Platelet Endothelial Cell Adhesion Molecule-1 in Neutrophil Emigration during Acute Bacterial Pneumonia in Mice and Rats

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Platelet endothelial cell adhesion molecule-1 (PECAM-1) (CD31) is an adhesion molecule believed to mediate transendothelial migration of neutrophils and other leukocytes after CD11/CD18-mediated adhesion. Our study evaluated the role of PECAM-1 in neutrophil emigration across the pulmonary capillaries and the bronchial microvasculature using blocking anti–PECAM-1 antibodies in mice and rats. Neutrophil emigration was induced by *Escherichia coli*, a stimulus eliciting CD11/CD18-dependent emigration, or *Streptococcus pneumoniae*, a stimulus inducing CD11/CD18-independent emigration. Although anti–PECAM-1 antibodies partially inhibited glycogen-induced neutrophil emigration into the peritoneum, neutrophil emigration across either the pulmonary capillaries or the bronchial microvasculature in response to either *E. coli* or *S. pneumoniae* was not prevented when the function of PECAM-1 was inhibited in either mice or rats. There was also no increase in the number of intravascular neutrophils within the bronchial vessels after treatment with anti–PECAM-1 antibody. These studies indicate that either CD11/CD18-dependent or -independent adhesion pathways may lead to PECAM-1–independent transendothelial migration through the pulmonary or the bronchial endothelium.

Keywords: rodent; lung; neutrophils; infectious immunity-bacteria; adhesion molecules

Neutrophil emigration into the alveolar space is one of the hallmarks of acute bacterial pneumonia (1). During bacterial pneumonia, neutrophils marginate within the microvasculature, adhere to endothelial cells, and transmigrate between endothelial cells into the alveolar space. Each step in this process is mediated by specific mechanisms and molecules.

Platelet endothelial cell adhesion molecule-1 (PECAM-1) (CD31) is a transmembrane protein and a member of immunoglobulin (Ig) gene superfamily structurally related to intercellular adhesion molecule-1 (2, 3). It is constitutively expressed at the junctions between endothelial cells and on the surface of leukocytes and platelets (2–5). Roles for PECAM-1 in modulating leukocyte adhesion and migration, angiogenesis, and thrombosis have been described (5–9). PECAM-1 is also an intracellular signaling molecule, and recent studies have suggested it is a member of the immunoreceptor tyrosine-based inhibitory motif subfamily of the Ig superfamily (3). Many studies have demonstrated that it plays a critical role in the transendothelial migration of neutrophils (10). *In vitro* studies show that anti–PECAM-1 antibody or soluble PECAM-1 inhibits neutrophil transmigration across the endothelium activated by tumor necrosis factor (TNF)-α (11). Similarly, *in vivo* studies show that anti–PECAM-1 antibody blocks neutrophil emigration in chemical-induced peritonitis (12–14).

PECAM-1 is expressed constitutively on pulmonary arterial, capillary, and venular endothelium, yet many questions regarding the role that this highly expressed cell adhesion molecule plays in pulmonary inflammation remain. An increase in PECAM-1 expression has been reported in mice exposed to 100% oxygen for 3 to 4 days, although the functional significance of this change was not studied (15). The only functional studies, to our knowledge, have been by Vaporciyan and colleagues who showed that IgG immune complex-induced lung injury, a process requiring neutrophils, interleukin (IL)-1, TNF-α, CD11/CD18, intercellular adhesion molecule-1, and E-selectin, also required PECAM-1 for neutrophil emigration into the alveolar space (12, 16–19).

The role of PECAM-1 in bacterial pneumonia has not been evaluated. In contrast to the microcirculation of many organs, neutrophil emigration through the pulmonary capillary bed into the distal airspaces of the lung during the acute inflammatory response can occur through adhesion pathways that do not require CD11/CD18 (20–22). The purpose of these studies was to examine the role of PECAM-1 in either *Escherichia coli*–induced pneumonia, where neutrophil emigration occurs through CD18-dependent adhesion pathways (22, 23), or *Streptococcus pneumoniae*–induced pneumonia, a stimulus eliciting CD18-independent emigration (20, 22). In addition, the function of PECAM-1 in the pulmonary and bronchial circulations was compared in the response of neutrophils with either stimulus.

METHODS

Antibodies

For the studies using rats, purified rabbit polyclonal anti-human PECAM-1 antibody that cross-reacted with rat PECAM-1 was used. This antibody has been carefully characterized and used *in vitro* and *in vivo* to block the function of rat PECAM-1 (12). Nonimmune rabbit IgG (Sigma I-4131; Sigma Chemical Co., St. Louis, MO) was used as a control antibody. For the studies using mice, two anti–PECAM-1 antibodies were used, a monoclonal rat antibody (mAb) against mouse PECAM-1 (Mec 13.3; PharMingen, San Diego, CA) (24), and an isotype matched control antibody (390) that was previously generated and characterized by us (25). We have shown previously that both antibodies inhibit *in vivo* angiogenesis in mice (8, 9), but only Mec 13.3 blocks *in vivo* neutrophil emigration during chemical peritonitis or after TNF injections in human skin grafts, on severe combined immunodeficiency

mice (14). For studies measuring PECAM-1 protein expression in immunoblots, the polyclonal goat anti-mouse PECAM-1 antibody M-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used. This antibody was raised against a peptide from the carboxy terminal region of murine PECAM-1.

Expression of PECAM-1 in *E. coli-* and *S. pneumoniae*–induced Pneumonia

To localize the expression of PECAM-1, Lewis rats (250–300 g; Harlan Sprague Dawley, Inc., Indianapolis, IN) and C57 BL/6 mice (18–26 g; Jackson Laboratories, Bar Harbor, ME) were given either no stimulus or an intratracheal instillation of *E. coli* or *S. pneumoniae* for 6 hours as described below. Optimum cutting temperature compound (Tissue-Tek) diluted 1:1 with normal saline was instilled through the trachea, and the lungs were frozen in liquid nitrogen. Rat hearts were also removed and frozen. Histologic frozen sections (7–9 μm) were cut. Immunohistochemistry was performed by incubating the frozen sections after fixation in acetone with the appropriate anti–PECAM-1 antibody (0.02 mg/ml) followed by a biotin-labeled rat anti-rabbit or goat anti-rat IgG secondary antibody (Kirkegaard and Perry Laboratories, Inc., Gaitherburg, MD). After incubation with streptavidin–alkaline phosphatase conjugates (Kirkegaard and Perry Laboratories, Inc.), the tissue sections were incubated with a substrate containing new fushcin, a red dye, to localize the antigen. In the negative control sections, the primary antibody was replaced by either nonspecific rabbit or rat IgG or Tris buffer. Anti-rat CD18 antibody was used as a positive control.

To quantitate PECAM-1 expression, protein was extracted from homogenates of noninfected lungs and lungs with either *S. pneumoniae* or *E. coli* pneumonia (n = 5 in each group). Immunoblots were performed by electrophoresis using 20 μg protein per lane, and the blots were stained with the polyclonal anti-murine PECAM-1 antibody M-20, which recognizes all PECAM-1 isoforms. PECAM-1 staining (MW 120,000–130,000) was measured using densitometry and expressed as a fold change relative to that observed in noninfected lungs.

Neutrophil Emigration in Glycogen-induced Peritonitis in Rats

Anesthetized rats (n = 4 in each group) received an intravenous injection of anti–PECAM-1 antibody (200 μg/rat) or rabbit IgG. After 15 minutes, oyster glycogen (25 ml of 0.1% solution) was instilled into the peritoneal space. Control rats received no glycogen. After 4 hours, the peritoneal space was lavaged with three aliquots of 50 ml phosphate-buffered saline. The leukocyte count in the peritoneal lavage fluid was quantitated using a hemocytometer, and neutrophil counts were determined using Wright-stained cytospin preparations. A blood sample was collected in heparin from the inferior vena cava to determine the circulating leukocyte counts.

Neutrophil Emigration in *E. coli-* and *S. pneumoniae*–induced Pneumonia in Rats

Induction of pneumonia. To examine neutrophil emigration into bronchi supplied by the bronchial circulation, as well as into the alveolar space through the pulmonary capillaries, both suspended single organisms and agar-embedded organisms were instilled into the airways. *E. coli* and *S. pneumoniae* were grown on trypticase soy agar plates overnight. The surface of agar was scraped and the organisms and fragments of agar were suspended in saline. The bacterial suspensions were diluted to 1.0 absorbance U/ml at 600 nm (2.5×10^9 *E. coli*/ml and 3.9×10^9 *S. pneumoniae*/ml). One control and one anti–PECAM-1 antibody-treated rat were studied using the same suspension to control for any day-to-day variability in the concentration of organisms.

The rats were anesthetized using ketamine hydrochloride (80–100 mg/kg intramuscularly) and acepromazine maleate (8–10 mg/kg). Additional anesthesia was given as required throughout the experiment. A ventral incision was made in the neck, and the left carotid artery and trachea were isolated. The carotid artery was cannulated using a 14-gauge catheter for drawing blood samples. A tracheotomy was performed. After a blood sample was obtained for measurement of circulating leukocyte counts and differentials, the rats (n = 4 in each group) received either anti–PECAM-1 antibody or rabbit IgG (200 μg/per rat, n = 4 in each group) through a tail vein. After 15 minutes, a blood sample was drawn, a neonatal feeding tube (3.5 Fr) was passed through

the trachea into a small bronchus, and 250 μl of the suspension of *E. coli* and *S. pneumoniae* mixed with 5% colloidal carbon was instilled. Blood samples were drawn from the carotid artery at 3 and 6 hours after instillation. After 6 hours, saturated potassium chloride was injected through the carotid catheter to stop the heart. The chest was opened immediately, and the heart was tied at the base to prevent loss of blood from the lungs. The lungs were removed *en bloc* from the chest cavity and fixed using 6% glutaraldehyde in phosphate-buffered saline at 25 cm H_2O.

Quantitation of neutrophil emigration. Lung tissue was cut from the blacked, colloidal carbon-containing regions, embedded in paraffin, and sectioned at 5 to 6 μm. The accumulation of neutrophils into the alveolar spaces was quantitated on hematoxylin and eosin-stained histologic sections using two methods:

Method 1: in *E. coli* pneumonia, the number of neutrophils that migrated into colloidal carbon-containing alveolar spaces was counted. Neutrophil emigration was calculated as the number of neutrophils per 100 alveoli (20).
Method 2: in *E. coli* and *S. pneumoniae* pneumonias, point-counting techniques were used to calculate the percent of the distal lung parenchyma that was occupied by intra-alveolar neutrophils. Five hundred points were observed for each slide (26, 27). Each point was classified as falling on a neutrophil within the alveolar space or the space itself. The percentage of alveolar neutrophils was calculated as: number of points on alveolar neutrophils/number of points on alveolar space.

Neutrophil emigration from the bronchial (systemic) circulation was evaluated by quantitating the number of neutrophils that migrated into the bronchial wall (26). In each animal, two to three large bronchi within the region of pneumonia were studied. The bronchial wall area was measured by digitizing the outer perimeter of the adventitia, the inner bronchial epithelial length, and the endothelial length of bronchial blood vessels within the bronchial wall and was calculated as: bronchial wall area = total area − airway luminal area − blood vessel luminal area. The number of neutrophils that migrated into the bronchial wall was counted and expressed as the number of neutrophils/1,000 μm² bronchial wall. The number of neutrophils sequestered within the bronchial vessels was also counted and expressed as neutrophils/1,000 μm² bronchial vessel lumen.

Neutrophil Emigration and Edema Formation during Bacterial Pneumonia in Mice

To determine the role of PECAM-1 in bacterial pneumonia in mice, anesthetized C57BL/6 mice were given an intravenous injection of either a blocking anti–PECAM-1 mAb (Mec 13.3; 200 μg/mouse) or a nonblocking anti–PECAM-1 mAb (390; 200 μg/mouse) 15 minutes before the intratracheal instillation of either *S. pneumoniae* (5.9×10^8 CFU/ml) or *E. coli* (1.1×10^8 CFU/ml) suspension (50 μl/22 g of body weight) mixed with 5% colloidal carbon as a tracer (n = 5 in each group [14, 24, 27–30]). Uninfected mice received no instillate (n = 5). Neutrophil emigration was quantitated morphometrically in histologic sections by quantitating the fraction of distal lung tissue that was occupied by intra-alveolar (emigrated) neutrophils using point-counting techniques, as described previously (26, 27, 29, 30). Edema formation was measured by determining the accumulation of extravascular ^{125}I-labeled albumin 6 hours after instillation of organisms using ^{51}Cr-labeled murine red blood cells to mark intravascular blood volumes and was expressed as microliters plasma equivalents/gram body weight, as described previously (29, 30).

Statistical Analysis

One-way analyses of variance were used to compare neutrophil accumulation between the groups (31). If this analysis showed any significant differences, multiple contrasts were used with a Bonferroni adjustment to determine which groups were significantly different (32). A p value less than 0.05 was considered to be statistically significant.

RESULTS

Expression of PECAM-1 in *E. coli*–induced and *S. pneumoniae*–induced Pneumonia in Rats and Mice

Immunohistochemical studies showed that the polyclonal rabbit anti-human PECAM-1 antibody bound to rat cardiac arteriolar
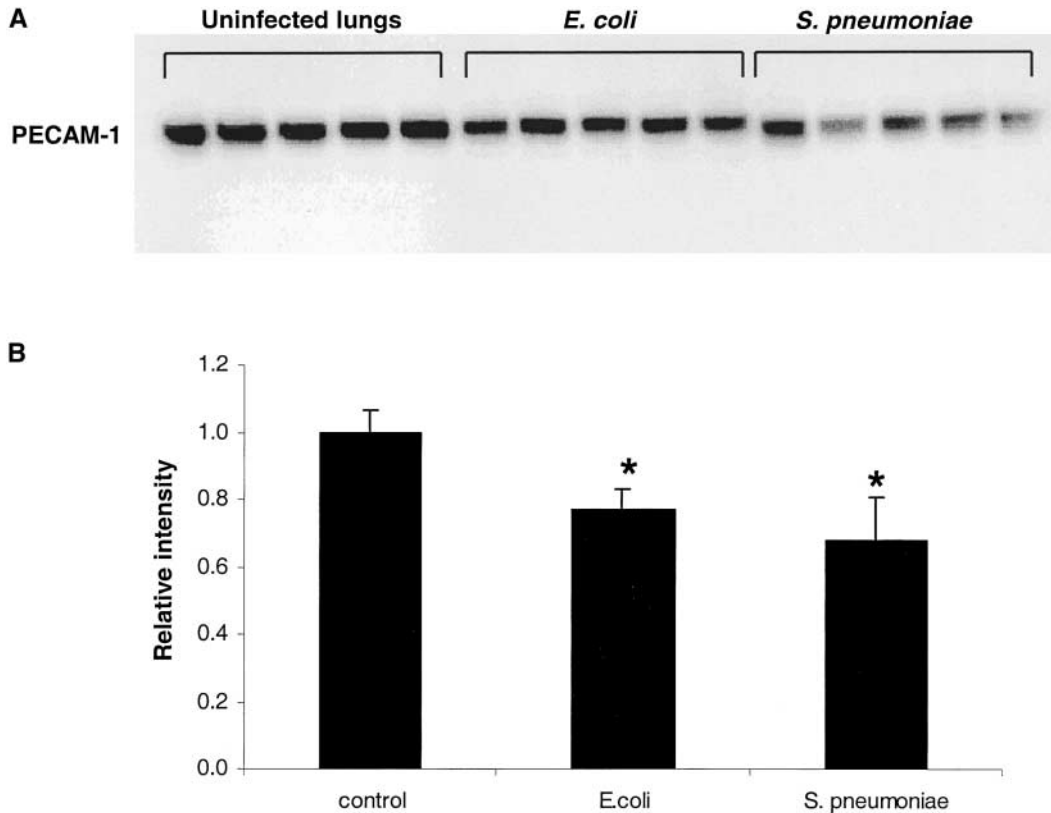
A

Figure 1. PECAM-1 protein expression in protein isolates from homogenates of noninfected murine lungs or lungs with *E. coli* or *S. pneumoniae* pneumonia. (*A*) Immunoblot comparing five lungs in each group. Each lane represents 20 μg protein from the lungs of one mouse. (*B*) Relative intensity of PECAM-1 expression expressed as a fold change from noninfected lungs; n = 5 mice in each group.

and venular endothelial cells. This antibody also recognized the endothelium lining the arterioles, venules, and alveolar capillaries in the lungs of uninfected rats, as reported previously (12). Using this technique, no change in the expression of PECAM-1 after instillation of either *E. coli* or *S. pneumoniae* could be identified. Similarly, using the rat anti-murine PECAM-1 antibody, the murine pulmonary and bronchial microvascular endothelial cells constitutively expressed PECAM-1, and no increase in staining over this bright baseline level could be identified during pneumonia (data not shown).

PECAM-1 protein expression was quantified in murine lungs using immunoblots (Figure 1). PECAM-1 expression was not increased and in fact was decreased slightly by 25 to 30% in both *E. coli* and *S. pneumoniae* pneumonia compared with noninfected lungs.

Neutrophil Emigration in Glycogen-induced Peritonitis in Rats

To confirm the activity of our anti–PECAM-1 antibody in rats, its effect on glycogen-induced peritonitis was studied. The number of neutrophils present within the peritoneal lavage fluid was measured 4 hours after instillation of glycogen into the peritoneum. Instillation of glycogen induced neutrophil emigration into the peritoneal cavity from $0.04 \pm 0.02 \times 10^6$/ml to $34.0 \pm 2.7 \times 10^6$/ml lavage fluid in rats given rabbit IgG. This emigration was inhibited by 60% when rats received anti–PECAM-1 antibody ($13.7 \pm 4.6 \times 10^6$ neutrophils/ml lavage fluid) compared with those given rabbit IgG (p < 0.05). The circulating neutrophil count in the anti–PECAM-1 antibody-treated animals measured $2.2 \pm 0.2 \times 10^6$/ml blood and was similar to that in the animals treated with rabbit IgG ($2.4 \pm 0.2 \times 10^6$/ml blood) at 4 hours.

Neutrophil Emigration in *E. coli*- or *S. pneumoniae*–induced Pneumonia in Rats

The role of PECAM-1 in neutrophil emigration into the alveolar space of rats was evaluated using two morphometric techniques. The percentage of the alveolar space that was occupied by neutrophils in either *S. pneumoniae*–induced or *E. coli*–induced pneumonia is shown in Figure 2. Pretreatment with anti–PECAM-1 antibodies did not inhibit neutrophil emigration in pneumonia induced by either stimulus. Using a completely different morphologic approach of counting the number of neutrophils per 100 cross-sectioned alveolar spaces, similar results were observed in *E. coli* pneumonia, where neutrophil emigration was similar in rats pretreated with anti–PECAM-1 antibody ($1,442 \pm 48$ neutrophils/100 alveoli) compared with rats pretreated with rabbit IgG ($1,587 \pm 36$ neutrophils/100 alveoli, p > 0.05).
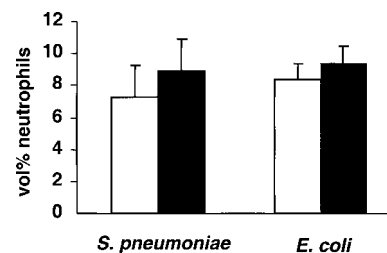

Figure 2. Neutrophil emigration in *S. pneumoniae* or *E. coli* in rats pretreated with either anti–PECAM-1 antibody or rabbit IgG. The percentage of the alveolar space that was occupied by neutrophils in the lungs of rats pretreated with the anti–PECAM-1 antibody (*solid bars*) was similar to that in rats given the control antibody (*open bars*) in either *S. pneumoniae* or *E. coli* pneumonias; n = 4 rats in each group.
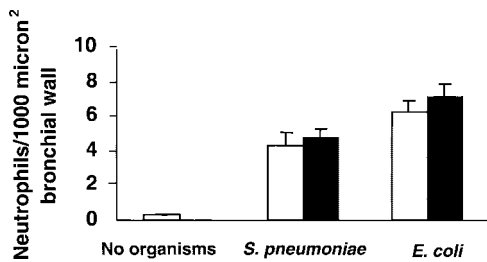
Figure 3. Neutrophil emigration into the bronchial wall induced by *S. pneumoniae* or *E. coli* or in rats pretreated with anti–PECAM-1 antibody or rabbit IgG. Both *S. pneumoniae* and *E. coli* induced a significant increase in the number of neutrophils that emigrated into the bronchial wall. This emigration was not inhibited when the rats were pretreated with the anti–PECAM-1 antibody. *Open bars*: rats pretreated with rabbit IgG. *Solid bars*: rats pretreated with anti–PECAM-1 antibody; n = 4 rats in each group.
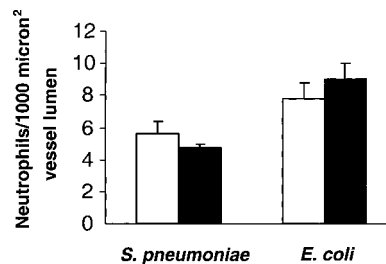

Figure 4. Neutrophil accumulation within the bronchial microvasculature in rats with either *S. pneumoniae* or *E. coli* pneumonia that were pretreated with anti–PECAM-1 antibody or rabbit IgG. The number of neutrophils sequestered within the bronchial microvasculature was not increased after administration of the anti–PECAM-1 antibody in pneumonia induced by either organism. *Open bars*: rats pretreated with rabbit IgG. *Solid bars*: rats pretreated with anti–PECAM-1 antibody; n = 4 rats in each group.

Neutrophil emigration into the bronchial walls is shown in Figure 3. The accumulation of extravascular neutrophils within the bronchial walls induced by either *E. coli* or *S. pneumoniae* was not inhibited by anti–PECAM-1 antibody. The number of intravascular neutrophils that were within the bronchial vessels was not increased after administration of anti–PECAM-1 antibody (Figure 4).

Injection of anti–PECAM-1 did not cause any change in either the circulating leukocyte or neutrophil counts (Table 1). The neutrophil counts increased within the first 3 hours of the experiment in animals with or without instillations of organisms. This increase was similar in IgG-treated compared with anti–PECAM-1 antibody-treated rats with either type of pneumonia.

Effect of Anti–PECAM-1 mAb on Bacterial Pneumonia in Mice

To extend the observations made in rats to a second species, neutrophil emigration into the airspaces of mice was examined 6 hours after intratracheal instillation of bacteria in animals treated with anti–PECAM-1 mAb or control antibody (Figure 5).

Both *S. pneumoniae* and *E. coli* induced neutrophil emigration within 6 hours because the volume fraction of airspaces that was occupied by neutrophils was only $0.02 \pm 0.02\%$ and their volume fraction of capillaries was $1.8 \pm 0.3\%$ in uninfected murine lungs. The anti–PECAM-1 antibody Mec 13.3 did not inhibit the number of emigrated or intravascular neutrophils for either *S. pneumoniae* or *E. coli*. For *S. pneumoniae* pneumonia, the number of emigrated neutrophils was in fact significantly greater in the mice treated with anti–PECAM-1 mAb compared with those given control antibody. The physiologic significance of this increase is unclear.

Pulmonary edema formation was also examined 6 hours after intratracheal instillation of bacterial suspension (Figure 6). Both *S. pneumoniae* and *E. coli* induced an increase in EVA within 6 hours from 0.6 ± 0.1 μl/g body weight in uninfected lungs. There was no significant difference in pulmonary edema between the mice treated with anti–PECAM-1 mAb and those administered control antibody after the instillation of either *S. pneumoniae* or *E. coli*.

Injection of anti–PECAM-1 mAb did not cause any change

TABLE 1. CIRCULATING NEUTROPHIL COUNTS IN UNINFECTED RATS AND RATS WITH PNEUMONIA

Time after Instillation (*h*)	Pretreated with Rabbit IgG (*neutrophils $\times 10^6$/ml blood*)	Pretreated with Rabbit Anti–PECAM-1 mAb (*neutrophils $\times 10^6$/ml blood*)
Streptococcus pneumoniae pneumonia		
−0.25	0.6 ± 0.06	0.4 ± 0.07
0	0.6 ± 0.13	0.6 ± 0.12
3	1.7 ± 0.09	2.0 ± 0.33
6	1.1 ± 0.09	1.2 ± 0.24
Escherichia coli pneumonia		
−0.25	0.8 ± 0.05	0.9 ± 0.31
0	0.7 ± 0.05	0.6 ± 0.12
3	2.6 ± 0.38	2.1 ± 0.41
6	1.2 ± 0.17	0.8 ± 0.05
No organisms		
−0.25	0.46 ± 0.22	
0	0.56 ± 0.08	
3	2.1 ± 0.07	
6	1.6 ± 0.13	

Definition of abbreviations: IgG = immunoglobulin G; mAb = monoclonal antibody; PECAM-1 = platelet endothelial cell adhesion molecule.

There are no significant differences in circulating neutrophil counts between noninfected rats and those with either infection or between rats treated with anti–PECAM-1 antibodies and those treated with control IgG.
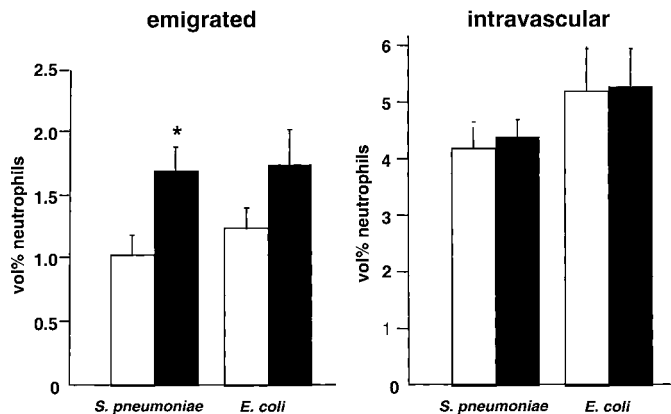
Figure 5. The percent of the distal lung occupied by either emigrated or intracapillary neutrophils in mice with *S. pneumoniae* or *E. coli* pneumonia that were pretreated with either a blocking (Mec 13.3) or nonblocking (390) anti–PECAM-1 antibody. The number of neutrophils that emigrated was significantly increased in *S. pneumoniae* pneumonia when the animals were pretreated with the blocking anti–PECAM-1 antibody (*solid bars*) compared with a nonblocking antibody (*open bars*). A similar trend was observed for *E. coli* pneumonia, but the differences did not reach significance. There was no significant difference in the numbers of intravascular neutrophils in pneumonia induced by either organism. n = 5 mice in each group. *p Values less than 0.05.

Figure 6. Edema formation in mice with *S. pneumoniae* or *E. coli* pneumonia and pretreated with either a blocking (Mec 13.3) or nonblocking (390) anti–PECAM-1 antibody. Edema formation was similar in mice treated with the blocking (*solid bars*) compared with the nonblocking antibody (*open bars*) in pneumonia induced by either organism; n = 5 mice in each group.

in either the circulating leukocyte or neutrophil counts, which were not different from those of uninfected mice (neutrophil counts in mice treated with the Mec 13.1 antibody: 1.07 ± 0.01, 390 control antibody: $1.04 \pm 0.01 \times 10^6$ neutrophils/ml blood in *S. pneumoniae* pneumonia and Mec 13.1 antibody: 1.17 ± 0.02, 390 control antibody: $1.18 \pm 0.02 \times 10^6$ neutrophils/ml blood in *E. coli* pneumonia).

DISCUSSION

In order to investigate the involvement of PECAM-1 in neutrophil emigration during bacterial pneumonia, the effects of antibodies against PECAM-1 were studied in both rats and mice given an airway instillation of either *S. pneumoniae* or *E. coli*. Anti–PECAM-1 antibodies that were capable of blocking neutrophil recruitment during glycogen-induced peritonitis or IgG immune complex-induced lung injury failed to inhibit neutrophil emigration out of either the bronchial or the pulmonary microvasculature. Moreover, no increase in neutrophil concentration was observed within either the pulmonary capillaries or the bronchial microvasculature, which would occur if transendothelial migration were prevented by PECAM-1 blockade. These data suggest that neutrophil recruitment in response to a bacterial challenge may occur independently of PECAM-1, whether emigration occurs through *S. pneumoniae*–elicited CD18-independent or *E. coli*–elicited CD18-dependent adhesion pathways. These studies provide further evidence for the presence of multiple pathways of neutrophil emigration in the lungs.

The function of PECAM-1 in neutrophil emigration into tissues other than the lungs has been examined in several *in vivo* systems. Antagonism of PECAM-1 function blocks neutrophil recruitment during chemical peritonitis (12–14, 33–35), mesenteric inflammation induced by IL-1β (36, 37), myocardial ischemia-reperfusion injury (33, 38), and cutaneous inflammation (12, 14, 39). In all of these studies, treatment with anti–PECAM-1 antibodies or blocking reagents resulted in diminished leukocyte extravasation. In many of these studies, neutrophils were ob-
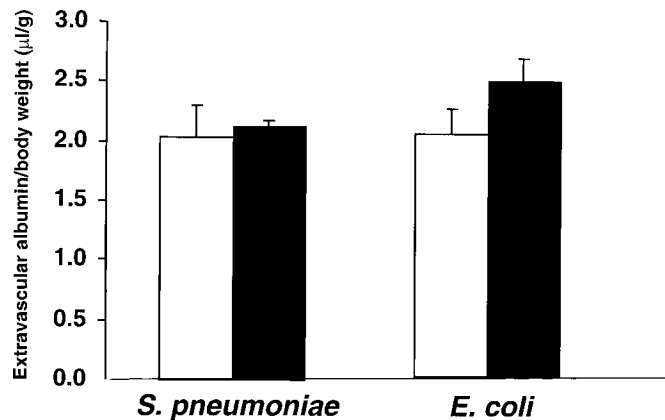
served to accumulate in the lumen of the vessels and within the vessel wall between the endothelium and the basement membrane (12, 13, 36, 37). These data suggest that, at least in some types of inflammatory responses, PECAM-1 plays an important role in the passage of neutrophils across the endothelium and/or through the basement membrane, a proposal that is consistent with *in vitro* studies of cultured endothelial cells (40). This hypothesis is also supported by recent findings from investigations of PECAM-1–deficient mice (41, 42).

The lack of a role for PECAM-1 during bacterial pneumonias is unlikely to be due to nonbioactive or ineffective antibody reagents. First, the antibodies stained endothelial cells in the vasculature of the myocardium and in the lung from both species of animals (data not shown). Second, the antibodies employed in this study to inhibit the function of PECAM-1 have been previously shown to block chemical peritonitis, as well as *in vivo* angiogenesis, in mice and rats (8, 9, 12, 14). Third, the anti–PECAM-1 antibody used in the rat experiments inhibited neutrophil emigration into the distal airways of the lung during IgG immune complex-induced injury (12). Fourth, the studies presented here reconfirmed that the anti-rat PECAM-1 antibody blocked neutrophil transendothelial migration after intraperitoneal injection of glycogen.

These data support recent observations that suggest that only certain types of inflammatory processes use PECAM-1–dependent mechanisms of neutrophil transmigration. Differential requirements for PECAM-1 may be due to the differential effects of these agents on neutrophil activation. Studies examining cytokine or chemoattractant-induced inflammation in the mesentery or the cremaster muscle show that PECAM-1 blockade is effective after IL-1β stimulation but not after exposure of blood vessels to TNF-α or to direct neutrophil activators such as formyl-methionyl-leucyl-phenylalanine (36, 37, 41, 42). Similar results were obtained in *in vitro* transmigration assays where anti–PECAM-1 antibodies block transmigration of IL-1–treated but not IL-8–treated transwells, as well as *in vivo* studies where PECAM-1 blockade prevented neutrophil extravasation after intradermal injections of TNF but not leukotriene B4 in mice (unpublished data). In the lungs, the anti–PECAM-1 antibody blocked neutrophil extravasation during IgG immune complex-induced injury (an IL-1, TNF-α, and CD11/CD18-dependent process [12, 16–

19]), but had no effect, as noted in this report, on neutrophil emigration triggered by *E. coli* (a process that is also mediated by IL-1, TNF-α, and CD11/CD18, [22, 30]) or *S. pneumoniae*. Coupled with previous reports indicating that PECAM-1 is not required for neutrophil extravasation triggered in response to formyl-methionylleucylphenylalanine (37, 42), these data suggest that bacterially derived products or mediators may induce PECAM-independent emigration that fully compensates for any role of PECAM-1. Admittedly, the *in vivo* situation is much more complex because multiple mediators may be active at sites of inflammation, and this hypothesis, although intriguing, is speculative until confirmed by additional studies.

Whether particular stimuli require PECAM-1 to elicit neutrophil emigration may be determined by which intracellular signaling cascades activate the processes involved in leukocyte extravasation induced by the stimulus. PECAM-1 ligation modulates a growing number of intracellular signaling pathways. For example, Reedquist and colleagues (43) have demonstrated in Jurkat cells that PECAM-1 interacts with and activates Rap1, a member of the Ras family of small guanosine triphosphatases that stimulates integrin-mediated adhesion in neutrophils (44). Rap1 can be activated by many molecules including formyl-methionylleucylphenylalanine, platelet activating factor, or granulocyte macrophage colony stimulating factor , and perhaps the requirement for PECAM-1 may be determined by whether a particular stimulus requires PECAM-1 ligation for modulating Rap1. PECAM-1 also interacts and activates phosphoinositide 3-kinase in neutrophils, leading to enhanced adhesion (45). Finally, PECAM-1 modulates events that are likely to be critical in the process of transendothelial migration, for example, the intracellular localization of phosphorylate β-catenin (46). The requirement for PECAM-1 may thus be determined by whether its ligation is necessary for activation of these pathways in response to a particular stimulus.

S. pneumoniae induced neutrophil emigration that did not require PECAM-1 and was, in fact, enhanced in mice given a blocking anti–PECAM-1 antibody compared with mice given a nonblocking antibody. The physiologic significance of this finding is unclear, as it was observed in only mice and not in rats. However, it is interesting to speculate that PECAM-1 may limit neutrophil emigration in some circumstances. Although the mechanism for this increase has not been examined in these studies, it may reflect an inhibitory role for PECAM-1 signaling through its cytoplasmic immunoreceptor tyrosine-based inhibitory motifs. When ligated, PECAM-1 is phosphorylated at tyrosine residues by Src or Csk family kinases, leading to binding of phosphotyrosine phosphatases recognizing Src homology 2 domains (47). These phosphatases include Src homology phosphatases-1 and -2, as well as SH2-containing inositol phosphatase and phospholipase C-γ1 (48, 49). Other signaling molecules that PECAM-1 may modulate include phosphoinositide 3-kinase in neutrophils (45) and the small guanosine triphosphatase, Rap1, as discussed previously (43, 45). Although increasing adhesion is generally considered to enhance migration, increased firm adhesion may actually prohibit crawling. We speculate that the balance of these complex signaling events initiated by PECAM-1 may act to limit neutrophil migration in response to particular stimuli such as *S. pneumoniae*. Regardless, however, these data do clearly suggest that PECAM-1 is not required for neutrophil emigration out of lung microvasculature or for edema formation in pneumonic lungs.

Taken together, these studies and others suggest that leukocyte emigration into the lungs can occur through at least three adhesion pathways that vary in their requirements for CD11/CD18 and PECAM-1. The first requires CD11/CD18 and PECAM-1 and includes neutrophil emigration induced by IgG immune complexes (12, 16–19), a second requires CD11/CD18 but not PECAM-1 and includes emigration induced by *E. coli*, and a third pathway mediates emigration through CD11/CD18- and PECAM-1–independent mechanisms, as demonstrated by emigration induced by *S. pneumoniae*. These pathways may reflect differences in pattern and nature of the mediators that are generated by the specific inflammatory stimulus.

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