A two-insult in vitro model of PMN-mediated pulmonary endothelial damage: requirements for adherence and chemokine release

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Wyman, Travis H., A. Jason Bjornsen, David J. Elzi, C. Wayne Smith, Kelly M. England, Marguerite Kelher, and Christopher C. Silliman. A two-insult in vitro model of PMN-mediated pulmonary endothelial damage: requirements for adherence and chemokine release. Am J Physiol Cell Physiol 283: C1592–C1603, 2002. First published July 24, 2002; 10.1152/ajpcell.00540.2001.—Lysophosphatidylcholines (lyso-PCs), generated during blood storage, are etiologic in a two-insult, sepsis-based model of transfusion-related acute lung injury (TRALI). Individually, endotoxin (LPS) and lyso-PCs prime but do not activate neutrophils (PMNs). We hypothesized that priming of PMNs alters their reactivity such that a second priming agent causes PMN activation and endothelial cell damage. PMNs were primed or not with LPS and then treated with lyso-PCs, and oxidase activation and elastase release were measured. For coculture experiments, activation of human pulmonary microvascular endothelial cells (HMVECs) was assessed by ICAM-1 expression and chemokine release. HMVECs were stimulated or not with LPS, PMNs were added, cells were incubated with lyso-PCs, and the number of viable HMVECs was counted. Lyso-PCs activated LPS-primed PMNs. HMVEC activation resulted in increased ICAM-1 and release of ENA-78, GRO, and IL-8. PMN-mediated HMVEC damage was dependent on LPS activation of HMVECs, chemokine release, PMN adhesion, and lyso-PC activation of the oxidase. In conclusion, sequential exposure of PMNs to priming agents activates the microbicidal arsenal, and PMN-mediated HMVEC damage was the result of two insults: HMVEC activation and PMN oxidase assembly.

Neutrophils (PMNs) are the most abundant phagocyte in circulation and are a vital part of host defense, especially against bacterial and fungal infections (11, 82). The normal function of PMNs involves a stepwise progression of events that results in PMNs migrating from the circulation through the vascular endothelium to the site of infection in the tissue (3, 12, 27, 75). At the site of infection, PMNs phagocytize the invasive microbes and kill them through both oxidative and nonoxidative methods. Importantly, the microbicidal functions of PMNs mostly occur in the tissues, and PMN priming by chemokines and other factors is part of the normal response to infection (3, 11, 12, 27, 75, 82). Priming of PMNs begins with their exposure to factors from activated vascular endothelium, both chemokines released by activated endothelial cells (EC) and the increased surface expression of EC adhesion molecules that initiate PMN adhesion, resulting in PMN priming that may continue during chemotaxis to the inflammatory site (2, 35, 39, 48). Primed PMNs have enhanced microbicidal capacity to a subsequent stimulus so that microbial invaders may be efficiently eradicated (2, 35, 39, 48). Whereas PMN priming is important for efficient killing of bacteria and fungi, priming agents have been implicated in the pathogenesis of syndromes of PMN-mediated organ damage, including acute lung injury (ALI) (59, 64, 74, 80).

Neutrophils are primed by a wide variety of stimuli that may be encountered during an inflammatory response (1, 2, 15, 16, 30, 49, 71, 80, 88). Exposure to small concentrations of bacterial endotoxin (lipopolysaccharide, LPS) is known to prime the respiratory burst and to augment, but not cause, elastase release from isolated PMNs (20–22, 30). Priming is defined operationally on the PMN NADPH oxidase such that agents that augment the oxidative burst to a subsequent stimulus but do not individually cause oxidative assembly are termed priming agents (1, 2, 15, 16, 30, 49, 71, 80, 88). Priming agents are chemoattractants and affect other PMN functions including changes in shape due to cytoskeletal rearrangements, firm adhesion mediated by a conformational change in the β2-integrins, and the release of small amounts of granule constituents (16, 42, 80). A number of compounds, including cytokines, the by-products of the complement cascade, and lipids, are priming agents and have been implicated in human disease; however, many of the well-described in vitro activators of the PMN oxidase,
e.g., phorbol esters, have little physiological relevance or may never achieve concentrations in vivo that are employed routinely in vitro (1, 13, 49, 88). PMN priming agents have been shown to be etiologic in animal models of ALI; however, two priming agents must be administered sequentially (59, 64, 74). Changes in PMN adherence, the enhanced release of cytotoxic products, and possible changes in PMN reactivity due to the “primed” state have been proposed as contributing to tissue injury in these conditions (70, 80).

Previous studies have demonstrated that the routine storage of blood components, both packed red blood cells and platelet concentrates, leads to the generation and accumulation of a potent PMN priming activity, identified as a mixture of lysophosphatidylcholines (lyso-PCs) (71, 72). In addition, a number of investigators have shown that lyso-PC may augment the respiratory burst in isolated human and rodent PMNs (13, 19, 26, 71–74). Animal models of the acute respiratory distress syndrome (ARDS) have postulated that two events are required; moreover, animal models have employed the sequential administration of agents that have the capacity to activate the vascular endothelium and prime the NADPH oxidase (11, 59, 64, 74). Because PMN priming agents have been implicated in ARDS, we postulated that the mixture of lyso-PCs may act as a second insult and cause pulmonary damage in patients with transfusion-related acute lung injury (TRALI), a syndrome virtually identical to ARDS (11, 59, 62, 64, 73, 74, 80). TRALI is thought to be secondary to the infusion of anti-leukocyte antibodies that result in pulmonary sequestration of PMNs, activation of the complement cascade, capillary leak, and pulmonary injury, similar to ARDS (43, 55, 78, 79). Because of the complement cascade, capillary leak, and pulmonary sequestration of PMNs, activation of the complement cascade, capillary leak, and pulmonary injury, similar to ARDS (43, 55, 78, 79). Because of these

Materials and Methods

Unless otherwise specified, all reagents were purchased from Sigma Chemical (St. Louis, MO). A Thermomax plate reader was purchased from Molecular Dynamics (Menlo Park, CA). Plastic microplates, manufactured by Nunc, were obtained from Life Sciences Products (Denver, CO). HMVECs of pulmonary origin and all media and tissue culture reagents were purchased from the Clonetics division of BioWhittaker (Walkersville, MD). T-25 tissue culture flasks, 12-well plates, sterile pipettes, and paraformaldehyde were obtained from Fisher Scientific (Pittsburgh, PA). A phycoerythrin-labeled monoclonal antibody to CD11b and an unlabeled monoclonal antibody to intercellular adhesion molecule-1 (ICAM-1) were purchased from PharMingen (Torrey Pines, CA), and a fluorescein isothiocyanate (FITC)-labeled monoclonal antibody to CD54 was procured from Immunotech (Marseille, France). Human PBMCs were isolated by standard techniques including dextran sedimentation, Ficoll-Hypaque gradient centrifugation, and hypotonic lysis of contaminating red blood cells (71). Isolated PBMCs were pretreated with anti-CD18 monoclonal antibody to CD18 and then incubated with lyso-PCs, mimicking transfusion of a septic patient with stored blood, to determine whether LPS-primed PMNs could be activated by lyso-PCs, a second priming agent. In the second portion of this study, to assess PMN-mediated damage of human pulmonary microvascular endothelial cells (HMVECs), we investigated LPS activation of these cells, including increased surface expression of adhesion molecules and chemokine release. Resting human PMNs were then added to both control and LPS-treated HMVECs, which were allowed to settle and were then activated with lyso-PCs or vehicle, and the number of viable HMVECs was counted. The roles of PMN adhesion to vascular endothelium, chemokine release, oxidase activation, and degranulation were investigated in this coculture model of PMN-mediated HMVEC damage.

Neutrophil isolation and oxidase priming. PMNs were isolated by standard techniques including dextran sedimentation, Ficoll-Hypaque gradient centrifugation, and hypotonic lysis of contaminating red blood cells (71). Isolated PMNs were pretreated for 30 min at 37°C with buffer control or LPS in concentrations varying from 2 ng/ml to 2 μg/ml. Assays of oxidase activation in response to lyso-PC or N-formylmethionyl-leucyl-phenylalanine (fMLP) control were determined by measurement of the SOD-inhibitable reduction of cytochrome c at 550 nm of light in a Thermomax microplate reader as described previously (71, 74). The priming activity of LPS was measured by first incubating the PMNs in the reaction mixture containing LPS or Krebs-Ringer phosphate with 2% dextrose (pH 7.35) (KRPD) control buffer for 3 min at 37°C, followed by activation of the oxidase with the addition of lyso-PC. fMLP was used as a positive control for these experiments to assess the integrity of the NADPH oxidase. Therefore, priming activity was measured as the augmentation of the maximal rate of O2 in response to fMLP.

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Determination of elastase release in isolated PMNs. PMNs (1.5 x 10^6) were warmed to 37°C in a shaking water bath and then primed with 0.02–2 μg/ml LPS or buffer control for 5 min. The PMNs were activated with buffer, 0.45–14.5 μM lyso-PCs, or 1 μM fMLP as the positive control. After a 5-min reaction time, the PMNs were pelleted and the supernatant was removed. Elastase release was determined spectrophotometrically on the supernatant by the reduction of the specific substrate methoxy-succinyl-alanyl-alanyl-prolyl-valyl p-nitroanilide (AAPVNA) at 405 nm in duplicate. To ensure that the reduction of AAPVNA was secondary to that of elastase, we ran identical wells containing 5 μM of the specific elastase inhibitor methoxy-succinyl-alanyl-alanyl-prolyl-valyl-chloromethyl ketone (AAPVCK) in conjunction with each treatment. Elastase release is reported as the percentage of total cellular elastase as determined by 0.1% Triton X-100 treated amount of an identical number of PMNs.

HMVEC activation. HMVECs were grown to ≥90% confluence on 12-well plates and incubated with LPS (2 ng/ml–2 μg/ml) for 2–12 h at 37°C, 7.5% CO_2. The supernatants were aspirated, aliquoted, and stored at −70°C for measurement of ENA-78, GRO_α, and IL-8 by employing enzyme-linked immunosorbent assay (ELISA) kits purchased from R&D Systems.

IL-8 priming of NADPH oxidase. PMNs were stimulated with IL-8 (10^{-12}–10^{-6} M) for 5 min at 37°C, and superoxide anion production was measured as the maximal rate of SOD-inhibitable reduction of cytochrome c at 550 nm of light as described previously (71, 74). PMNs were also incubated for 5 min at 37°C with IL-8 (10^{-12}–10^{-9} M) and then activated with 1 mM fMLP, and the maximal rate of superoxide anion production was measured as described above. The data (nmol O_2·ml^{-1} min^{-1}) are expressed as means ± SE or as the relative increase over buffer-treated controls activated with fMLP.

HMVEC damage assay. HMVECs were grown to ≥90% confluence in 12-well plates. Half of the wells were incubated with LPS (2 ng/ml–2 μg/ml) and the other half with buffer for 6 h at 37°C, 7.5% CO_2. PMNs (1 x 10^6) were added, at a 10:1 effector cell-to-target cell ratio, and allowed to settle for 30 min. After settling, the PMNs were exposed to buffer, 200 ng/ml phosphor 12-myristate 13-acetate (PMA), or 0.45–14.5 μM lyso-PCs for 60 min. The supernatants were forcefully aspirated, and the supernatants were removed. Elastase release was determined spectrophotometrically on the supernatant by the reduction of the specific substrate methoxy-succinyl-alanyl-alanyl-prolyl-valyl-chloromethyl ketone (AAPVCK) in conjunction with each treatment. Elastase release is reported as the percentage of total cellular elastase as determined by 0.1% Triton X-100 treated amount of an identical number of PMNs.

Inhibition of PMN-mediated EC damage with antibodies to CD18, ICAM-1, GRO_α, ENA-78, and IL-8 and inhibitors of the oxidase and PMN elastase. Inhibition of EC damage from the described priming activity was due to lyso-PCs and not PAF or like compounds with an acetyl group in the sn-2 position. We ran identical wells containing 5 μM AAPVCK, or intracellular inhibitors of the oxidase, 1 μM resveratrol or 1–10 μM DPI, were added either to the wells 30 s before the addition of PMNs or to the PMNs 30 min before their addition to the coculture, respectively (63, 67). The employed concentrations of resveratrol and DPI were determined by inhibition of PMA-mediated oxidase activation, and these inhibitors of the oxidase also effectively blocked superoxide anion production to fMLP, PAF-primed PMNs stimulated with fMLP, and LPS-primed PMNs stimulated with lyso-PCs (results not shown). The 1 μM concentration of resveratrol and 1–10 μM concentrations of DPI inhibited activation of the oxidase by 50–75% without affecting cellular integrity. Finally, to block lyso-PC-mediated changes in cytosolic Ca^{2+} concentration, we loaded PMNs for 30 min with BAPTA, a cell-permeable, rapid chelator of cytosolic Ca^{2+}, which has been demonstrated to inhibit priming of the PMN oxidase (18).

PMN adherence to HMVECs. HMVECs were grown to ≥90% confluence in 12-well plates and stimulated with buffer or LPS for 6 h at 37°C, 7.5% CO_2. In selected wells, 1 mg/ml of neutralizing antibodies or isotype controls to ENA-78, GRO_α, and IL-8 were added 10 min before the inclusion of PMNs. PMNs (1 x 10^6) were then added and allowed to adhere for 60 min. An aliquot of the identical number of PMNs was set aside. At the completion of incubation, an adhesive covering was placed over the 12-well plates, the plates were centrifuged inverted at 200 g for 5 min, and the supernatant was discarded. The adherent cells were lysed with 0.01% Triton X-100, and the total amount of PMN elastase per well was determined as mentioned previously and compared with the total cellular elastase from the identical number of PMNs added to each well. The data are the percentage of adherent PMNs expressed as means ± SE.

Statistical analysis. The means, SD, and SE were calculated using standard techniques. Statistical differences among groups were determined by a paired analysis of variance followed by a Tukey post hoc analysis for multiple comparisons. Statistical significance was determined at the P < 0.05 level.

RESULTS

Priming and activation of the PMN oxidase. Previous work demonstrated that the mixture of lyso-PCs generated during routine blood storage was capable of priming the respiratory burst of PMNs (71–74). Based on these data, lyso-PC concentrations that primed the NADPH oxidase were employed (0.45–14.5 μM). No concentrations of the lyso-PC mixture caused activation of the PMN oxidase (albumin: 0.2 ± 0.2 vs. 14.5 μM; lyso-PCs: 0.2 ± 0.2 nmol O_2/min). To ensure that the described priming activity was due to lyso-PCs and not PAF or like compounds with an acetyl group in the sn-2 position, we incubated the lyso-PC mixture for 30
activated respiratory burst was consistently increased over the lower doses and became maximal at the 2 μg/ml LPS dose. Notably, concentrations of LPS <2 ng/ml and lyso-PC <0.45 μM displayed no evidence of priming or activation of the PMN oxidase, respectively, and at all concentrations of LPS employed (2 ng/ml–2 μg/ml), there was no evidence of PMN lysis or loss of viability (PMNs were 99% viable by trypan blue exclusion). Finally, pretreatment of PMNs with 50 μM BAPTA, an effective chelator of cytosolic Ca2+, inhibited lyso-PC activation of the respiratory burst in LPS-primed PMNs (92 ± 5 to 97 ± 3%) for all concentrations of LPS and lyso-PCs employed.

Elastase release by PMNs. The ability of LPS to augment the lyso-PC-elicited release of elastase from isolated PMNs was evaluated over a range of LPS concentrations from 2 ng/ml to 2 μg/ml and lyso-PC concentrations from 0.45 to 14.5 μM (Fig. 2). Compared with vehicle-treated controls, 0.45 μM lyso-PC did not cause any elastase release, and this concentration was not augmented by pretreatment with any concentration of LPS. In addition, concentrations of 2 ng/ml LPS (Fig. 2) to 200 ng/ml LPS (results not shown) did not result in the augmentation of elastase release in response to either lyso-PCs or fMLP. At the 2 μg/ml concentration, LPS priming resulted in a direct increase in the release of elastase by treatment of PMNs with 14.5 μM lyso-PCs, but not 4.5 μM lyso-PCs, compared with both buffer-primed controls and LPS-treated PMNs with buffer (Fig. 2). Lyso-PCs at 4.5 μM and 14.5 μM did cause increased amounts of elastase release was measured as the percentage of elastase released into the supernatant compared with Triton X-100-lysed PMNs, and data are expressed as means ± SE. Notably, statistical significance (*P < 0.05) was attained only at an LPS concentration of 2 μg/ml and an L-PC concentration of 14.5 μM. Data are representative of 7 separate experiments.

Fig. 2. Elastase released from LPS-primed PMNs in response to L-PC stimulation. Isolated PMNs were treated with buffer or L-PC (0.45–14.5 μM), or 1 μM fMLP (positive control). Total elastase release was measured as the percentage of elastase released into the supernatant compared with Triton X-100-lysed PMNs, and data are expressed as means ± SE. Notably, statistical significance (*P < 0.05) was attained only at an LPS concentration of 2 μg/ml and an L-PC concentration of 14.5 μM. Data are representative of 7 separate experiments.
Fig. 3. LPS-mediated increased surface expression of ICAM-1 on human pulmonary microvascular endothelial cells (HMVECs). The surface expression of ICAM-1 on pulmonary HMVECs as quantified by flow cytometry is represented as a function of the dose of endotoxin, and data are expressed as means ± SE. Pulmonary HMVECs were incubated with endotoxin (0.002–2 μg/ml) or vehicle control (C) for 6 h at 37°C, 7.5% CO2. The supernatants were aspirated, and the cells were removed with trypsin and incubated for 30 min at 4°C with a FITC-labeled monoclonal antibody (MAB) to ICAM-1. MFI, mean fluorescence intensity. Data represent a sample size of 8. *P < 0.05 compared with the vehicle-treated control pulmonary HMVECs.

Adherence and chemokine release compared with albumin-treated controls (Fig. 2), similar to reports of other priming agents including PAF (80). Moreover, the positive control, 1 μM fMLP, showed similar results.

Activation of pulmonary HMVECs. HMVECs were incubated with 2 μg/ml LPS from 2 to 12 h, and the surface expression of ICAM-1 was measured by employing a FITC monoclonal antibody to ICAM-1 (CD54) and flow cytometry. Increases in ICAM-1 began at 2 h (2.4 ± 0.6-fold increase, P < 0.05 compared with media-treated controls) and at 6 h appeared similar to TNF-α-stimulated positive controls (LPS: 5.1 ± 0.6-fold increase vs. TNF-α: 6.7 ± 1.1-fold increase, both compared with media-treated controls). At 12 h there was a slight, but not statistically different, increase compared with the 6-h incubation with LPS (6.7 ± 1.1 vs. 7.4 ± 1.5-fold increase compared with media-treated controls). Thus 6-h incubations were used for all experiments to assess LPS activation of HMVECs. The changes in ICAM-1 surface expression after 6 h of LPS incubation (2 ng/ml−2 μg/ml) are shown in Fig. 3. LPS caused an increase in ICAM-1 surface expression at concentrations from 20 ng/ml to 2 μg/ml (P < 0.05) but did not cause an increase in CD54 at an LPS concentration of 2 ng/ml (Fig. 3). In addition, activation of endothelium, which is associated with chemokine release, was measured in the incubation media (ELISA) from the same HMVECs employed for the ICAM-1 surface expression before the HMVECs were removed (trypsin) from the 12-well plates. LPS concentrations from 20 ng/ml to 20 μg/ml were assessed for their ability to cause production and release of ENA-78, GROα, and IL-8. Concentrations of LPS that caused an increase in ICAM-1 surface expression also caused significant chemokine release for all three chemokines measured (Fig. 4). In these experiments, the concentration response was taken an order of magnitude higher to ensure that the HMVECs were “fully” stimulated by LPS for chemokine production. The amount of ENA-78 released from HMVECs was statistically different from control HMVECs at concentrations of 2–20 μg/ml LPS, although the amount of ENA-78 released at the 20 μg/ml concentration was not different from that at 2 μg/ml (Fig. 4A). The release of GROα became significant at LPS doses of 20 ng/ml and was maximal at 200 ng/ml; higher concentrations of LPS did not induce an increased release of this chemokine from HMVECs (Fig. 4B). Conversely, IL-8 was released maximally from the LPS activation of HMVECs at an LPS concentration of 20 ng/ml; higher concentrations of LPS did not augment the amount of IL-8 released by HMVECs (Fig. 4C). Therefore, LPS concentrations that caused increased ICAM-1 surface expression also caused chemokine release of GROα and IL-8, and only LPS concentrations of 2 μg/ml and higher caused release of significant amounts of ENA-78 compared with media-stimulated controls.

To determine whether the concentrations of chemokines caused priming or oxidative activation of PMNs in vitro, we incubated PMNs with IL-8 for 5 min at 37°C and measured superoxide anion production. IL-8 (100 nM–1 μM) caused activation of the oxidase compared with saline-treated controls (saline: 0.1 ± 0.5; IL-8 (1 μM): 1.0 ± 0.5; IL-8 (100 nM): 0.9 ± 0.2; IL-8 (10 nM): 0.1 ± 0.2; and 1 μM fMLP: 2.0 ± 0.2 nmol O2−·ml−1·min−1 (P < 0.05 compared with saline-treated controls, n = 6)]. At concentrations of 1–10 nM, IL-8 significantly primed the fMLP-activated respiratory burst 2.5 ± 1.0 to 6.2 ± 2.0-fold (P < 0.05 compared with buffer-primed fMLP-activated controls, n = 8), and lesser concentrations (<1 nM) did not prime fMLP activation of the PMN oxidase. Similar results have been reported with respect to ENA-78 and GROα (5, 16, 25, 28, 29, 37, 50, 81). These data provide supportive evidence that concentrations of the chemokines produced by LPS-activated HMVECs (1–5 ng/ml IL-8 <1 nM) do not cause activation of the NADPH oxidase and are related to priming of PMNs, which includes adhesion.

PMN-mediated HMVEC damage. Pulmonary HMVECs were activated with buffer or LPS (2 ng/ml–2 μg/ml) for 6 h. PMNs were added, allowed to settle (30 min), and then activated with buffer or lyso-PCs over a range of concentrations (0.45–14.5 μM) and incubated for 60 min. The number of viable HMVECs, trypan blue negative, was counted over a 4-mm2 surface area. In all cases, 99 ± 2% of the adherent HMVECs were trypan blue negative; conversely, the detached HMVECs in the supernatant were 99 ± 2% trypan blue positive. LPS did not affect HMVEC viability for any concentration employed (2 ng/ml–2 μg/ml) (Fig. 5). Quiescent, buffer-treated HMVECs, incubated with lyso-PCs at
0.45–14.5 μM, did not display any evidence of killing of HMVECs; similarly, quiescent, buffer-treated HMVECs incubated with PMNs and lyso-PCs (0.45–14.5 μM) also exhibited no evidence of killing. Furthermore, even the addition of 200 ng/ml PMA, a robust activator of the NADPH oxidase, to PMNs coincubated with quiescent HMVECs did not result in a decreased number of viable HMVECs per 4 mm² [HMVECs/PMNs: 1,166 ± 77 (mean ± SE) vs. HMVECs/PMNs/PMA: 984 ± 159 HMVECs/4 mm² (n = 6)]. The lowest dose of LPS, 2 ng/ml, did not result in any observed PMN-mediated cytotoxicity when combined with lyso-PC-activated PMNs for all lyso-PC concentrations tested (results not shown). Also, importantly, lyso-PCs or PMA alone did not affect either quiescent HMVECs or LPS-activated HMVECs in the absence of PMNs. However, PMN-mediated HMVEC damage became readily apparent at LPS concentrations of 20 ng/ml–2 μg/ml when followed by the addition of PMNs activated with 4.5 or 14.5 μM lyso-PCs (Fig. 5, A and B). At an LPS concentration of 2 μg/ml, all lyso-PC concentrations caused significant PMN-mediated HMVEC damage compared with unstimulated HMVECs, LPS-activated HMVECs coincubated with quiescent PMNs, and buffer-primed HMVECs treated with PMNs and lyso-PCs (P < 0.05) (Fig. 5C). In addition, LPS-activated HMVECs incubated with PMNs and treated with PMA also evidenced significant PMN-mediated HMVEC cytotoxicity [HMVECs/PMNs: 1,166 ± 77; HMVECs + PMNs + PMA: 984 ± 159; and HMVECs + 20 ng/ml LPS + PMNs + PMA: 520 ± 123 viable HMVECs/4 mm² (P < 0.05 compared with the other two groups, n = 6)]. Thus lyso-PC activation of PMNs adhered to LPS-stimulated HMVECs resulted in destruction of HMVECs in a concentration-dependent fashion (Fig. 5).

To determine whether firm adherence is required in this in vitro EC damage assay, we preincubated PMNs with a monoclonal antibody to CD18 at saturating concentrations (1 μg/ml). Preincubation inhibited PMN-mediated HMVEC damage in HMVECs stimulated with 2 μg/ml LPS and incubated with PMNs activated with 14.5 μM lyso-PCs (Fig. 6A, P < 0.05, n = 4). Moreover, to investigate the role of EC adhesion molecules in this model, HMVECs were preincubated (10 min) with a monoclonal antibody (1 μg/ml) to ICAM-1 (CD54). Similar to blockade of the PMN CD18 adhesion molecule, blockade of ICAM-1 also abrogated PMN-mediated HMVEC damage in this model (Fig. 6B, P < 0.05, n = 4). These results indicate that firm adhesion through β2-integrins on the PMN and ICAM-1 on the EC is required for PMN-mediated cytotoxicity in this in vitro model of EC damage.

To characterize the cytotoxic agent responsible for the observed PMN-mediated HMVEC damage, we employed the selective elastase inhibitor AAPVCK and two intracellular inhibitors of the NADPH oxidase, resveratrol and DPI (63, 67). To inhibit elastase, we added AAPVCK to the reaction mixture 30 s before the addition of PMNs. Previous work with this chloromethyl ketone has demonstrated that it does not in-

Fig. 4. LPS-mediated release of chemokines from pulmonary HMVECs. Chemokine release (ENA-78, A; GROα, B; and IL-8, C) is represented as a function of LPS concentration. The chemokines were measured in the media from HMVECs incubated for 6 h with differing doses of LPS (0.02–2 μg/ml) or vehicle control (C) by using specific ELISA assays in duplicate, and concentrations were calculated from a standard curve. Data are expressed as means ± SE and represent a sample size of 16. *P < 0.05 compared with media of control HMVECs incubated for 6 h with saline. #P < 0.05 compared with lower doses and vehicle control.
hibit the PMN oxidase or other signaling pathways in PMNs (7). AAPVCK inhibition of PMN elastase had no effect on PMN-mediated damage of LPS-primed HMVECs coincubated with lyso-PC-activated PMNs (results not shown). Conversely, resveratrol and DPI inhibited PMN-mediated destruction of activated HMVECs (Table 1). Such inhibition of PMN-elicited cytotoxicity by resveratrol and DPI strongly suggests that the oxidase played an important role in this model.

Inhibition of PMN-mediated HMVEC damage with antibodies to chemokines. To determine the role of the chemokines released from LPS-activated HMVECs, we added monoclonal antibodies to ENA-78, GRO, and IL-8 to the HMVEC reaction media after 6 h of LPS (2 μg/ml) stimulation before the addition of PMNs. As shown in Table 2, incubation with neutralizing, monoclonal antibody to one or two chemokines attenuated the PMN-mediated cytotoxicity compared with isotypic monoclonal antibody- or media-treated controls. When antibodies to all three chemokines were used, total abrogation of PMN-mediated HMVEC damage was observed.

Because the addition of neutralizing antibodies to the coculture inhibited PMN cytotoxicity in this two-event in vitro model, we examined the possibility that these antibodies inhibited chemokine-mediated adhesion of PMNs to the activated HMVECs (Table 3). HMVECs were incubated in the presence or absence of 2 μg/ml LPS, the neutralizing antibodies were added to selective wells 10 min before the addition of PMNs, and the PMNs were then added and allowed to adhere for 60 min. Activated HMVECs caused significant PMN adhesion that was abrogated by preincubation with the chemokine antibodies, determined by employing an assay, similar to previously published data (6). Also, importantly, inverted centrifugation of HMVECs at 200 g did not cause significant detachment of these cells.

BAPTA inhibition of PMN cytotoxicity. Because changes in cytosolic Ca\(^{2+}\) concentration are required for lyso-PC signaling in PMNs (69), PMNs were incubated with BAPTA for 30 min before their addition to the coculture to chelate the cytosolic Ca\(^{2+}\) and make it

Fig. 5. PMN-mediated damage of pulmonary HMVECs. The damage caused by L-PC-activated PMNs on HMVECs primed with LPS at differing concentrations: 20 ng/ml LPS (A), 200 ng/ml LPS (B), and 2 μg/ml LPS (C), all after stimulation for 6 h at 37°C, 7.5% CO\(_2\). PMNs were added, allowed to settle, and stimulated with buffer or L-PCs (450 nM–14.5 μM) for 60 min. The amount of viable, adherent HMVECs, as determined by the ability to exclude trypan blue, was counted via a dissecting microscope over a surface area of 4 mm\(^2\). Four different observers were employed to alleviate observer bias, and the results, expressed as means + SE, were within 2.4 ± 2% of one another for all measurements. These damage assays demonstrated dose dependence of both the first event, LPS, and the second event, L-PCs, for PMN-mediated HMVEC damage. \(* P < 0.05\), HMVEC damage was statistically significant compared with HMVECs alone and HMVECs + PMNs. In selected experiments the supernatants were aspirated and stained, and the discernible HMVECs were 99 ± 4% trypan blue positive. Data are representative of 7 separate experiments for each L-PC concentration.
biologically unavailable. As shown in Fig. 7, BAPTA chelation of cytosolic Ca\(_{2+}\) totally inhibited the lyso-PC-mediated cytotoxicity of LPS-activated HMVECs, compared with dimethyl sulfoxide (DMSO)-treated PMNs, which caused significant HMVEC damage. Notably, upon visual inspection, BAPTA pretreatment did not noticeably decrease the number of adherent PMNs compared with PMNs preincubated with DMSO, although it abrogated HMVEC damage.

**Table 1. Inhibition of PMN-mediated killing of LPS-activated pulmonary endothelial cells by resveratrol and DPI**

<table>
<thead>
<tr>
<th>Antibody Pretreatment</th>
<th>Buffer</th>
<th>PMNs</th>
<th>PMNs + Lyso-PCs</th>
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<tr>
<td>DMSO</td>
<td>1,088 ± 22</td>
<td>1,073 ± 66</td>
<td>668 ± 26 (\ast)</td>
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<tr>
<td>Resveratrol</td>
<td>1,027 ± 38</td>
<td>1,033 ± 41</td>
<td>885 ± 24 (\ast)</td>
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<td>DPI (10 (\mu)M)</td>
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<td>1,012 ± 44</td>
<td>968 ± 38 (\ast)</td>
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Data are expressed as means ±SE of the number of viable human pulmonary microvascular endothelial cells (HMVECs)/4 mm\(^2\) for 4 separate experiments. Only the highest concentration of diphenyleneiodonium chloride (DPI) was used for both buffer treatment and neutrophils (PMNs) and HMVECs alone. Lyso-PCs, lysoosphatidylcholines. \(\ast\) \(P < 0.05\) compared with buffer and PMNs alone. \(\dagger\) \(P < 0.05\) compared with PMNs + lyso-PCs.

**DISCUSSION**

TRALI is identical to ARDS and is postulated to be the result of infusing anti-leukocyte antibodies with transfusion of blood components (43, 55). These anti-leukocyte antibodies are directed against recipient antigens and cause pulmonary sequestration, activation of the complement cascade, capillary leak, and pulmonary injury (43, 55, 78, 79). Because a number of observed TRALI reactions did not have an immune etiology, a two-event model was proposed identical to animal models of ARDS (9, 10, 73, 74). This model hypothesized that susceptible patients must have an underlying clinical condition that causes activation of the pulmonary endothelium, resulting in sequestration but not activation of host PMNs (70, 73, 74). Infusion of biological response modifiers, including lipids or even immunoglobulins directed against specific granulocyte antigens, that activate these primed sequestered PMNs could then result in activation of the microbi-
Table 3. Neutralizing antibodies to chemokines block PMN adherence to activated HMVECs

<table>
<thead>
<tr>
<th>Endothelial Treatment</th>
<th>Adherent PMNs, %</th>
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<tbody>
<tr>
<td>Buffer</td>
<td>1.3 ± 0.9</td>
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<tr>
<td>LPS</td>
<td>13.3 ± 3.9*</td>
</tr>
<tr>
<td>LPS + antibodies</td>
<td>2.0 ± 1.0†</td>
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</tbody>
</table>

Data are expressed as means ± SE of 4 separate experiments employing pulmonary HMVECs and the PMNs from 4 separate healthy donors. *P < 0.05 compared with buffer controls. †P < 0.05 compared with LPS-activated HMVECs.

dal arsenal focused at the points of adherence, pulmonary endothelial cell damage, capillary leak, and pulmonary damage (70, 73, 74). Patients with acute infection or recent surgery may be predisposed to TRALI, and the infusion of stored, but not fresh, blood with high concentrations of lyso-PCs could cause activation of sequestered PMNs and pulmonary damage (73). This hypothesis was tested in an animal model of TRALI in which the first insult, intraperitoneal LPS, caused pulmonary leukostasis of PMNs, and the second insult, plasma and lipids from stored packed red blood cells including purified lyso-PCs solubilized in albumin, caused acute lung injury (74). Because of these findings, we investigated the cellular physiology of the observed two-event lung injury.

Prior work both in vitro and in animal models has postulated that the first event in ARDS causes activation of the pulmonary endothelium, causing increased surface expression of adhesion molecules and chemokine release that prime PMNs, changing their phenotype to adhesive, resulting in pulmonary vascular leukostasis, a prerequisite for ALI (8, 11, 14, 29, 37, 46, 74, 75). In this light, both endothelial cell adhesion molecules and their PMN ligands, β2-integrins, have been reported to be essential in many models of PMN-mediated acute lung injury (8, 9, 36, 38, 41, 42, 46, 51). However, the lung contains many small, tortuous capillaries that may entrap rigid, primed PMNs, and such nondistensible leukocytes would then be unable to traverse the pulmonary vasculature, resulting in pulmonary leukostasis with points of direct contact between the primed PMNs and the endothelium (17, 31). The second insult causes activation of the microbicidal arsenal of these adherent PMNs, which focuses the release of cytotoxic agents at the points of PMN-endothelial cell adhesion or contact, culminating in endothelial damage cell, capillary leak, and pulmonary injury (11, 59, 64, 70, 74, 80). Acute lung injury, whether it be TRALI or ARDS, is based on this two-event model (59, 62, 64, 74, 75, 84).

The data presented in this report confirm that PMN priming not only causes adhesion of PMNs to integrin consensus (RGD) ligands of activated HMVECs but also alters the reactivity of PMNs such that these primed PMNs could be activated by the addition of a second priming agent, lyso-PCs, in vitro (56). Moreover, neither LPS nor lyso-PCs given as single agents were able to cause oxidase assembly, and activation of the oxidase and augmentation of elastase release were dependent on the concentrations of LPS, the first insult, and lyso-PCs, the second insult. In the second portion of the study, the initial priming stimulus is not LPS but, rather, the chemokines released as a function of HMVEC activation, because all three of these agents are effective primers of the PMN oxidase and directly cause PMN adhesion, presumably through a conformational change in the β2-integrins (5, 15, 16, 25, 28, 37, 50, 81). Thus these data provide supportive evidence that the exposure of PMNs to two sequential priming agents may activate their microbicidal arsenal and cause PMN-mediated cytotoxicity.

The second part of this report examined PMN-mediated damage of pulmonary HMVECs. These studies demonstrated that two sequential events or insults may lead to PMN-mediated HMVEC damage in vitro. The first event consisted of LPS activation of HMVECs, resulting in increased surface expression of ICAM-1 and the release of chemokines ENA-78, GROα, and IL-8, which are effective PMN-priming agents at the concentrations released from HMVECs, as shown in Fig. 3 and as demonstrated for IL-8 (5, 15, 16, 25, 28, 37, 50, 81). These chemokines were required for PMN adherence and, together with firm adherence, a known priming event, most likely altered the reactivity of these PMNs to a subsequent insult (5, 15, 16, 25, 28, 37, 50, 81). The addition of lyso-PCs, the second event, caused activation of these primed PMNs that resulted in the focused release of cytotoxic agents, at the points of firm adherence, that damaged and/or destroyed the activated HMVECs. Inhibition of PMN-HMVEC adhesion with monoclonal antibodies to ICAM-1 (CD54) or CD18 abrogated PMN-mediated HMVEC damage. In-
turbation of lyso-PC priming of the oxidase with BAPTA abrogated PMN-mediated HMVEC damage by inhibiting activation of the oxidase and attenuating adherence. Furthermore, the inclusion of neutralizing, monoclonal antibodies to all three chemokines abrogated HMVEC damage in this model, including the adherence of PMNs to activated HMVECs. These adherence assays do not invite comparison to other leukocyte adherence assays, only for PMNs that remain adherent when the plates are inverted and subjected to 200 g for 5 min. Thus only firmly adherent PMNs remained attached to the HMVECs. Incomplete inhibition of PMN-mediated damage was demonstrated when a single neutralizing chemokine antibody or any combination of two chemokine antibodies was added. Moreover, the addition of primed PMNs to quiescent HMVECs or the addition of quiescent PMNs to activated HMVECs had no effect on HMVEC integrity, but the latter group did elicit PMN adherence to the LPS-activated HMVECs. Without PMNs, none of the stimuli employed alone or in combination affected HMVEC integrity, including the LPS/lyso-PC combination.

In addition, we investigated the components of the microbicidal arsenal responsible for PMN-mediated damage of activated HMVECs. Although both elastase release and oxidase activation could cause HMVEC damage, preincubation of the PMN/HMVEC coculture with a selective elastase inhibitor, AAPVCK, did not affect PMN-mediated damage, data that are opposed to other adherence-based killing of PMN targets, including previous work from this laboratory (6, 83). Conversely, inhibitors of the respiratory burst, both DPI and resveratrol, inhibited PMN-mediated HMVEC damage without affecting the cellular integrity of PMNs or the qualitative adhesion of PMNs to activated HMVECs. Taken together, these data suggest that oxidase activation is important for PMN-mediated HMVEC damage. Future experiments exploring the individual roles of the chemokines released from activated HMVECs may provide more insight into the cellular physiology of PMN-mediated HMVEC damage.

In the presented model, PMN-mediated HMVEC damage occurred in a static environment without blood flow. Furthermore, pulmonary HMVEC activation resulted in increased surface expression of ICAM-1 but not vascular cell adhesion molecule-1 (VCAM-1). Conversely, we were able to show an increase in VCAM-1 on the surface of activated, human umbilical vein endothelial cells (HUVECs) (results not shown). Other in vitro models of endothelial cell damage have implicated VCAM-1 or other β2-integrin ligands, but many of these models employed flow chambers or HUVECs that may have little physiological relevance to the human lung (7, 23, 36, 60, 61, 77). Although previous work from this and other laboratories has demonstrated that lyso-PCs can prime PMNs, a number of investigators have asserted that lyso-PCs are inactive with respect to both leukocytes and platelets (4, 33, 44, 45, 52, 57). Similar to PAP, lyso-PCs require an albumin carrier and do not prime the PMN oxidase at concentrations <0.45 μM (results not shown) (85). Moreover, the addition of these compounds to fresh human plasma resulted in 1.7 ± 0.2-fold priming of the PMN oxidase compared with fresh plasma-pretreated controls (16, 25, 28, 29, 32, 37, 38, 40, 41, 47, 51, 54, 65, 68, 71 76, 84, 87).

In conclusion, one of the largest studies of ALI (70, 74) demonstrated that blood transfusion was the most commonly associated event; however, this transfusion requirement was deemed a marker of clinical injury and not a possible etiology (24). In traumatically injured patients, transfusion is a robust, independent predictor of the postinjury multiple organ failure syndrome (MOF), which includes ALI (66). More importantly, the infusion of older stored blood, which contains significant amounts of lyso-PCs, into trauma patients was also associated with the development of ALI/MOF, indicating that TRALI may be more common than previously reported (70, 86).

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