The $\beta 2$, $\alpha 4$, $\alpha 5$ Integrins and Selectins Mediate Chemotactic Factor and Endotoxin-Enhanced Neutrophil Sequestration in the Lung

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Intravascular chemotactic factor activation of neutrophils (polymorphonuclear leukocytes; PMNLs), associated with actin polymerization resulting in PMNL stiffening, induces rapid and transient sequestration in the pulmonary vasculature and lung dysfunction. Recent studies have proposed that this sequestration is mediated by physical lodging of PMNLs because of loss of deformability. To examine the contribution of cell adhesion molecules in this process, we used blocking monoclonal antibodies (mAbs) to rat selectins and integrins in a model of PMNL margination (reflected by acute blood neutropenia) induced by *N*-formyl-met-leu-phe (FMLP) chemotactic factor infusion in normal or lipopolysaccharide (LPS)-primed rats. Blood PMNL levels dropped by 70% within 1 minute and for the duration of FMLP infusion (20 minutes) in normal or by 90% in LPS-primed rats. Pretreatment with mAbs to $\beta 2$ (WT.3), VLA-4(TA-2 F(ab)₂), and VLA-5 (HM $\alpha 5$ F(ab)₂) in combination inhibited the decrease by 50% and to a greater degree than $\beta 2$ blockade alone (35% inhibition). F(ab)₂ mAbs to L-(HRL-3), P-(RMP-1), plus E-(RME-1) selectins had no effect but they potentiated inhibition by anti- $\beta 2$ + anti-VLA-4 + anti-VLA5 mAb treatment (69% inhibition, $P < 0.05$). Similar results were observed in the first 6 minutes in LPS-primed rats with complete inhibition of sequestration thereafter by combined selectin and integrin blockade. These results indicate that besides PMNL stiffening because of actin polymerization, both selectins and integrins substantially contribute to activated PMNL sequestration in the lung. (*Am J Pathol* 2001, 158:1809–1819)

An important component of the inflammatory reaction is the migration of leukocytes from the blood into the ex-

travascular space. At sites of inflammation, mediators such as the cytokines interleukin-1 and tumor necrosis factor- α are produced and activate the endothelium to increase expression of cellular adhesion molecules (CAMs)¹ and chemoattractants generated in the tissue, eg, C5a and chemokines, traverse the vessel wall to the luminal side. The CAMs initiate leukocyte capture and rolling on the postcapillary vascular endothelium and allow leukocyte activation by the chemotactic factors resulting in firm adhesion to endothelial cells. The CAMs belonging to the selectin family (E-, P-, and L-selectin) and the $\alpha 4$ (CD49d) integrins mediate capture of leukocytes from the flowing blood and rolling along the vessel wall. The $\beta 2$ (CD11/CD18) and also $\alpha 4$ (CD49d) integrins, after leukocyte activation, mediate firm attachment of these cells to the vascular endothelium by association with their ligands of the immunoglobulin (Ig) superfamily (ICAM-1, ICAM-2, and VCAM-1) on the endothelium. The integrins, along with additional interactions with PECAM-1, mediate migration of the leukocyte across the vessel wall, presumably guided by chemotactic factor gradients.^{2,3}

When inflammation extends beyond localized tissue sites as during disseminated infection⁴ or during blood-derived inflammatory mediator release, such as during extracorporeal circulation (eg, cardiopulmonary bypass^{5–7}), inflammatory mediators such as C5a and/or bacterial products such as endotoxin (lipopolysaccharide; LPS) and bacterial peptides analogous to the F-met-leu-phe (FMLP) chemotactic factor are released into the bloodstream. These bind to receptors on leukocytes including polymorphonuclear leukocytes (PMNLs) and on vascular endothelium, thereby activating these cells. Under these conditions of PMNL and endothelial activation, in the absence of a chemotactic factor gradient to guide the emigration of PMNL, a reversible, intravascular mar-

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gination or adhesion of the PMNLs occurs.^{3,8} A major site of this sequestration is in the pulmonary microvasculature.⁸⁻¹⁰ During this margination, activated PMNLs and their products (O_2^- , proteases, and NO) may contribute to lung dysfunction and even to the adult respiratory distress syndrome.¹¹⁻¹³

The mechanisms of PMNL sequestration in the pulmonary vasculature in response to intravascular chemotactic factors do not conform to the paradigm of localized inflammation in peripheral vessels. In the pulmonary capillary bed, selectin-mediated rolling may not occur, likely because the average diameter of these capillaries is smaller than that of PMNLs.^{14,15} This requires the PMNLs to deform to flow through the vessel and intimate contact of the PMNLs with the vascular endothelium must occur, thereby minimizing a requirement for the initial tethering of the leukocyte from the flowing blood. Thus initial integrin-Ig superfamily adhesion may be achieved. However, it has been proposed that selectins may, under the conditions of low shear flow in the pulmonary capillaries, mediate firm adhesion of leukocytes in the lung or at least provide important outside-in signaling for activation of leukocyte integrins.¹⁶

It has also been proposed that increased PMNL rigidity resulting from actin polymerization secondary to PMNL activation is a primary mechanism for PMNL sequestration in the pulmonary capillary bed.^{14,15,17-19} Chemotactic mediators such as FMLP, C5a, interleukin-8, and others, bind to receptors on PMNLs and initiate events leading to actin polymerization required for migration in a chemotactic gradient.²⁰⁻²² When the chemotactic factor is present in the blood, no such gradient exists but the intrinsic activation of actin polymerization still occurs. This results in a shift to a nonspherical shape as well as an increase in cell volume and a decrease in cell deformability. This loss of deformability and the cell shape change has been proposed to result in physical lodging of PMNLs in the relatively narrow capillary bed of the lung and markedly prolong the transit time of PMNLs through the pulmonary vasculature.^{15,18} However, whether this is the sole mechanism of pulmonary PMNL sequestration has yet to be clarified.

It has recently been reported that activated PMNLs express and functionally use not only the $\beta 2$ family of integrins in adhesion and migration but also several $\beta 1$ integrins including $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, and $\alpha 9\beta 1$.²³⁻²⁶ To date, there have been very few studies examining the role of CAMs in pulmonary sequestration of PMNLs induced by chemotactic factors. However, studies examining the role of the $\beta 2$ integrins in PMNL sequestration induced by the infusion of complement-activated plasma in rabbits have suggested that the initial sequestration is $\beta 2$ -integrin-independent but that at later time points there may be a role for $\beta 2$ integrins.^{27,28} Other studies have implicated a limited role for L-selectin in PMNL sequestration in the pulmonary vasculature.^{27,29} Our goal was to systematically examine the involvement of the $\beta 2$ and $\beta 1$ integrins and the role of selectins in PMNL pulmonary sequestration.

Materials and Methods

Animals

Male Lewis rats, weighing 250 to 350 g were purchased from Charles River Canada Corporation (St. Constant, QC, Canada) and used in all experiments. The experimental protocols were approved by the university committee of laboratory animal care.

Reagents

LPS (*Escherichia coli* 0111:B4) was obtained from List Biologics (Campbell, CA). Hespan-6% hetastarch in 0.9% sodium chloride was obtained from DuPont Canada (Scarborough, Ontario, Canada). Percoll was from Pharmacia Biotech (Uppsala, Sweden) and FMLP peptide was from Sigma Chemical Co. (St. Louis, MO.).

Blood Neutrophil Isolation and Radiolabeling of Neutrophils (PMNLs) and Red Blood Cells (RBCs)

Blood PMNLs were purified from rats immunized subcutaneously with *Mycobacterium butyricum* at the base of the tail 10 to 12 days before harvesting blood by a Hespan exchange transfusion and purification by Percoll gradient centrifugation as previously described³⁰ and modified.³¹ These rats provided a higher yield of PMNLs than unimmunized rats, yet the PMNLs were functionally comparable *in vivo* to PMNLs from normal donors as previously shown.³⁰ The donor animals were anesthetized with 0.3 ml of a 2:1 mixture of ketamine (50 mg/ml; Warner-Lambert Canada, Scarborough, Canada) and Innovar (50 μ g/ml fentanyl citrate and 2.5 mg/ml droperidol; Janssen Pharmaceuticals, Mississauga, Canada) by subcutaneous injection. A 25-gauge needle was inserted into the femoral vein, and the blood of the donor rat was slowly exchanged with 50 ml of Hespan containing 5 μ /ml heparin. The RBCs were allowed to sediment and the leukocyte-rich plasma was collected. Leukocytes were harvested by centrifugation from the leukocyte-rich plasma, resuspended in calcium-magnesium-free Tyrode's solution with 5% platelet-poor plasma (PPP) and PMNLs were isolated by separation ($400 \times g$, 30 minutes) on a 63%/74% Percoll-10% PPP gradient. The purified PMNLs were consistently >95% pure and >95% viable. The PMNLs in Tyrode's solution with 5% PPP at a cellular density of 5×10^7 cells/ml were then labeled with $Na_2^{51}CrO_4$ (Amersham, Oakville, Ontario, Canada) (1 μ Ci/ 10^6 cells at 37°C for 30 minutes). PMNLs were then washed twice in Tyrode's-5% PPP and resuspended in Tyrode's with 10% PPP for injection, each animal receiving 8×10^6 PMNLs labeled with $\sim 6.5 \times 10^4$ cpm. RBCs were obtained after the initial Hespan sedimentation, washed twice with Tyrode's solution, and resuspended at 5×10^8 /ml. ^{111}In -oxine was used to label (1 μ Ci/ 10^8) these cells for 10 minutes at 20°C. The cells were then washed twice with Tyrode's and resuspended at $5 \times$

10^8 /ml in Tyrode's-10% PPP for intravenous injection of 5×10^7 RBCs carrying 1×10^5 cpm of ^{111}In per rat.

Monoclonal Antibody Treatments

The mouse monoclonal antibody (mAb) WT.3 (IgG1; a gift from M. Miyasaka, Osaka, Japan) recognizes and functionally blocks the rat $\beta 2$ chain of the CD11/CD18 integrins.³² WT.3 was used as an intact antibody whereas all other antibodies were used as F(ab)_2 fragments generated by pepsin digestion as previously described.³³ The mouse TA-2 mAb (IgG1) generated in this laboratory, reacts with and functionally blocks the rat $\alpha 4$ (CD49d) chain of VLA-4.³⁴ The hamster mAbs HM $\alpha 5$ (IgG1; a gift from H. Yagita, Tokyo, Japan) reacts with and blocks the mouse and rat α -chain of VLA-5^{35,36} and HRL-3 (IgG1; a gift from D. Anderson, Pharmacia Upjohn, Kalamazoo, MI; and M. Miyasaka, Osaka, Japan) reacts with and blocks rat L-selectin mediated adhesion.³⁷ The mouse mAbs RMP-1 (IgG2a) to rat and mouse P-selectin and RME-1 (IgG1) to rat and mouse E-selectin were generated in our laboratories, and each specifically blocks adhesion mediated by these receptors.^{38,39} Other mAbs used were HM $\beta 1$ and HM $\beta 3$ (hamster IgG1) that block function of mouse $\beta 1$ and $\beta 3$ integrins and also recognize rat $\beta 1$ and $\beta 3$ (a gift from H. Yagita, Tokyo, Japan)^{35,40} and irrelevant control murine mAb B9 (IgG1) anti-pertussis toxin.

The effect of these mAbs on PMNLs was examined in the sequestration experiments as well as in studies examining their effect on FMLP- and LPS-induced PMNL shape and volume change. For *in vivo* assays, animals were pretreated with 1 mg of each antibody 20 minutes before any other manipulation of the animals. Measurement of the concentration of antibody in the serum of recipient animals at sacrifice by immunofluorescent flow cytometry showed that this dose of mAb was sufficient to produce blood levels of antibody at least 5 times higher than necessary to saturate the receptors on PMNLs or on endothelium (40 to 80 $\mu\text{g}/\text{ml}$). For *in vitro* assays, at least four times the saturating concentrations of the mAb (20 to 30 $\mu\text{g}/\text{ml}$) were used.

Chemotactic Factor-Induced PMNL Sequestration

A 25-gauge needle was inserted into the jugular vein under anesthesia by subcutaneous injection of 0.5 ml of a 2:1 mixture of xylazine (20 mg/ml; Bayer Inc., Etobicoke, Ontario, Canada) and Innovar and a slow infusion (50 $\mu\text{l}/\text{minute}$) of 0.9% saline containing 5 U/ml heparin was commenced. When indicated, the saline solution was replaced with a 6×10^{-4} mol/L solution of FMLP infused at 0.5 ml/minute for 30 seconds to clear the tubing dead space and an initial bolus of 10 nmol/100 g of this FMLP solution was given intravenously for a 30-second period, followed by a reduction of the rate of infusion to 0.1 ml/minute for 20 minutes. Some animals were injected intravenously with mAbs 20 minutes before initiation of the infusion. Blood samples were collected

from the tail vein into ethylenediaminetetraacetic acid (0.2%) anticoagulant for enumeration of leukocytes by hemocytometer counting before mAb injection and the FMLP infusion and at the indicated times thereafter.

In another group of animals, an intravenous injection of 50 $\mu\text{g}/\text{kg}$ of LPS in saline was given and, 4 hours later, a single bolus of 10 nmoles/100 g of FMLP was injected intravenously. In these experiments, tail blood samples were collected before LPS injection, and at the indicated times before and after mAb treatment and FMLP injection.

In some FMLP infusion experiments in normal or LPS-primed animals, the protocol was altered as follows: 20 minutes before FMLP infusion, ^{51}Cr -labeled purified PMNLs were injected intravenously. The FMLP infusion was as above and tail blood samples were obtained as indicated for quantitation of PMNL counts and ^{51}Cr -PMNL content in the samples using a γ counter (Wallac LKB1280; Fisher Scientific, Mississauga, Ontario, Canada). In each sample the specific activity (SA) or ratio of unlabeled to labeled PMNLs was determined as follows: $\text{SA}(\text{PMNL}/\text{cpm}) = (\text{PMNL}/\text{ml blood}) \div (^{51}\text{Cr cpm on PMNL}/\text{ml blood})$.

Determination of Sites of FMLP-Induced PMNL Sequestration

^{51}Cr -labeled PMNLs and ^{111}In -labeled RBCs were injected into recipient animals 20 minutes before the commencement of a 10-minute FMLP infusion via the jugular vein. Blood samples were obtained from the tail before the injection of the labeled cells and again before starting the FMLP infusion. Blood samples were also collected at 1, 3, and 6 minutes after beginning the FMLP infusion and a sample was collected immediately after completion of the infusion. After the last blood sample, 1 ml of saturated KCl was injected intravenously to immediately arrest the circulation. Various organs were then dissected and removed for γ -radiation counting. Blood samples were analyzed for PMNL count and ^{51}Cr and ^{111}In content with automatic spill-over correction.

The ^{111}In -RBC cpm content/ml blood was used to determine the blood volume by dividing it into the total ^{111}In -RBCs injected intravenously. RBC contamination of the purified PMNLs used in each experiment was corrected for by quantitation of the ^{111}In content in a given organ. Based on NH_4Cl lysis of residual contaminating RBCs (<1 RBC per 4 PMNLs) in the ^{51}Cr -PMNL preparation, the number of ^{51}Cr counts on RBCs in the purified PMNLs was determined. By calculating the percentage of the total injected ^{111}In -labeled RBCs in a given organ, the percentage of the total injected ^{51}Cr -labeled RBC content in the organ was determined and subtracted from the total ^{51}Cr counts in that organ. This yielded a small (10%) correction to the total ^{51}Cr in the organ and allowed calculation of the tissue ^{51}Cr attributable to PMNLs present in the organ. Results are expressed as a percentage of the total ^{51}Cr -PMNLs injected per organ.

Histology

Lung tissue for histology from animals receiving FMLP, with or without LPS-priming with or without mAbs as indicated was collected between 10 and 20 minutes after start of FMLP administration. Control animals received an infusion of phosphate-buffered saline (PBS). The abdomen was opened, a blood sample was obtained from the inferior vena cava, and a rapid infusion of Tyrodes solution (Ca^{++} , Mg^{++}) containing 10^{-8} mol/L FMLP was begun as the abdominal aorta was transected. After 25 ml was perfused, the chest was opened and a further 25 ml of this solution was infused into the inferior vena cava above the diaphragm until the lungs were white. This was followed by infusion of 10 ml of buffered formalin (3.7%) for 10 minutes and removal of the lungs into formalin. After overnight fixation, wedge segments from each lobe were paraffin-embedded and 5- μm sections were stained with hematoxylin and eosin. Tissue was also embedded in epoxy and semithin sections (1.5 μm) were stained with toluidine blue.

Analysis of PMNL Shape Change and Volume

To assess the effect of mAb treatment on the response of PMNLs to FMLP and LPS in terms of shape and volume changes, purified rat PMNLs (10^5) in 400 μl of Tyrode's solution with Ca^{++} , Mg^{++} -5% PPP were treated with mAbs as indicated (20 minutes, 22°C) followed by addition at 37°C of FMLP (10^{-7} mol/L final concentration). In some cases, the cells were first pretreated with LPS (100 ng/ml) for 20 minutes. The cells were allowed to be stimulated with FMLP for 1, 3, or 10 minutes and then the cells were either fixed with an equal volume of 2% glutaraldehyde for shape change analysis, expressed as the percent aspherical PMNLs with ruffles and pseudopod formation. For volume quantitation, PMNLs were directly diluted in PBS and analyzed with a Coulter counter (Coulter Electronics Inc., Hialeah, FL)

Statistical Analysis

All data are reported as the mean $\pm$ SEM. Differences between means at each time point were analyzed by analysis of variance with Bonferroni corrections for multiple comparisons.

Results

Localization and Dynamics of PMNL Margination Induced by Intravascular Chemotactic Factor

Previously, it was shown in animal models in other species that intravascular infusion or intravascular generation of chemotactic factors induces PMNL margination primarily in the pulmonary vasculature.⁸ Before evaluating the mechanisms used by PMNLs to marginate in the pulmonary vasculature of rats, we first examined whether

Table 1. Effect of Intravenous FMLP on ^{51}Cr -PMNL Distribution in the Rat

Organ	^{51}Cr -PMNL injected/organ, %	
	Control	FMLP*
Lung	26.3 [†]	44.5
Liver	14.8	19.1
Spleen	18.9	14.9
Kidney	3.5	4.3
Blood (total)	21.2	2.3

*Rats received ^{51}Cr -labeled blood PMNLs and ^{111}In -RBC intravenously followed 30 minutes later by 10^{-7}M FMLP or PBS (control) for 10 minutes under anesthesia, prior to sacrifice with KCl.

[†]Values are corrected for RBC content. Representative of two experiments with similar results.

PMNLs marginate selectively in the pulmonary vasculature in a Lewis rat model. ^{51}Cr -labeled purified blood PMNLs and ^{111}In -labeled RBCs from a Lewis rat donor were injected intravenously into an untreated Lewis rat. Twenty minutes later, the animal was anesthetized and a 10-minute infusion of FMLP was commenced. During the infusion, blood samples were taken from the tail vein to determine the drop in blood PMNLs (data not shown). After infusion of FMLP for 10 minutes, the animals were sacrificed, and organs of interest were dissected and evaluated for radioactivity. Control animals received PBS instead of FMLP.

The ^{111}In -RBC content was used to determine the intravascular blood pool in each organ and to adjust for any RBC contamination of the purified ^{51}Cr -PMNLs ($<10\%$), and thus any ^{51}Cr -RBC contribution to the ^{51}Cr content of the organs. The data presented in Table 1 show the percentage of the injected ^{51}Cr -PMNLs in each organ. As expected, under control (PBS) conditions the lung vascular bed accounts for the largest proportion of the marginated PMNL pool and the four organs analyzed accounted for 63.5% of the total injected PMNLs. The total circulating blood pool accounted for 21.2% in the control. In response to FMLP, there was a marked increase in the total ^{51}Cr -PMNLs in the lung, accounting for 44.5% of all of the ^{51}Cr -PMNLs and a decrease in the total ^{51}Cr PMNLs in the circulating blood. In absolute terms, the lung sequestration of injected PMNLs increased an additional 18.2%, the increase in liver was only 4.3% of total and the blood pool dropped by 18.9% of the injected dose. The spleen content decreased by 4%, likely because of the decline in the circulating pool, because this organ is extremely vascular. The blood and organ content of ^{51}Cr -PMNLs in the control and FMLP-treated animals accounted for 84.7 and 85.1% of the total injected ^{51}Cr -PMNLs, respectively. During FMLP infusion, redistribution seems to have occurred primarily between these compartments and primarily from blood to lung.

To visualize these changes in the lung, histological sections from normal animals that received a FMLP infusion or PBS as control were taken after perfusion of the pulmonary vasculature with Tyrode's- Ca^{++} , Mg^{++} to wash out RBCs. These sections are shown in Figure 1. The alveolar capillaries of an animal that received intravenous FMLP (Figure 1B) showed a marked increase in intravascular PMNLs and prominent septae as compared

to the PBS treated rat in which there are many leukocyte-free capillaries visible in cross-section (Figure 1A). This PMNL accumulation is active and selective because a proportional increase in intravascular RBCs is not observed (<1 RBC per PMNL). Occasionally a few RBCs were observed in the extravascular space, which may be secondary to vascular injury as reported during induced pulmonary PMNL sequestration in other models.^{11,14,19} There was no observed emigration of the PMNL into the extravascular or air spaces when semithin toluidine blue-stained sections were examined at high power (not shown).

To determine whether the PMNL margination is transient, as has been previously reported in other species,⁸ we injected animals with ^{51}Cr -labeled blood PMNLs before initiation of PMNL margination by the intravenous infusion of FMLP. The rats received a 20-minute FMLP infusion and blood samples were collected before and during the infusion. In the samples obtained PMNLs were enumerated by hemocytometer counting and for content of ^{51}Cr -PMNLs. As Figure 2 shows, blood PMNL counts dropped within 1 minute of FMLP infusion as did the blood ^{51}Cr -PMNL, indicating that the ^{51}Cr -labeled cells margined as did the unlabeled blood PMNL. Furthermore, the ^{51}Cr -PMNL returned to the circulating blood in parallel with the total PMNL count and the ratio of labeled to unlabeled PMNLs; ie, the specific activity did not change. No significant changes in the mononuclear cell count were observed. Taken together, the data indicate that PMNLs transiently marginate in the pulmonary vasculature in response to FMLP and that the blood PMNL count is a good indicator of this dynamic, reversible process.

Role of Integrins and Selectins in FMLP-Induced Pulmonary PMNL Sequestration in Normal Animals

To evaluate the role of selectins and integrins in chemotactic factor-induced PMNL sequestration, we infused normal rats with FMLP for 20 minutes and examined the effect of pretreatment with mAbs to specific integrins and selectins to block their adhesive interactions. Figure 3 shows the results in animals that received an infusion of FMLP and pretreatment with either PBS or isotype control mAb (B9) (results pooled). Blood PMNL levels decreased by 70% within 1 minute of FMLP infusion, remained at this level for the 20-minute duration of infusion and returned to baseline levels within 10 minutes of the termination of the infusion. When animals were first treated with anti- β_2 (CD18) integrin (WT.3) mAb, the decrease in blood PMNLs was significantly ($P < 0.05$) attenuated at all time points, the decrease being only 45% from baseline.

Figure 1. Histology of the lung during PMNL margination. Partially inflated, perfusion-fixed lungs from control (PBS) and FMLP-infused rats were epoxy embedded and semithin sections were stained with toluidine blue. **A:** Control animal that received PBS infusion, and **B:** received FMLP intravenously. Note patent alveolar capillaries in **A** and occlusion of these by PMNLs in **B**. Representative sections from two animals are shown. Original magnification, $\times 500$.

Figure 2. Dynamics of total and ^{51}Cr -labeled blood PMNLs in response to a 20-minute FMLP infusion in normal rats. Rats were given blood PMNLs labeled with ^{51}Cr intravenously 20 minutes before the FMLP infusion. Blood PMNL and ^{51}Cr -PMNL concentrations were determined at the times shown. Representative of two experiments.

When animals were pretreated with both mAbs to $\beta 2$ and to $\alpha 4$ (TA-2) integrins, there was no change in attenuation relative to anti- $\beta 2$ mAb alone (Figure 3). However, the drop in blood PMNLs in response to the FMLP infusion was diminished to only 35% from baseline when anti- $\alpha 5$ mAb was also added (Figure 3). This was significant compared to anti- $\beta 2$ with or without $\alpha 4$ mAb-treated animals at 3 and 20 minutes ($P < 0.05$) and at 6 and 10 minutes ($P < 0.01$). The decrease in PMNL counts was further attenuated by selectin blockade with the combination of mAbs to L- and P-selectin along with the anti-integrin mAbs above ($\beta 2$ and $\alpha 4$ with or without $\alpha 5$). Here, the drop in PMNL levels was only 20% below baseline at 3 minutes and 25% from baseline at 1 and 20 minutes during FMLP infusion. This attenuation was significant compared to the effects observed with anti-integrin ($\beta 2$, $\alpha 4$, and $\alpha 5$) treatment ($P < 0.001$ at all time points.). Studies were also conducted to evaluate the role of $\beta 1$ integrins ($\alpha 4$ and $\alpha 5$) and selectins (L- and P-selectin) when the $\beta 2$ integrins were not blocked. No significant attenuation of the margination was observed in

Figure 3. Effect of anti-integrin and selectin treatment on peripheral blood neutropenia induced by intravenous infusion of FMLP. Rats were injected intravenously with mAbs to $\beta 2$, $\alpha 4$, $\alpha 5$, L- and P-selectins, or anti-pertussis (B9) control as in Materials and Methods 20 minutes before the FMLP infusion as indicated. Blood PMNL counts were obtained by cytometer counting and values are expressed as percent change from baseline before FMLP infusion. Shown are the means of three to six experiments $\pm$ SEM. Control shows pooled results of PBS and isotype control mAb-treated rats. There was no difference between anti- $\beta 2$ and anti- $\beta 2$ plus anti- $\alpha 4$ groups so, for clarity, these results were pooled.

Figure 4. Effect of mAb treatment on blood neutropenia induced by intravenous FMLP injection in rats primed with LPS. Rats were given LPS ($50 \mu\text{g}/\text{kg}$ i.v.) 4 hours before FMLP. Twenty minutes before FMLP, mAbs to $\beta 2$, $\alpha 4$, $\alpha 5$, and L-, P-, and E-selectin, or control mAb were injected intravenously. Blood PMNL counts are expressed as percent change from baseline immediately before the FMLP injection and are the means $\pm$ SEM of three to six animals in each group. The baseline PMNL concentration was $11.2 \times 10^6 \pm 3.2 \times 10^6$ c/ml in the control rats and there was no significant difference between the baseline PMNL count in the control and mAb-treated rats.

two separate experiments each by the mAbs to the selectins alone or to the $\alpha 4$ and $\alpha 5$ integrins(not shown).

Mechanisms of PMNL Sequestration in Response to FMLP in LPS-Primed Rats

Pathological PMNL margination in the lung is frequent during gram-negative sepsis and LPS is believed to contribute to this response.^{4,10,13} Therefore we evaluated the mechanism used by PMNLs to marginate in the pulmonary vasculature in rats first primed by the intravenous route with LPS in a dose that did not induce shock or significant neutropenia but did induce a delayed (3 to 4 hours) neutrophilia of the order of 3.5-fold to sevenfold increase in baseline PMNL levels (to $11.2 \times 10^6 \pm 3.1 \times 10^6$ c/ml). This neutrophilia is prolonged, lasting at least 18 hours (data not shown). These studies were conducted in much the same way as those described in the previous section, however a continuous infusion of FMLP was not necessary because a single dose of FMLP resulted in a prolonged decrease in PMNL levels. In Figure 4, it can be seen that not only is the single dose of FMLP sufficient to cause a prolonged margination but it also results in a more marked decrease in peripheral PMNL levels (ie, as much as 90% from baseline within 3 minutes of FMLP).

When animals were pretreated with anti- $\beta 2$ mAb, the initial PMNL decrease was not attenuated but the peripheral blood PMNLs recovered more quickly ($P < 0.001$ from 6 to 30 minutes after intravenous administration of FMLP). Additional pretreatment with anti- $\alpha 4$ mAbs did not further inhibit the margination and thus this data are pooled with $\beta 2$ mAb blockade alone. Pretreatment of animals with an anti- $\beta 2$ plus anti- $\alpha 4$ and anti- $\alpha 5$ mAbs, however, resulted in both a more rapid recovery ($P < 0.01$ from 10 minutes to 45 minutes after FMLP) and an attenuation of the initial drop in the PMNL count ($P < 0.001$ at 1 minute as compared to both control or anti- $\beta 2$

treated animals; and $P < 0.01$ at 3 minutes as compared to control and $P < 0.05$ at 3 minutes as compared to $\beta 2$ blockade). However, at 6 minutes, there was no difference between $\beta 2$ mAb-treated animals and those treated with mAbs to $\beta 2$ plus $\alpha 4$ and $\alpha 5$.

To define any contribution of selectins to PMNL margination in LPS primed rats, selectin blockade alone or in combination with integrin blockade was examined. Treatment of animals with mAbs to L-, P-, and E-selectins as a combination had no significant effect (not shown). However, when combined with anti- $\beta 2$ and anti- $\alpha 4$ mAbs, in the presence or absence of anti- $\alpha 5$ mAbs, an attenuation of the initial blood PMNL drop was observed ($P < 0.001$ as compared to control or anti- $\beta 2$ + anti- $\alpha 4$ treated animals at 1 and 6 minutes; $P < 0.001$ as compared to anti- $\beta 2$ plus anti- $\alpha 4$ -treated animals at 6 minutes). There was also a complete inhibition of the neutropenia by 10 minutes, which represented a significantly increased rate of recovery compared to control or integrin (anti- $\beta 2$, anti- $\alpha 4$, and anti- $\alpha 5$ mAbs) blockade ($P < 0.01$ for 10- to 30-minute time points). Interestingly, the effects of anti-selectin antibody treatment on PMNL sequestration was only observed in the presence of multiple integrin blockade. For example, when the three selectin antibodies were used, even in the presence of anti- $\alpha 4$ mAb, no significant attenuation of the margination was observed as compared with control animals, despite the fact that selectins and $\alpha 4$ integrins are the predominant CAMs mediating leukocyte rolling³ (not shown).

To examine whether the rapid recovery of circulating PMNLs observed in animals treated with mAbs to selectins (L-, P-, and E-selectin) and integrins ($\beta 2$, $\alpha 4$, and $\alpha 5$) could have been the result of the release of PMNLs from the bone marrow in response to mAb blockade, in two experiments, blood samples were monitored for 60 minutes after administration of mAbs. The samples indicated a steady PMNL level throughout this time course (not shown). Furthermore, in two animals blood samples were collected for up to 65 minutes after administration of FMLP. At these late time points, the blood PMNL count did not exceed the initial baseline pre-FMLP values (not shown).

Further experiments addressed the possibility that PMNLs released from the bone marrow accounted for the PMNL recovery in mAb-treated rats. To examine this, ^{51}Cr -labeled PMNLs were used for kinetic studies in LPS-primed, FMLP-injected rats. As shown in Figure 5, the ratio of ^{51}Cr -PMNLs to total PMNLs in blood remained quite constant throughout the period of PMNL margination and re-entry into the circulating blood pool. The blood PMNL counts responded as in Figure 4 for these groups. This excludes a substantial release of unlabeled PMNL accounting for the recovery in the PMNL count even in the mAb-treated rats.

Finally, in Figure 6A, a lung section from a LPS-primed animal 20 minutes after FMLP injection shows the significant accumulation of PMNLs in the alveolar capillary. However, in Figure 6B, the section from a LPS-primed animal 20 minutes after FMLP, which received pretreatment with mAbs to $\beta 2$, $\alpha 4$, and $\alpha 5$ integrins and L-, P-, and

Figure 5. Dynamics of total and ^{51}Cr -labeled blood PMNLs in response to a FMLP bolus in LPS-primed rats. LPS-primed rats were given ^{51}Cr -labeled blood PMNLs followed by intravenous mAbs to $\beta 2$, $\alpha 4$, $\alpha 5$, and L-, P-, and E-selectin (squares) or control mAb (diamonds). Twenty minutes later FMLP was given intravenously and blood PMNL and ^{51}Cr -PMNL concentrations were determined at times shown. Representative of two experiments.

E-selectin shows much diminished PMNL adherence in capillaries, similar to the PBS control shown in Figure 1A.

Effect of Antibodies on FMLP- and LPS-Induced Shape and Volume Changes

It has been proposed that a possible mechanism for the sequestration of PMNLs in the pulmonary vasculature after chemotactic factor or LPS activation may be an increase in cellular rigidity resulting from actin polymerization. We therefore examined whether the antibody treatments found to inhibit pulmonary PMNL sequestration inhibit shape and volume changes induced by FMLP. This was achieved by direct visualization of shape change in purified PMNLs after FMLP and glutaraldehyde fixation or by Coulter counter analysis of PMNL volume. Results from these experiments, shown in Figure 7, indicate that FMLP induces a shift from spherical to a ruffled shape. These shape changes were not inhibited by pretreatment of the cells with single mAbs or with a combination of mAbs to $\beta 2$, $\alpha 4$, $\alpha 5$, and L-selectin. FMLP also induced a 6 to 8% increase in cell volume and this was also not inhibited by pretreatment with these mAbs (not shown).

Discussion

Most studies examining chemotactic factor-induced PMNL margination in the lung, primarily in the rabbit, have focused on physical lodging induced by actin polymerization.^{14,15} The examination of CAM mechanisms have focused on $\beta 2$ integrins or selectins,²⁷ studies that have suggested only a limited role for these CAMs. Our goal was to examine the margination induced primarily in the lungs of rats by intravenously administered chemotactic factors, and to evaluate the contribution of CAMs to this process. Our findings indicate a role for $\beta 2$ integrins in the sequestration of PMNLs in normal rat lungs with significant contributions also by the $\alpha 4$ and $\alpha 5$ integrins and to a lesser extent, the selectins. The relative contribution to PMNL margination of the CAMs studied was

influenced by previous exposure of the rat to LPS as a priming stimulus. The differences in CAM-mediated margination observed may be because of the activation and up-regulation of several CAMs on PMNLs and on the endothelium in the LPS-primed animals. A substantial portion of the PMNL margination in normal animals is mediated by $\beta 2$ integrins, because blockade of these CAMs markedly attenuated both the immediate (1 minute) and sustained neutropenia (Figure 3). However, the role of $\beta 2$ integrins in the LPS-primed model is less evident. In LPS-primed animals, $\beta 2$ integrin blockade resulted in a more rapid recovery from the FMLP-induced neutropenia but an inhibition of the initial margination was not seen (Figure 4). This observation could be related to newly released PMNLs from the bone marrow, mobilized by LPS stimulation, being less deformable, and preferentially sequestering in pulmonary microvessels as reported by van Eeden and colleagues.⁴¹ However, additional CAM interactions also contribute as discussed below.

The role of $\beta 1$ integrins also varies between the two models. In normal animals, the additive inhibitory effect of blocking $\alpha 4$ and $\alpha 5$ in combination with $\beta 2$ -integrins on margination is not seen in the first minute after FMLP infusion. At 3 minutes and for the remainder of the infusion, an additive effect is observed when $\alpha 4$ plus $\alpha 5$, but not $\alpha 4$ alone (Figure 4), are blocked in combination with $\beta 2$ integrins (Figure 3). This indicates that there is a role for $\alpha 5$, likely functioning in concert with $\alpha 4$, in $\beta 2$ -independent margination. Neither $\alpha 4$ nor $\alpha 5$, when blocked alone or in combination, had any inhibitory effect (not shown) unless $\beta 2$ integrins were also blocked. Similarly, in LPS-primed animals, blocking of $\alpha 4$ alone or in combination with $\alpha 5$ had no inhibitory effect (not shown). However, when $\alpha 4$ and $\alpha 5$ were blocked in combination with $\beta 2$ integrins, there was a significant reduction in the initial neutropenia and a markedly expedited recovery from neutropenia (Figure 4). Thus one effect of LPS exposure is the increased involvement of $\beta 2$ -independent mechanisms both in the immediate and sustained PMNL margination and this is in part because of enhanced involvement of $\alpha 4$ - and $\alpha 5$ -dependent mechanisms. We believe these findings are validated by a number of controls including: 1) irrelevant control mAb and anti- $\alpha 4$ plus $\alpha 5$ mAb treatments had no effect; 2) these effects were observed with F(ab)₂ fragments of the mAbs, ruling out Fc receptor or complement effects; and 3) other mAbs, as F(ab)₂ fragments, reactive with rat $\beta 3$ integrin had no effect on FMLP-induced neutropenia (not shown).

After LPS administration at the doses used, there was little effect on the blood PMNL count initially, but within 2 to 3 hours the PMNL count increased and remained elevated for as long as 24 hours. It was important that the

Figure 6. Effect of antibody blockade on lung histology in rats primed with LPS and given FMLP. Sections were prepared as for Figure 1 and stained with toluidine blue. **A:** Control mAb-treated animal 20 minutes after FMLP injection (i.v.). **B:** Rat receiving mAbs to $\beta 2$, $\alpha 4$, and $\alpha 5$ integrins plus mAbs to L-, P-, and E-selectin. Note marked capillary plugging with leukocytes in **A** and much less so in **B** with patency of many capillaries despite the elevated absolute PMNL count in the LPS-primed rat. Original magnification, $\times 250$.

Figure 7. Effect of mAb treatment on FMLP activation of PMNL shape change *in vitro*. Rat blood PMNLs were pretreated with PBS or control mAb (results pooled) or mAbs to integrins and selectins as shown for 20 minutes (22°C). FMLP at 10^{-7} mol/L was added to induce PMNL shape change, which was quantified by light microscopy. Values are representative of one of four experiments performed with duplicate replicates.

PMNL counts during the neutrophilia stage of the LPS response be constant because a slow increase in blood PMNL levels would lead to an increase in blood PMNLs regardless of the effect of antibody treatment. However, PMNL counts in blood reached a plateau by 4 hours after LPS priming and were maintained at this level for the duration of the experiments. Thus, a steady state of PMNLs in the circulating pool was observed. Furthermore, during and after PMNL margination, mAbs did not mobilize PMNL from the bone marrow nor were they mobilized in response to the neutropenia but rather marginated PMNLs returned to the circulating pool. This conclusion is supported by several experiments using ^{51}Cr -labeled PMNL and monitoring of the unlabeled-to-labeled PMNL ratio, or specific activity (Figure 2).

In addition to α 4 β 1 and α 5 β 1, and the β 2 integrins, the selectins also seemed to contribute to the mechanism of PMNL margination, although this was only observed when these integrins were blocked. During FMLP infusion, in LPS-primed animals, the combination of integrin and selectin blockade resulted in complete recovery to baseline PMNL levels within 10 minutes of FMLP infusion. In normal animals, there was a significant attenuation of the neutropenia over and above that seen by integrin blockade alone. Thus selectin interactions likely contribute to PMNL margination but are overshadowed by integrin-mediated adhesion which is dominant. This conclusion is in accordance with observations in E- and P-selectin-deficient mice where PMNL margination was unaffected and only a limited decrease of PMNL margination in noncapillary microvessels in L-selectin-deficient animals was observed.^{29,42} Our results suggest that although selectins are mediators of leukocyte rolling in venules, in the pulmonary capillaries they may not be required for rolling because the average diameter of a PMNL is equal to or greater than that of the pulmonary capillaries.^{14,19} However, selectins can mediate static adhesion *in vitro* and in pulmonary capillaries under low shear, they may stabilize or enhance adhesion secondary to ligand binding by integrins. It is, however, also possible that in the pulmonary bed, ligand engagement by selectins contributes to activation or up-regulation of other adhesion molecules such as the above studied integrins. Thus selectin blockade may be disrupting such

outside-in signaling with secondary effects on PMNL margination.

It has been reported that actin polymerization after stimulation of the cells with chemotactic factors such as FMLP results in increased PMNL rigidity^{15,20–22} and lodging of activated PMNLs in the pulmonary capillaries.^{14,15} Our findings are not necessarily in conflict with this hypothesis. In both models of FMLP-induced PMNL margination presented here, there is a degree of acute margination that cannot be inhibited by blocking antibodies to α 4, α 5, and β 2 integrins and L-, P-, and E-selectins. However, blockade of these CAMs markedly attenuated the neutropenia by at least 50% even within 1 minute of FMLP administration. Thus even at this early time point and subsequently, a combination of adhesion molecule and physical factors may be operative in pulmonary PMNL margination. In this context, it is noteworthy that none of the mAb treatment combinations altered FMLP-induced PMNL shape change (Figure 7). Thus the observed mAb effects are unlikely to have altered the physical trapping mechanisms.

In contrast to normal rats in which PMNL margination lasts only minutes after FMLP injection unless FMLP is continuously infused, in animals primed with LPS, FMLP injection induces prolonged margination. Under these conditions, after the initial 1 to 3 minutes, during which integrins and selectins only moderately contribute to the sequestration, the sustained margination seems to be essentially completely β 2-, α 4-, α 5-, and selectin-dependent since by 10 minutes neutropenia was completely reversed when these CAMs were all blocked (Figure 4). We propose that in this model, the initial tethering event that leads to a more prolonged margination may be primarily mediated by physical trapping of PMNLs in capillaries. This mechanism seems to be enhanced by LPS, possibly because of priming effects on PMNL responsiveness to FMLP and other chemotactic factors^{17,43,44} and/or decreased deformability of newly released PMNLs from bone marrow.^{41,45} This initial physical lodging may allow integrins and selectins to engage their ligands and support sustained adhesion beyond the transient actin polymerization and PMNL shape change.^{20–22,46}

In both models we were surprised by the inhibitory effect of CAM blockade on even the initial PMNL margination, given the reports that CAMs may have little role in the acute response. These previous studies examined the effect of functional inhibition (by mAb or by knock-out of the CAM gene of interest) of only one or two CAMs and may not have accounted for redundant mechanisms in the sequestration. One such study examined the effect of mAb blockade of Mac-1 (α M β 2) in a model of lung inflammation resulting from intestinal ischemia and reperfusion. Results from this study showed that although a reduction in lung injury was observed, no inhibition of lung leukosequestration was found.^{47,48} Other studies have shown similar results when P-selectin was blocked.⁴⁹ There have also been reports of a role for L-selectin in late neutrophil sequestration (≥ 5 minutes) using L-selectin-deficient mice. However the effect of blockade of other CAMs in these knockout mice was not examined.²⁹ Our data indicate that during neutrophil se-

questration in the lungs, multiple CAMs are involved with extensively redundant roles.

Our studies indicate that there is a major role for adhesion molecules, especially for the $\beta 2$, $\alpha 4$, and $\alpha 5$ integrins in conjunction with selectins in chemotactic factor-induced PMNL margination in the pulmonary capillary bed. These mechanisms appear to be highly overlapping and thus require interruption of multiple CAM-ligand interactions to be detected. These mechanisms are in addition to physical trapping of activated and/or aggregated PMNLs in these vessels as previously proposed. The results suggest that approaches to regulate this pathological process in circumstances such as sepsis, cardiopulmonary bypass, and other forms of extracorporeal circulation that can lead to adult respiratory distress syndrome will require a dual approach of inhibition of PMNL stiffening and, in particular, integrin activation and ligand binding.

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