Activated polymorphonuclear cells increase sickle red blood cell retention in lung: role of phospholipids

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Received 9 March 2001; accepted in final form 6 September 2001

Activated polymorphonuclear cells increase sickle red blood cell retention in lung: role of phospholipids. Am J Physiol Heart Circ Physiol 282: H122–H130, 2002.—This study investigates the role of the activated polymorphonuclear cell (APMN) products on sickle red blood cell (SRBC) retention/adherence in the pulmonary circulation. Isolated rat lungs were perfused with $^{51}$Cr-labeled normal RBCs (NRBC) or SRBCs (10% hematocrit) suspensions ± PMNs. Specific activities of lung and perfusate were measured and retention (the number of SRBC/g lung) was calculated. SRBC retention was 3.5 times greater than NRBC retention. PMN activation was required to increase SRBC retention. Supernatants from APMN increased SRBC retention, which suggested soluble products such as oxidants, PAF, and/or leukotriene (LTB$_4$) are involved. Heat inactivation of PMN NADPH oxidase had no effect on retention. Whereas neither platelet-activating factor (PAF) nor LTB$_4$ (secreted by APMN) increased SRBC retention, PAF+LTB$_4$ did. The PAF antagonist, WEB-2170, attenuated SRBC retention mediated by PAF+LTB$_4$ and APMNs. Similarly, zileuton (5-lipoxygenase inhibitor) attenuated APMA-mediated SRBC retention. We conclude the concomitant release of PAF and LTB$_4$ from APMN is involved in the initiation of microvascular occlusion by SRBCs in the perfused rat lung.

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platelet-activating factor; leukotriene B$_4$; zileuton; WEB-2170

CENTRAL TO MICROVASCULAR OCCLUSION in sickle cell disease (SCD) is the polymerization of deoxyhemoglobin S, which results in increased rigidity of the sickle red blood cell (SRBC). Because hemoglobin polymerization does not occur immediately after deoxygenation, most RBCs pass through the capillary bed before sickling, thus making microvascular occlusion uncommon (29, 50). On the basis of this observation, hemoglobin S polymerization is not likely the exclusive cause of microvascular occlusion and suggests that factors that increase the capillary transit time are also operative. Factors demonstrated in experimental models to increase transit time of the SRBC through the microcirculation are random precapillary obstruction by rigid dense SRBCs (contain irreversible SRBCs) and increased adhesion of sickle reticulocytes to vascular endothelium (37–39). Hebbel and colleagues (26) found that SRBCs, particularly reticulocytes, have an increased tendency to adhere to vascular endothelial cells (27) and that increased adherence can be correlated with clinical severity of vasoocclusion. Of the sickle reticulocytes, $\sim$25% express adhesion molecules, making them capable of adhering to vascular endothelial cells (35, 60). Regarding dense SRBCs, their correlation to clinical severity of vasoocclusion is less clear. Ballas (4) has described a subset of patients with sickle cell anemia who have mild disease, as related to pain, with increased numbers of rigid, dense SRBCs, leg ulcers, and lower mortality compared with patients with highly deformable SRBCs and low numbers of dense SRBCs. In contrast, Powars et al. (52) has shown that SCD patients with the most severe disease produce twice the number of dense SRBCs compared with patients with disease of minimal severity.

Nonetheless, dense SRBCs and reticulocytes are present during steady-state conditions and during clinical disease. This raises an interesting question as to whether or not other blood cells, such as polymorphonuclear cells (PMNs), play a role in the initiation of microvascular occlusion. Indeed, PMN activation has been implicated in the pathophysiology of SCD (44, 49). Several investigators (9, 18) have demonstrated that PMNs from some patients in crisis are more adherent to vascular endothelium than during steady state and possibly contribute to vasoocclusion. Clinical studies show that elevated total white blood cell counts are common in SCD (8, 14) and that a white blood cell count $>$15,000 cells/ml is associated with an increased risk of early death (51). Cytokines such as interleukin-6 have been correlated positively with the number of sickle PMNs adherent to fibronectin (36). Interleukin-8 increases SRBC adherence to endothelial cells via fibronectin, upregulates $\alpha_4\beta_1$-integrin receptors, and is a chemoattractant for PMNs (12, 3). These studies cumulatively suggest that PMN activation and the release of cytokines in response to infections, endothelial cell activation, and other injurious agents may prove to play a vital role in the pathophysiology of microvascular occlusion in SCD.

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Ibe et al. (33) studied the interaction between SRBCs and autologous platelets in an isolated perfused lung model and suggested that lipid mediators may play a role in the pathophysiology of sickle lung disease. Lungs perfused with SRBCs and SRBCs plus autologous platelets revealed high perfusate levels of the arachidonate metabolites, thromboxane A2, and prostaglandin E2. Products of the arachidonate and 5-lipoxygenase were not measured. In our study, we propose that PMN activation, as may occur in response to infections or other injurious agents, increases SRBC retention/adherence in the pulmonary circulation of isolated perfused rat lung via a mechanism(s), which involve the release of platelet-activating factor (PAF) and leukotriene B4 (LTB4) from the activated PMN.

**METHODS**

**Materials**

Phorbol myristate acetate (PMA) and bovine serum albumin (BSA; 66,000 mol wt) were purchased from Sigma (St. Louis, MO). PAF and LTB4 were purchased from Cayman Chemical (Ann Arbor, MI). Zileuton was purchased from the University of South Alabama Pharmacy (Mobile, AL). WEB-2170 base was a kind gift from Boehringer Ingelheim Pharmaceuticals. PMA, WEB-2170, and PAF were dissolved in DMSO. LTB4 and zileuton were dissolved in 95% ethanol.

**Isolated Perfused Lung**

Male Sprague-Dawley rats (275–350 g) were anesthetized with nembutal sodium (25 mg ip), and lungs were removed for extracorporeal perfusion as previously described (25, 47). A tracheostomy was performed that permitted ventilation with a Harvard rodent ventilator (model 683) at 55 breaths/min with a tidal volume of 2.5 ml and 2.0 cmH2O-positive end-expiratory pressure. The inspired gas mixture was 21% O2-5% CO2-balance N2 (room air gas). A median sternotomy was performed, heparin sodium (100 IU) was injected in the right ventricle, and cannulas were placed in the pulmonary artery and left ventricle. Heart, lungs, and mediastinal structures were removed en bloc and placed into a humidified chamber. Lungs were perfused with the use of a Gilson Minipuls 2 peristaltic pump at a constant flow of 0.03 ml/g body wt−1·min−1. Lungs were perfused with a physiological salt solution (PSS) containing bovine serum albumin (BSA). The PSS-BSA perfusate contained (in mM) 119 NaCl, 4.7 KCl, 1.17 MgSO4, 22.5 NaHCO3, 1.18 KH2PO4, 3.2 CaCl2, 5.5 glucose, and 4 g/100 ml BSA. PSS-BSA (100 ml) was perfused through the lungs in a nonrecirculating fashion to remove residual blood cells and plasma. The perfusate was then changed to a PSS-BSA perfusate that contained RBCs from individuals homozygous for sickle cell anemia. The perfusate hematocrit was ~10%. Pulmonary arterial pressure and pulmonary venous pressure were continuously monitored with pressure transducers (model 041–500–503, Cope) and recorded on a polygraph recorder (model 7E, Grass Instruments). Zone 3 flow conditions (arterial > venous > alveolar pressures) were maintained throughout all experiments.

**Blood Collection**

All participants gave informed consent and the Human Use Committee approved the protocol. During steady state, blood samples (~40 ml) were obtained from normal (Hemoglobin A) human volunteers and from individuals with homozygous sickle cell anemia in the sickle cell clinics at the University of South Alabama. Samples were collected in syringes containing heparin sodium and utilized within 48 h of collection. Individuals who had received blood transfusions within the previous 6 wk were excluded from this study. Sickle cell anemia was documented by hemoglobin electrophoresis in each subject.

**RBC Isolation and 51Cr Labeling**

Each whole blood sample was centrifuged at 4,100 rpm for 10 min, followed by the removal of the plasma and buffy coat. Packed RBCs were then mixed at a 1:3 dilution with normal saline and washed three times. With each wash, the RBC suspension was centrifuged for 10 min at 3,000 rpm, followed by removal of the supernatant. After the third wash, complete removal of platelets and PMNs was verified by microscopic examination of stained smears. Packed RBCs were resuspended in phosphate-buffered saline (PBS; 1 ml PBS and 1 ml packed RBCs) and incubated with 100 μCi 51Cr for 60 min in a shaker water bath at 37°C. After incubation, the 51Cr-labeled RBCs were washed once with normal saline and resuspended in PSS-BSA to obtain a hematocrit of ~10%.

**Neutrophil Isolations**

Heparinized whole blood (20 ml) from individuals with homozygous sickle cell anemia was diluted 1:1 with normal saline. PMNs were then isolated after dextran sedimentation and centrifugation on histopaque 1077 cushions as described previously (10). Briefly, dextran 500 was added to the 1:1 mix at one-fourth the volume of the 1:1 mix. This was allowed to incubate for 45 min at 37°C. The supernatant (contains PMNs) was aspirated into a 50-ml centrifuge tube and underlayered with 10-ml histopaque 1077. This was centrifuged at 1,250 rpm for 30 min. The supernatant was aspirated and discarded, leaving the PMN pellet. Sterile H2O (3 ml) was added to the PMN pellet and agitated for 30 s to lyse any residual RBCs. PBS (45 ml) was added immediately thereafter to stop the reaction. This was centrifuged at 1,250 rpm for 10 min. The supernatant was aspirated and discarded. This process of RBC lysis, followed by PBS, was repeated until the PMN pellet was without visual evidence of RBC contamination. The PMN pellet was suspended in 1 ml of sterile Hank’s balanced salt solution (HBSS). A total PMN count was obtained using the Cell-DYN 900 Coulter counter. Final preparations contained 95% PMN and viability was >95% as assessed by trypan blue exclusion.

**Heat inactivation of neutrophils.** Purified PMNs, suspended in 1 ml of sterile HBSS (pH 7.4), were transferred to a siliconized glass tube and placed in a 48°C water bath with constant agitation for 10 min (17, 21). Aliquots of the heat-treated PMN suspension were immediately added to a 10% sickle RBC suspension to obtain a final PMN concentration of ~200,000/ml of perfusate.

**RBC Retention**

Cell counts of the 51Cr-labeled RBC suspension were obtained in a Cell-DYN 900 Coulter counter. The specific activity of the 51Cr-labeled RBC perfusate was calculated after gamma counts at 162–176 Kev for 1 min and expressed as counts per minute (cpm)/RBC. The isolated lung was then perfused with the 51Cr-labeled RBC perfusate for 30 min. After this, the lung was washed with 100 ml of RBC-free PSS-BSA under identical hemodynamic conditions. Perfusion was stopped, and the lung was dissected into lobes and weighed. Each lobe was gamma counted for 1 min. Retention (R) was calculated as
Specific Protocols

Comparison of normal RBC and SRBC retention/adherence in the isolated lung. Initially, studies were done in the isolated rat lung perfused with 10% hematocrit RBC suspensions, which contained either SRBCs (n = 8) or normal human (Hemoglobin A) RBCs (NRBC; n = 6). Lungs were ventilated with a room air gas mixture, as described earlier in methods. After a 30-min period of perfusion with the RBC-containing perfusates, SRBC and NRBC retention/adherence was determined as previously described and compared.

Comparison of unactivated PMN to activated PMN effect(s) on SRBC retention/adherence. Subsequently, in isolated SRBC-perfused rat lungs ventilated with a 21% O2-5% CO2-balance N2 gas mixture, the effects of PMN activation with PMA on altering SRBC retention in the pulmonary circulation were assessed. All perfusate hematocrits were ~10% and paired SRBC samples were utilized in each study except the study comparing NRBC and SRBC retention. Paired SRBC samples were used to minimize patient-to-patient variability as well as within-patient variability relative to time. In studies utilizing PMNs, PMNs were added to the perfusate at ~200,000/ml of perfusate. The activation of PMNs was achieved in the test tube by the addition of PMA (20 ng/ml) to PMNs suspended in 1 ml of HBSS. Activated polymorphonuclear cells (APMNs) were then added to the perfusate reservoir at the initiation of lung perfusion in experiments, which investigated SRBC and APMN interactions. Lungs were perfused in a recirculating fashion under constant flow conditions for 30 min and the retention/adherence of SRBCs was determined as previously described. The following comparisons of SRBC retention relative to PMN activation were made: 1) SRBC versus SRBC + unactivated PMNs (UPMN; n = 6 each); 2) SRBC + UPMN versus SRBC + APMN (n = 11 each); 3) SRBC + APMN versus SRBC + APMN supernatant (n = 8 each); and 4) SRBC versus SRBC + PMA (n = 9 each).

The comparison of lungs perfused with SRBC + APMNs versus SRBC + AMN supernatant suggested either a soluble product(s) of the APMN and/or mechanical impedance by entrapped APMNs increased SRBC retention/adherence in the pulmonary circulation. PMN activation results in stimulation of NADPH oxidase and superoxide production (2, 13) and in the production of phospholipid products (7, 34).

Heat inactivation of PMN NADPH oxidase: effect on SRBC retention/adherence. To assess the potential role of NADPH oxidase, the following studies were performed. Purified PMNs were heated at 48°C for 10 min to inactivate NADPH oxidase (17, 21). In lungs perfused with 51Cr-labeled SRBCs, retention/adherence was assessed in the presence of heat-treated UPMNs (∼200,000/ml perfusate) versus UPMN control (no heat treatment).

Effect of PAF and LTB4 on SRBC retention/adherence. The phospholipid products assessed in this study were PAF and LTB4. To assess the role of PAF (100 ng/ml) and LTB4 (100 ng/ml) in SRBC retention/adherence, the following groups were compared: 1) SRBC versus SRBC + PAF 30 (PAF added to perfusate reservoir at the initiation of lung perfusion and allowed to recirculate for 30 min; n = 6 each); 2) SRBC versus SRBC + LT B4 (n = 5 each); 3) SRBC versus SRBC + PAF + LTB4 (n = 9 each); 4) SRBC + PAF + LTB4 versus SRBC + WEB-2170 (PAF antagonist) pretreatment + PAF + LTB4 (n = 8 each); 5) SRBC + APMN versus WEB-2170 pretreatment + SRBC + APMN (n = 6 each); and 6) SRBC + APMN versus SRBC + zileuton (5-lipoxygenase inhibitor) pretreated APMN (n = 8 each). In group 2, LT B4 was added to the perfusate at the initiation of perfusion. In group 3, PAF was added to the reservoir at the initiation of perfusion and allowed to recirculate for 30 min. LT B4 was then added to the perfusate for an additional 30 min of lung perfusion. In group 4, WEB-2170 (10 μM final perfusate concentration) was added to the perfusate reservoir at the initiation of perfusion and allowed to recirculate for 20 min before PAF administration and 50 min before the administration of LT B4. This protocol was the same as in group 3, except for the 20-min pretreatment of lungs with vehicle or WEB-2170. In group 5, WEB-2170 (10 μM) (19, 28, 56) was added to the SRBC perfusate 20 min before the addition of APMNs (∼200,000/ml perfusate). In the study using zileuton, PMNs were incubated with zileuton (2.5 μM) for 30 min before activation with PMA in the test tube. Zileuton (2.5 μM) was used based on the finding reported by Guidot et al. (20) and preliminary studies performed in our laboratory, which demonstrated an ~50% inhibition of plasma LT B4 production in A-23187-stimulated whole blood from individuals with homozygous sickle cell anemia. APMNs were subsequently added to the perfusate reservoir at the initiation of lung perfusion. Lungs were perfused in a recirculating fashion for 30 min and the SRBC retention/adherence was determined as previously described.

Statistics

All results are presented as means ± SE. Statistical analyses were performed using the unpaired Student’s t-test and one-way analysis of variance (ANOVA). Tukey’s test (62) was used for multiple comparisons when ANOVA indicated statistically significant differences between or within groups. Differences were considered to be significant when P < 0.05.

RESULTS

SRBC Retention/Adherence in Isolated Perfused Lung is Greater Than NRBC

When comparing RBC retention in lungs perfused with SRBCs with lungs perfused with NRBCs, we found that SRBC retention was 3.5 times greater than that observed in NRBC-perfused lungs (Table 1).

PMN Activation is Required to Increase SRBC Retention/Adherence

In lungs perfused with SRBCs alone compared with lungs perfused with a SRBC suspension that contained UPMNs, no significant increase in retention/adherence in the lung was observed in the presence of UPMNs. In lungs perfused with SRBCs alone and in lungs perfused with SRBC + UPMN, the number of retained
Table 1. Effect of polymorphonuclear cells on sickle red blood cell retention in pulmonary circulation

<table>
<thead>
<tr>
<th>Perfusates</th>
<th>n</th>
<th>Retained RBC/g Lung (10^8)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRBC alone</td>
<td>8</td>
<td>4.9 ± 0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>vs. SRBC</td>
<td>6</td>
<td>1.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>SRBC alone</td>
<td>6</td>
<td>3.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>vs. SRBC + UPMN</td>
<td>6</td>
<td>4.3 ± 0.4</td>
<td>0.32</td>
</tr>
<tr>
<td>SRBC + UPMN</td>
<td>11</td>
<td>4.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>vs. SRBC + APMN</td>
<td>11</td>
<td>6.1 ± 0.4</td>
<td>0.004</td>
</tr>
<tr>
<td>SRBC + APMN</td>
<td>8</td>
<td>5.7 ± 0.2</td>
<td>0.99</td>
</tr>
<tr>
<td>vs. SRBC + supernatant</td>
<td>8</td>
<td>5.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>SRBC alone</td>
<td>9</td>
<td>4.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>vs. SRBC + PMA</td>
<td>9</td>
<td>5.0 ± 0.4</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of lungs studied. RBC, red blood cell; SRBC, sickle red blood cell; UPMN, unactivated polymorphonuclear cell; AP, activated PMN; supernatant, supernatant from AP; PMA, phorbol myristate acetate. Retained RBC/g of lung, the number of red bloods remaining in the lung after a 30-min period of perfusate recirculation, followed by 100-ml nonrecirculating wash with a physiological salt solution containing BSA.

SRBCs as expressed in RBC/g of lung was 3.9 ± 0.3 × 10^8 and 4.3 ± 0.4 × 10^8, respectively. In SRBC-perfused lungs with a perfusate that contained either UPMN or APMN, the presence of APMN resulted in a 31% increase in retention/adherence in the pulmonary circulation (Table 1). From Table 1, data were pooled from lungs perfused with SRBCs alone (n = 23), SRBC + UPMN (n = 17), and SRBC + APMN (n = 19), and compared. SRBC retention/adherence was 4.6 ± 0.2 × 10^8, 4.3 ± 0.2 × 10^8, and 5.9 ± 0.3 × 10^8 SRBC/g of lung, respectively. As reported using paired SRBC samples (see specific protocol), there was no significant difference in SRBC retention in lungs perfused with SRBC alone and SRBC + UPMN, SRBC retention in lungs perfused with SRBC + APMN was also significantly (P < 0.001) greater than that seen in the SRBC alone and SRBC + UPMN groups.

**Supernatant From APMN Increases SRBC Retention/Adherence**

To determine whether the increase in retention/adherence of SRBCs seen in the presence of APMN was due to the release of a product(s) of the APMN, SRBC retention/adherence in lungs perfused with SRBC + APMN was compared with lungs perfused with SRBC + the supernatant of APMN. No significant difference in SRBC retention/adherence was noted in SRBC perfused lungs that contained APMN and supernatant from APMMNs.

**PMA Had No Effect on SRBC Retention/Adherence**

Because all of the perfusates containing PMA and phorbol esters have been reported (43) to activate the αβ1-integrins on the surface of sickle reticulocytes and promote increased adherence of the reticulocyte to endothelial cells via fibronectin, SRBC retention/adherence in lungs perfused with SRBC alone was compared with lungs perfused with SRBC + PMA. Lungs perfused with PMA did not significantly increase SRBC retention/adherence (Table 1). To further assess whether PMA increased SRBC retention/adherence in the lung, or whether it was a product of the APMN, lungs perfused with SRBC + UPMN were compared with a lung perfused with SRBC + washed APMN (PMNs were activated with PMA then washed before adding to the perfusate, which contained SRBCs). Washed APMN caused a 19% (P = 0.033) increase in SRBC retention/adherence compared with SRBC + UPMN-perfused lungs.

**Heat Inactivation of PMN NADPH Oxidase Had No Effect on SRBC Retention/Adherence**

The potential role of PMA activation of PMN NADPH oxidase with superoxide production in SRBC retention/adherence was assessed next. When we compared lungs perfused with SRBC + APMN and SRBC + heat-treated APMMNs, no significant (P = 0.885) difference in SRBC retention/adherence was observed between groups. In SRBC-perfused lungs with APMN, retention/adherence was 4.5 ± 0.4 × 10^8 SRBC/g of lung and 3.9 ± 0.4 × 10^8 SRBC/g of lung in lungs perfused with heat-treated APMN (n = 9 each).

**PAF +LTB4 in Tandem is Required to Increase SRBC Retention/Adherence**

The effect(s) of PAF and LTB4, which are soluble products of the APMN, was assessed (Fig. 1–5). In lungs perfused with SRBC alone compared with SRBC + PAF for 30 min, retention/adherence was 5.0 ± 0.7 × 10^8 and 4.1 ± 0.6 × 10^8 SRBC/g of lung, respectively. No significant difference in SRBC retention/adherence was observed (Fig. 1). When comparing SRBC retention/adherence in control lungs (5.1 ± 0.6 × 10^8 SRBC/g of lung) to lungs perfused with SRBC + LTB4 (4.7 ± 0.4 × 10^8 SRBC/g of lung) for 30 min (n = 5 each), there was no significant difference in SRBC retention/adherence (Fig. 2). Interestingly, in
lungs perfused with SRBC + PAF for 30 min, followed by the addition of LTB₄ to the perfusate for an additional 30 min, there was a significant (P = 0.02) increase in SRBC retention/adherence compared with SRBC control (n = 9, each, Fig. 3). SRBC retention/adherence in the SRBC control and SRBC + PAF + LTB₄ groups was 4.4 ± 0.2 × 10⁸ and 5.5 ± 0.2 × 10⁸ SRBC/g of lung, respectively.

WEB-2170 and Zileuton Attenuate APMN-Mediated SRBC Retention/Adherence

To further assess the role of PAF and LT₄ as potential mediators of PMN-enhanced SRBC retention/adherence, the effects of the PAF antagonist, WEB-2170 (28, 61) and the 5-LO enzyme inhibitor zileuton (6, 20) were assessed. When we compared SRBC retention/adherence in lungs perfused with SRBC + LT₄ with lungs perfused with SRBC + PAF + LT₄, which were pretreated with WEB-2170, SRBC retention/adherence was 4.9 ± 0.5 × 10⁸ and 3.3 ± 0.5 × 10⁸, respectively. Pretreatment with WEB-2170 resulted in a 33% decrease in SRBC retention/adherence compared with SRBC + PAF + LT₄-perfused lungs (Fig. 4). Comparison of SRBC retention/adherence in lungs perfused with SRBC + APMN ± pretreatment with WEB-2170 resulted in a 19% decrease in SRBC retention/adherence compared with the SRBC + APMN control. SRBC retention was then assessed in lungs perfused with SRBC with PAF + APMN and compared with lungs perfused with SRBC + PMN, which were pretreated with zileuton before PMA activation. In the SRBC + APMN-perfused lungs, SRBC retention/adherence was 5.95 ± 0.6 × 10⁸ compared with 3.40 ± 0.3 × 10⁸ in the SRBC + zileuton + APMN group. Zileuton pretreatment resulted in a 43% decrease in SRBC retention/adherence compared with the SRBC + APMN control (Fig. 5).

DISCUSSION

The concentration of PMNs in pulmonary capillary blood is 40–80 times higher than seen in the blood of large vessels (5, 15). Many individuals with SCD have chronically elevated peripheral white blood cell counts in the absence of an acute crisis, which suggest the marginal pool of PMNs in the pulmonary circulation may be higher than that reported in normals (8, 14). Boghossian et al. (9) and Fadlon et al. (18) have demonstrated that PMNs from patients in crisis are more adherent than control PMNs and suggest that enhanced PMN adhesion to vascular endothelium could contribute to the initiation of vasoocclusion in the acute crisis of SCD. However, we postulate in this study that PMN activation, as seen with inflammation, infection (57), or possibly in relation to SRBC adherence to PMNs (30), initiates vaso-occlusion via the
release of phospholipid products, which increase SRBC retention/adherence in the pulmonary circulation.

In this study, the major findings are the following: 1) SRBC retention/adherence is 3.5 times greater than that seen in lungs perfused with NRBCs; 2) for PMNs to increase SRBC retention/adherence in the pulmonary circulation, PMN activation is required; 3) increased SRBC retention/adherence is not due to PMA; and 4) PAF in tandem with LTB₄ production from the APMN are at least partly responsible for the increased pulmonary microvascular retention/adherence of SRBCs observed in this study.

Pulmonary microvascular occlusion by abnormally adherent and/or nondeformable SRBCs has been reported (1, 25) as a potential factor in the initiation of acute lung injury. Similar to this study, which demonstrated SRBC retention/adherence to be 3.5 times greater than that seen with NRBCs in lungs ventilated with a room air gas mixture, Aldrich et al. (1) reported a threefold increase in SRBC retention compared with NRBCs in the isolated rat lung ventilated with 95% O₂. This study further demonstrated that regional ventilation with 0% O₂ resulted in a 25-fold increase in SRBCs compared with NRBCs. Unlike the study by Aldrich et al. (1), which reported that the retention of SRBCs in pulmonary microvasculature was primarily due to the mechanical entrapment of nondeformable SRBCs, our study provides data supporting a nonmechanical mechanism, which involves the release of PAF and LTΒ₄ from the APMN in the increased retention/adherence seen in the isolated rat lung model.

The requirement of PMN activation to increase SRBC retention/adherence supports our hypothesis that PMN-SRBC-endothelial cell interactions may initiate vasoocclusion. However, this finding did not allow for distinguishing between mechanical properties of the APMN and/or the release of a soluble product(s), which could contribute to the observed increase in SRBC retention. Activation of the PMN in response to inflammatory mediators such as interleukin-8, C5a, PAF, LTΒ₄, tumor necrosis factor-α, N-formylmethionyl-leucyl-phenylalanine, or PMA causes an increase in PMN f-actin, which results in decreased PMN deformability and increased endothelial cell adhesion (15, 22–24, 42). The same mediators cause neutropenia and pulmonary microvascular PMN sequestration when infused intravascularly (15). Decreased PMN deformability, increased PMN adhesion to vascular endothelium and the larger PMN size relative to the RBC (15, 22–24, 42) are likely important determinants of increased microvascular resistance. Each of these PMN properties could prolong RBC transit time through the pulmonary microcirculation and contribute to the increased SRBC retention/adherence observed in this study (31, 42). To exclude the potential contribution of these biomechanical properties of the APMN in increased SRBC retention, studies that compared SRBC retention in lungs perfused with APMNs to lungs perfused with the supernatant of APMNs were performed. In these studies, SRBC retention was essentially identical, which strongly suggested that in part the product of the activated PMN was responsible for increased SRBC retention in the pulmonary circulation. However, before this could be concluded, an additional study investigating a potential role of PMA on the upregulation of adhesion molecules on the sickle reticulocytes and/or endothelial cells was performed. Kumar et al. (43) reported that phorbol esters activate the α₂β₁ integrins on the surface of sickle reticulocytes and increase the adherence of the reticulocyte to endothelial cells via fibronectin. In contrast, incubation of endothelial cells with the phorbol esters did not increase adhesion of sickle reticulocytes. The authors concluded that phorbol esters increase sickle reticulocyte adhesion to endothelium via activation of α₂β₁ integrins and this effect was independent of endothelial cell vascular adhesion molecule-1 (VCAM-1). In our study, PMA added to the perfusate did not increase SRBC retention when compared with SRBC control. This finding excludes any significant role of the integrin receptor α₂β₁ or endothelial cell vascular adhesion molecule-1 in the observed increased SRBC retention seen.

Beyond the biomechanical changes seen with PMN activation, a series of enzymes [phospholipases C (PLC), D (PLD), and A₂ (PLA₂)] involved in the generation of phospholipid products by the PMN and NADPH oxidase is activated (2). Of particular interest are oxidants and the products of membrane phospholipids and PLA₂, lyso-PAF-acether (when acetylated results in PAF), and free arachidonic acid (AA) (2, 11, 58).

Mild heating of human PMNs inactivates NADPH oxidase. This inactivation of NADPH oxidase produces a defect in superoxide production without altering PMN adhesion or chemotaxis (17, 21). In this study, heat-treated APMNs did not result in a decreased SRBC retention compared with the APMN control. This suggested that APMN-related increased SRBC retention/adherence was not due to NADPH oxidase...
activation. This led to further studies, which examined the role of PAF and LTβ₄ as products released by the APMN, which could increase SRBC retention in the pulmonary circulation.

AA, lyso-PAF-acether, and other metabolites are released from the membrane phospholipids of stimulated PMNs by PLC, PLD, and PLA₂ (2, 7). The major product of AA, which is secreted by human PMNs, is the 5-lipoxygenase product LTβ₄ (20). The lyso-PAF-acether is inactive, but when acetylated by an acetyltransferase, its metabolically active product PAF is formed (7, 58). Both PAF and LTβ₄ can cause the release of other inflammatory mediators, and influence vascular permeability, cell infiltration, and PMN adhesion to vascular endothelium (7, 45). Perhaps a similar interaction between PAF and LTβ₄ on SRBC-endothelial interaction also occurs. Interestingly, PAF priming of human umbilical vein endothelial cells increases LTβ₄-mediated PMN adhesion (46). The LTβ₄ effect on PMN-endothelial cell adhesion is dose dependent with a maximum at 1 μM, requires no more than 1 min of preincubation, and decays linearly over 30 min once LTβ₄ is removed (32). There are no data currently available that describe LTβ₄ having any effects on SRBC-endothelial cell interactions. Our study supports that LTβ₄ alone has no effect on SRBC retention/adherence in the rat lung circulation. In contrast, there are data demonstrating that PAF increases SBC adhesion to rat ex vivo mesocoeum vasculature through a mechanism that involves PAF induction of endothelial αVβ3. This effect is blocked by monoclonal antibodies to αVβ3 (40). In addition, Sultana et al. (59) reported that the PAF receptor antagonist CV-3988 inhibits CoCl₂ and hypoxia-induced SRBC adhesion to vascular endothelium. Whereas these data using CV-3988 provide indirect evidence that PAF plays a role in increasing SRBC adhesivity to vascular endothelium, they alone do not exclude the potential interaction of PAF and LTβ₄ in tandem as proposed in this study. In our study, PAF alone did not increase SRBC retention/adherence. Possible explanations for why no effect of PAF on SRBC retention/adherence was seen are: 1) the time course was too long, resulting in the metabolism of PAF and a loss of the transient period of hyperadhesiveness; 2) the ⁵¹Cr-labeled SRBC assay was not sensitive enough to demonstrate lower degrees of increased SRBC retention/adherence; and 3) PAF alone does not increase SRBC retention/adherence in the rat lung circulation.

Whereas the rheology of the APMN has been studied, the effects of the release of its phospholipid mediators PAF + LTβ₄ on SRBC retention/adherence in the pulmonary circulation has not. In the present study, lungs perfused with PAF and LTβ₄ in tandem increased the retention of SRBCs in the pulmonary circulation compared with control. Increased SRBC retention/adherence was partially reversed compared with controls in lungs perfused with PAF + LTβ₄ + WEB-2170 (PAF antagonist), and in lungs pretreated with WEB-2170 before the addition of APMNs. The decrease in SRBC retention/adherence seen in the PAF + LTβ₄ + WEB-2170 group compared with the PAF + LTβ₄ control paralleled studies utilizing LTβ₄ alone. Whereas PMN activation results in the release of PAF and LTβ₄ and APMNs increase SRBC retention/adherence, lungs pretreated with WEB-2170 before the addition of APMNs attenuated APMN-mediated SRBC retention/adherence. Similarly, in lungs where PMNs were pretreated with the 5-lipoxygenase inhibitor zileuton before activation with PMA, decreased SRBC retention/adherence was observed compared with APMN controls. 5-lipoxygenase inhibition with zileuton results in decreased production of LTβ₄ (6, 16), which is the principal leukotriene released by human PMN (20). These observations with WEB-2170 and zileuton support the hypothesis that both PAF and LTβ₄ are required to increase SRBC retention and link the APMN to the SRBC-endothelium interactions reported in this study. The decrease in SRBC retention/adherence seen with WEB-2170 caused a 33% decrease in SRBC retention/adherence in PAF + LTβ₄-treated lungs and zileuton treatment of PMNs before activation decreased PMN-mediated SRBC retention/adherence 43%. In additional studies (n = 4), combination treatments of WEB-2170 (in perfusate) and zileuton pretreatment of PMNs before activation significantly decreased PMN-mediated SRBC retention/adherence by 45% but did not demonstrate synergism compared with WEB-2170 or zileuton treatments alone (data not shown). These findings, coupled with those demonstrating that neither PAF nor LTβ₄ when individually perfused increased SRBC retention in the pulmonary circulation, provide convincing evidence that APMN secretion of PAF and LTβ₄ in tandem is at least partly responsible for the observed increase in SRBC retention/adherence seen in the pulmonary circulation of the isolated-perfused rat lung model. Although these findings have not been reported previously, Setty et al. (55) have reported in a static incubation system that AA metabolites are involved in mediating basal adhesion of normal and SRBCs to endothelium via lipoxygenase metabolites. In subsequent studies, Setty et al. (53, 54) found that SRBCs stimulate endothelial cell production of AA and diacylglycerol and that the lipoxygenase product 12(s)-hydroxy-5,8,10,14-eicosatetraenoic acid increased hypoxia-induced SRBC-endothelial adherence via the upregulation of VCAM-1 on endothelial cells. These studies are similar to the present study in that products of membrane phospholipids, related in part to the lipoxygenase enzyme system, appear to be involved in mediating SRBC retention/adherence. These studies differ in that Setty et al. analyzed RBC-endothelial cell interaction in a static system while this study investigated PMN-SRBC-endothelial cell interactions in a dynamic flow model. Further studies will be required to determine whether the primary effect of PAF + LTβ₄ is on the endothelium or SRBC.

In conclusion, our results support a potential role of the APMN in the initiation of pulmonary microvascular occlusion, which involves the phospholipid, proinflammatory mediators, PAF and LTβ₄. This observation possibly explains how PMN activation in response
to infections or other injurious insults, such as that seen in reperfusion injury, precipitates vasoocclusion in SCID and the acute chest syndrome. Furthermore, it supports potential therapeutic approaches employing PAF antagonist and/or 5-lipoxygenase inhibitors in the prevention of SRBC-endothelium interactions.

We thank Marilyn Chancellor for the preparation of this manuscript and Dr. Betty Pace for reviewing this manuscript.

This study was supported by the National Heart, Lung, and Blood Institute Comprehensive Sickle Cell Program Grant P60 HL-38639 and the Florence Foundation Research Career Development Grant.

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