase in neutrophils adhering to VCAM-1.

The recruitment factor (R factor) is an indicator we previously used to demonstrate the propensity of a specific leukocyte population to be recruited from the circulation onto the cover slip; it is calculated as: (% of cell type on cover slip)/(% cell type in circulation)[16]. In the control groups, the percentage of neutrophils on VCAM-1 cover slips was less than 10%, though 60% of the circulating leukocytes were neutrophils. The R factor was calculated as 0.12. In contrast, the R factor on VCAM-1 for neutrophils from septic patients was elevated 7–8-fold, to 0.92. This indicates a large increase in adhesion to VCAM-1 of neutrophils from septic patients.

The ligand for VCAM-1 on mononuclear cells is α_4-integrin. The recruitment of all leukocytes including neutrophils on VCAM-1 was completely blocked with a HP1/2α, monoclonal antibody specific for α_4-integrin (Fig. 3*c*), showing a functional induction of α_4-integrin on neutrophils in human disease. Neutrophil precursors are known to have α_4-integrin, so this could simply reflect a maturation defect in bone marrow of septic patients, or mobilization of very immature neutrophils. However, when control leukocytes were incubated with plasma from septic patients, the number of neutrophils recruited onto VCAM-1 was comparable to that seen in septic patients (45.2 ± 8.4%; Fig. 4). Furthermore, the R factor of the normal neutrophils in septic plasma ($R = 0.76$) was markedly elevated compared to the control plasma ($R = 0.12$). The recruitment of control leukocytes in septic plasma on VCAM-1 was also completely blocked with HP1/2 (data not shown). Interestingly, the reverse experiment of adding septic neutrophils to normal plasma revealed a lower percentage of neutrophils (~30%) adhering to VCAM-1. Although still significantly higher than control values, this decrease in α_4-integrin function indicates that neutrophils might require continuous exposure to the pro-inflammatory milieu to maintan α_4-integrin expression.

Septic neutrophils express α_4-integrin.

We studied α_4-integrin levels on the surface of neutrophils from eight septic patients and eight matched controls (Fig. 5*a*). Although α_4-integrin was not present in detectable quantities on normal human neutrophils, neutrophils from septic patients showed a clear and consistent increase in surface α_4-integrin (8/8 patients). On average, approximately 30–40% of neutrophils stained positive for α_4-integrin, whereas control neutrophils revealed minimal (0–5%) α_4-integrin expression. These data almost certainly underestimate the amount of α_4-integrin on the surface of neutrophils from septic patients, as α_4-integrin seems to be rapidly shed or internalized following removal of pro-inflammatory stimuli. Nevertheless, these data are entirely consistent with the functional adhesion data found on VCAM-1. In additional experiments, blood was drawn from patients on three consecutive days, and patients with clinical improvements showed diminishing α_4-integrin values over the 72 hours (Fig. 5*b*). There was no change in α_4-integrin expression on neutrophils of healthy control subjects over the three day period. Finally, to determine whether the systemic aspect of sepsis syndrome was required, we examined α_4-integrin expression in five patients with acute bacterial pneumonia without evidence of severe sepsis syndrome or septic shock (Fig. 5*a*). Neutrophils from only 1 of these patients showed any increase in α_4-integrin expression which was at the lower range of α_4-integrin values detected on neutrophils of septic patients.

Discussion

We show here that neutrophils from septic but not control patients express α_4-integrin and functionally bind to the ligand VCAM-1. To date, only β_2-integrin has been shown to have a role in neutrophil adhesion in humans. In fact, neutrophils are generally assumed not to express α_4-integrin. Previously, α_4-integrin had only been implicated in the recruitment of mononuclear cells and eosinophils to sites of inflammation[13]. Here we have

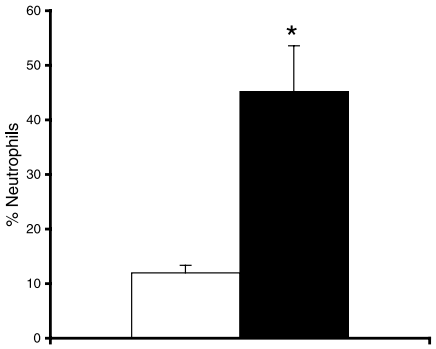

Fig. 4 The percent of neutrophils from healthy volunteers adhering to VCAM-1 in the presence of healthy (□, $n = 4$) or septic plasma (■, $n = 5$). *, $P < 0.05$ relative to group without septic plasma.

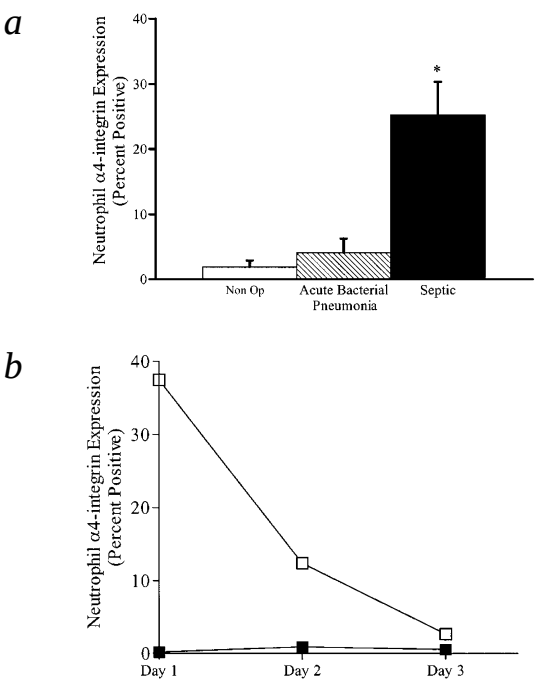

Fig. 5 α_4-integrin expression on neutriophils from control, acute bacterial pneumonia and septic patients. ***a***, Individual results for neutrophil α_4-integrin expression on non-operative control patients (□, n = 8), septic patients (■, n = 8) or patients with acute bacterial pneumonia (▨, n = 5). **, $P < 0.05$ relative to control group. ***b***, One example of a patient whose α_4-integrin expression levels were monitored for 3 days (□, septic patient; ■, control patient). As the septic patient improved, α_4-integrin decreased on the neutrophil surface.

identified a new pathway through which neutrophils may be inappropriately recruited in septic syndrome and septic shock in humans. Moreover, the property of septic plasma responsible for inducing the α_4-integrin/VCAM-1 pathway on neutrophils is transferable, given that normal neutrophils adhered to VCAM-1 via α_4-integrin with 30 minutes of exposure to septic plasma. Stable, soluble mediators within the plasma of septic patients might induce this pathway of neutrophil adhesion. Moreover, the data suggest that the α_4-integrin pathway is rapidly induced by septic plasma in normal neutrophils, and does not necessarily require a bone marrow response in septic patients (that is, release of immature α_4-integrin–positive neutrophils). The latter observation is important, as neutrophil precursors are known to use α_4-integrin to adhere to bone marrow sinusoids[21]. Finally, the data demonstrate that a systemic response is necessary, as acute bacterial pneumonia was not sufficient to induce this pathway of neutrophil recruitment.

Presently, no single mediator has been found that can directly invoke a functional α_4-integrin response in human neutrophils. We have tested bacterial products (fMLP), chemokines (IL-8), phospholipids (LTB_4) and cytokines (TNF-α) without seeing α_4-integrin–dependent adhesion. Multiple pro-inflammatory molecules, or an initial priming event followed by multiple pro-inflammatory molecules may be required for α_4-integrin expression. Indeed, we previously reported that maximal pharmacological stimulation of neutrophils with pro-inflammatory mediators plus cytochalasins (which perhaps maximize membrane receptor number) will induce α_4-integrin–dependent neutrophil binding to VCAM-1 under static and flow conditions[22,23]. Although these were non-physiologic stimuli, the data prompted the hypothesis that under certain severe pathophysiologic conditions, a sufficient pro-inflammatory milieu could induce neutrophils to adhere to VCAM-1. Indeed, our finding that septic plasma stimulates neutrophils from healthy individuals to bind to VCAM-1 supports this hypothesis. This observation may have numerous implications with respect to human sepsis. It may explain the β_2-integrin-independent, inappropriate neutrophil adhesion in various microvascular beds [24–27]. More importantly, it may provide a potential therapeutic target which could reduce inappropriate neutrophil adhesion without altering the normal yet critical adhesive function of neutrophils. This would be most useful considering that inhibition of β_2-integrin in animal models of sepsis worsens rather than improves outcome[28,29].

The discovery of functional α_4-integrin on neutrophils from septic patients is also important when considering the versatility of this integrin. Unlike the β_2-integrin, which cannot tether the neutrophil to substrata under normal flow (requires a selectin), the α_4-integrin can perform the function of both a selectin (tether and roll) and an integrin (adhere). Our data indicate that neurotrophils express sufficient functional α_4-integrin to roll and adhere on VCAM-1 independent of selectins and β_2-integrin. Therefore, therapeutically targeting the selectins or β_2-integrin would not necessarily inhibit neutrophil recruitment in sepsis. In addition, the normal rolling on P-selectin and enhanced β_2-integrin adhesion of neutrophils from septic patients are also important findings. Altered L-selectin and β_2-integrin expression have been reported[5,10,30]. These expression studies can be difficult to interpret because absence of L-selectin does not preclude rolling[31], and an increase in β_2-integrin can occur without increased adhesion[32]. Functional studies have revealed altered adhesiveness in neutrophils from septic patients. However, those studies used inert substrates under static conditions and therefore did not focus on the molecular adhesion mechanisms. In fact, results were equivocal: groups reported decreased[15] and increased[8,9] adhesion of isolated septic neutrophils to substrata such as nylon wool, glass and albumin-coated latex beads.

Studying neutrophil behavior in human disease using whole blood under flow conditions has revealed a novel role for neutrophils in sepsis. Increased β_2-integrin activity and, more importantly, an unexpected functional presence of α_4-integrin on septic human neutrophils greatly enhance the adhesion of these cells in the pathogenesis of this disease. We suggest that targeting the β_2-integrin will not be useful in sepsis, as properly functioning neutrophils are essential for bacterial clearance; however, the dysregulated α_4-integrin–dependent neutrophil adhesion could conceivably be a target to reduce excessive inflammation induced by inappropriate neutrophil adhesion.

Methods

Patients with sepsis. Adult ICU patients were included if they had a systemic inflammatory response and met the clinical criteria for septic shock or severe sepsis syndrome[33]; all patients had a confirmed infection[34,35]. Pneumonia was diagnosed based on accepted standards[36,37]. Intra-abdominal sepsis required the presence of transmural bowel infarction, free intestinal perforation with frank peritoneal soiling or direct visualization on an abscess cavity with microbiologic confirmation. Bacteremia was defined as 2 'positive' blood cultures drawn from 2 separate sites (one requiring venipuncture) at least 20 min apart. Necrotizing fasciitis was determined by direct surgical inspection, and pathological and microbiologic confirma-

tion. All patients also had either hypotension unresponsive to adequate fluid resuscitation (> 2 l intravenous crystalloid, a pulmonary artery wedge pressure > 14 mm Hg and central venous pressure > 10 mm Hg, and requiring vasopressors to maintain systolic blood pressure > 90 mm Hg), or the presence of acute dysfunction in at least 2 organ systems (metabolic acidosis pH < 7.30 or a base deficit > 5 or a serum lactate > 2.5 mmol/L, PaO2/FiO2 < 250 mm Hg and requiring mechanical ventilation, oliguria < 0.3 ml/kg bodyweight/hour ×2 consecutive hours despite volume resuscitation, > 1.5-fold increase in either international normalized ratio or partial thromboplastin time relative to control, > 50% drop in platelet count or absolute count < 50×10^9 cells/L, or acute metabolic encephalopathy without other adequate explanation). All subjects must have met criteria for entry within a consecutive 24-h period prior to enrollment. All patients were enrolled within 36 h of presenting to the ICU. Patients with leukopenia (< 4.0×10^9 cells/L), human immunodeficiency infection or immunosuppression from chemotherapeutic agents were excluded. The local human subject research ethics board approved the study, and informed consent was obtained from subject's families in accordance with local guidelines. Normal healthy volunteers served as controls. Because many of the septic patients had undergone surgery, a postoperative control group who underwent laparotomy, thoracotomy or mediastinoscopy/bronchoscopy was studied. Blood was drawn within 24 h after the operation to coincide with times used in the experimental group.

Substrata. Two separate substrata were used to assess leukocyte rolling and adhesion in the laminar flow chamber. To examine neutrophil rolling and adhesion, a P-selectin and CD18 ligand system was used. The simplest and most consistent system is a platelet monolayer immobilized to collagen coated glass cover slips as described by us[17] and others[18,19]. To study rolling and adhesion of mononuclear leukocytes, VCAM-1 was immobilized to glass cover slips as previously described[16].

Whole blood collection. Whole blood (5 ml) was collected into 15 ml polypropylene vials with 30 U/ml heparin from healthy volunteers, post operative patients and septic ICU patients. The whole blood was then diluted 1:10 with HBSS and used within 120 min of collection. We have demonstrated that the concentration and type of heparin used does not affect leukocyte adhesion in this system[16] whereas other anti-coagulants affected leukocyte recruitment. In addition, there are minimal numbers of secondary leukocyte–leukocyte interactions in this blood system allowing us to assess the direct importance of, for example, leukocyte VCAM-1 interactions. Blood smears were made from all samples for the determination of WBC counts and differentials.

Protocol. A perspex parallel-plate flow chamber described by Lawrence and Springer[20] was used. The cover slips with platelets or VCAM-1 were used as the chamber's bottom plate. The flow chamber was placed on a stage of an inverted microscope (Zeiss Canada) and the cover slips observed using phase contrast imagery (×200) as previously described[16]. The diluted whole blood was perfused at a shear force of 4 dynes/cm^2 for 5 min and followed by a perfusion with HBSS. Interacting leukocytes were either rolling or adhered to the surface of the cover slip. Leukocytes which remained stationary for less than 10 s were defined as adherent. In additional experiments, diluted whole blood was perfused with monoclonal antibodies HP1/2 (against α_4-integrin), G1 (against P-selectin) or IB_4 (against β_2-integrin). All experiments were video recorded for playback analysis using a CCD camera (Hitachi Denshi, Japan) and video cassette recorder. The data for each patient was calculated as the average from 4 separate fields of view. At the end of each experiment all cover slips were stained (Wright–Giemsa stain) for the determination of leukocyte differentials on the cover slips. In some experiments plasma from the septic patients was added to the cellular portion of healthy controls and vice versa. The samples were then incubated for 30 min at 37 °C, diluted to 1/10 in HBSS and perfused over VCAM-1 in the laminar flow chamber.

Flow cytometry measurements. Blood was drawn and primary antibodies directed against (α_4-integrin, HP1/2, 5 μg/ml) were added. HP1/2 was used instead of the more commonly used HP2/1 as the letter was reported to have a contaminant that interfered with α_4-integrin expression[38]. Indeed, our results did indicate complications with some but not all batches of HP2/1. After 20 min at 4 °C, the red blood cells were lysed and leukocytes were simultaneously fixed in 1% formalin and then labeled with FITC-conjugated rat IgG against mouse (Cedar Lanes Laboratories, Hornby, Ontario) and measured on a FACScan flow cytometer (Becton Dickinson). Appropriate controls (no primary, an isotype control and no secondary antibody) were completed for each set of experiments.

Statistics. The data are presented as mean ± s.e.m. Means were then compared using the *t*-test with Bonferroni's correction for multiple comparisons. Statistical significance was set at $P < 0.05$.

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