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Organism-Specific Neutrophil-Endothelial Cell Interactions in Response to *Escherichia coli*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*[1]

Jessica G. Moreland,[2]*† Gail Bailey,*† William M. Nauseef,†‡ and Jerrold P. Weiss†‡

The recruitment of polymorphonuclear leukocytes (PMNs) from the vascular space into the lung interstitium and airspace is an early step in the host innate immune response to bacterial invasion of these sites. To determine the ability of intact bacteria to directly elicit PMN migration across an endothelial monolayer, we studied in vitro migration of PMNs across a monolayer of human pulmonary microvascular endothelial cells in response to *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Escherichia coli*, as well as to purified *E. coli* LPS. Bacterial induction of PMN migration was dose dependent and elicited by $\geq 10^4$ bacteria/ml of each of the species tested. Pretreatment of PMNs with blocking Abs to CD18 significantly inhibited migration of PMN in response to all stimuli tested, but had the most profound effect on migration to *S. pneumoniae* and *S. aureus*. Intact *E. coli* were 10 times more potent in inducing transmigration of PMNs than a corresponding amount of purified LPS. Bacterial induction of PMN migration did not correlate with up-regulation of surface endothelial ICAM-1 expression (purified LPS $\gg$ intact *E. coli* > *S. aureus* and *S. pneumoniae*) nor up-regulation of VCAM-1 and E-selectin. Neutralizing Ab to ICAM-1 had no effect on PMN migration to any of the bacteria or to purified LPS. These findings demonstrate that diverse bacterial pathogens induce PMN migration across a pulmonary microvascular endothelial cell monolayer in a fashion that appears to be organism specific. In addition, intact bacteria elicit PMN-endothelial cell interactions distinct from those seen when purified bacterial products are used as agonists. ***The Journal of Immunology*****, 2004, 172: 426–432.**

Bacterial pneumonia continues to be a prevalent problem in both the community and the hospital and exacts significant morbidity and mortality (1). Specific pulmonary manifestations of pneumonic disease vary significantly according to its microbial cause. Bacteremia is a frequent consequence of pneumonia caused by certain pathogens (e.g., *Streptococcus pneumoniae* and *Escherichia coli*) (2, 3), suggesting that bacterial pathogens interact with host cells lining the airways and residing in the alveolar spaces, as well as with the pulmonary microvascular endothelium. The acute influx of polymorphonuclear leukocytes (PMNs)[3] into the alveolar space is a feature common to all bacterial pneumonias and an early element of the innate immune response to bacterial invasion of the airspace. Studies of neutrophil recruitment from the vasculature suggest a requirement for the coordinated expression of adhesion molecules by the leukocyte and counter ligands on the endothelium (4–6).

Whereas the paradigm for neutrophil migration to nonpulmonary sites of infection has been extensively studied and fairly well defined (reviewed in Ref. 7), the movement of neutrophils out of the pulmonary circulation appears to be mechanistically different. Leukocytes emigrate predominantly across the pulmonary capillary endothelium, rather than across postcapillary venules as is the case at other sites of PMN transmigration (8, 9). In addition, pathways of migration vary dependent on the nature of the bacterial or inflammatory stimulus.

The established concepts of neutrophil-endothelial cell interactions as applied to the lung are derived from fairly limited studies. Bacterial pneumonic processes have been studied in the whole animal using intratracheal instillation of intact bacteria or bacterial products, and neutrophil migration has been categorized as CD18 dependent or CD18 independent, reflecting the requirement for β_2 integrins by the neutrophil (10–13). Whereas both pathways may be operative in response to a specific stimulus, the relative importance of each may vary depending on the nature of the inflammatory stimulus (e.g., bacterial species). In addition, the role of specific endothelial cell adhesion molecules in pneumonia caused by microbial pathogens has been difficult to define precisely in whole-animal models despite studies focused on members of the ICAM family (14–16).

In vitro studies have recently established that PMN migration across the vascular endothelium in a simplified model also demonstrates variable requirements for CD18, with migration to leukotriene B_4 and fMLP showing a far greater CD18 dependence than migration to IL-8 (17). There are no in vitro studies to date that focus on the neutrophil-endothelial cell interactions in response to intact bacteria, which likely provide a more complex array of molecular stimuli.

Hence, little is known about the capacity of intact bacteria to directly induce migration of PMNs across the pulmonary endothelium. Therefore, the current study focuses specifically on the interaction of the neutrophil and the pulmonary microvascular endothelium as two early components of the host innate immune

*Division of Pediatric Critical Care, Department of Pediatrics, †The Inflammation Program, and ‡Department of Medicine, University of Iowa, and Veterans Affairs Medical Center, Iowa City, IA 52242

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[2] Address correspondence and reprint requests to Dr. Jessica G. Moreland, Division of Pediatric Critical Care, Department of Pediatrics, University of Iowa, Iowa City, IA 52242. E-mail address: jessica-moreland@uiowa.edu

[3] Abbreviations used in this paper: PMN, polymorphonuclear leukocyte; PMVEC, pulmonary microvascular endothelial cell; IFM, immunofluorescence microscopy; MCP-1, monocyte chemoattractant protein-1; GRO-α, growth-related oncogene-α; PECAM, platelet endothelial cell adhesion molecule; HMVEC-L, human lung microvascular endothelial cell; EBM, endothelial basal medium; RT, room temperature.

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response to bacterial infection in the lung. An in vitro transmigration model was used to study migration of human neutrophils across intact monolayers of human pulmonary microvascular endothelial cells (PMVEC) in response to intact live *E. coli*, *Staphylococcus aureus*, and *S. pneumoniae*, all well-described pathogens for lower respiratory tract infection, as well as migration to purified *E. coli* LPS. Interestingly, neutrophil migration in response to *S. pneumoniae* and *S. aureus* exhibited the greatest requirement for CD18, whereas *E. coli*-induced migration was substantially independent of CD18. The expression of several endothelial adhesion molecules (ICAM-1, ICAM-2, VCAM-1, and E-selectin) in response to these pathogens was characterized using intact cell ELISA and immunofluorescence microscopy (IFM), and chemokine expression by the endothelium or the neutrophils was quantitated by ELISA of the supernatant for IL-8, monocyte chemoattractant protein-1 (MCP-1), IL-6, RANTES, TNF-α, and growth-related oncogene-α (GRO-α). Purified LPS markedly upregulated endothelial cell surface expression of ICAM-1, VCAM-1, and E-selectin, whereas intact bacteria had minimal effect on surface expression of these molecules. Intact *E. coli* elicited PMN migration with much greater potency than purified *E. coli* LPS at corresponding LPS concentrations, suggesting that, in addition to the LPS component of the bacteria, there may be other bacterial factors interacting with the neutrophil or the endothelium. The data demonstrate that intact bacteria potently elicit PMN migration across the endothelial monolayer in an organism-specific manner by mechanisms that do not seem linked to up-regulation of several endothelial adhesion molecules.

Materials and Methods

Materials

Tissue culture medium was purchased from Clonetics (San Diego, CA). Transwell filters (12 and 24 mm) were purchased from Costar (Cambridge, MA). Blocking Abs used in transmigration studies include anti-CD18 (clone L130) from BD PharMingen (San Diego, CA), anti-CD18 (clone YFC118.3) purchased from BioSource (Camarillo, CA), and anti-ICAM-1 $F(ab')_2$ purchased from Caltag Laboratories (Burlingame, CA). Abs used for ELISA and IFM studies include anti-platelet endothelial cell adhesion molecule (PECAM)-1 (clone hec 2.7) (a generous gift from Dr. W. Muller (Weill Medical College, New York, NY)), anti-ICAM-1 (clone 6.5B5) purchased from DAKO (Glostrup, Denmark), anti-E-selectin (clone 68-5H11) and anti-VCAM-1 (clone 51-10C9) from BD PharMingen, and anti-ICAM-2 (clone CBR-IC2/2) purchased from Research Diagnostics (Flanders, NJ). Control Abs include mouse IgG1 and IgG2a (Sigma-Aldrich, St. Louis, MO) and rat IgG2b (BioSource). Cycloheximide was purchased from Calbiochem (San Diego, CA).

Human PMN purification

Human PMNs were isolated according to standard techniques from heparin anti-coagulated venous blood from healthy consenting adults in accordance with a protocol approved by the Institutional Review Board for Human Subjects at the University of Iowa. PMN were isolated using dextran sedimentation and Hypaque-Ficoll (Amersham Biosciences, Piscataway, NJ) density-gradient separation, followed by hypotonic lysis of erythrocytes as previously described (18). Purified PMNs were resuspended in 0.9% saline before use in migration experiments.

Endothelial cell culture

Human lung microvascular endothelial cells (HMVEC-L) were purchased from Clonetics and cultured on collagen-coated flasks (type VI; human placental collagen; Sigma-Aldrich) using endothelial growth medium 2 (Clonetics) with added bovine brain extract, vascular endothelial growth factor, epidermal growth factor, gentamicin, and hydrocortisone according to the manufacturer's specifications. Cells were received from Clonetics at passage 5 and used between passages 5 and 9. Cells were passed from T-75 flasks into experimental plates when ~70–80% confluent. HMVEC-L were detached using trypsin-EDTA and cultured on collagen-coated Transwell (Costar) 12-mm filters (transmigration experiments), Transwell 24-mm filters (IFM), or 96-well plates (whole-cell ELISA). Cell monolayers on Transwell filters were monitored by measuring resistance changes across the endothelial cell monolayer using an End-Ohm epithelial voltohmmeter (World Precision Instruments, Sarasota, FL). The microvascular endothelial cell monolayers reached an average resistance of 18–20 Ω/cm^2 across the monolayer within 5–6 days. Medium was changed on the monolayer every 48 h.

Bacterial cell cultures

S. pneumoniae (strain 6303; American Type Culture Collection, Manassas, VA) were stored at −80°C, and an aliquot was streaked on blood agar plates 22–24 h before use. Bacteria were resuspended in endothelial basal medium (EBM), and bacterial concentration was determined by measuring OD_{520}. *S. aureus* (strain RN450) and *E. coli* (strain MC4100) were grown overnight from frozen stocks in tryptic soy broth (*S. aureus*) or nutrient broth (*E. coli*) at 37°C with continuous shaking. Subcultures were set up the morning of each experiment at $OD_{0.1}$ and grown for 2 h to mid-log phase. Bacterial concentration was determined by measuring OD_{520}, and confirmed by measuring CFU in tryptic soy agar or nutrient broth agar plates. Bacteria were added to the Transwell lower chambers at known OD, and samples from $t = 0$ and $t = 4$ h (just before the addition of the PMNs) were plated for CFU determination to determine the growth of each bacterium during the initial 4-h bacteria/endothelial cell incubation. In specified experiments, bacteria were prepared as described and incubated for 6 h in EBM at 37°C. Medium was collected and sterile filtered with a 0.2-μm low-protein binding filter, and then added to the lower chamber of the Transwell for migration experiments using conditioned medium.

Exposure to purified LPS

Purified *E. coli* LPS was purchased from List Chemicals (Campbell, CA) and reconstituted in sterile water according to the manufacturer's instructions. LPS was preincubated with LPS binding protein (0.5 μg/ml) and soluble CD14 (2.5 μg/ml) for 30 min at 37°C, before adding to the Transwell lower chamber at the described concentrations.

Ab treatments

In experiments using mAbs, PMNs were coincubated with the specified Ab at room temperature (RT) for 15 min, just before addition of the PMNs to the HMVEC-L monolayer in the Transwell filter. For endothelial cell mAb treatment, mAbs were added to the HMVEC-L in the Transwell filter 30 min before the addition of the PMNs and remained throughout the incubation period. The concentration of Ab used was 2× the concentration with the greatest inhibitory effect, based on initial studies using each Ab over a wide range of concentrations.

PMN transendothelial migration

Migration assays were performed across HMVEC-L monolayers as follows. Transwell filters with attached endothelial cell monolayers were transferred to clean 12-well plates, and washed twice with HBSS. After removal of the HBSS, 400 μl of EBM (Clonetics) was added to the Transwell filter compartment. Whole bacteria or purified LPS dispersed at the indicated concentrations (or bacterial conditioned medium), were added to the lower compartment in a total volume of 1 ml and incubated with the endothelial cells for 4 h at 37°C. At the end of this 4-h incubation, 10-μl samples were removed from the lower chamber for quantitation of the bacterial CFU, and 2×10^6 PMNs were added to the Transwell (upper chamber) in a volume of 100 μl. PMNs were allowed to migrate over a 3-h period at 37°C. At the conclusion of the incubation, migrated cells were collected from the lower chamber for counting in a hemacytometer in triplicate. In preliminary experiments, PMNs recovered from above and below the endothelial monolayer account for ~90% of the initial number added, indicating that there are not a significant number of tightly adherent PMNs that are not recovered. Therefore, PMNs were counted from the lower chamber only. In specified experiments, cycloheximide (10 or 100 μg/ml) was added to the monolayer in EBM 1 h before the addition of bacteria (both doses of cycloheximide used gave similar results).

Assays of surface expression of endothelial cell adhesion molecules

To assess endothelial cell surface expression of adhesion molecules, monolayers were grown on 6.5-mm Transwell filters to peak resistance as previously described. Whole bacteria or purified LPS were added to the lower chamber of the Transwell apparatus and incubated for either 6 or 16 h. For all experiments using purified LPS, the LPS was preincubated for 30 min with LPS binding protein (0.5 μg/ml) and soluble CD14 (2.5 μg/ml). At the completion of the incubation, bacteria or LPS were removed, and cells were washed twice with HBSS and then blocked for 30 min with HBSS

containing 0.5% BSA. In ELISA for E-selectin, cells were then fixed with 0.5% glutaraldehyde at 4°C for 30 min, before addition of the primary Ab. In ELISA for ICAM-1, ICAM-2, and VCAM-1, primary Ab was added immediately following the blocking step. Primary Abs were added (100 μl/well), diluted in PBS plus 0.1% BSA, and incubated for 1 h at RT on a rotating shaker. The concentrations of the primary Abs were as follows: E-selectin, 2 μg/ml; ICAM-1, 0.375 μg/ml; ICAM-2, 0.5 μg/ml; and VCAM-1, 0.5 μg/ml. Following this incubation, cells were washed five times with HBSS/0.1% BSA and incubated with peroxidase-conjugated goat anti-mouse secondary Ab (dilution of 1/1000) for 1 h at RT. After the secondary Ab, cells were washed an additional five times and liquid tetramethylbenzidine substrate (Sigma-Aldrich) was added. OD_{650} was read after 30 min. For each ELISA, an isotype-matched control Ab was used in place of the primary Ab in three wells, and this background was subtracted from the signal.

Analysis of chemokine expression

Supernatants from the lower chambers of transmigration experiments were collected after PMN migration and filtered to remove bacteria and neutrophils before storage at −20°C. IL-8, MCP-1, RANTES, IL-6, GRO-α, and TNF-α levels were assayed in the supernatant using standard ELISA techniques. Briefly, 96-well plates were coated with a mAb to the protein of interest (R&D Systems, Minneapolis, MN). Fifty-microliter samples from the collected supernatant were added to the plate followed by a biotinylated detection Ab. HRP-conjugated streptavidin (Pierce, Rockford, IL) was then added to the plate, followed by a color substrate tetramethylbenzidine (Sigma-Aldrich). Standard curves were made using recombinant protein for each of the proteins of interest (R&D Systems). Absorbance at 450 nm was read, and protein concentrations of unknown samples were extrapolated based on the standard curve.

Immunofluorescence microscopy

HMVEC-L were grown on Transwell 24-mm filters (Costar) to peak resistance. Filters with attached endothelial cells were rinsed twice with HBSS. After removal of the HBSS, 1500 μl of EBM (Clonetics) was added to the Transwell filter compartment. Bacteria suspended at the described concentrations in EBM were added to the lower compartment in a total volume of 2.5 ml. Bacteria and endothelial cells were coincubated for 6 h at 37°C. Following the 6-h incubation, bacterial suspensions were removed, and filters were washed twice with PBS, fixed in 10% formalin at RT for 15 min, and blocked overnight with PBS plus 0.5% BSA. Primary Ab was applied to the luminal surface of the endothelial cells on the filter for 1 h at RT (anti-PECAM (1/50), anti-ICAM-1 (1/250), and anti-E-selectin (1/250)). After washing three times with PBS plus 0.5% BSA, Texas Red-labeled goat anti-mouse Ab (Molecular Probes, Eugene, OR) was applied (1/1000 dilution) for 1 h at RT. Specificity of staining was assessed by use of isotype control mouse Abs (Sigma-Aldrich). Filters were washed and mounted on glass slides. Samples were viewed using a Zeiss Axioplan 2 photomicroscope (Zeiss, Thornwood, NY), and digital images were obtained using a Zeiss AxioCam and AxioVision 3.1 software.

Results

Transmigration of PMN across PMVEC

PMN migration across intact PMVEC monolayers was assessed in response to a wide range of concentrations of *S. pneumoniae*, *S. aureus*, and *E. coli*, each added to the lower chamber of the Transwell system. In the absence of added bacteria or other stimulus, there was minimal migration of PMNs across the intact endothelial monolayer (<10,000 cells or <0.5% of the added PMNs). All three bacterial species tested induced a significant dose-dependent increase in PMN migration over a range of 10^4–10^6 CFU/ml bacteria added to the lower chamber (Fig. 1). The maximal percentage of PMNs migrating in response to each of the three bacterial species was similar, and exceeded 50% of the added PMN. Resistance across each endothelial monolayer was maintained following the initial 4-h incubation with bacteria, confirming that incubation of endothelial cells with bacteria within this dose range did not disrupt the integrity of the endothelial monolayer. Requirements for new protein synthesis in the transmigration process were assessed by pretreatment of the endothelial monolayers with cycloheximide (10–100 μg/ml) for 1 h before addition of bacteria to the lower

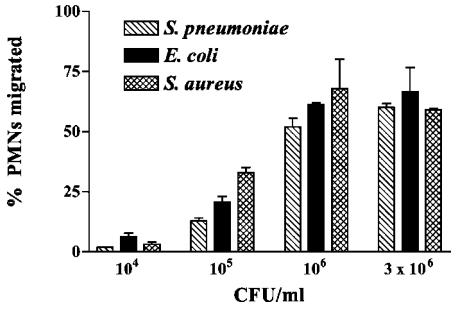

FIGURE 1. Migration of human neutrophils across a PMVEC monolayer in response to intact *S. pneumoniae*, *S. aureus*, and *E. coli*. The *x*-axis represents bacterial CFUs present in the lower chamber of the Transwell system at the time PMNs were added to the assay, after 4 h of bacterial coincubation with the endothelial monolayer. All three bacterial species elicited PMN migration in a dose-dependent manner.

chamber. Interestingly, the effects of cycloheximide varied substantially according to the stimulus. Migration to intact *S. pneumoniae* did not require new protein synthesis with migration after cycloheximide at 98.9% of nontreated monolayers. Migration to *E. coli* was substantially inhibited (69.3% of control), as was migration to *S. aureus* (59.7% of control) by cycloheximide pretreatment of endothelium ($n = 3$ experiments) (Fig. 2). ELISA performed on the supernatant for IL-8 and MCP-1 confirmed that cycloheximide treatment inhibited new protein synthesis. Transmigration of PMN in response to purified *E. coli* LPS was studied for comparison (Fig. 3). After normalizing bacteria and LPS by LPS content of *E. coli* (i.e., 10^5 bacteria contain 1 ng of LPS (19)), intact *E. coli* was 10-fold more potent than was purified LPS in inducing PMN transmigration. Given the differential potency between intact *E. coli* and purified LPS, we assessed the requirement for intact bacteria in eliciting PMN transendothelial migration. Migration was assessed in response to each of the intact bacteria vs bacterial conditioned medium recovered from each of the bacterial suspensions. Conditioned media were prepared by incubation of log-phase bacteria at 5×10^6 CFU/ml for 6 h in EBM, followed by sterile filtration to

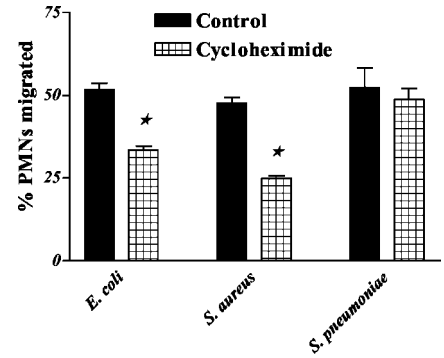

FIGURE 2. Requirements for new protein synthesis during PMN transendothelial migration to intact *S. pneumoniae*, *S. aureus*, and *E. coli*. Cycloheximide (10 μg/ml) was added to the endothelial monolayer for 1 h before the addition of 1×10^6 CFU/ml of each bacterial species to the lower chamber of the Transwell. In each experiment, duplicate wells for each condition (control vs cycloheximide) were compared for each bacterial species. There was differential inhibition of migration following blockade of new protein synthesis with no effect on PMN migration in response to *S. pneumoniae*, but significant reduction in migration to *S. aureus*, and *E. coli*. *, $p \leq 0.05$ as compared with migration under control conditions (no cycloheximide).

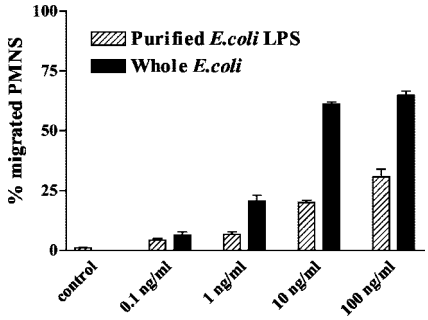

FIGURE 3. Migration of human neutrophils in response to intact *E. coli* vs purified *E. coli* LPS (10^5 bacteria contain 1 ng of LPS). LPS content of intact bacteria was normalized to purified LPS based on CFU counts for *E. coli* after initial 4-h coincubation of bacteria with the endothelial cell monolayer, just before addition of PMNs.

remove bacteria before addition of the medium to the lower chamber of the Transwell system. Migration to *E. coli*- and *S. pneumoniae*-conditioned medium was substantially less than migration to similar numbers of intact bacteria (54.6 and 41.5%, respectively). In contrast, PMN migration to *S. aureus*-conditioned medium was quantitatively similar to migration to intact bacteria, suggesting that secreted or shed bacterial products account for much of the induction of PMN transmigration.

β_2 Integrin requirements for PMN migration

Neutrophil adhesion molecules involved in PMN migration have been extensively studied using animal models of pneumonia. There are well-described differences in CD18 requirements for migration based on the migratory stimulus (e.g., bacterial species, chemical irritant) in whole-animal studies. We assessed the role of β_2 integrins during in vitro transmigration in response to intact bacteria or purified LPS. PMNs were pretreated with two blocking Abs, L130 and YFC118.3, each recognizing a different epitope on the CD18 β-chain. Both Abs reduced PMN transmigration in response to each of the bacteria and also to purified LPS (Fig. 4). Migration to *S. pneumoniae* demonstrated the greatest requirement for CD18 with transmigration reduced by 79% (clone L130) and 64% (YFC 118.3) as compared with transmigration in response to *S. pneumoniae* by PMNs pretreated with an isotype control Ab. Migration was also significantly reduced in response to *S. aureus* following pretreatment of PMNs with L130 (46% reduction) or YFC 118.3 (74% reduction). PMN migration elicited by intact *E. coli* was slightly reduced by pretreatment with anti-CD18 Abs (<25% reduction); however, the contribution of the β_2 integrins during neutrophil migration in response to purified *E. coli* LPS appears to be much more substantial.

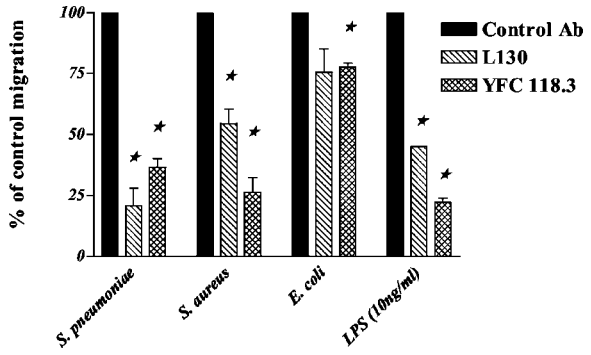

FIGURE 4. Effects of two different CD18 blocking Abs, L130 and YFC118.3, on neutrophil migration across a pulmonary microvascular endothelial monolayer in response to intact *S. pneumoniae*, *S. aureus*, *E. coli*, or purified LPS (10 ng/ml). Bacterial CFU at time of PMN addition was 1×10^6 CFU/ml for each species. Migration to isotype control Ab was set at 100%, and the effects of blocking Abs were normalized for comparison; $n = 3$ transmigration assays for each of the blocking Abs. *, $p \leq 0.05$ as compared with migration to the isotype control Ab.

Expression of endothelial cell adhesion molecules

After demonstrating organism-specific β_2 integrin requirements for PMN transmigration to intact bacteria, we focused on elucidating endothelial counter ligands that might be involved in neutrophil movement across the monolayer. Surface expression of endothelial adhesion molecules was studied using two approaches, whole-cell ELISA for quantitative data and IFM. Following either 6- or 16-h exposure of PMVECs to either *S. aureus* or *S. pneumoniae*, no increase in surface expression of ICAM-1, ICAM-2, VCAM-1, or E-selectin was observed by intact-cell ELISA. Intact, live *E. coli* elicited a 1.8-fold up-regulation of ICAM-1 surface expression, whereas purified LPS induced a >4-fold increase in ICAM-1 (Fig. 5*a*). Purified LPS induced surface expression of VCAM-1 and E-selectin, but there was no up-regulation by intact *E. coli* (Fig. 5, *b* and *c*). None of the stimuli significantly increased ICAM-2 expression under the conditions tested (Fig. 5*d*). Treatment of the endothelial cells with each of the bacteria for 5 h followed by 1 h in the presence of PMNs at the luminal surface of the monolayer gave similar results (data not shown). These data demonstrate no obvious correlation between neutrophil integrin requirements and endothelial adhesion molecule expression during PMN transmigration.

Similar observations were made by examination of the treated endothelial cell monolayers by IFM. Exposure to bacteria did not disrupt the integrity of the monolayers, as demonstrated by PECAM-1 staining (Fig. 6*a*). Surface ICAM-1 was up-regulated after exposure of the endothelial monolayer to intact *E. coli*, but not after exposure to *S. aureus* or *S. pneumoniae*. Monolayers exposed to purified LPS had further increases in the total number of cells displaying ICAM-1 expression as compared with monolayers exposed to intact *E. coli* (Fig. 6*b*). Expression of E-selectin and VCAM-1 could not be detected in monolayers exposed to any of the three bacterial species tested, but was clearly up-regulated following exposure to purified LPS (data not shown).

Effects of ICAM-1 blockade on PMN migration

The possible role of ICAM-1 in bacteria- or purified LPS-induced PMN transmigration was tested by examining the effect of a blocking anti-ICAM-1 mAb. Pretreatment of the endothelial cell monolayer with an ICAM-1 $F(ab')_2$ had no effect on PMN migration in response to *E. coli*, *S. aureus*, *S. pneumoniae*, or purified LPS. In contrast, in response to the bacterial chemoattractant fMLP, PMN migration was reduced by 53% (Fig. 7). Hence, up-regulation of surface expression of endothelial adhesion molecules did not correlate with a functional role in PMN transmigration across the monolayer.

Chemokine expression by endothelium and neutrophils

The paradigm described for neutrophil recruitment from the vascular space in response to bacteria in the lung suggests a significant role for chemokines in initiating the migratory process. Levels of chemokines secreted into the medium in response to each of the bacterial stimuli and purified LPS were studied by ELISA of the filtered supernatant from the lower Transwell chamber following PMN migration (Table I). None of these chemokines were identified in medium collected from the abluminal surface of unstimulated endothelial monolayers. IL-8, MCP-1, and RANTES were

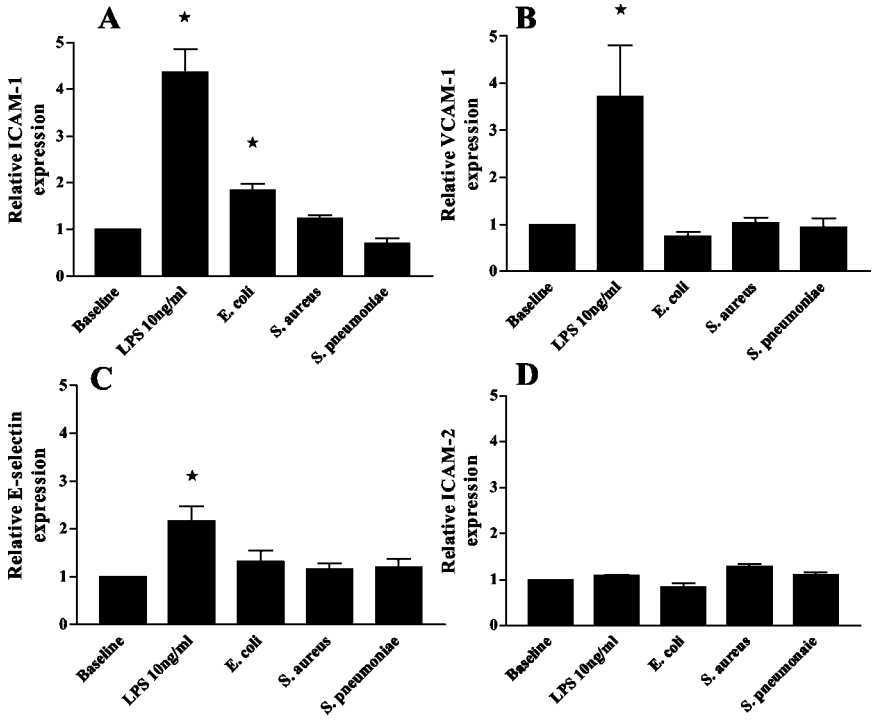

FIGURE 5. Surface expression of endothelial cell adhesion molecules, ICAM-1 (*a*), VCAM-1 (*b*), E-selectin (*c*), and ICAM-2 (*d*), by intact-cell ELISA in response to 6-h exposure to intact *S. pneumoniae*, *S. aureus*, *E. coli* (1×10^6 CFU/ml), or purified LPS (10 ng/ml). Baseline or constitutive expression was normalized to 1, and expression in response to bacteria expressed as compared with control. $*$, $p \leq 0.05$ as compared with baseline expression.

significantly up-regulated in response to all three bacterial stimuli studied. Up-regulation of expression of IL-8 and MCP-1 was more dramatic following purified LPS than the intact bacteria. IL-6 and GRO-α were significantly up-regulated during transmigration to LPS, but much less so, or not at all, in response to the intact organisms. TNF-α was not detected in any of the samples.

Discussion

Neutrophil migration from the bloodstream to the alveolar space during acute bacterial pneumonia represents the initial phase of the host innate immune response and thus has been the focus of intense study. However, much of our understanding of this process comes from data from animal models that instill bacteria or bacterial products into the trachea, and monitor the arrival of neutrophils in the lung (3, 10, 15). Although many of these studies have suggested that the mechanisms and requirements for PMN migration across the endothelium into the lung are both organism specific and different from the well-defined paradigm for systemic inflammation, use of in vitro models to more closely assess cellular interactions has been limited in number. The direct effects of whole bacteria on endothelial cell responses are likely to be relevant in a disease process where certain types of bacteria (e.g., *S. pneumoniae*) have been demonstrated to move across the respiratory epithelium by transcytosis (20). In addition, the frequency of bacteremia as a consequence of pneumonia suggests that the endothelium interacts directly with certain pathogens.

In this study, we demonstrate potent induction of PMN transmigration in vitro across a PMVEC monolayer in response to several species of intact bacteria, all well-described lower respiratory pathogens. Resting PMNs added to the luminal surface of the

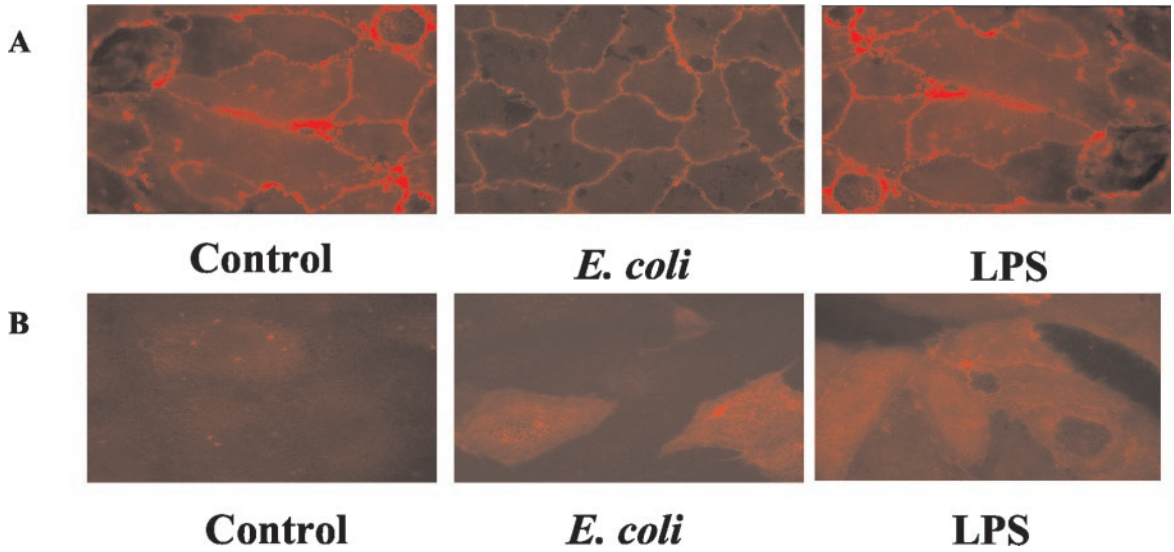

FIGURE 6. Surface expression of PECAM-1 (*a*) and ICAM-1 (*b*) by PMVEC monolayers at baseline (control) and after 6-h exposure to *E. coli* (1×10^6 CFU/ml) or purified LPS (10 ng/ml).

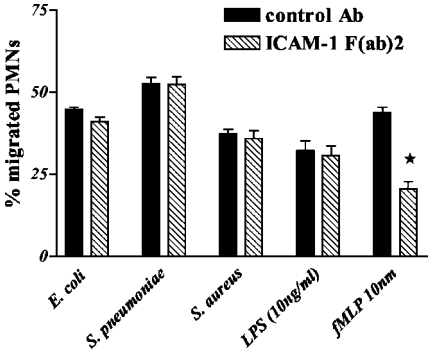

FIGURE 7. Effect of ICAM-1 F(ab′)$_2$ blocking Ab on PMN migration elicited by intact *S. pneumoniae*, *S. aureus*, *E. coli*, purified LPS (10 ng/ml), or fMLP (10 nM). Bacterial CFU at time of PMN addition was 1×10^6 CFU/ml for each species. ∗, $p \leq 0.05$ as compared with control Ab.

endothelial monolayer were stimulated to migrate in response to as few as 10^4 *S. aureus*, *S. pneumoniae*, or *E. coli* added to the abluminal side of the monolayer. The similar induction of PMN migration by both Gram-positive and Gram-negative bacteria suggests that there is not a single stimulus that initiates this response. Interestingly, when normalized for LPS content, intact *E. coli* elicited markedly greater PMN migration than did purified *E. coli* LPS, suggesting that there may be multiple bacterial factors interacting with host cells to initiate the migration cascade. PMN migration across the PMVEC monolayer in response to intact bacteria and purified LPS occurred in a dose-dependent manner in contrast to migration across monolayers of HUVEC, where as few as 10^5 bacteria of any species elicited the full PMN migratory response. In addition to this pattern of less discriminatory PMN migration, the electrical resistance across HUVEC monolayers was substantially less than the resistance that developed across PMVEC monolayers, suggesting a difference in organization of intercellular junctions (our unpublished observations).

Up-regulation and/or activation of neutrophil and endothelial cell adhesion molecules have been postulated as the mechanism(s) by which bacteria induce PMN movement into the lung. The β_2 integrins have been observed in several animal models to have a significant role in airway accumulation of PMN in response to intratracheally administered Gram-negative bacteria and/or purified Gram-negative bacterial products (e.g., LPS), whereas PMN migration elicited by *S. pneumoniae* and *S. aureus* has been categorized as predominantly CD18 independent (11, 12). In contrast to previously reported data from whole-animal models, pretreatment of PMNs with either of two monoclonal blocking Abs to CD18 in our in vitro model had the greatest inhibitory effect on migration elicited in response to *S. pneumoniae* and *S. aureus*. Migration to *E. coli*, previously described as a CD18-dependent stimulus, was substantially less affected by CD18 blockade. Our model focuses only on neutrophil movement across the endothelial monolayer, suggesting that subsequent interactions important for PMN movement across the pulmonary interstitium and/or airway epithelium may explain the different organism-specific CD18 dependence previously observed in vivo.

Previous in vitro data have suggested that neutrophil transendothelial migration has variable requirements for CD18, depending on the stimulus for migration (17). However, the stimuli used in that study were various chemoattractants rather than intact bacteria or even bacterial products. Recent in vitro data have demonstrated that there are different routes of migration used by neutrophils during an acute pulmonary inflammatory process as compared with a chronic inflammatory state (21), suggesting that both the nature of the inflammatory stimulus and the functional state of the neutrophil may affect the pathway of migration. Our results demonstrate a role for the β_2 integrins in movement of neutrophils across the endothelium in response to intact bacteria, but these requirements appear to be organism specific, and, at least in response to certain microorganisms, additional neutrophil adhesion proteins are likely to participate as well. In addition, because both Abs used blocked epitopes on the common β-chain, the relative contribution of each of the heterodimers (CD11a/CD18, CD11b/CD18, and CD11c/CD18) needs to be addressed.

This potent induction of PMN migration across an endothelial monolayer also suggests an active role for endothelial proteins in the migration process. Our data using cycloheximide to inhibit new protein synthesis suggest that constitutively expressed endothelial proteins may have a substantial role. The extent of this role appears to depend on the specific organism used as stimulus. Based on previously published data, a logical candidate counter ligand for CD18-dependent migration is a member of the ICAM family. Anti-ICAM-1 mAbs (10, 15) or antisense oligonucleotides against ICAM-1 (16) partially block neutrophil migration to the lung in response to intratracheal endotoxin or intact *Pseudomonas aeruginosa* in mice. In vitro studies of neutrophil transendothelial migration to C5a or IL-8 (14) demonstrate a role for both ICAM-1 and ICAM-2 in CD11a/CD18-mediated migration, but not for CD11b/CD18-mediated migration. We studied endothelial cell surface expression of both ICAM-1 and ICAM-2 as potential ligands involved in CD18-dependent movement. VCAM-1 and E-selectin expression were also studied as potential participants in neutrophil transendothelial migration. Our results indicate that induction of neutrophil migration does not correlate with up-regulation of these adhesion molecules. There was no up-regulation of any of these molecules in response to either of the Gram-positive bacteria. Intact *E. coli* significantly increased surface expression of ICAM-1 but did not affect expression of ICAM-2, VCAM-1, or E-selectin, whereas purified LPS induced a much more dramatic increase in ICAM-1 up-regulation, and also induced increased expression of VCAM-1 and E-selectin.

If induction of neutrophil migration by the bacteria we studied is not linked to up-regulation of ICAM-1, ICAM-2, VCAM-1, or

Table I. *Chemokine expression during PMN transendothelial migration to intact bacteria or purified LPS*[a]

	IL-8 (pg/ml)	MCP-1 (pg/ml)	RANTES (pg/ml)	IL-6 (pg/ml)	GRO-α (pg/ml)
S. pneumoniae	430 (±14)	127 (±6.1)	762 (±5.2)	12 (±1.5)	<15
S. aureus	353 (±16)	39 (±5.1)	346 (±3.5)	<12	<15
E. coli	477 (±13)	164 (±12.1)	680 (±11.1)	48 (±2.1)	<15
Purified LPS	1037 (±87)	288 (±36.3)	Not tested	482 (±16.4)	1933 (±31.6)

[a] Supernatants were collected from the abluminal surface of the endothelial monolayer at the completion of the migration experiments and filtered to remove bacteria and PMNs. Chemokine expression was assayed by standard ELISA techniques. $n = 4$ experiments.

E-selectin, what are the inducing factors from the endothelium? Several possibilities are imaginable, including participation of alternative adhesion molecules (not yet tested) or a role for ICAM-1 or -2 that is constitutively expressed on the endothelial surface. To test the latter possibility, we used blocking ICAM-1 F(ab) in our transmigration model. Our data (Fig. 6) demonstrate no change in neutrophil migration in response to *E. coli*, *S. pneumoniae*, *S. aureus*, or purified LPS following endothelial blockade of ICAM-1 alone, despite evidence of constitutive ICAM-1 expression in all of the endothelial monolayers, significant up-regulation in the *E. coli*- and purified LPS-treated endothelial monolayers, and the ability of the F(ab) used to inhibit fMLP-induced neutrophil migration. Our results indicate that neither constitutive nor induced ICAM-1 is needed in the migration process.

More directed studies to assess the role of CD11a/CD18 vs CD11b/CD18 in migration to each stimulus may suggest alternative endothelial counter ligands that have functional significance. Recent data have established that junctional adhesion molecule-1, a member of the junctional adhesion molecule family, is a ligand for CD11a/CD18 (22), suggesting that interactions between integrins and alternative endothelial Ig superfamily proteins may be functional in our system. The regulation of expression of endothelial adhesion molecules in response to whole bacteria using an in vitro model has not previously been described. Endothelial cell studies have primarily focused on the response to cytokines (23) or purified endotoxin (24) often using HUVEC, rather than effects of intact bacteria on a microvascular endothelium.

The importance of studying intact bacteria rather than just purified bacterial products is further underscored by the striking differences in endothelial and neutrophil responses to purified *E. coli* LPS vs intact *E. coli*. Whereas purified LPS induced marked up-regulation of multiple endothelial adhesion molecules believed to have significant roles in the transmigration process, LPS alone was a far less potent stimulus of PMN migration than were intact bacteria. Previous in vitro data using *Salmonella typhimurium* LPS and porins from the same organism have demonstrated that other components of Gram-negative bacteria can elicit neutrophil migration (25). Similarly, using a rat model of Gram-negative pneumonia, and several *E. coli* mutant strains, pulmonary neutrophil influx was demonstrated to vary based on the presence of the capsular polysaccharide (3), again suggesting that the endotoxin component is only one of several inflammatory mediators in Gram-negative infection. Gram-positive pneumonitis is also likely to elicit a host inflammatory response based on multiple organism-specific components. A recent report from Yipp et al. (26) demonstrated distinct neutrophil-endothelial cell responses using purified lipoteichoic acid and peptidoglycan from *S. aureus*; however, there were far more profound neutrophil-endothelial interactions in response to the intact bacteria. Our data further support the importance of defining host cellular interactions in response to intact organisms in addition to purified bacterial products at the in vitro level.

The current studies demonstrate an in vitro approach to studying neutrophil-endothelial cell interactions in response to living bacteria. Future studies will focus both on alternative endothelial adhesion molecules as well as other endothelial responses induced by exposure to intact bacteria. In addition, the distinct neutrophil and endothelial responses to purified *E. coli* LPS vs intact *E. coli* suggest that study of purified bacterial components may not fully model disease caused by intact bacteria. Future work will focus on assessing specific bacterial proteins/virulence factors involved in the induction of neutrophil and endothelial cell responses. The reduced complexity of this in vitro approach as compared with whole-animal models should permit a better definition of the molecular determinants of neutrophil transmigration across pulmonary microvascular endothelium in response to invading bacterial pathogens.

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