Selectins and Neutrophil Traffic: Margination and Streptococcus Pneumoniae-induced Emigration in Murine Lungs

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Summary

The roles of selectins in the pulmonary margination and emigration of neutrophils were investigated by using mice genetically deficient in both E- and P-selectins (E/P mutants) and/or by intravenous injections of fucoidin (inhibiting both L- and P-selectins). E/P mutants were neutrophilic (14.7 ± 4.9 × 10⁶ vs. 0.8 ± 0.1 × 10⁶ neutrophils/ml). This neutrophilia was associated with increased margination of neutrophils within pulmonary capillaries (39.7 ± 9.4 vs. 4.6 ± 1.1 neutrophil profiles per 100 red blood cell profiles) but no change in margination within noncapillary pulmonary microvessels. After intratracheal instillation of Streptococcus pneumoniae, lungs of E/P mutants displayed increased neutrophil emigration (564 ± 92 vs. 116 ± 19 neutrophils per 100 alveolar profiles), edema (5.3 ± 1.5 vs. 1.5 ± 0.4 μl/g body weight), and histologic evidence of lung injury compared with those in wild-type (WT). Fucoidin treatment did not affect neutrophil emigration during streptococcal pneumonia in WT or E/P mice. During pneumonia, the number of white blood cells (WBC) tethered to or spread upon the noncapillary vessel endothelium increased in both WT and E/P lungs. These are the first data demonstrating that neutrophil margination in uninfected pulmonary capillaries does not require E- and P-selectins; that streptococcal pneumonia induces an E- and P-selectin-independent increase in WBC interactions with noncapillary endothelium; and that migration of neutrophils to alveoli can occur despite deficiency or inhibition of all of the known selectins.

Selectins are critical to inflammatory interactions of neutrophils with the endothelium of the systemic circulation (for reviews see references 1–3). However, many aspects of the margination and emigration of neutrophils in the pulmonary circulation are different from these processes in the systemic circulation (4–6). For example, Streptococcus pneumoniae or C5a complement fragments deposited in the alveoli elicit CD18-independent emigration from pulmonary capillaries, whereas the same stimuli injected into the peritoneum or skin induce CD18-dependent emigration from systemic venules (4, 7).

Margination of neutrophils within pulmonary capillaries is thought to result from geometric constraints and the biomechanical properties of neutrophils and capillary walls (8–12). To the authors’ knowledge, the role of selectins in neutrophil margination within pulmonary capillaries has never been investigated. Although the majority of the marginated neutrophils are in capillaries, neutrophils also roll along the endothelium of pulmonary arterioles and venules after placement of a thoracic window (12, 13). This neutrophil rolling in pulmonary venules is abrogated by fucoidin (12), demonstrating a role for selectins in margination within noncapillary microvessels.

The emigration of neutrophils during pneumonia occurs primarily from the capillaries of alveolar septae (14), where the respective sizes of neutrophils and capillary lumina suggest that selectin-mediated rolling is unlikely (9, 10). In fact, rolling white blood cells (WBCs)¹ are not observed in alveolar capillaries by intravital microscopy (12, 13). However, neutrophil emigration in response to alveolar deposition of IgG immune complexes is diminished by intravenous administration of selectin inhibitors (15–18), suggesting that selectins play a role in this injury through mechanisms other than rolling.

Mice made genetically deficient in the selectins expressed on endothelial cells (E- and P-selectins) have recently become available (19, 20). These mice (E/P mutants) exhibit susceptibility to superficial infections, failure

¹Abbreviations used in this paper: ANOVA, analysis of variance; E/P mutants, mice genetically deficient in both E- and P-selectins; gbw, gram of body weight; WBC, white blood cell; WT, wild-type.
to thrive, cervical lymphadenopathy, hypergammaglobulinemia, neutrophilia, elevated levels of hematopoietic cytokines in the blood, and decreased L-selectin expression (15% of normal) on circulating neutrophils (19, 20). They demonstrate impaired neutrophil-endothelial interactions in the systemic circulation (19, 20), including the complete inhibition of neutrophil rolling in venules of exteriorized cremaster muscle over 2 h and the complete inhibition of peritoneal neutrophil accumulation 4 h after intraperitoneal injection of S. pneumoniae.

The present study investigates the roles of selectins in neutrophil traffic within the lungs by comparing wild-type (WT) and E/P mutant mice with and without intravenous administration of fucoidin to inhibit the remaining L-selectin (21). In mice without experimental pneumonia, the size of the marginalized pool in the pulmonary capillaries and noncapillary microvasculature is examined. In mice with streptococcal pneumonia, neutrophil emigration, pulmonary edema, and WBC attachments to endothelium are examined.

Materials and Methods

Animals. The E/P mutant line of mice was created as previously described (20). WT mice were from the same genetic background as the mutants (mixed 129/Sv and C57BL/6). The ages of E/P mutants ranged from 7 to 21 wk. All mice were housed in the Harvard University Center for Animal Resources (Boston, MA), and all experiments received institutional approval.

Margination. Mice were killed by inhalation of a lethal overdose of halothane. The thoracic and peritoneal cavities were opened, the heart was tied off at the base to prevent escape of pulmonary blood content, and a blood sample was drawn from the inferior vena cava for measurement of circulating WBC and neutrophil counts. The heart and lungs were removed en bloc and fixed by tracheal instillation of 2.5% glutaraldehyde at 22 cm H2O pressure.

Margination of neutrophils within pulmonary capillaries was examined by electron microscope. Lungs were diced, post-fixed in OsO4, and embedded in Epox 812 (Ernest F. Fullam, Inc., Hazelwood, MO). Thin sections (80 nm) were stained with uranyl acetate in the pulmonary blood volume and the hematocrit for each animal and venous plasma were measured (Cobra Quantum gamma counter; Packard Instruments, Meriden, CT) before dicing of lungs for morphometry. Blood and plasma volumes of lungs were determined from 51Cr-labeled RBC counts and 125I-albumin counts, respectively (5). Pulmonary edema (extravascular plasma in the lung) was calculated as total lung plasma minus intravascular lung plasma (5). Intravascular lung plasma was calculated from the pulmonary blood volume and the hematocrit for each animal (5). Pulmonary edema was expressed as μl/gbw.

Neutrophils in alveoli and pulmonary microvessels. Lung regions stained black with colloidal carbon were processed for light microscopy as described above. The number of neutrophil profiles per 100 alveolar profiles was counted in regions in which alveolar macrophages contained carbon particles. Leukocytes within noncapillary microvessels were scored as free, tethered, or spread, as described above.

Statistics. Data were compared by analysis of variance (ANOVA) using Tukey's honest significant difference for unequal sample sizes (22) to compare groups post hoc. Data that failed the Bartlett's χ2 test for homogeneity of variances (23) were log-transformed before analysis. Sets of data containing only two groups were compared by independent t test or Mann-Whitney U test. For all tests, differences were considered significant when P < 0.05. Mean and SEM are reported throughout. In E/P mutants with pneumonia, because median circulating WBC values differed markedly from the means, medians and ranges are reported in addition to means and SEM.
Peripheral blood was drawn from wild-type mice (WT) and mutants lacking E- and P-selectins (E/P) in three groups: control (three WT, six E/P), pneumonia (seven WT, nine E/P), and fucoidin plus pneumonia (seven WT, eight E/P). Mean ± SEM cells/ml blood (X 10^6) are reported for all groups. Because of large variability in the data, medians are also reported for the E/P mutant mice (in parentheses).

* Significant effect of genetic deficiency (P <0.05 compared with WT).
* Significant effect of fucoidin (P <0.05 compared with pneumonia with no fucoidin).

**Results**

**Neutrophil Margination in Mice without Pneumonia.** Circulating WBC counts were elevated in E/P mutants compared with those in WT mice, owing largely to neutrophilia (Table 1). The neutrophilia of E/P mutants was associated with increased pulmonary capillary margination compared with WT mice. There were 4.6 ± 0.1 neutrophil profiles per 100 RBC profiles in pulmonary capillaries of uninfected WT mice (n = 3), compared with 39.7 ± 9.4 neutrophil profiles per 100 RBC profiles in pulmonary capillaries of uninfected E/P mutants (n = 7; P <0.05 compared with WT).

The increase in circulating WBC counts was also reflected in sections of pulmonary noncapillary microvessels. More free WBCs were present within the lumina of noncapillary microvessels of E/P mutants than in WT mice (Table 2). However, neither tethered nor spread numbers of WBCs differed between uninfected WT and E/P mice (Table 2), in contrast with capillary margination.

Where there were few neutrophils in alveoli of mice without experimental infections, and no differences in the numbers of neutrophil per 100 alveoli between uninfected WT and E/P mice (Table 3).

**S. Pneumoniae-induced Alveolitis.** Significant neutrophil emigration in response to S. pneumoniae occurred in both WT and E/P mice (Table 3). Surprisingly, more neutrophils migrated to the alveoli of E/P mutants than to WT alveoli (Table 3). Furthermore, fucoidin treatment did not affect neutrophil emigration in WT or E/P mice instilled with S. pneumoniae (Table 3).

In parallel with the increased neutrophil emigration, pulmonary edema was greater in E/P mutants than in WT mice with streptococcal pneumonia. Lungs of E/P mutants (n = 9) contained 5.3 ± 1.5 μl/gbw edema fluid, whereas lungs of WT mice (n = 7) contained 1.5 ± 0.4 μl/gbw (P <0.05, t test).

Lung damage was prominent in histologic sections from E/P mutant mice instilled with S. pneumoniae, but little lung damage was observed in WT mice (Fig. 1). Lungs of E/P mutants exhibited necrosis, loss of alveolar integrity, and perivascular edema.

**Circulating Cells During Streptococcal Pneumonia.** E/P mice with streptococcal pneumonia were neutrophilic compared with WT mice with pneumonia (see Table 1). There was wide variability among E/P mutants' circulating neutrophil counts, with a median (number/ml) of 15.1 ± 10^6 and
P > 0.05 compared with pneumonic WT mice without fucoidin or LPS), suggesting that the leukocytemia was not an artifact of LPS contamination. Fucoidin did not significantly alter circulating WBC or neutrophil counts in E/P mice (see Table 1), although the variability was large.

*S. pneumoniae*-induced Tethering and Spreading of WBCs in Noncapillary Microvessels. Streptococcal pneumonia increased both tethering and spreading along endothelium in WT mice without significantly affecting the number of free WBCs within noncapillary microvessels (see Table 2). Fucoidin treatment of WT mice with pneumonia had no significant effect.

Interestingly, tethering and spreading were also increased during pneumonia in E/P mutants (see Table 2). The number of tethered WBCs in fucoidin-treated E/P mutants fell between the values observed for E/P mice with and without pneumonia, not differing significantly from either group. The number of free WBCs was not affected by pneumonia or fucoidin treatments (Table 2).

Free WBC counts within microvessel lumina were elevated in E/P mutants compared with those in WT mice with and without pneumonia (see Table 2). There were no statistically significant differences between WT and E/P mice in the numbers of WBCs tethered to or spread upon the endothelium in any group (see Table 2).

Discussion

Neutrophil dynamics in the lungs of mice lacking E- and P-selectin differed from normal (WT) in several ways. There was an increase in neutrophils marginated within the pulmonary capillaries of uninfected E/P mice, and this increase was associated with peripheral neutrophilia. In contrast, the pool of tethered and spread WBCs within noncapillary pulmonary microvessels did not increase in parallel with the pool of circulating cells. After instillation of *S. pneumoniae*, pneumonia was more severe in E/P than in WT mice, as assessed by neutrophil emigration, edema, and histopathology. This pneumonia was not abrogated by inhibition of the remaining L-selectin in E/P mice by injections of fucoidin. The tethering and spreading of WBCs on noncapillary blood microvessel endothelium increased in response to bacterial instillation, both in WT and in E/P mutant lungs.

The pool of marginated neutrophils within pulmonary capillaries was increased despite the complete lack of E- and P-selectin and the partial lack of L-selectin. These are the first data demonstrating that margination of neutrophils within alveolar capillaries occurs independently of endothelial selectins. These results are consistent with data showing neutrophil margination within normal (noninflamed) alveolar capillaries is due to hemodynamic forces and the biomechanical properties of neutrophils and capillary walls (8, 10, 11). The findings further suggest that the sizes of the circulating and marginated pools are associated.

In contrast with the capillary margination of neutrophils, margination within noncapillary blood microvessels of the lungs was not increased in parallel with the neutrophilia.
The neutrophilia was reflected in increased free cells within microvessel lumina in E/P mutants, but the greater number of circulating neutrophils did not increase the numbers of neutrophils attached to noncapillary endothelium. These data suggest that selectins regulate the margination of WBC within noncapillary microvessels of uninfected lungs, corroborating and extending earlier investigations using intravital microscopy in the uninfected canine lung (12).

The fact that neutrophils emigrated into the alveoli without contacting E- or P-selectin, and despite inhibition of L-selectin, demonstrates that selectins are not required for pulmonary emigration of neutrophils in response to S. pneumoniae. These results are the first evidence that neutrophil emigration into the lungs can occur despite a deficiency or inhibition of all known selectins. The data contrast with several previous studies. Selectins are critical to maximal pulmonary emigration of neutrophils in response to alveolar deposition of IgG immune complexes in rats (15-18), bacterial LPS or IL-1 in rats (24), and phorbol ester in rabbits (25). S. pneumoniae is different from the above stimuli (IgG immune complexes, LPS, IL-1, and phorbol ester) in that it elicits neutrophil emigration that is independent of the integrins CD11/CD18 (4). In addition, the role of selectins in the lungs of mice may vary from their role in rats or rabbits, although we are aware of no studies demonstrating such differences. Finally, many of the growing number of mice with genetic deficiencies in adhesion molecules behave differently from WT mice acutely treated with adhesion molecule inhibitors, suggesting that alternative pathways may be used in the genetically deficient strains (26). Regardless, the present results establish that neutrophils can migrate to the lungs independently from selectins.

The lack of inhibition of acute neutrophil emigration in the lungs in response to S. pneumoniae contrasts with observations of peritonitis in E/P mutants, where acute (4 h) neutrophil emigration in response to S. pneumoniae was completely inhibited (20). These data are similar to observations made with P-selectin/ICAM-1 double mutant mice, in which peritoneal but not pulmonary neutrophil emigration in response to S. pneumoniae was inhibited at 4–6 h of infection (5). The data again demonstrate that adhesion molecule requirements critical for acute neutrophil emigration in the lung differ from those in the systemic vasculature.

The increased number of neutrophils in alveoli of E/P mice with pneumonia (as compared with the numbers in WT) is intriguing. This increase might be a direct result of lacking E- and P-selectins, for example if soluble selectins play anti-inflammatory roles (27, 28). Alternatively, the increased number of neutrophils/alveoli might secondarily result from physiological changes induced by the missing selectins, such as neutrophilia and increased margination within the pulmonary capillaries. Insight may be gained from previous studies with P/I mutants. These mice exhibit a comparatively mild neutrophilia (less than fivefold increase over WT, as opposed to 15-fold in E/P mutants) (5), and their pulmonary capillary pool of marginated neutrophils is not grossly exaggerated like that of E/P mutants (29). When P/I mutants are treated with anti-E-selectin Ab, pulmonary inflammation in response to bacterial instillation does not increase compared with that in pneumonia WT mice without Ab treatments (30). This suggests that the increase in neutrophil emigration observed in E/P mutants likely resulted from the extraordinary increase in peripheral blood and pulmonary capillary neutrophils rather than from the missing selectins per se. These data support the hypothesis that the size of the pool of marginated neutrophils within pulmonary capillaries, the primary site of neutrophil emigration (14), is a critical determinant of the extent of alveolitis.

During streptococcal pneumonia, the number of tethered and spread WBCs attached to the endothelium of noncapillary pulmonary microvessels increased in both E/P and WT mice. The increase in tethering and spreading observed in WT mice confirms that the morphometric technique is adequate to detect the increased rolling and firm adhesive interactions of neutrophils with endothelium induced by inflammatory stimuli. The increased tethering and spreading in E/P mice suggests that rolling and rolling-to-firm adhesion in pulmonary noncapillary microvessels can be amplified independently of selectins. Selectin-independent rolling has been demonstrated under experimental conditions that decrease shear stress (31). If selectin-ligand interactions are inhibited (by fucoidin administration), very little rolling occurs in rat mesenteric veins under normal flow conditions. However, if local blood flow is then decreased by partial closure of the vessel (reducing shear stress by 50%), WBC rolling increases severalfold, and this low flow-rolling is mediated by CD18 (31). Since arteriolar blood flow to rabbit lung regions with focal pneumonia is diminished by approximately 50% (32), shear stresses may be decreased and rolling in pulmonary noncapillary microvessels may be mediated by CD18. The fact that fucoidin completely eliminates rolling in uninjured pulmonary microvessels in the dog (12), but that fucoidin (and even fucoidin plus the deficiency of E- and P-selectins) does not inhibit rolling in pneumatic mouse noncapillary microvessels, further supports the notion that changes in blood flow resulting from pneumonia may affect neutrophil interactions with the endothelium.

Tethering and spreading of neutrophils in E/P mice were increased by streptococcal pneumonia, but these values did not differ from those of WT mice with pneumonia. In contrast, E/P mice with pneumonia had dramatic increases in all of the other neutrophil parameters measured (circulating counts, pulmonary capillary margination, and alveolar emigration) when compared with WT animals. It is unclear exactly what the expected values for tethering and spreading in E/P mice with pneumonia should have been. The lack of increase in tethered and spread cells compared with that in WT, despite a 15-fold increase in circulating neutrophils per ml, may result from a loss of selectin-mediated rolling in these vessels. The data certainly demonstrate selectin-independent tethering (see above), but they do not preclude an important role for selectin-dependent tethering during streptococcal pneumonia as well.
These studies establish that selectins are not required for neutrophil margination within pulmonary capillaries or for *S. pneumoniae*-induced emigration of neutrophils to alveoli. Leukocyte margination within noncapillary pulmonary microvessels is significantly mediated by selectins, but selectin-independent tethering and spreading is induced during streptococcal pneumonia.

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