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The Role of Leukocyte Emigration and IL-8 on the Development of Lipopolysaccharide-Induced Lung Injury in Rabbits[1]

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Leukocyte emigration and alveolar macrophage-derived cytokines may contribute to lung microvascular injury associated with adult respiratory distress syndrome. We have used mAbs against cell adhesion molecules on leukocytes (anti-CD18 and anti-CD49d) or against IL-8 to investigate these contributions. Intratracheal (i.t.) instillation of LPS (50 μg/kg) caused a significant increase in bronchoalveolar lavage polymorphonuclear leukocytes (PMNs) without an increase in mononuclear cells (MNCs) or an increase in lung permeability. Injection of LPS (10 μ/kg) i.v. at 24 h after i.t. LPS caused significant increases in bronchoalveolar lavage PMNs, MNCs, IL-8, and monocyte chemotactic protein-1, as well as increases in lung permeability. Rabbits that were administered i.t. LPS followed by i.v. LPS and treated with anti-CD18 mAb had a significantly lower lung permeability index and emigration of fewer PMNs but no change in MNC emigration compared with saline treatment. Anti-IL-8 mAb treatment resulted in a significantly lower lung permeability index with no change in PMN emigration compared with no treatment. These results suggest that PMN emigration is necessary but not sufficient for the development of LPS-induced lung injury, and that IL-8 plays a significant role in PMN-dependent lung injury, independent of PMN emigration. *The Journal of Immunology*, **1998, 161: 5704–5709.**

Acute inflammatory disorders of the lung, including acute bacterial pneumonia and adult respiratory distress syndrome (ARDS)[3], are characterized by leukocyte accumulation and increased microvascular permeability. ARDS is of particular interest because of the severity of the associated lung injury and its high mortality rate (50–60%). Multiple events are implicated as risk factors for the development of ARDS, and two or more of these risk factors result in a synergistic interaction (1, 2). Endotracheal intubation is a frequent occurrence in severely ill patients who may develop ARDS, and can lead to bacteria and/or LPS being introduced into the lung. This initial introduction of bacteria can "prime" the lung for a second insult, such as bacteremia or endotoxemia. In this paper, we modeled this process by intratracheal (i.t.) instillation of LPS followed 24 h later by an i.v. injection of LPS.

Polymorphonuclear leukocytes (PMNs) have long been suspected of causing the acute lung injury associated with ARDS (3). In addition, it has been proposed that cytokines produced within the lung play an important role in the pathogenesis of ARDS either by directly injuring the lung, activating leukocytes, increasing the expression of leukocyte-endothelial cell adhesion molecules, or acting as chemoattractants to PMNs (4–6). The transendothelial migration of PMNs occurs in response to released cytokines that provide a chemotactic gradient signal for PMN movement from the vascular space to the site of inflammation. The cytokine IL-8 has been proposed as the major chemoattractant for PMNs (7, 8) and is released by activated macrophages and endothelial cells (9). This cytokine also can stimulate PMN activation, resulting in elastase release (10), oxidant production (10, 11), increased integrin expression (12), and activation of CD18 (12). Alveolar macrophages are a source of other cytokines in the alveolar space in response to instillation of LPS through the interaction of LPS-LPS binding protein and CD14 on macrophages (13). Activation of alveolar macrophages results in the release of TNF as well as other cytokines that can cause the induction of cell adhesion molecules on endothelial cells (13, 14). LPS alone is also capable of causing increased expression of some of these same adhesion molecules on endothelial cells (reviewed in Ref. 15).

The transendothelial migration of PMNs requires their adherence to the endothelium before they can complete the emigration process along the chemotactic gradient. The integrins CD49d/CD29 (mononuclear cells (MNCs)) and CD11/CD18 (MNCs and PMNs) are thought to provide the major adherence mechanism for the firm adhesion of leukocytes to the vascular endothelium (reviewed in Ref. 16). However, we have shown previously that a CD18-independent pathway for PMN emigration exists in the lung (17) and peritoneum (18) following some stimuli. Likewise, neutrophil migration to delayed-type hypersensitivity reaction in joints was CD18-independent (19). In the peritoneum, mAb blocking both CD18 and CD49d resulted in a significant reduction in PMN emigration (18). The resting PMN does not express CD49d; however, PMNs that were treated with dihydrocytochalasin B or allowed to emigrate across an endothelial barrier were shown to express CD49d and adhere to protein-coated plastic under static conditions and to endothelial cells following stimulation under flow conditions via CD49d, suggesting a role for this molecule in

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[3] Abbreviations used in this paper: ARDS, adult respiratory distress syndrome; i.t., intratracheal; MNC, mononuclear cell; MCP-1, monocyte chemotactic protein-1; BAL, bronchoalveolar lavage; PMN, polymorphonuclear leukocyte; HSA, human serum albumin.

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PMN emigration in vivo (20, 21). The CD18-independent pathway of emigration is of interest, because we have shown in septic rabbits that lung permeability changes are not blocked by the administration of CD18 mAbs alone (22).

In the present study, we tested the hypothesis that increased lung permeability resulting from two challenges with LPS (one i.t. and the other i.v.) could be prevented by treatment with mAbs directed to either CD18 plus CD49d or by blocking the effect of IL-8. We proposed that the permeability increase due to i.t. plus i.v. LPS would result in CD18-independent increased lung permeability, and that treatment with CD18 and CD49d mAbs would ameliorate the increase. In addition, we predicted that anti-IL-8 mAbs would block PMN emigration into the lung by blocking the chemotactic ability of IL-8, and that blocking emigration would likewise prevent increased permeability. We found that our initial hypotheses were not true; the increased permeability was primarily CD18-dependent, and anti-IL-8 mAb had little effect on PMN emigration but was very effective in preventing the increase in lung permeability.

Materials and Methods

Animals and general experimental protocol

Experiments were performed using New Zealand white rabbits weighing 2.0–2.5 kg. The rabbits were anesthetized with i.v. ketamine (10 mg/kg) and xylazine (1 mg/kg) via a catheter in a marginal ear vein. Blood samples were drawn to determine total and differential leukocyte counts at the beginning of the experiment. All experimental protocols and euthanasia procedures were approved by the Institutional Animal Care and Use Committee of the University of Washington and performed under the National Institutes of Health guidelines for the care and use of experimental animals.

An uncuffed endotracheal tube (2.5 mm inner diameter and 3.6 mm outer diameter; Sheridan Catheter Corporation, Argyle, NY) was placed into the right mainstem bronchus (15–16 cm in depth) of all animals; next, rabbits were ventilated with 95% O_2 and 5% CO_2. A balloon-tipped catheter was advanced through the endotracheal tube into the right lower lobe (18 cm in depth), and the endotracheal tube was pulled back 2 cm. The balloon was inflated with 0.2 ml of air, and 1 ml of saline containing 50 μg/kg of *Escherichia coli* LPS was instilled into the right lower lobe. Artificial ventilation was continued for 10 min, the balloon catheter was withdrawn, and ventilation was continued for an additional 10 min. The endotracheal tube was removed, and rabbits were allowed to recover from anesthesia and then returned to their cages with free access to food and water.

At 24 h after i.t. instillation of LPS, the lung permeability index (see below) and leukocyte counts in bronchoalveolar lavage (BAL) fluid were determined in two group of animals (one with no treatment and one treated with IL-8 mAb at the time of i.t. LPS instillation). In all other study groups (see below), animals were given an i.v. injection of LPS (10 μg/kg) at 24 h after i.t. LPS and followed for an additional 6 h before lung permeability index and leukocyte counts in BAL were determined. Blood samples were withdrawn from all animals before they were killed with an overdose of ketamine.

At 1 h before death, 50 mg/kg of human serum albumin (HSA) was injected i.v. for the determination of the lung permeability index. This index was defined as the ratio of HSA in the BAL fluid to that in plasma. This technique will result in a measurement of the permeability index in the lung at one point in time during the experiment and may not measure the peak value. Lung permeability indices for the right and left lower lobes were determined separately in all rabbits. Confirmation of right lower lobe instillation of LPS was made by visual inspection, with findings of local hemorrhage, atelectasis, and consolidation. Postmortem BAL was performed with 10 ml of saline containing 10 U/ml of heparin after placing a catheter in either the right or left lower lobe. The saline was lavaged in and out four times, and the HSA concentration in the lavage fluid was determined by ELISA. The plasma HSA concentration was determined from the blood drawn just before death.

Specific experimental protocol

There were seven experimental groups. One group did not receive LPS and served as a normal control group (normal). Two groups received i.t. LPS and 24 h of observation, with one of these groups receiving anti-IL-8 mAb (i.t. LPS plus anti-IL-8) and the other saline (i.t. LPS). These animals allowed for the determination of changes in permeability and PMN emigration in a subsequent 6-h period following an i.v. dose of LPS (see the following). The remaining four groups all received i.v. LPS at 24 h after i.t. LPS (i.t. plus i.v. LPS). The four groups that received both i.t. and i.v. LPS were treated with saline (control group), the CD18 mAb 60.3 (60.3 group), both mAb 60.3 and the CD49d mAb HP1/2 (60.3 plus HP1/2 group), or the IL-8 mAb ARIL8.2 (IL-8 mAb group). Treatments with Abs were administered i.v. in a bolus dose of 1 mg/kg at the time of i.t. instillation of LPS and again just before i.v. LPS.

Increased expression of the CD18 adhesion complex occurs as a result of the fusion of the cytoplasmic granules with the cellular membrane as degranulation occurs (23). To determine whether the IL-8 mAb could block activation, we evaluated CD18 expression following in vitro stimulation of PMNs with rabbit IL-8 in the presence or absence of the IL-8 mAb. In these experiments, blood was drawn from a marginal ear vein of rabbits into heparinized syringes. Rabbit IL-8 (30 nM final concentration) was added to the blood and incubated at 37°C, following which a fluorescent-labeled CD18 mAb, MHM23, was added (250 μg/ml final concentration); subsequently, RBCs were lysed, and white blood cells were fixed in a one-step procedure. CD18 expression was determined by flow cytometry.

ELISA for HSA determination

Standard 96-well plates were incubated for 24 h at 4°C with unlabeled anti-HSA Ab (Dako, Carpinteria, CA) (0.1 μg/well in 0.025 M sodium carbonate buffer, pH 9.6). Nonspecific binding was blocked with 200 μl of specimen diluent (Genetic Systems, Redmond, WA) for 1 h and then washed with saline containing 0.1% Tween 20 (NaCl/Tween) buffer. Test samples were diluted with specimen diluent to bring sample concentrations into equivalent range of the standard curve. Next, 100 μl/well of diluted plasma, BAL fluid, or standard solution of HSA was added, incubated for 2 h, and then washed with NaCl/Tween. A total of 100 μl/well of anti-HSA-peroxidase conjugate (diluted 1–2000) was added, incubated for 1 h, and then washed with NaCl/Tween. Chromogen (Genetic Systems) was added, incubated for 90 s at room temperature, and subsequently blocked with 100 μl of 1N H_2SO_4. Plates were analyzed within 1 h with a microplate spectrophotometer at 450/650 nm.

ELISA for rabbit IL-8 and monocyte chemotactic protein-1 (MCP-1) determination

BAL concentrations of IL-8 and MCP-1 were determined by ELISA as described previously (24). Briefly, goat anti-rabbit IL-8 or MCP-1 Abs were diluted in 0.1 M bicarbonate buffer (pH 9.6), adsorbed to 96-well plates, incubated at 4°C, and then washed with PBS. Nonspecific binding to IL-8 or MCP-1 Abs was blocked by placing 5% nonfat milk in Dulbecco's PBS in each well and incubating at 37°C for 1 h. Samples were diluted as necessary, and 100 μl was added to each well; next, the plates were incubated at 37°C for 2 h. Plates were washed with PBS containing 0.05% Tween 20, and a biotinylated goat anti-rabbit IL-8 or MCP-1 Ab was added to the well and incubated at 37°C for 2 h. The plates were then incubated with streptavidin-biotin-peroxidase complex at 37°C for 1 h. Finally, the washed plates were incubated with the peroxidase substrate 3,3,5,5′-tetramethylbenzidine for 1 h at 37°C, the reaction was stopped with 1.0 M phosphoric acid, and OD was read by spectrophotometer.

Monoclonal Abs

Anti-human mAbs that recognize functional epitopes on rabbit CD18 and CD49d were used in these experiments. The mAb HP1/2 recognizes CD49d (very late activation Ag-4) on rabbit monocytes (18) and was a gift of Dr. R. Lobb (Biogen, Cambridge, MA). The mAb 60.3 was a gift of Dr. P. Beatty (University of Utah, Salt Lake City, Utah) and recognizes rabbit CD18 (25, 26). The anti-rabbit mAb ARIL8.2 was a gift of Dr. C. Hebert (Genentech, San Francisco, CA). The anti-IL-8 mAb inhibits the chemotactic activity of rabbit IL-8 in vitro (27) and attenuates PMN emigration and PMN-dependent lung injury in vivo in rabbits (27, 28).

Statistical analysis

Data are expressed as means ± SEM. Multiple comparisons were performed by one-way ANOVA with Dunnett's (post hoc) test for the determination of significant differences between groups. The statistical significance of the correlation between MNCs and PMNs in alveolar lavage fluid and the cytokines IL-8 and MCP-1 was tested using the z-test for correlation coefficients. Differences in CD18 expression following IL-8 activation in the presence or absence of anti-IL-8 mAb were determined by more robust nonparametric tests. We initially used the Kruskal-Wallis to test for differences among multiple groups and then determined differences between two groups using the Mann-Whitney *U* test. All statistical analyses

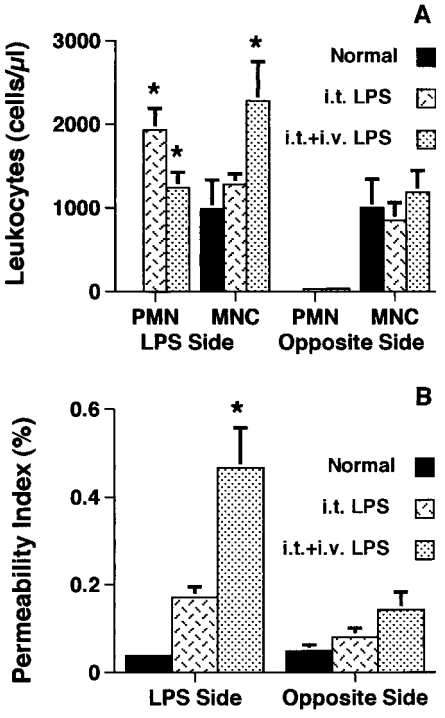

FIGURE 1. *A*, PMN and MNC counts were determined in BAL fluid from either the right lower lobe (LPS-instilled side) or the left lower lobe (opposite side) in normal ($n = 5$), i.t. LPS ($n = 10$), or i.t. plus i.v. LPS ($n = 7$) groups. Data are means ± SEM; *, $p < 0.05$ compared with the same side of the normal group. *B*, Lung permeability index measured as the accumulation of HSA in BAL fluid. HSA (50 mg/kg) was injected i.v. 1 h before lung lavage. Data are means ± SEM; *, $p < 0.05$ compared with the same side of the normal group.

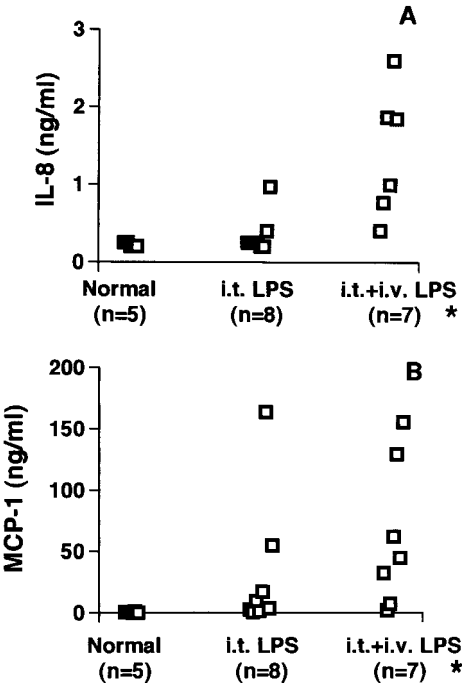

FIGURE 2. *A*, IL-8 concentration in BAL fluid from the LPS-instilled side. The number of animals for each group is indicated in parentheses. *B*, MCP-1 concentration in BAL fluid from the LPS-instilled side. The number of animals for each group is indicated in parentheses.

were completed using the computer program StatView (Abacus Concepts, Berkeley, CA).

Results

LPS-induced leukocyte emigration and increased lung permeability (Fig. 1)

There was a significant increase in BAL PMN counts but not MNC counts in the LPS-instilled lobe compared with either the uninoculated lobe or BAL from normal rabbits at 24 h after i.t. LPS (Fig. 1*A*). The addition of i.v. LPS at 24 h resulted in BAL PMN counts in the inoculated lobe that were somewhat less than the i.t. LPS group, whereas MNC counts increased to almost twice those in either the normal lungs or the i.t. LPS only group. However, the addition of i.v. LPS did not affect either PMN or MNC counts in BAL fluid from the uninoculated lobe.

The permeability index for normal rabbit lungs was 0.038 ± 0.006% in the right lung and 0.049 ± 0.014% in the left lung (Fig. 1*B*). At 24 h after i.t. LPS, only the lung permeability index on the LPS-instilled lobe was 0.171 ± 0.024%; the difference compared with normal rabbit lungs was not significant. The addition of i.v. LPS further increased the permeability index to levels that were 12 times greater than normal lungs in the LPS-inoculated lobe (0.466 ± 0.091%). This increase was ~2.7 times greater than the increase in the i.t. LPS only group for the LPS-inoculated lobe. There were no significant changes in the permeability index of the uninoculated lobe (0.081 ± 0.20% i.t. LPS, 0.142 ± 0.041% i.t. plus i.v. LPS).

*LPS-induced expression of IL-8 (Fig. 2*A*) and MCP-1 (Fig. 2*B*)*

No IL-8 was detected in normal rabbits, and MCP-1 was detected in only one of five normal animals. IL-8 was detected in the BAL from the inoculated side of two of eight rabbits treated only with i.t. LPS. MCP-1 was detected in all rabbits treated with i.t. LPS (31.4 ± 19.9 ng/ml). IL-8 and MCP-1 were detected in all animals in the i.t. plus i.v. LPS group. These values averaged 1.41 ± 0.34 ng/ml for IL-8 and 66.5 ± 21.0 ng/ml for MCP-1. The IL-8 and MCP-1 concentrations were significantly higher in the i.t. plus i.v. LPS group compared with normal rabbits ($p < 0.05$). The MCP-1 concentration in the anti-IL-8 group that was given both i.t. plus i.v. LPS was measured in only three animals and averaged 17.0 ± 7.2 ng/ml (data not shown). The IL-8 concentration in these same three animals averaged 1.75 ± 0.99 ng/ml (data not shown).

LPS-induced leukocyte emigration and BAL cytokine concentrations (Fig. 3)

The correlation between BAL PMNs and IL-8 was not significant for the i.t. LPS group, whereas this correlation was significant for the i.v. plus i.t. group. However, in this case, the correlation was unexpectedly negative (Fig. 3*A*). The correlation between BAL MNCs and MCP-1 was not significant for the i.t. group but was positive and significant for the i.t. plus i.v. LPS group (Fig. 3*B*).

Effect of rabbit IL-8 on rabbit PMNs

The addition of rabbit IL-8 to rabbit blood resulted in a significant increase in CD18 expression on rabbit PMNs ($p < 0.05$). The average of the mean fluorescence of normal PMNs was 3.97 ± 0.32 and increased to 9.19 ± 1.86 with the addition of rabbit IL-8. The average mean fluorescence following rabbit IL-8 in the presence of anti-IL-8 mAb was 4.82 ± 0.65; this fluorescence was significantly different from rabbit IL-8 alone but was not significantly different from normal PMNs. These results show that rabbit IL-8 activates rabbit PMNs, and that the anti-IL-8 mAb used in these experiments can block this activation.

Effects of antiadhesion and anti-IL-8 therapy

The mechanism of phagocyte emigration into the alveolar space of rabbits receiving i.t. plus i.v. LPS was investigated by treatment

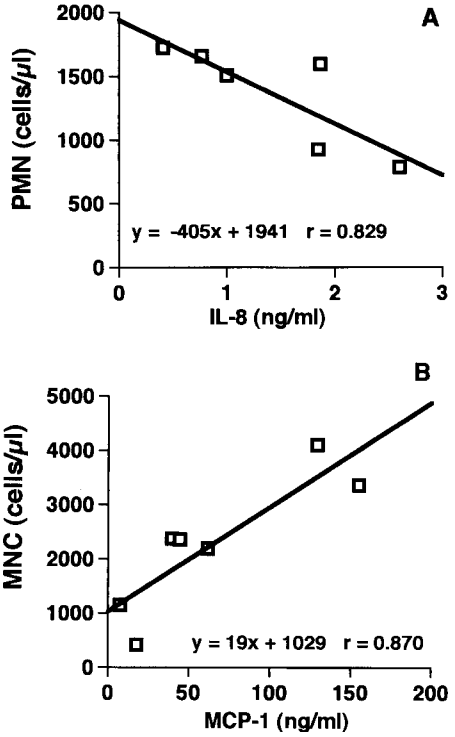

FIGURE 3. *A*, Correlation between IL-8 concentration and emigrating PMN counts in BAL fluid; *B*, Correlation between MCP-1 concentration and emigrating MNC counts in BAL fluid from the LPS-instilled side using the data from the i.t. plus i.v. LPS group.

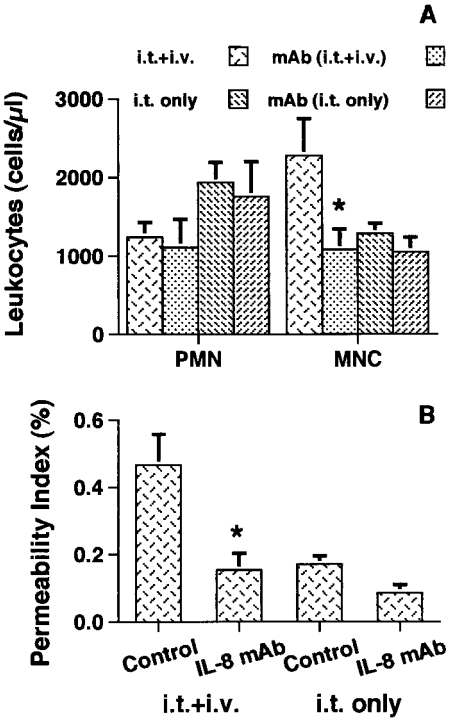

FIGURE 5. *A*, Effects of anti IL-8 mAb on leukocyte composition in BAL from the LPS-instilled side. *B*, Effects of anti-IL-8 mAb on the lung permeability index. Data are expressed as means ± SEM of six to seven animals; ∗, $p < 0.05$.

with anti-CD18 mAb 60.3 or a combination of anti-CD49d mAb HP1/2 and mAb 60.3. The mAb was administered before i.t. instillation and again before i.v. LPS. Fig. 4, *A* and *B* demonstrate

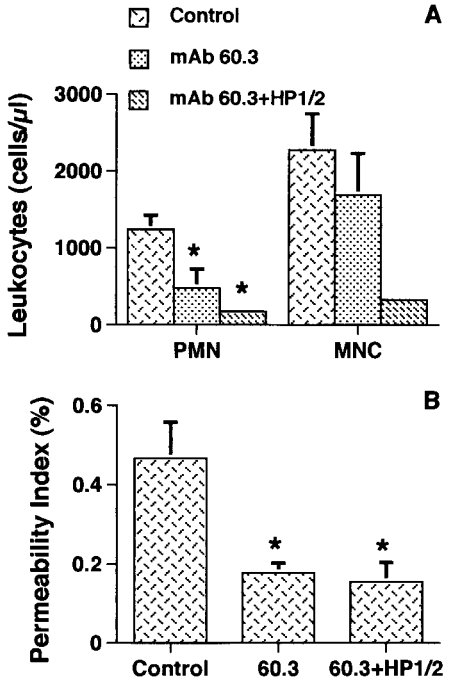

FIGURE 4. *A*, Effects of anti-CD18 mAb and anti-CD49d mAb on leukocyte composition in BAL from the LPS-instilled side. *B*, Effects of anti-CD18 mAb and anti-CD49d mAb on the LPS-induced lung permeability index. Data are expressed as means ± SEM with five to seven animals per treatment group; ∗, $p < 0.05$ compared with control.

that treatment with mAb 60.3 ameliorated LPS-induced PMN emigration by 61% ($p < 0.05$) but had little effect on the emigration of MNCs (NS). Combination therapy with mAb 60.3 and mAb HP1/2 significantly ameliorated the influx of both PMNs and MNCs in BAL fluid compared with untreated animals. The PMNs were reduced by ~86%, and MNCs were reduced to levels below those found in normal lungs. The LPS-induced lung permeability index was ~62% of control with mAb 60.3 treatment (Fig. 4*B*). The lung permeability index was ~68% of control in the combination therapy. These results are consistent with the hypothesis that PMNs are at least partly responsible for increased lung permeability following i.t. plus i.v. LPS. Since the permeability was not different between mAb 60.3 treatment and mAb 60.3 plus mAb HP1/2 treatment, even with the reduction in monocyte emigration, we conclude that monocytes did not contribute to permeability changes.

Treatment with the anti-IL-8 mAb had no effect on the number of PMNs in the BAL in either the group receiving i.t. LPS alone or the group receiving i.t. plus i.v. LPS (Fig. 5*A*). In the rabbits given i.t. LPS alone, the PMN counts in the BAL were 1934 ± 254 cells/μl at 24 h; in the group given i.t. LPS and anti-IL-8 mAb, these counts were 1755 ± 443 PMN/μl. These two groups were not statistically different. There were 1241 ± 188 PMN/μl in the i.t. plus i.v. LPS control group and 1109 ± 358 PMN/μl in the i.t. plus i.v. LPS anti-IL-8 mAb group. However, there was a surprising reduction in the number of MNCs in the anti-IL-8 mAb group (1079 ± 263 MNC/μl) compared with controls (2280 ± 470 MNC/μl). Anti-IL-8 mAb treatment resulted in a lung permeability index that was ~67% of control (Fig. 5*B*). Thus, a significant reduction in the permeability index with anti-IL-8 mAb treatment occurred without a decrease in the number of PMNs in the BAL.

Discussion

In the current experiments, i.t. instillation of LPS with or without subsequent i.v. injection of LPS resulted in significantly increased leukocyte emigration into the alveolar space. The change in lung permeability in the i.t. plus i.v. LPS group was accompanied by an increase in PMNs in the BAL, compared with the normal group. This observation, coupled with mAb 60.3, which blocks both the PMN emigration and the increased permeability index, is consistent with the hypothesis that PMNs are responsible for a portion of the increased permeability. The protection afforded by anti-IL-8 mAb in the i.t. plus i.v. LPS group despite PMN emigration being approximately equal to the group receiving no treatment (i.e., the i.t. plus i.v. LPS group), suggests that PMNs alone are not sufficient for increased permeability.

Isotype-matched control Abs were not used in these experiments, and there is the possibility that some of the results were due to nonspecific protective effects of the Abs. However, mAb 60.3 (anti-CD18) and mAb ARIL8.2 (anti-IL-8) are the same isotype (IgG2a), and their use resulted in significantly different effects on PMN emigration into the lung, suggesting that the effect of each mAb was due to specific interactions. Also, we have used mAb 60.3 in experiments in which lung injury was induced by devascularization of the appendix and found the mAb to be without effect in preventing an increased permeability index (22). Thus, we believe that the action of each mAb is due to specific effects of the Ab (i.e., mAb 60.3 blocking CD18 function, mAb HP1/2 blocking CD49d function, and mAb ARIL8.2 blocking IL-8 function).

We have shown previously that the combination of mAb 60.3 and mAb HP1/2 was more effective in preventing both MNC and PMN emigration into the peritoneum than was mAb 60.3 alone (18). Therefore, we examined the effect of mAb 60.3 plus mAb HP1/2 administration on PMN and MNC emigration as well as on lung permeability. The combined treatment with mAb HP1/2 and mAb 60.3 resulted in fewer emigrating MNCs compared with treatment with mAb 60.3 alone; however, there was no statistical difference in the lung permeability index or in PMN emigration between the two groups. These findings suggest that the CD11/CD18 complex is necessary for PMN emigration, and that blocking both the CD49d/CD29 complex and the CD11/CD18 complex prevents MNC emigration. CD11/CD18 blockade alone was as effective in preventing LPS-induced lung injury as was combination therapy. Thus, blocking PMN emigration is associated with the decreased lung injury following the combination of i.v. and i.t. LPS, whereas blocking MNC emigration did not provide additional protection. We conclude from these results that the recruitment of additional MNCs in the mAb 60.3 only treated group compared with the mAb 60.3 plus HP1/2 group does not result in additional injury.

As an alternative approach to ameliorate LPS-induced lung injury, we used anti-IL-8 mAb in an effort to prevent IL-8-induced PMN emigration and/or activation. IL-8 has been proposed to be a major chemotactic factor for the recruitment of PMNs into the lungs (8). Anti-IL-8 mAbs have been used to investigate the role of IL-8 in acute lung injury resulting from acid aspiration or from ischemia-reperfusion of pulmonary circulation (28, 29). In both of these cases, there were increased concentrations of IL-8 in BAL fluid, and anti-IL-8 mAb treatment inhibited both PMN emigration and lung injury (28, 29). In the current study, there was an increase in the BAL IL-8 concentration following i.t. plus i.v. LPS; however, the correlation between the concentration of IL-8 in BAL fluid and the number of emigrating PMNs was negative. Therefore, increased IL-8 concentration does not predict increased PMN emigration. Furthermore, anti-IL-8 mAb failed to inhibit PMN emigration, although it was effective in reducing lung injury. These findings suggest that IL-8 is not the major chemotactic factor for PMNs in this type of LPS-induced lung injury, but rather may play a more important role in regulating PMN activation.

IL-8 is known to act as a chemotactic factor as well as an activating factor for PMN functions (8). PMNs stimulated with IL-8 will cause elastase release from cytochalasin B-pretreated cells (11), a respiratory burst (30) and phagocytosis of IgG-coated E (12), and increased CD18 expression (12). Thus, in the present study, PMNs migrating by an IL-8-independent mechanism may still be activated by IL-8 to produce proteases and/or toxic oxygen products that injure the surrounding tissues. Our in vitro results examining the ability of the anti-IL-8 mAb to block an increased expression of CD18 are consistent with the ability of this mAb to block activation. Further investigations are necessary to define the precise role of IL-8 in the progression of LPS-induced lung injury and were beyond the scope of this work.

The mechanisms of anti-IL-8 mAb reduction of emigrating MNCs remain unclear. This observation may be a secondary effect of preventing lung injury by anti-IL-8 mAb. Alternatively, MCP-1, which is both a chemotactic and activating factor for monocytes (31–34), may also contribute to MNC emigration in our experiments, since there was a significant correlation between MCP-1 concentration and emigrating MNC counts. A recent report indicates that activated PMNs produce MCP-1 (35); thus, the action of the anti-IL-8 mAb may be through its ability to prevent IL-8 activation of PMNs, thereby resulting in an inhibition of MCP-1 production by PMNs. The MCP-1 concentration in the anti-IL-8 group was less than in the i.t. plus i.v. LPS group, which is consistent with the above hypothesis. However, care must be taken, because measurements of MCP-1 in the anti-IL-8 group were limited.

In summary, the combination of i.t. plus i.v. LPS caused an increased lung permeability in rabbits that was associated with PMN recruitment and activation. The inhibition of PMN recruitment by antiadhesion therapy or the prevention of PMN activation by anti-IL-8 therapy are strategies that may ameliorate LPS-induced lung injury by independent mechanisms. The results reported here show that PMN emigration into the lungs is not sufficient to cause lung injury, because anti-IL-8 mAb did not block emigration but did prevent lung injury.

References

1. Pepe, P. E., R. T. Potkin, D. Holtman, L. D. Hudson, and C. J. Carrico. 1982. Clinical predictors of the adult respiratory distress syndrome. *Am. J. Surg. 144: 124.*
2. Hudson, L. D., J. A. Milberg, D. Anardi, and R. J. Maunder. 1995. Clinical risks for development of the acute respiratory distress syndrome. *Am. J. Respir. Crit. Care Med. 151:293.*
3. Wortel, C. H., and C. M. Doerschuk. 1993. Neutrophils and neutrophil-endothelial cell adhesion in adult respiratory distress syndrome. *New Horiz. 1:631.*
4. Shenkar, R., W. F. Coulson, and E. Abraham. 1994. Hemorrhage and resuscitation induce alterations in cytokine expression and the development of acute lung injury. *Am. J. Respir. Cell Mol. Biol. 10:290.*
5. Suter, P. M., S. Suter, E. Girardin, P. Roux-Lombard, G. E. Grau, and J. M. Dayer. 1992. High bronchoalveolar levels of tumor necrosis factor and its inhibitors, interleukin-1, interferon, and elastase, in patients with adult respiratory distress syndrome after trauma, shock, or sepsis. *Am. Rev. Respir. Dis. 145:1016.*
6. Goodman, R. B., R. M. Strieter, D. P. Martin, K. P. Steinberg, J. A. Milberg, R. J. Maunder, S. L. Kunkel, A. Walz, L. D. Hudson, and T. R. Martin. 1996. Inflammatory cytokines in patients with persistence of the acute respiratory distress syndrome. *Am. J. Respir. Crit. Care Med. :602.*
7. Standiford, T. J., S. L. Kunkel, K. Kasahara, M. J. Milia, M. W. Rolfe, and R. M. Streiter. 1991. Interleukin-8 gene expression from human alveolar macrophages: the role of adherence. *Am. J. Respir. Cell Mol. Biol. 5:579.*
8. Baggiolini, M., B. Dewald, and B. Moser. 1994. Interleukin-8 and related chemotactic cytokines–CXC and CC chemokines. *Adv. Immunol. 55:97.*
9. Harada, A., N. Mukaida, and K. Matsushima. 1996. Interleukin 8 as a novel target for intervention therapy in acute inflammatory diseases. *Mol. Med. Today 2:482.*
10. Clark-Lewis, I., C. Schumacher, M. Baggiolini, and B. Moser. 1991. Structure-activity relationships of interleukin-8 determined using chemically synthesized

analogs: critical role of NH2-terminal residues and evidence for uncoupling of neutrophil chemotaxis, exocytosis, and receptor binding activities. *J. Biol. Chem. 266:23128.*
11. Peveri, P., A. Walz, B. Dewald, and M. Baggiolini. 1988. A novel neutrophil-activating factor produced by human mononuclear phagocytes. *J. Exp. Med. 167: 1547.*
12. Detmers, P. A., D. E. Powell, A. Walz, I. Clark-Lewis, M. Baggiolini, and Z. A. Cohn. 1991. Differential effects of neutrophil-activating peptide 1/IL-8 and its homologues on leukocyte adhesion and phagocytosis. *J. Immunol. 147:4211.*
13. Martin, T. R., J. C. Mathison, P. S. Tobias, D. J. Leturcq, A. M. Moriarty, R. J. Maunder, and R. J. Ulevitch. 1992. Lipopolysaccharide binding protein enhances the responsiveness of alveolar macrophages to bacterial lipopolysaccharide: implications for cytokine production in normal and injured lungs. *J. Clin. Invest. 90:229.*
14. Ishii, Y., Y. Wang, A. Haziot, P. J. del-Vecchio, S. M. Goyert, and A. B. Malik. 1993. Lipopolysaccharide binding protein and CD14 interaction induces tumor necrosis factor-α generation and neutrophil sequestration in lungs after intratracheal endotoxin. *Circ. Res. 73:15.*
15. Carlos, T. M., and J. M. Harlan. 1994. Leukocyte-endothelial adhesion molecules. *Blood 84:2068.*
16. Harlan, J. M., R. K. Winn, C. M. Doerschuk, N. B. Vedder, and C. L. Rice. 1992. In vivo models of leukocyte adherence to endothelium. In *Adhesion: Its Role in Inflammatory Disease.* J. M. Harlan and D. Liu, eds. W. H. Freeman and Company, New York, p. 117–150.
17. Doerschuk, C. M., R. K. Winn, H. O. Coxson, and J. M. Harlan. 1990. CD18-dependent and -independent mechanisms of neutrophil emigration in the pulmonary and systemic microcirculation of rabbits. *J. Immunol. 144:2327.*
18. Winn, R. K., and J. M. Harlan. 1993. CD18-independent neutrophil and mononuclear leukocyte emigration into the peritoneum of rabbits. *J. Clin. Invest. 92: 1168.*
19. Gao, J. X., A. C. Issekutz, and T. B. Issekutz. 1994. Neutrophils migrate to delayed-type hypersensitivity reactions in joints, but not in skin: mechanism is leukocyte function-associated antigen-1-/Mac-1-independent. *J. Immunol. 153: 5689.*
20. Kubes, P., X. F. Niu, C. W. Smith, M. E. Kehrli, Jr., P. H. Reinhardt, and R. C. Woodman. 1995. A novel β1-dependent adhesion pathway on neutrophils: a mechanism invoked by dihydrocytochalasin B or endothelial transmigration. *FASEB J. 9:1103.*
21. Reinhardt, P. H., J. F. Elliott, and P. Kubes. 1997. Neutrophils can adhere via $\alpha 4\beta 1$-integrin under flow conditions. *Blood 89:3837.*
22. Thomas, J. R., J. M. Harlan, C. L. Rice, and R. K. Winn. 1992. Role of leukocyte CD11/CD18 complex in endotoxic and septic shock in rabbits. *J. Appl. Physiol. 73:1510.*
23. Singer, I., S. Scott, D. W. Kawka, and D. M. Kazazis. 1989. Adhesomes: specific granules containing receptors for laminin, C3bi/fibrinogen, fibronectin, and vitronectin in human polymorphonuclear leukocytes and monocytes. *J. Cell. Biol. 109:3169.*
24. Kajikawa, O., R. B. Goodman, M. C. Johnson, II, K. Konishi, and T. R. Martin. 1996. Sensitive and specific immunoassays to detect rabbit IL-8 and MCP-1: cytokines that mediate leukocyte recruitment to the lungs. *J. Immunol. Methods 197:19.*
25. Beatty, P. G., J. A. Ledbetter, P. J. Martin, T. H. Price, and J. A. Hansen. 1983. Definition of a common leukocyte cell-surface antigen (Lp95–150) associated with diverse cell-mediated immune functions. *J. Immunol. 131:2913.*
26. Price, T., P. G. Beatty, and S. R. Corpuz. 1987. In vivo inhibition of neutrophil function in the rabbit using monoclonal antibody to CD18. *J. Immunol. 139:4174.*
27. Broaddus, V. C., A. M. Boylan, J. M. Hoeffel, K. J. Kim, M. Sadick, A. Chuntharapai, and C. A. Hebert. 1994. Neutralization of IL-8 inhibits neutrophil influx in a rabbit model of endotoxin-induced pleurisy. *J. Immunol. 152: 2960.*
28. Folkesson, H. G., M. A. Matthay, C. A. Hebert, and V. C. Broaddus. 1995. Acid aspiration-induced lung injury in rabbits is mediated by interleukin-8-dependent mechanisms. *J. Clin. Invest. 96:107.*
29. Sekido, N., N. Mukaida, A. Harada, I. Nakanishi, Y. Watanabe, and K. Matsushima. 1993. Prevention of lung reperfusion injury in rabbits by a monoclonal antibody against interleukin-8. *Nature 365:654.*
30. Wymann, M. P., V. von-Tscharner, D. A. Deranleau, and M. Baggiolini. 1987. The onset of the respiratory burst in human neutrophils: real-time studies of H_2O_2 formation reveal a rapid agonist-induced transduction process. *Anal. Biochem. 165:371.*
31. Carlos, T., N. Kovach, B. Schwartz, M. Rosa, B. Newman, E. Wayner, C. Benjamin, L. Osborn, R. Lobb, and J. Harlan. 1991. Human monocytes bind to two cytokine-induced adhesive ligands on cultured human endothelial cells: endothelial-leukocyte adhesion molecule-1 and vascular cell adhesion molecule-1. *Blood 77:2266.*
32. Zachariae, C. O., A. O. Anderson, H. L. Thompson, E. Appella, A. Mantovani, J. J. Oppenheim, and K. Matsushima. 1990. Properties of monocyte chemotactic and activating factor (MCAF) purified from a human fibrosarcoma cell line. *J. Exp. Med. 171:2177.*
33. Yoshimura, T., and E. J. Leonard. 1990. Identification of high-affinity receptors for human monocyte chemoattractant protein-1 on human monocytes. *J. Immunol. 145:292.*
34. Sozzani, S., W. Luini, M. Molino, P. Jilek, B. Bottazzi, C. Cerletti, K. Matsushima, and A. Mantovani. 1991. The signal transduction pathway involved in the migration induced by a monocyte chemotactic cytokine. *J. Immunol. 147:2215.*
35. Burn, T. C., M. S. Petrovick, S. Hohaus, B. J. Rollins, and D. G. Tenen. 1994. Monocyte chemoattractant protein-1 gene is expressed in activated neutrophils and retinoic acid-induced human myeloid cell lines. *Blood 84:2776.*