Dissociation between alveolar transmigration of neutrophils and lung injury in hyperoxia

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OXYGEN TOXICITY IS A COMMON problem observed in patients with acute lung injury (ALI) and acute respiratory distress syndrome (31). The high concentrations of inspired oxygen used in these patients may result in increased reactive oxygen metabolites in the lung that may amplify the inflammatory response. Hyperoxia-induced lung injury patterns include endothelial and alveolar epithelial cell injury, pulmonary edema, alveolar hemorrhage, and inflammatory cell infiltration, followed by fibrin deposition (3, 10). Pulmonary neutrophil (PMN) sequestration and transmigration in the lung are typical manifestations of hyperoxic exposure (2, 27). However, specific mechanisms for and pathological significance of PMN accumulation in the lung during hyperoxia are still not completely understood.

Recruitment of inflammatory cells to a site of injury depends in part on local expression of specific cell adhesion molecules (CAMs) and chemotactic agents (22, 45, 46). The pulmonary circulation differs from the systemic circulation, however, in that the initial sequestration of leukocytes in response to inflammation may be caused by generalized activation of the cells and changes in cell deformability that lead to trapping of neutrophils within the pulmonary capillary bed (14, 15, 17, 52). Therefore, in contrast to the systemic circulation, the initial interaction between leukocytes and endothelium may be relatively independent of initial tethering reactions because of increased expression of selectins on the activated pulmonary vascular surface. Which inflammatory mediators signal this interaction in the early stages of oxygen exposure remains unclear, although mechanisms underlying initial PMN activation and sequestration within the pulmonary microvasculature may include interactions of CXCL chemokines with their CXCR2 receptor (47).

Sustained margination and transmigration of PMNs across the pulmonary vascular barrier involve specific PMN interactions with endothelial adhesion molecules, including P-selectin, ICAM-1, and platelet-endothelial cell adhesion molecule-1 (PECAM-1) (1, 39). Both CD11/CD18-dependent and -independent adhesion pathways are present in the lung (16). CD11/CD18-dependent pathways appear to be associated with constitutively expressed and overexpressed ICAM-1, which is the inducible endothelial ligand for the leukocyte β₂-integrins CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1) (41). Independent pathways work through other mechanisms that remain unclear (13). The relative importance of these two pathways for PMN emigration during hyperoxia and the exact contribution of endothelial CAMs in leukocyte binding and facilitation of transmigration are still unknown.

Elevated expression of P-selectin, ICAM-1, and PECAM-1 within the lung in response to hyperoxia has been demonstrated

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by semiquantitative methods; however, the exposure of these CAMs on the luminal surface has yet to be quantitatively assessed. Other groups have evaluated changes in CAM expression using in situ hybridization techniques (38, 50, 53) or densitometric analyses of Western blots of lung homogenates (50, 53). These methods are not quantitative, do not distinguish CAMs expressed by different cell types within the lung (e.g., endothelial vs. epithelial), and do not identify specific subpopulations of CAMs expressed in the vascular lumen that may be involved in functional interactions with PMNs. Electron microscopic evaluation with immunogold (5) is semiquantitative at best and too time and labor intensive for analysis of diverse groups of animals and conditions, whereas immunohistochemical evaluation using immunofluorescence (7) is limited by its inability to identify vascular accessibility of CAMs.

Epiphaner and Granger (21) have used a technique that uses intravenous injection of dual-radiolabeled monoclonal antibodies (MAbs) to CAMs to quantify vascular CAM expression in murine and rat models of sepsis. Using this approach, we examined changes in the luminal expression of P-selectin, ICAM-1, and PECAM-1 in the pulmonary vasculature in response to hyperoxia. On the basis of the results of this evaluation, we further examined the relative role of these CAMs in PMN recruitment and the development of lung injury in response to hyperoxia by two different strategies: using either blocking anti-CAM MAbs or mutant mice deficient in these CAMs.

The role of the inflammatory cell in hyperoxic lung injury is controversial. Hyperoxia is usually associated with PMN infiltration, which occurs during the development of injury, but whether this response is adaptive or further aggravates the injury is unclear. For example, patients with neutropenia develop acute respiratory failure with signs and symptoms similar to those in immune-competent patients with acute respiratory failure (35). Previous studies that used neutrophil-depleted animals in hyperoxic lung injury have shown both protection and no effect (4, 42, 44), although these studies varied with regard to species, age of the animals, degree of neutropenia, and agent used to deplete neutrophils. Therefore, in this study, we interrogated the role of PMNs in our model of hyperoxic lung injury 1) by comparing changes in PMN trafficking with CAM blockade with changes in lung injury and 2) by depleting PMNs before oxygen exposure.

MATERIALS AND METHODS

Antibodies. The following antibodies were used: a rat anti-mouse CD62P (P-selectin) (clone RB40.34; BD Biosciences PharMingen, San Diego, CA) (29); a rat MAb (Mec 13.3) reacting with murine PECAM-1 (CD31), a generous gift of Dr. Vecchi (Istituto Clinico Humanitas, Milan, Italy) (48); a rat anti-mouse ICAM-1 (YN1) MAb (28); obtained from the American Type Culture Collection (Manassas, VA); and rat anti-mouse IgG2a control antibody (clone R35.95; BD Biosciences PharMingen).

Radiolabeling of antibodies. MAbs and control rat IgG were labeled, respectively, with 125Iodine or 131Iodine (NEN Dupont) using Iodogen (Pierce, Rockford, IL), as described previously (8) according to standard manufacturer’s instructions. The MAb and control IgG are designated here as follows: 125I-P-selectin, 125I-anti-ICAM-1, 125I-anti-PECAM-1, and 131I-IgG.

Animal studies. BALB/C mice (ages 8–12 wk old) were purchased from Charles River. PECAM-1 knockout mice were obtained from Dr. Tak Mak (20), knockout mice for P-selectin and ICAM-1 were obtained from Jackson Laboratories, and control mice [wild-type (WT)] were C57/B16-Jax mice from Jackson Laboratories. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Induction of hyperoxic lung injury. First, to establish the kinetics of ALI, BALB/C mice were exposed to O2 concentrations of >95% in a hyperoxic chamber for up to 96 h as described previously (8). Mice were killed after 0, 2, 4, 6, 8, 24, 48, 60, 72, and 96 h of hyperoxia. In the interventional part of the study, all animals treated with blocking antibody, as well as PMN-depleted mice and CAM knockout mice, were evaluated at 72 h of hyperoxic exposure, a time determined to be the period of sublethal yet measurable ALI.

In vivo CAM quantification with dual-radiolabeled MAb technique. The dose of each MAb injected per animal was first determined by Scatchard analysis: 5 μg/animal P-selectin MAb, 100 μg/animal ICAM-1 MAb, and 10 μg/animal PECAM-1 MAb. Radiolabeled MAb (1–2 μg) were combined with cold MAbs to saturate binding sites (total injected radioactivity of ~500,000 cpm/animal), as described previously (32).

Mice were simultaneously injected via the tail vein with binding anti-CAM monoclonal antibodies (131I-radiolabeled) and nonbinding control antibody (131I-radiolabeled, isotype-matched, nonimmune rat IgG). Simultaneous injection of a mixture of both anti-CAM and control IgG labeled with different isotopes permitted a direct comparison of both counterparts in the same animal, thus allowing an accurate estimation of the specificity of anti-CAM targeting, while reducing the number of animals needed for the studies. One hour after injection, lungs were harvested and radioactivity was measured in a gamma counter. The resulting net percent injected dose/gram of lung tissue was calculated by subtracting the control, nonbinding IgG counts from the counts from the binding MAb, thus correcting for nonspecific leakage of antibody from injury and edema.

CAM blockade and PMN depletion. For blockade studies on separate BALB/C mice, antibodies to endothelial CAMs (100 μg/injection) including P-selectin (BR33.1), ICAM-1 (YN1), or PECAM-1 (Mec 13.3) were injected via tail vein just before and 24 h after O2 exposure was started.

To further explore the role of PMNs in the development of lung injury, we depleted circulating PMNs by injecting an anti-granulocyte MAb (RB6–8C5, Pharmingen; 100 μg iv or 150 μg ip, respectively) (30) 72 h before and at the start of the 72 h exposure to >95% O2 (days −3 and 0). Depletion of circulating PMNs was confirmed by peripheral blood smears before and after 72 h of O2 exposure. Control mice in hyperoxia received a volume of intravenous saline similar to the PMN-depleted mice.

Evaluation of lung injury. Immediately after each mouse was killed, a subjective global lung injury score on a scale of 1 (no injury) to 10 (maximal hemorrhagic injury) was assigned by inspecting the lungs en bloc as previously described (9). The lungs of predetermined mice were then used for either bronchoalveolar lavage (BAL) to determine 1) BAL fluid protein concentrations and differential cell counts; 2) wet and dry lung weights, from which a wet-to-dry weight ratio could be calculated; or 3) histopathology. Lungs from different mice were used for each of these measurements of lung injury. BAL was performed by exposing and cannulating the trachea with a 20-gauge angiocatheter (Becton Dickinson, Sandy, UT) and then lavaging three times with 0.5 ml PBS containing a protease inhibitor cocktail (Sigma, St. Louis, MO) at 10 μl/ml. Recovery of infused fluid was >95%. The lavage fluid was spun at 2,000 rpm for 3–4 min; the supernatant was collected, aliquoted, and frozen at −70°C, after which the cell pellet was resuspended in serum-containing PBS. When further processing of the cells was delayed, an equal volume of cyto spin collection fluid (Shandon, Pittsburgh, PA) was added to the samples as a preservative. The cells were spun on a Shandon Cyto spin-3 cell preparation system (Thermo Electron, Waltham, MA) at 1,500 rpm for 10 min and stained with a standard Diff-Quick (Hemacolor) protocol from EM Diagnostic Systems (Gibbstown, NJ).
Protein concentrations were later measured in the thawed supernatant of the BAL fluid by use of a standard BCA protein assay kit (Pierce). Histopathological evaluation was performed on paraffin-embedded tissues. Before removal from the animal, the lungs were instilled with 0.75 ml of buffered formalin through a 20-gauge angiocatheter placed in the trachea. The lungs were then immersed in buffered formalin overnight and processed for conventional paraffin histology. The sections were stained with hematoxylin and eosin and examined by light microscopy.

**Determination of lung myeloperoxidase content.** A myeloperoxidase (MPO) ELISA was used as a measure of PMN accumulation in the lung (12). Briefly, mice were killed, and the pulmonary vasculature was perfused via the right ventricle with PBS (10 ml). The lungs were then quickly removed and placed in 2 ml of 0.1 M K2HPO4 buffer (pH 7.0) on ice. Lungs were then homogenized for 30 s on ice and sonicated twice for 30 s each time. The slurry was centrifuged for 10 min at 2,000 rpm, and the supernatant containing the active MPO enzyme was aliquoted into 100-μl samples and assayed via a mouse MPO ELISA kit according to the manufacturer’s directions (Cell Sciences, Canton, MA).

**Statistical methods.** Unpaired t-tests (radiolabeled antibody data) and ANOVA (performed by Prism 3.0, GraphPad Software, San Diego, CA) were used to determine whether a statistically significant difference existed between the mice groups for the therapeutic experiments. A two-way ANOVA with Bonferroni-Dunn test (experiment number and treatment) was used so that all five rescue experiments could be analyzed together while still accounting for potential differences in the susceptibility of various litters of mice to hyperoxic lung injury.

**RESULTS**

**Characterization of the time-dependent hyperoxic lung injury.** Lung injury and edema formation were grossly evident by 48–72 h of exposure to >95% O2, and mice usually expired shortly after 96 h under these conditions. Although normal lungs appeared pink to slightly red, hyperoxic lungs were increasingly congested and hemorrhagic. Microscopic examination of the lungs (Fig. 1) revealed patchy vascular congestion and mild edema along with modestly increased cellularity as early as 2 h of exposure to hyperoxia. These parameters increased in severity over the first 8 h of hyperoxia. The histological appearance of the lungs then remained relatively stable until 48–72 h, at which time generalized severe vascular congestion and edema were evident. Florid pulmonary edema, diffuse hemorrhage, and an acute inflammatory infiltrate were inevitably present by 96 h (not shown).

Edema formation, indicative of injury to the pulmonary endothelial side of the alveolar-capillary barrier, was measured as an increase in lung wet-to-dry weight ratios and was significantly increased (P < 0.01) by 60 h of hyperoxia (Fig. 2). A significant increase in BAL protein concentration (P < 0.00001) occurred by 72 h, indicating an increase not only in endothelial permeability but also alveolar epithelial injury.

**Neutrophil margination lags extravasation into the interstitium and alveolar space.** The presence of PMNs in the BAL fluid was compared with that of perfused lung tissue to determine the kinetics of PMN transition from the pulmonary vascular lumen to the alveolar compartment during prolonged hyperoxic exposure. This was done by comparing the PMN counts in BAL fluid over time and the MPO levels in non-lavaged, perfused lungs. PMN began to appear in the BAL fluid at 24–48 h into the hyperoxic challenge (Fig. 3, A and B; P < 0.03). However, MPO levels of perfused (blood-free) lungs were significantly elevated 1.7-fold (P < 0.04) within the first 8 h of hyperoxia (Fig. 3C), consistent with the early increase in cellularity within lung tissue sections detected on histology (Fig. 1); levels continued to increase throughout the 72-h exposure. This implies that a sequential recruitment of PMN occurs during hyperoxia and that early adherent or interstitial PMN do not transmigrate into the alveolar compartment until 24–48 h of hyperoxic challenge, coincident with the escalation of the lung injury (Fig. 2).

**Expression of endothelial CAMs (P-selectin, ICAM-1, PECAM-1) in hyperoxia using a dual-radiolabeled system (simultaneous intravenous injections of 125I-labeled antibody and 131I-labeled IgG).** Quantitative measurements of the expression of P-selectin, ICAM-1, and PECAM-1 on pulmonary endothelium were made by using a dual-radiolabeled antibody technique (Fig. 4). Results are given as percentage of injected dose per lung (in grams).

No changes in luminal expression of any of the investigated CAMs occurred at relatively early (24 h) time points, a time subsequent to initial PMN sequestration but before emigration into the air space (data not shown). Luminal expression of P-selectin was significantly increased by 48 h of hyperoxia from 0.932 ± 0.059 (n = 11) to 1.51 ± 0.011 (n = 8) (mean ± SE; P < 0.0001) and expression continued to increase at 72 h (to 3.55 ± 0.42, n = 13; P < 0.00001), whereas significant increases in ICAM-1 [from 2.75 ± 0.21 (n = 24) to 6.03 ± 0.56 (n = 13); P < 0.00001] and PECAM-1 [from 9.59 ± 0.46 (n = 7) to 11.2 ± 0.3 (n = 7); P < 0.05] occurred by 72 h. **Blocking endothelial CAMs with systemic anti-CAM MAbs.** To determine the functional role of CAMs in leukocyte [white blood cell (WBC)] recruitment and development of O2-induced
lung injury, we injected BALB/C mice with blocking MAbs directed against P-selectin (RB40.34), ICAM-1 (YN-1), and PECAM-1 (Mec 13.3) or control IgG \((n/H11005/14)\) before exposure to \(95\% \text{ O}_2\) for 72 h. BAL fluid was harvested to determine changes in cellular trafficking into the alveolar space using BAL WBC and PMN counts (Fig. 5) and lung injury using BAL protein accumulation (Fig. 6). Although luminal expression of P-selectin was significantly increased at 48 and 72 h of \(O_2\) exposure, administration of an anti-P-selectin antibody had no effect on PMN emigration into the alveolar space (Fig. 5) \((n/H11005/13)\) or on the development of lung injury (Fig. 6), compared with mice receiving IgG alone. In contrast, ICAM-1 blockade with an anti-ICAM antibody \((n/H11005/11)\) significantly decreased leukocyte influx by 50\% \((P < 0.03)\) and PMN influx by 87\% but failed to prevent lung injury.

CAM knockout mice showed no decrease of lung injury in hyperoxia compared with WT mice. To further evaluate the role of CAMs in \(O_2\)-induced changes in leukocyte emigration and

**Fig. 2.** Kinetics of development of injury in response to \(>95\% \text{ O}_2\). Kinetics of hyperoxic lung injury were evaluated in BALB/C mice from 0 to 96 h. \(A\): lung wet-to-dry ratio. \(B\): bronchoalveolar lavage (BAL) protein levels. Each time point represents pooled data from several experiments (minimum \(n = 6\) and maximum \(n = 27\) mice/time point). Values are means \(\pm\) SE. *\(P < 0.05\) from baseline.

Fig. 3. Kinetics of cellular trafficking in response to \(>95\% \text{ O}_2\). Kinetics of lung inflammation parameters from hyperoxia were evaluated in BALB/C mice from 0 to 96 h (A and B) and from 0 to 72 h (C). \(A\): BAL white blood cell (WBC) counts. \(B\): pulmonary neutrophils (PMN) in BAL fluid. \(C\): lung myeloperoxidase (MPO) (expressed as fold change from baseline). Each time point represents pooled data from several experiments (minimum \(n \geq 6\) and maximum \(n = 27\) mice/time point). Values means \(\pm\) SE. An *\(P < 0.05\) from baseline.
ICAM-1 genetic deficiency did not alter PMN extravasation. WBC influx was actually increased 3.09-fold ($P < 0.02$ vs. WT mice) in mice with P-selectin genetic deficiency, suggesting alternative compensatory pathways in these mutant mice. Similar to the findings from experiments using blocking antibodies, we found no amelioration in the signs of lung injury in these mutant mice in response to hyperoxia. Genetic deficiency of PECAM-1 blockade completely blocked hyperoxia-induced PMN transmigration into the alveolar space ($P < 0.03$) vs. WT mice (Fig. 7A); however, PECAM-1 deficiency and blocking of PMN alveolar traffic provided no protection against lung injury (Fig. 7B).

PMN depletion does not decrease hyperoxic lung injury and inflammation. To more specifically evaluate the role of PMNs in the development of the O$_2$-induced lung injury, circulating PMNs were depleted by administration of an anti-granulocyte antibody (RB6) 3 days before and at the institution of the O$_2$ exposure. Depletion was confirmed by peripheral blood smears before and after 72 h of O$_2$ exposure in BALB/C mice (Fig. 8A). Treatment with RB6 effectively prevented the accumulation of PMNs in the BAL fluid in response to hyperoxia, with PMN counts within the BAL approximating 0%, although overall cell counts were not different between RB6 and saline-treated mice (Fig. 8B). Macrophage counts were not different between the two groups of mice, suggesting a relative increase in mononuclear cell influx in the RB6-treated mice.

Wet-to-dry ratios, as a measure of edema formation, were not significantly different between saline-treated and RB6-treated BALB/C mice (6.7 + 0.1 and 6.3 + 0.1, respectively, mean ± SE) (Fig. 8C). In addition, a significant increase in BAL protein concentration ($P < 0.05$), indicative of injury to the alveolar-capillary barrier, occurred after 72 h of O$_2$ exposure in both saline- and RB6-treated mice with no difference in the degree of injury noted between the two groups (Fig. 8D). Similar results were found in separate experiments using C57BL6 mice (data not shown), suggesting that the dissociation between PMN recruitment and the development of the lung injury is not strain specific.

**DISCUSSION**

O$_2$-induced lung injury is characterized by alveolar and endothelial cell damage, leukocyte accumulation within the
pulmonary microvasculature, and subsequent extravasation and emigration of inflammatory cells, predominantly PMNs, into the air space, in conjunction with activation of alveolar macrophages and rapid escalation of the morphological signs of lung injury (3, 10). Increased retention of intrapulmonary neutrophils, suggesting a change in neutrophil-endothelial interaction, has been reported before emigration into the air space during O2 exposure (40). However, the mechanisms leading to the initial sequestration of neutrophils in the pulmonary microvasculature, subsequent extravasation in response to hyperoxia, and the pathophysiological role of these events remain unclear.

In this study, we found evidence for pulmonary sequestration of PMNs within a few hours of hyperoxia, both by histological analysis (Fig. 1) and MPO activity assay (Fig. 2). The first approach is not quantitative, whereas the second cannot distinguish between intravascular sequestration, extravasation into the interstitial compartment, and transmigration into the alveolar space. However, BAL analysis showed that inflammatory cell infiltration into the alveolar space occurred after 24–48 h (Fig. 2), temporally associated with the development of full-scale lung injury (Figs. 1 and 3). Reutershan et al. (39), using a flow-cytometry based technique to assess in vivo trafficking, recently showed a similar sequential recruitment of PMNs into the lung in response to intratracheal LPS challenge in mice.

Several authors have reported increased expression of vascular adhesion molecules, including P-selectin, ICAM-1, and PECAM-1, after 48–72 h of O2 exposure (38, 50, 53). However, the relative importance of these CAMs to sequential changes in cellular trafficking during prolonged O2 exposure is still unknown. It has been suggested that PMN influx during hyperoxic exposure occurs through a predominantly CD11/CD18-independent pathway (19, 26). Keeney et al. (26), using an MAb directed against the CD18 complex in adult guinea pigs, found no change in neutrophil influx or lung injury after 72 h of hyperoxia, suggesting a predominantly CD18-independent pathway for emigration. However, these authors did not investigate changes in leukocyte trafficking within the pulmonary vasculature. In contrast, Nishio et al. (34) found a significant increase in ICAM-1 expression in the pulmonary capillaries of rat lungs after 48 h of hyperoxic exposure. The frequency of leukocytes tethered firmly to the endothelium was increased in the hyperoxia group, and this difference was significantly reduced by administration of an ICAM-1 antibody. Welty et al. (50) found a significant increase in lung ICAM-1 expression in mice after 48 h of hyperoxic exposure, in association with increased neutrophil influx into the alveoli. They found that administration of anti-ICAM antibodies significantly attenuated, but did not prevent, lung injury and neutrophil influx in response to hyperoxia (49). Further work from this group (37, 51) and others (25) suggests that the type II alveolar epithelial cells, not the pulmonary endothelium, is the major site of increased ICAM-1 expression during O2 exposure.

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In this study, we quantitatively examined changes in the luminal expression of P-selectin, ICAM-1, and PECAM-1 within the pulmonary vasculature and found no evidence for upregulation of these adhesion molecules until 48–72 h of O2 exposure (Fig. 4). This result implies that either constitutively expressed ICAM-1 or PECAM-1 is sufficient for the initial PMN sequestration or that these play little role in the process. However, both constitutive and newly exposed CAMs seem to play a role in later emigration into the interstitial and alveolar space during hyperoxia. Interestingly, the first morphological signs of injury occur on the endothelial side of the alveolar-capillary barrier (10), although the primary toxic route of entry is inhalational during O2-induced lung injury. Studies utilizing both antibody-blocking and mutant mice showed that PECAM-1, located at the junctions between endothelial cells, is required for O2-induced alveolar transmigration of leukocytes (Figs. 5 and 7). The mechanism(s) for this dependence is not yet known. It could represent a defect in the actual transmigration process or a deficit in the ability of tissues to provide proper signals to initiate transmigration. For example, Albelda et al. (1) have demonstrated that the dependence on PECAM-1 for leukocyte transmigration in an alveolar immune complex model was due to a defect in macrophage Fc receptor signaling.

The increase in luminal CAM expression within the pulmonary vasculature that we found at later time points is especially interesting, given that previous studies by Crapo et al. (11) demonstrated a 50–60% decrease in endothelial cell surface area in rats exposed to 100% O2 for 60 h. This endothelial destruction would be expected to attenuate any measurable changes seen in CAM expression because of upregulation on the endothelial cell surface. For example, although previous work from our group (36) failed to show a change in bulk PECAM-1 protein within the lung in response to 48 h of hyperoxia, a small, but significant, increase in pulmonary luminal expression was found in the present study after 72 h of O2 exposure. It is plausible that elevated uptake of the radiolabeled anti-CAM in the lungs in hyperoxia-treated mice reflects increased expression of CAMs on the surface of the pulmonary endothelium. However, pulmonary sequestration of CAM-expressing leukocytes or platelets may also contribute to this effect. Thus PECAM-1 expressed on the surface of adherent leukocytes or platelets may contribute to the increase in luminal expression seen in these studies. Similarly, P-selectin expressed on infiltrating platelets and ICAM-1 expressed on infiltrating leukocytes could contribute to increased pulmonary uptake of these anti-CAMs.

Despite blocking of PMN alveolar transmigration by anti-ICAM in WT mice, ICAM-1 was not apparently required for this process in our ICAM-1 mutant mice. Doerschuk et al. (18) found a similar disparity in the function of adhesion molecules during cellular trafficking when using blocking antibodies compared with mutant mice in a cobra venom factor model of ALI. One possible explanation for this discrepancy in the present experiments is the particular strain of mouse that was used and the targeted mutation that these mice express. After completion of the experiments, it was discovered that, although these mice have a single exon deletion (mutated exon 5), alternative splicing of the RNA can cause this mutated exon to be skipped, resulting in residual amounts of three alternative
ICAM isoforms that may still be expressed in the thymus and lung (43) and may compensate for the missing ICAM molecules. Another possible explanation, as postulated by Doerschuk et al., is that mutant mice deficient in specific adhesion molecules may utilize alternative compensatory pathways, possibly by upregulating the expression of other CAMs and thereby the activity of other pathways that are normally present. This possibility was not specifically examined in these studies, although studies done with fucoidin, which blocks both P- and L-selectin, failed to prevent neutrophil influx in similar experiments (data not shown). Interestingly, P-selectin mutant mice demonstrated an increase in leukocyte infiltration relative to WT mice. The reason for this enhanced migration is unknown but may be due to changes in platelet function, which also express P-selectin. If platelets or activation of the coagulation cascade play a role in modulating endothelial injury or leukocyte recruitment, decreased platelet accumulation within the pulmonary microvasculature could alter leukocyte kinetics. Alternatively, these mice may upregulate other compensatory pathways as discussed above. Mice treated with anti-P-selectin antibody did not have a similar increase in leukocyte recruitment, although this may be because of insufficient antibody delivery to sites of platelet sequestration.

Although blockade of both ICAM-1 and PECAM-1 attenuated PMN recruitment into hyperoxic lung tissues, this had no protective effect on lung injury. Similarly, PMN depletion failed to prevent the development of injury. These results emphasize the controversial role of the inflammatory cell in hyperoxic injury. Leukocyte infiltration usually occurs during the course of injury, but the extent to which this inflammation is adaptive or contributes to further injury is unclear. During exposure to 100% oxygen, pulmonary capillary endothelial damage begins shortly after the onset of the inflammatory stage. The time course of the neutrophil infiltration into the airway during O2 exposure has suggested that PMNs are required for the development of severe lung injury in the response to O2, although our data support the idea that the lung injury itself could result in production or release of factors that attract neutrophils to the area and they do not cause the lung injury. The present experiments were ended after 72 h of O2 exposure because of relatively low rates of survival after 96 h. Therefore, it is not known how attenuation of neutrophil recruitment may affect indexes of lung injury or survival at these later time points.

It has been suggested that the ability to recruit PMNs to the circulation during periods of inflammation may be important in limiting the severity of ALI and promoting recovery of normal pulmonary vascular function (6). In addition, there may be large differences in the role played by PMNs sequestered within the pulmonary vasculature vs. those extravasating into the interstitium and those transmigrating into the alveolar space. Nemzek et al. (33), using a “2 hit” model of acid aspiration, were able to decrease PMN influx into the alveolar space with antibodies directed against the chemokine KC, although this did not have an effect on increases in MPO, a measure of PMN accumulation within the lung. Decreasing PMN emigration did not affect lung injury, as measured by albumin concentrations in BAL. It has been suggested that emigrating PMNs may actually be important for the removal of alveolar debris, including dying cells (24).

In conclusion, we found that early PMN retention in the hyperoxic lung is independent of CAM overexpression, although constitutively expressed ICAM-1 and PECAM-1 could still be involved. Subsequent PMN extravasation into the alveoli is supported by ICAM-1 and PECAM-1, possibly from their upregulation on the vascular luminal surface. Attenuation of PMN emigration into the alveolar space did not attenuate O2-induced lung injury, suggesting that neutrophil accumulation may not be directly contributing to the lung injury and may play an adaptive function in response to the injury. The relative role of other inflammatory cell types, including platelets and macrophages, in hyperoxia remains a subject of further investigation.

REFERENCES


