EXPRESSION OF LEUKOCYTE-ENDOTHELIAL ADHESION MOLECULES DURING ACUTE INFLAMMATION IN THE LUNG

by

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THE UNIVERSITY OF BRITISH COLUMBIA

January 1993

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The University of British Columbia
Vancouver, Canada

Date April 19/93

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Neutrophil (PMN)-endothelial cell adhesion is a critical step in the response of PMNs to inflammation. In the systemic circulation, the leukocyte adhesion molecule, L-selectin, facilitates PMN adhesion to inflamed endothelium while CD11/CD18 is required for PMN emigration into extravascular tissues. An inducible endothelial ligand for CD18 is intercellular adhesion molecule-1 (ICAM-1). In the pulmonary circulation, PMNs emigrate by either a CD18-independent or CD18-dependent mechanism. The objective of this thesis was to quantitate and compare the surface expression of L-selectin, CD18, and ICAM-1 during CD18-independent and CD18-dependent emigration. Rabbits and mice received airway instillates of *Streptococcus pneumoniae* or *Escherichia coli* endotoxin to induce CD18-independent or CD18-dependent emigration, respectively. Ultrathin cryosections of frozen lung were immunogold labeled for L-selectin, CD18, and ICAM-1. Gold particles on the plasma membranes were quantitated by transmission electron microscopy. In rabbits, CD18-independent emigration was associated with L-selectin downmodulation and CD18 upmodulation on intravascular PMNs. A similar alteration of L-selectin and CD18 expression was observed during CD18-dependent emigration but only after PMNs emigrated into the interstitium. Alterations in L-selectin and CD18 expression were only observed on PMNs within the inflammatory focus. In mice, capillary endothelial ICAM-1 expression was unchanged during CD18-independent emigration. During CD18-dependent emigration, ICAM-1 expression increased 4.2-fold and this increase bordered on statistical significance, suggesting that the mechanism of adhesion may be regulated by the expression of endothelial rather than PMN adhesion molecules. ICAM-1 was also constitutively expressed on alveolar Type I but not Type II pneumocytes, the precursors
of Type I cells. During pneumonia, Type II but not Type I pneumocytes showed increased ICAM-1 expression, suggesting that ICAM-1 expression represents an early event in differentiation preceding proliferation. In vitro studies of unstimulated human PMNs showed that L-selectin was preferentially expressed on the PMN surface microvilli that mediate initial contact with endothelium. During transendothelial migration, L-selectin downmodulation is temporally correlated with PMN-endothelial contact. These studies describe the ultrastructural localization of adhesion molecules in normal and inflamed lungs and increase our understanding of the correlation between expression and function of adhesion molecules.
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<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>µg</td>
<td>Microgram(s)</td>
</tr>
<tr>
<td>60.3</td>
<td>Anti-CD18 MAb</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>C3bi</td>
<td>Split product of the third component of complement</td>
</tr>
<tr>
<td>C5a</td>
<td>Split product of the fifth component of complement</td>
</tr>
<tr>
<td>C5f</td>
<td>Fragment of the fifth component of complement</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation designation</td>
</tr>
<tr>
<td>CD11a/CD18</td>
<td>A leukocyte β₂-integrin</td>
</tr>
<tr>
<td>CD11b/CD18</td>
<td>A leukocyte β₂-integrin</td>
</tr>
<tr>
<td>CD11c/CD18</td>
<td>A leukocyte β₂-integrin</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cell line</td>
</tr>
<tr>
<td>COS</td>
<td>Monkey kidney cell line</td>
</tr>
<tr>
<td>CSLEX-1</td>
<td>Anti-sLex MAb</td>
</tr>
<tr>
<td>DREG-200</td>
<td>Anti-L-selectin MAb</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>E-selectin</td>
<td>Endothelial-selectin</td>
</tr>
<tr>
<td>g</td>
<td>Gram(s)</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>HL60</td>
<td>Myeloid/monocytic cell line</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>ICAM-2</td>
<td>Intercellular adhesion molecule-2</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram(s)</td>
</tr>
<tr>
<td>LAD</td>
<td>Leukocyte adhesion deficiency</td>
</tr>
<tr>
<td>LRP</td>
<td>Leukocyte-rich plasma</td>
</tr>
<tr>
<td>L-selectin</td>
<td>Leukocyte-selectin</td>
</tr>
<tr>
<td>LTB4</td>
<td>Leukotriene B4</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram(s)</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>NZW rabbit</td>
<td>New Zealand White rabbit</td>
</tr>
<tr>
<td>N₂</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>P-selectin</td>
<td>Platelet-selectin (also found on endothelial cells)</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-FCS</td>
<td>Phosphate buffered saline containing 5% fetal calf serum</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocyte (or neutrophil)</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td><em>Streptococcus pneumoniae</em></td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard Error</td>
</tr>
<tr>
<td>sLex</td>
<td>Sialylated Lewis X antigen</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>YN1/1/7/4</td>
<td>Anti-ICAM-1 MAb</td>
</tr>
<tr>
<td>ZAP</td>
<td>Zymosan activated plasma</td>
</tr>
<tr>
<td>ZAS</td>
<td>Zymosan activated serum</td>
</tr>
</tbody>
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DEDICATION

To my best friend and wife, Elizabeth Donnachie.
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1.1 INTRODUCTION: OVERVIEW

Inflammation can be broadly classified as being either acute or chronic. Chronic inflammation differs from acute inflammation not only in duration, but also in the nature of the response. By definition, chronic inflammation is persistent. It is generally, but not necessarily, preceded by acute inflammation and it is characterized by the histological presence of lymphocytes, monocytes, and plasma cells. Moreover, it is associated with a proliferation of fibroblasts and vascular endothelial cells (Cotran et al., 1989). In contrast, acute inflammation is of short duration lasting from minutes to several days (Ibid). In response to focal injury or infection, it is characterized by an increased passage of fluid and leukocytes, mainly polymorphonuclear leukocytes (PMNs), out of the blood and into the affected tissues (Lackie and Smith, 1980; Harlan 1985). The PMNs must first adhere to the vascular endothelium in preparation for emigration. This adherence is mediated by the complex interaction of surface adhesion molecules expressed on both the PMN and the endothelial cell (Smith, 1990). The expression of surface adhesion molecules can be modulated by the release of chemotactic mediators and inflammatory cytokines from injured tissues (Carlos and Harlan, 1990). The purpose of this thesis is to investigate the expression of adhesion molecules in the lung during acute inflammation.

The following introduction is intended to highlight those studies and observations on leukocyte biology and acute inflammation that prompted this investigation. The introduction will focus on some of the historical observations that led to the concept of endothelial- and leukocyte-dependent mechanisms of adhesion and emigration. This will be followed by a discussion of the proposed role of proinflammatory adhesion molecules on both the PMN and the endothelium. Evidence gathered from the literature will be used to show that PMN
emigration in the pulmonary circulation is fundamentally distinct from PMN emigration in the systemic circulation. Based on this distinction, a working hypothesis will be presented along with a statement of the specific aims of this thesis.

1.2 THE HISTORY OF LEUKOCYTE ADHESION AND EMIGRATION

1.2.1 Leukocytes adhere preferentially to veins, not arteries

The history associated with the study of leukocyte adhesion and emigration is a distinguished one with origins that can be traced back to the late 17th century and the discovery of the blood leukocyte by Antoni van Leeuwenhoek in 1669 (Schierbeek, 1962). The early investigators who studied the phenomenon of adhesion and emigration, did so without any knowledge of the existence of adhesion molecules. In fact, it is only within the last 10 years that adhesion molecules have been demonstrated to play a role in PMN adhesion and emigration (Harlan et al., 1992).

The Marquis Henri Dutrochet, is frequently cited as being the first investigator to describe the adherence and emigration of blood leukocytes in the year 1824 (Atherton and Born, 1972; Grant, 1973; Williams et al. 1984; Harlan, 1985; Harlan et al., 1992). However, a re-examination of the literature shows that Dutrochet was not the first observer of leukocyte emigration. In fact, by his own admission, he never actually observed the phenomenon of leukocyte emigration with which he is credited. To begin with, the evidence favouring Dutrochet as the discoverer of leukocyte emigration is based upon the following passage from his manuscript of 1824:

"... the vesicular globules contained in the blood are added to the tissues of the organs and become fixed there to augment and repair them so that nutrition consists of a
veritable intercalation of fully formed and extremely tiny cells. This opinion, though it may seem strange, is however well founded, since observation favours this view. Many times I have seen blood globules leaving the blood stream, being arrested and becoming fixed to the organic tissue. I have seen this phenomenon, which I was far from suspecting, when I observed the movement of the blood in the transparent tail of young tadpoles under the microscope... Observing the movement of the blood, I have seen many times a single globule escape laterally from the blood vessel and move in the transparent tissue... with a slowness which contrasted strongly with the rapidity of the circulation from which the globule had escaped. Soon afterwards, the globule stopped moving and remained fixed in the transparent tissue. A comparison with the granulations which this tissue contained showed that they were in no way different. There is no doubt that these semi-transparent granulations were also blood globules which had previously become fixed."[Dutrochet, 1824; italics added].

The "vesicular globules" have been interpreted as white blood cells (Grant 1973); however, this interpretation is probably incorrect and it is more likely that the blood globules, to which Dutrochet refers, were in fact red blood cells and not white blood cells. In his later (1837) memoirs on the subject, Dutrochet wrote:

"...The blood globules are composed of two parts, to be more specific: a central nucleus which is opaque and a semi-transparent and very delicate envelope which dissolves rapidly in water, and which contains the coloured material to which the blood owes its red colour..."[Dutrochet, 1837].

This is an excellent description of what we would recognize today as the nucleated red blood cell of a submammalian vertebrate, such as a tadpole (Andrew, 1965). More importantly, these later memoirs also contain Dutrochet's own admission that he had misinterpreted what he saw in 1824. He wrote:

"Some observations lead me to think that the blood globules are fixed to the organs by an intercalary aggregation, and it is these fixed globules that form the elementary globules that give form to all the organs; but this theory seems inadmissible to me today. I saw, it is true, often enough the blood globules suspending their movement and remaining fixed in the transparent organic tissue of the tails of tadpoles, but that observation probably resulted from those globules that were engaged in very small vessels; the point being that this wasn't a phenomenon of nutrition and growth." [Dutrochet, 1837; italics added].

It is clear that Dutrochet's final thoughts on the matter were that the blood globules (red
blood cells) were still within the microvasculature (capillaries) of the tadpole tail and had not emigrated into the tissues. Therefore, it seems unreasonable to continue to credit Dutrochet with the distinction of being the first investigator to observe leukocyte adhesion and emigration.

If Dutrochet was not the first investigator to observe leukocyte adhesion and emigration, then who was? A re-examination of the literature suggests that Albrecht von Haller rightfully deserves this distinction and that he made his observations almost 70 years before Dutrochet's publication of 1824. Von Haller documented the adhesion of blood cells to the inner wall of a mesenteric vein in a frog when he wrote:

"Experiment CXXIV. On four frogs. 22 July.

I followed with my eyes for a long time the movement of blood, in an artery and vein of considerable size: it was almost as swift in the vein as in the artery. It was similarly swift in the small vessels of the venous network, where the globules followed in single file and far enough apart that they were separated from each other. The globules stuck out along the edge of the veins, they gave rise to a half semicircular circumference, and took the shape of a string of beads, because the membrane of the veins is so thin as to become invisible: this same phenomenon does not exist in arteries, where the membranes are thicker." [von Haller, 1756; italics added].

It is well established that, during inflammation, leukocytes preferentially adhere to the veins and not arteries (for a review, see Harlan et al., 1992). While von Haller failed to describe the colour of these blood globules, his morphological description of blood globules sticking to veins and not arteries is reason enough to think that he was observing leukocyte adhesion. In 1757, in a dissertation on the movement of blood and the effects of hemorrhage in the mesenteries of various species of animals, von Haller described leukocyte emigration when he reported that:

"he was struck with the appearance of globules coating the veins like a chaplet of beads and the extravascular appearance of spherical and yellow cells." [From Grant, 1973; italics added].

Grant (1973) correctly points out that red cells appear pale yellow when viewed individually
and he therefore concludes that von Haller’s "yellow cells" were probably red blood cells and not leukocytes at all (Grant, 1973). However, von Haller may not have been describing the extravascular appearance of spherical yellow cells, but rather the extravascular appearance of spherical cells (white cells) and yellow cells (red cells). The amphibian white blood cell is spherical, but the red blood cell is flat and ellipsoid (Andrew, 1965). The suggestion that von Haller did not assign a colour to the spherical cells is in keeping with the idea that they have no colour. Indeed, years later, Cohnheim would refer to white blood cells as "colourless corpuscles" (Cohnheim, 1882).

In conclusion then, there is strong evidence to support the idea that von Haller described the adhesion and emigration of leukocytes from the mesenteric veins of frogs. This conclusion is important for two reasons. First, it gives von Haller priority on the point of being the first investigator to describe leukocyte adherence and emigration. Secondly, and more importantly, it establishes that the process of leukocyte adhesion and emigration preferentially occurs in the veins and not the arteries of the systemic circulation; an observation that is repeatable even to this day (Harlan et al., 1992).

1.2.2 The mechanism of leukocyte adherence and emigration

By the beginning of the 1840’s, the early English investigators had begun to use an experimental approach to show that leukocytes adhere to and emigrate from blood vessels in response to inflammatory stimuli (reviewed by Grant, 1973). However, Grant (1973) notes, that no explanation was forthcoming as to the mechanism of leukocyte adhesion and emigration until Cohnheim (1882) and Metchnikoff (1893) considered the problem.

Julius Cohnheim’s writings elegantly describe leukocyte adhesion and emigration in the
frog (mesentery and tongue) and rabbit (ear). Like von Haller, he draws the reader’s attention to the fact that leukocytes primarily and preferentially adhere to and emigrate from veins rather than arteries, although he acknowledges that they sometimes emigrate from small capillaries as well (Cohnheim, 1882). But perhaps Cohnheim’s most influential contribution to the study of inflammation was not what he saw but how he interpreted it. In 1882, he suggested that:

"Inflammation is the expression and consequence of a molecular alteration in the vessel walls...it is only and solely the vessel wall which is responsible for the entire series of events..." [Cohnheim, 1882].

This suggestion is remarkable in light of the fact that evidence of a molecular alteration would not be documented for almost another 100 years. However, Cohnheim’s theory is only partially correct; both the endothelium and the leukocyte are now known to undergo molecular alterations that contribute to the adhesion and emigration process (Smith, 1990). Interestingly, Cohnheim argued so strongly for the role of the endothelium in this process, that he considered leukocyte emigration to be the result of mechanical filtration. He did not believe that the leukocyte actively participated in adhesion or emigration.

In contrast, Elias Metchnikoff believed that the whole process of adhesion and emigration could be attributed to the activity of the leukocyte and he constructed elaborate and logical arguments to disprove Cohnheim’s theory about the role of the endothelium in this process. Metchnikoff believed that the accumulation of leukocytes at sites of inflammation was effected by their attraction (sensibility) towards a chemotactic substance, a theory first advanced by Leber in 1888, to whom he gives full credit. Metchnikoff wrote:

"...leukocytes are not sticky and do not become attached on account of their consistency but solely by means of their amoeboid properties." [Metchnikoff, 1893].

"...the leukocytes, led by their sensibility and by means of their amoeboid movements, themselves proceed towards the injured spot instead of passively filtering through a vessel-wall." [Metchnikoff, 1893].
Thus, by the end of the 19th century, the study of leukocyte adhesion and emigration was dominated by two seemingly incompatible theories. However, as Harlan and colleagues (1992) point out, it would turn out that both theories are correct and it is now appreciated that there are endothelial- and leukocyte-dependent mechanisms of adhesion and emigration.

Some progress was made in this direction in 1935 by Eliot and Eleanor Clark. In a study of inflammation in the tadpole tail and the rabbit ear they reported that:

"...individual leukocytes were seen to stick at localized points of a vessel wall, to approach such a point without sticking, and when dislodged by the blood stream to move on once more without sticking, demonstrated that a change in the endothelium itself is an essential preliminary to the sticking of leukocytes. Our studies showed also that although the migration of leukocytes through the vessel wall is carried out through the activity of individual leukocytes, a further change in the wall, beyond that of stickiness alone appears to be necessary before such cells can successfully penetrate the endothelium, as was shown that a reversal from this latter phase may occur so abruptly as to trap leukocytes in the act of emigrating, in addition to preventing further diapedesis of the leukocytes adherent to the inner wall." [Clark and Clark, 1935; italics added].

Their conclusions brought together Cohnheim’s views on the importance of an alteration in the constitution of the endothelium with Metchnikoff’s concept of the amoeboid nature of the leukocyte. Twenty years later, Allison and colleagues made the additional observation that the leukocytes also became more adhesive during the inflammatory response. They reported that:

"...during the course of the inflammatory reaction leukocytes were frequently seen to stick to one another, indicating that the increased adhesiveness characteristic of the inflammatory response is not limited to the endothelium." [Allison, et al., 1955; italics added].

The critical observation that the leukocyte also undergoes important adhesive changes that enable it to adhere to and migrate across an inflamed endothelium would have to await the discovery and characterization of leukocyte-endothelial adhesion molecules.
1.3 LEUKOCYTE-ENDOTHELIAL ADHESION MOLECULES

1.3.1 An introduction to the leukocyte-endothelial adhesion molecules

To facilitate the reading of this thesis, an adhesion molecule diagram is shown in Figure 1 and it illustrates the leukocyte-endothelial molecules that are considered to be important for PMN adhesion and migration during acute inflammation. It is hoped that this reference diagram will prove useful to the reader as one reads through the body of this thesis.

1.3.2 PMN adhesive glycoproteins that bind to endothelial cells

An intravital microscopy study in the rabbit mesentery showed that PMNs adhere to inflamed (activated) postcapillary and collecting venules in a two-step process (von Andrian et al., 1991). First, PMNs within the flowing blood make transient contacts (adhesions) with the vascular endothelium which causes them to slowly roll and slide along it (Atherton and Born, 1972, 1973; Arfors et al., 1987; von Andrian et al., 1991; Ley et al., 1991). In the second step of this process, the PMNs cease rolling and become more firmly attached to the endothelium. The firm attachment of PMNs to the endothelium is essential to PMN emigration and it is mediated by the leukocyte integrins (Harlan, 1985; see below). The rolling of PMNs along the venular endothelium is largely (80%) dependent upon the leukocyte adhesion molecule L-selectin (von Andrian et al., 1991; Ley et al., 1991).

L-selectin (also known as LECAM-1, Leu 8 Ag, MEL-14, Dreg-56, TQ1, gp90MEL, or LAM-1) is expressed on the surface of PMNs, lymphocytes, monocytes, and eosinophils (Lewinsohn et al., 1987) and it is a member of the selectin family of adhesion molecules (reviewed by Paulson, 1992). Selectins are a unique family of adhesion molecules...
characterized by the juxtaposition of an N-terminal C-type lectin domain, an epidermal growth factor (EGF) domain, and variable numbers of complement regulatory protein (CRP)-like repeating units (Siegelman et al., 1989; Lasky et al., 1989; Bevilacqua et al., 1989; Johnston et al., 1989). L-selectin is a 37 kD polypeptide and when expressed as a glycoprotein, it has a relative molecular mass (under reducing conditions) of 90-100 kD and 74 kD on PMNs and lymphocytes, respectively (reviewed by Kansas et al., 1991). L-selectin was originally identified in the mouse by the rat monoclonal antibody, MEL-14 (Gallatin et al., 1983); MEL-14 binds to the amino terminus of the lectin binding domain of L-selectin (reviewed by: Kansas et al., 1991 and Lasky, 1992). In vitro, MEL-14 specifically blocked the adherence of lymphocytes to frozen sections of the peripheral lymph node high venule endothelium, but not to Peyer's patches; in vivo, it inhibited lymphocyte migration into peripheral lymph nodes, and this suggested that the lymphocyte cell surface antigen (L-selectin) recognized by the MEL-14 antibody functions as a "homing" receptor for peripheral lymph nodes (Gallatin et al., 1983; Kansas et al., 1991). Significantly, the MEL-14 antibody also binds to PMNs suggesting that PMN L-selectin may allow PMNs to home into sites of acute inflammation (Lewinsohn et al., 1987). In vivo, the MEL-14 antibody reduced PMN migration into sites of acute inflammation in the peritoneum and skin of the mouse by 50-70% (Lewinsohn et al., 1987; Jutila et al., 1989, 1991). This reduced migration is likely related to an inhibition of PMN margination along the inflamed endothelium (Smith, 1992) as suggested by the in vitro findings that anti-L-selectin antibodies inhibit PMN adhesion to interleukin-1-stimulated (Smith et al., 1991) and endotoxin-stimulated (Abbassi et al., 1991) endothelial monolayers by 60% under conditions of flow. Moreover, anti-L-selectin antibodies did not inhibit the transendothelial migration of adherent PMNs in vitro (Smith et al., 1991; Abbassi et al., 1991).
Within seconds, L-selectin is rapidly downmodulated on the PMN cell surface following activation with a variety of inflammatory agents (Kishimoto et al., 1989a; Jutila et al., 1990, 1991; Smith et al., 1991; von Andrian et al., 1991). Downmodulation is caused by the rapid shedding of the L-selectin molecule from the cell surface (Kishimoto et al., 1989a). In mouse, the apparent molecular mass of the shed L-selectin molecule is only about 4 kD smaller than the plasma membrane-associated form of L-selectin (Ibid) suggesting that L-selectin is proteolytically cleaved at a site very close to the transmembrane domain of the molecule (reviewed by Lasky, 1992). Collectively, these observations predict that if after adhering to the vascular endothelium, PMNs become activated by tissue-based inflammatory stimuli, then L-selectin will be shed from the PMN cell surface. Significantly, PMN adhesion to and migration across an interleukin-1 (IL-1)-stimulated endothelial monolayer in vitro is associated with L-selectin downmodulation (shedding) (Kuijpers et al., 1992a, 1992b). IL-1 is an inflammatory cytokine that stimulates the endothelial monolayer to produce platelet activating factor (PAF) and interleukin-8 (IL-8) (Kuijpers et al., 1992a). Significantly, both PAF and IL-8 induce L-selectin downmodulation (Smith et al., 1991; Kuijpers et al., 1992a) and PMNs that have transmigrated an IL-1-stimulated endothelial monolayer are essentially L-selectin negative (Kuijpers et al., 1992a, 1992b). However, the transmigrated PMN is an endpoint and the dynamics of L-selectin shedding have not been studied in this model. It is unclear as to whether PMNs lose their L-selectin prior to, during, or shortly after transendothelial migration. Normally, PMNs begin transmigration within a few minutes after adhering to the IL-1-stimulated endothelium. However, when transendothelial migration is intentionally blocked, sustained (30-60 min) PMN adhesion to the IL-1-stimulated endothelium results in a significant, but incomplete, 4-fold loss of L-selectin from the PMN cell surface (Smith et al., 1991). To
what extent transendothelial migration is associated with L-selectin shedding remains to be determined.

Significantly, studies conducted both *in vivo* and *in vitro* show that the same activating agents that cause L-selectin shedding also induce an increased expression and functional upregulation in another class of PMN adhesion molecules. These adhesion molecules are known as leukocyte integrins and they appear to strengthen the adhesive interaction between activated PMNs and the activated endothelium (Arfors et al., 1987; von Andrian et al., 1991). Leukocyte integrins are essential for PMN emigration in the systemic circulation. This point is best illustrated by a group of patients that suffer from a rare disease known as leukocyte adhesion deficiency (LAD) (reviewed by: Kishimoto et al., 1989b and Arnaout, 1990a, 1990b). In these patients, PMNs fail to emigrate from the systemic circulation in response to infection or injury and despite having a chronic leukocytosis, these patients suffer from life threatening, recurrent bacterial and fungal infections (Ibid). The molecular basis for this disease is an inherited deficiency in the expression of a high molecular weight leukocyte surface protein (Crowley et al., 1980). In 1984, several investigators simultaneously reported that leukocytes from LAD patients are deficient in a class of heterodimeric proteins that are now referred to as the leukocyte integrins (Kishimoto et al., 1989b; Arnaout, 1990a, 1990b).

Leukocyte integrins are evolutionarily related to the integrin receptors that mediate cell-cell and cell-matrix adhesion (Kishimoto et al., 1989b; Arnaout, 1990a, 1990b; Hynes, 1992). The term integrin signifies that "...these are membrane receptors that integrate information from the extracellular environment (extracellular matrix or other cells) with the intracellular cytoskeletal network." (Kishimoto et al., 1989b). As many as twenty different integrins have been reported and all of them are $\alpha\beta$ heterodimers (Hynes, 1992). Integrins are found on a
wide variety of cell types and to date, 8 different β-subunits and 14 different α-subunits have been identified (Ibid). Most α-subunits only associate with one particular type of β-subunit (the exceptions are α4, α6, and αv) and based on the identity of the β-subunit, 8 subfamilies (β1 to β8) of integrins are currently recognized (Ibid). Most integrin subfamilies recognize extracellular matrix proteins (one exception is α4β1, which also recognizes an endothelial ligand known as vascular cell adhesion molecule-1 (VCAM-1)) and they are considered to be important in cell-substrate adhesion (Ibid). However, the leukocyte integrins (β2-integrins) are largely involved in the recognition of cell surface determinants and have a well established role in cell-cell adhesion (see below). The β2-integrin subfamily consists of 3 different heterodimers with a variable α-subunit non-covalently linked to a common β-subunit. The CD (cluster of differentiation designation) nomenclature assigned to each α/β subunit combination is CD11a/CD18, CD11b/CD18, and CD11c/CD18 (also known as LFA-1, Mac-1, and p150,95, respectively) (Kishimoto et al., 1989b; Arnaout, 1990b; Hynes, 1992). CD11a/CD18 and CD11b/CD18 are important in PMN adhesion and migration (reviewed by Smith, 1992). The basal expression of CD11a/CD18 and CD11b/CD18 on the PMN is sufficient for adhesion (Vedder and Harlan, 1988; Schleiffenbaum et al., 1989), but the total CD11/CD18 expression can increase 2-10 fold upon stimulation with PMN activating agents such as formyl peptides, phorbol esters, and the complement split product C5a (Arnaout et al., 1984; Bainton et al., 1987; Detmers et al., 1987). This rapid (minutes) increase in CD11/CD18 expression occurs through the exocytosis of an intracellular granular pool of CD11b/CD18 and CD11c/CD18 that is stored within the PMN (Arnaout et al., 1984; Springer et al., 1986; Bainton et al., 1987; Jones et al., 1988); the majority of this increase is thought to be accounted for by CD11b/CD18 (Bainton et al., 1987). The contribution of CD11c/CD18 to PMN adhesion and
emigration is largely unknown. Although endothelial cells stimulated with interleukin-1 (IL-1) or endotoxin for 18h show increased binding to purified CD11c/CD18 absorbed to plastic, minimal binding was observed when the CD11c/CD18 density fell to $< 1000$ binding sites/µm² (Stacker and Springer, 1991). The current evidence suggests that CD11c/CD18 plays a minor role in the process of PMN adhesion to endothelial cells (reviewed by Smith, 1992).

1.3.3 Endothelial adhesive glycoproteins that bind to PMNs

Stimulation of the vascular endothelium by inflammatory mediators and cytokines results in both transient (minutes) and sustained (hours) increases in endothelial adhesiveness for PMNs. Transient increases in endothelial adhesion are largely attributable to P-selectin, whereas sustained increases are effected by E-selectin and ICAM-1. P- and E-selectin are classed as vascular selectins and together with the leukocyte selectin, L-selectin, they comprise the three known members of the selectin family (reviewed by Paulson, 1992). ICAM-1, or intercellular adhesion molecule-1, belongs to the immunoglobulin supergene family (Staunton et al., 1988). The distinct roles played by P- and E-selectin and ICAM-1 in leukocyte-endothelial interactions will now be considered.

P-selectin (also known as PADGEM, GMP-140, and CD62) was first identified by a MAb to a surface membrane protein expressed on activated platelets, but subsequent studies have confirmed that it is also expressed by endothelial cells (reviewed by McEver, 1991). Immunocytochemistry of normal human tissues demonstrated that endothelial P-selectin is found in specialized endothelial cytoplasmic granules known as Weibel-Palade bodies (Bonfanti et al., 1989; Hattori et al., 1989a, 1989b; McEver et al., 1989). In vitro stimulation of human umbilical vein endothelial cells with thrombin, histamine, calcium ionophore A23187,
complement proteins C5b-9, or phorbol ester results in a rapid translocation of P-selectin onto
the cell surface (Hattori et al., 1989a, 1989b; Geng et al., 1990). The time course for
endothelial P-selectin surface expression is transient; following histamine stimulation, P-selectin
expression is maximal by 3 minutes and declines to near-basal levels by 20-30 minutes as a
result of endocytosis (Hattori et al., 1989b). Unstimulated PMNs rapidly bind to P-selectin
in vitro and this binding can be blocked by anti-P-selectin antibodies directed against the lectin-
binding domain of P-selectin (McEver, 1991) or by fluid phase P-selectin (Geng et al., 1990).
These data suggest that P-selectin expression by activated endothelial cells might promote rapid
targeting of unstimulated PMNs to sites of acute inflammation (Geng et al., 1990; McEver,
1991). As mentioned above, during an inflammatory response in the systemic circulation,
PMNs preferentially adhere to and emigrate from veins rather than arteries (von Andrian et al.,
1991). Significantly, immunoperoxidase localization of P-selectin shows that it is synthesized
by a wide variety of tissues and that the majority of P-selectin is found in small veins and
venules rather than arteries, arterioles, or capillaries (McEver et al., 1989). Although the role
of P-selectin in PMN adhesion and emigration in vivo has not yet been determined, it is
tempting to speculate that the characteristic expression of P-selectin by veins and not arteries
explains the PMN’s preferred adherence to these vessels. The PMN counter-receptor for P-
selectin is unknown (reviewed by Zimmerman et al., 1992), but a possible candidate for this
function is PMN L-selectin (Picker et al., 1991; see below).

E-selectin, also known as endothelial leukocyte adhesion molecule-1 (ELAM-1), was
first identified by MAbs generated against cytokine stimulated endothelial cells (Pober et al.,
1986a, 1986b; Bevilacqua et al., 1987); unstimulated endothelial cells do not express E-selectin
(Cotran et al., 1986; Munro et al., 1991). E-selectin is expressed on the surface of
endothelium only after the induction of E-selectin mRNA, an event that is preceded by
activation of the transcription factor NF-κB (Montgomery et al., 1991). A wide range of
inflammatory mediators have been shown to induce E-selectin expression and these include:
interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), lymphotxin, bacterial endotoxin, and
the neuropeptide, substance P (reviewed by: Bevilacqua and Gimbrone, 1990, Bevilacqua et
al., 1991, and Paulson, 1992). Following the exposure of endothelial monolayers to IL-1,
TNF-α, or endotoxin, E-selectin is on the endothelial surface by 1 h, is maximally expressed
by 4-8h, and is no longer detectable by 24-48h (Pober et al., 1986a, 1986b; Bevilacqua et al.,
1987). E-selectin expression on cultured endothelial cells supports PMN adhesion \textit{in vitro}
(Bevilacqua et al., 1987); however, studies on the role of E-selectin in PMN transmigration
have produced conflicting results (Smith, 1992). One study provides evidence that PMN
migration across an IL-1-stimulated endothelial monolayer is maximal at 4h and can be
inhibited by >90% with anti-E-selectin, anti-ICAM-1, or anti-CD18 antibodies. When anti-E-
selectin and anti-ICAM-1 antibodies were used together, they were found to have an additive
inhibitory effect on PMN adhesion (Luscinskas et al., 1991). Moreover, when the endothelium
was stimulated with IL-1 for 24h, a time-point when E-selectin expression is low and ICAM-1
expression is maximal, the level of PMN transendothelial migration had returned to baseline
(Ibid). These findings indicate that PMN adhesion is dependent upon both E-selectin and
ICAM-1 and that successful PMN transendothelial migration requires the participation of E-
selectin, ICAM-1, and CD18 (Ibid). However, in contrast to this study, several other studies
show that anti-E-selectin antibodies have little or no effect on PMN transmigration and that transmigration is only CD18- and ICAM-1-dependent (reviewed by Smith, 1992). The reason
for these different results is not known, but it may relate to technical differences between the
The most convincing argument that E-selectin does not play a role in PMN migration and that CD18/ICAM-1 interactions are more important is the finding that CD18-deficient PMNs migrate poorly, if at all, across a 4h endotoxin-stimulated endothelial monolayer, while 80% of normal PMNs successfully transmigrate the endothelium (Smith et al., 1988).

Immunohistochemical studies of human tissues from a variety of disease states have shown that E-selectin, like P-selectin, is preferentially expressed by venules (Cotran et al., 1986). In vivo, the time course of E-selectin expression appears to correlate with PMN adhesion and emigration. E-selectin was induced on subcutaneous venules in baboons 2 h after an injection of bacterial endotoxin into the skin; 2 h after the endotoxin injection, adherent and extravasated PMNs were associated with these venules (Munro et al., 1991). In response to an intraperitoneal injection of glycogen, PMN emigration was reduced (70%) in rats treated systemically with CL-3, an anti-E-selectin antibody (Mulligan et al., 1991). Moreover, CL-3 also reduced PMN emigration by approximately 50% into the skin and alveolar airspaces in response to IgG immune complex deposition (Ibid). Significantly, immunohistochemistry revealed that 3-4 h after IgG immune complex deposition in the lung, there was a striking upregulation of E-selectin not only in the pulmonary venules, but also in the alveolar capillaries (Ibid). Alveolar capillaries are an important site of PMN emigration in the lung (see below, Section 1.3.5). A functional role for E-selectin in acute inflammation in the lung is further strengthened by the finding that in a cynomolgus monkey model of extrinsic asthma, PMN emigration in the lung is blocked by pretreatment with the anti-E-selectin antibody CL-2, but not with the anti-ICAM-1 antibody RR1/1 (Gundel et al., 1991). The in vivo studies demonstrate that E-selectin plays a prominent role in acute inflammation; however, E-selectin
expression has also been detected in certain chronic inflammatory diseases including rheumatoid arthritis, psoriasis, and allergic cutaneous inflammation (reviewed by Paulson, 1992). The role of E-selectin in chronic inflammation is unknown, but it may be related to its ability to bind to a variety of different types of leukocytes including T-cells, monocytes, eosinophils, and PMNs (Ibid). The PMN counter-receptor for E-selectin is unknown (reviewed by Zimmerman et al., 1992), but one possible candidate is PMN L-selectin (Picker et al., 1991; see below).

ICAM-1, also known as CD54, was identified by Rothlein and colleagues during a study of phorbol ester induced lymphocyte aggregation. This type of aggregation is mediated by CD11a/CD18 and it does not involve an increase in CD11a/CD18 surface expression, showing that CD11a/CD18-dependent lymphocyte aggregation is regulated by some other mechanism (Rothlein and Springer, 1986). In addition, the mechanism does not involve like-like interactions between CD11a/CD18 adhesion molecules on adjacent cells because CD11a/CD18 deficient cells from LAD patients do not aggregate in the presence of phorbol ester, but they will aggregate with CD11a/CD18 positive cells (Ibid). This finding demonstrated that the ligand for CD11a/CD18 was on CD11a/CD18 deficient cells and therefore, monoclonal antibodies (MAbs) were raised against these cells and screened for their ability to inhibit CD11a/CD18-mediated aggregation (Rothlein et al., 1986). One MAb, designated as RR1/1, inhibited cell aggregation and identified a novel cell surface molecule with a molecular weight of 90kD (Ibid). This molecule was termed intercellular adhesion molecule 1 (ICAM-1) (Ibid).

Dustin and colleagues (1986) used the RR1/1 antibody to isolate and purify ICAM-1 from a variety of cell types. They found that the apparent molecular mass of ICAM-1 varied between 90 and 114 kD and that it was heavily glycosylated. Immunohistochemistry
established that both hematopoietic as well as non-hematopoietic cells expressed ICAM-1. ICAM-1 expression on dermal fibroblasts increased 3 to 5 fold after IL-1 or IFN-γ stimulation. Moreover, they found that this increase was dependent on both mRNA and protein synthesis. Of importance to the study of PMN adhesion and emigration was the observation that ICAM-1 is constitutively expressed on large and small blood vessels. A substantial body of evidence shows that ICAM-1 is upregulated on endothelial cells, both in vitro and in vivo, in response to inflammatory cytokines (IL-1, TNF-α, and IFN-γ) or endotoxin (reviewed by Lobb, 1992). In vitro, the time course for ICAM-1 expression initially parallels that of E-selectin and PMN adherence to and migration across an IL-1 stimulated endothelium is maximal at 4h (Smith et al., 1988, 1989; Luscinskas et al., 1991). ICAM-1 appears to mediate CD18-dependent adhesion and is essential to transendothelial migration (Smith et al., 1988, 1989), whereas E-selectin mediates CD18-independent PMN adhesion (Luscinskas et al., 1991) and, as mentioned above, there is some controversy over whether it plays a role (Ibid) or not (reviewed by Smith, 1992) in transendothelial migration. Unstimulated PMN adherence to ICAM-1 is mediated by CD11a/CD18, whereas PMNs stimulated with a chemotactic agent (e.g. formyl peptide) show enhanced adherence to ICAM-1 and this enhancement is mediated by the additional engagement of CD11b/CD18 with ICAM-1 (Smith et al., 1989). Significantly, in vivo studies in the rabbit mesentery have established a role for ICAM-1 in the rapid attachment of the PMN to the endothelium following superfusion of the mesentery with zymosan-activated serum (ZAS), a rich source of the complement split product C5a (Argenbright et al., 1991). This study showed that pretreatment with an anti-ICAM-1 antibody, or antibodies directed against the CD18 glycoprotein complex, inhibits ZAS-induced PMN adhesion (Ibid). Because of the short duration of their experiment (10 minutes) and their unpublished observation that exposure of
human umbilical endothelial cells to ZAS for 24h did not induce ICAM-1 expression, they suggest that basal expression of ICAM-1 is sufficient for stimulated PMN adhesion (Ibid). CD11a/CD18 and CD11b/CD18 bind to different regions of the ICAM-1 molecule. ICAM-1 has 5 tandem immunoglobulin domains (Staunton et al., 1988) and CD11a/CD18 binds to the first N-terminal domain while CD11b/CD18 binds to the third N-terminal domain (Staunton et al., 1990; Diamond et al., 1991). Similar observations on the related adhesion molecule ICAM-2 confirm the specificity and importance of these immunoglobulin domains. ICAM-2 contains only 2 immunoglobulin domains and these domains are 34% homologous to the first 2 immunoglobulin domains of ICAM-1 (Staunton et al., 1989); significantly, it binds only to CD11a/CD18 and not to CD11b/CD18 (Staunton et al., 1989; Diamond et al., 1990). Current evidence shows that although ICAM-2 is constitutively expressed on endothelial cells, it does not show enhanced expression in response to inflammatory stimuli (reviewed by Lobb, 1992; Staunton et al., 1989).

1.3.4 L-selectin as a counter-receptor for P- and E-selectin

A recent review article suggests that the PMN carbohydrate-bearing ligands for P- and E-selectin are unknown and that they still await characterization at the molecular level (Zimmerman et al., 1992). However, a potential ligand candidate for E-selectin is PMN L-selectin. The evidence is that blocking MAbs to L-selectin or E-selectin inhibit unstimulated CD18-deficient PMN binding to 3h IL-1 stimulated endothelium in vitro (Kishimoto et al., 1991). In combination, the effects of these blocking MAbs were not additive, implying that they inhibit a common adherence mechanism (Ibid). Formyl peptide stimulation of these CD18-deficient PMNs reduced the percentage of adherent cells from 74% to 29%, suggesting
that the PMN ligand for E-selectin was downmodulated (Ibid). Finally, normal unstimulated
PMNs bound avidly to E-selectin-transfected mouse fibroblasts and this binding was equally
inhibitable by either anti-E-selectin or anti-L-selectin blocking MAbs (Ibid). The mechanism
by which L-selectin binds to E-selectin was not determined. However, E-selectin (Bevilacqua
et al. 1987, 1989; Munro et al., 1989) and P-selectin (Moore et al., 1991, 1992) are thought
to facilitate PMN adhesion via recognition of sialylated derivatives of the Lewis X (sLex)
oligosaccharide (reviewed by Paulson, 1992). In fact, recognition of the sLex oligosaccharide
is a property shared by all three members of the selectin family, including L-selectin (Foxall
et al., 1992). The importance of the sLex oligosaccharides to selectin-mediated adhesion is
clearly illustrated in a recent report on two patients with a rare congenital defect in fucose
metabolism (Etzioni et al., 1992). These patients suffer from recurrent soft tissue infections
without the formation of pus (Ibid). The failure of PMNs to emigrate is likely explained by
the finding that these cells do not express the sLex (a fucosylated oligosaccharide) determinant;
in vitro, sLex-deficient PMNs do not adhere to E-selectin (Ibid). This type of leukocyte
adhesion deficiency (LAD) has been termed LAD II to distinguish it from LAD caused by a
deficiency in $\beta_2$-integrin expression (Ibid; see page 12).

Interestingly, P- or E-selectin transfected COS cells bind to purified PMN L-selectin
and not to purified lymphocyte L-selectin; this binding can be inhibited up to 70% by an anti-
L-selectin MAb (Picker et al., 1991). Similarly, when chymotrypsin was used to selectively
cleave PMN L-selectin from the cell surface, PMN adhesion to E-selectin transfected COS cells
was inhibited by 68% (Ibid). Significantly, PMN L-selectin, unlike lymphocyte L-selectin, is
modified with the sLex oligosaccharide and this observation suggested that PMN L-selectin
may "present" this carbohydrate to these vascular selectins (Ibid). However, L-selectin-
associated sLex accounts for less than 5% of the total cell surface sLex (Ibid); sLex is also expressed by a variety of glycolipids and glycoproteins found on the surface of the PMN (reviewed by Paulson, 1992). Collectively, these data imply that if L-selectin were to function as an important presenter of sLex to the vascular selectins, then sLex determinants on other cell surface molecules might be functionally less efficient or relatively inaccessible to the vascular selectins (Kishimoto et al., 1991; Picker et al., 1991). The investigation of this latter possibility, that L-selectin may have a unique topographical distribution on the surface of the PMN favouring its sLex presentation to the vascular selectins, is one of the specific aims of this thesis (see below).

1.3.5 PMN margination and emigration in the pulmonary circulation

As mentioned above, intravital microscopy studies in the systemic circulation have shown that PMNs can roll (i.e. marginate) along the vascular endothelium of post-capillary and collecting venules and this phenomenon is greatly increased during acute inflammation (Smith, 1992). L-selectin is largely responsible for this phenomenon (Arfors et al., 1987; von Andrian et al., 1991; Ley et al., 1991) that allows the PMNs to leave the blood stream and marginate along the vascular endothelium (Atherton and Born, 1972, 1973). Under conditions of flow and shear, both in vitro (Lawrence et al., 1990) and in vivo (Arfors et al., 1987; von Andrian et al., 1991; Ley et al., 1991) L-selectin makes a significantly larger contribution to PMN margination than CD18.

A different type of PMN margination occurs in the pulmonary circulation and the role of PMN adhesion molecules in this phenomenon is less clear. In the lungs of normal humans (Peters et al., 1985) and animals (Doerschuk et al., 1987; Martin et al., 1987) there is a large
pool of margined PMNs and this pool is estimated to contain 0.6-3 times the total number of circulating PMNs (Doerschuk et al., 1987). In vivo studies in animals have determined that the marginated pool of PMNs is located within the capillary bed (Doerschuk et al., 1987; Lien et al., 1987, 1990). Using direct in vivo microscopy, PMNs are observed to temporarily stop moving (marginate) at discrete locations within the pulmonary capillary network, rather than moving through the capillaries at constant rate (Lien et al., 1990). In humans, the diameter of the PMN ranges from 4.9 to 8.1 μm (Schmid-Schonbein et al., 1980a). In comparison, the diameter of the pulmonary capillaries ranges from 1 to 15 μm (Weibel et al., 1963) and approximately half of the capillaries have diameters smaller than that of the PMN (Hogg, 1987). Although, PMNs and red blood cells are similar in diameter (Schmid-Schonbein et al., 1980a), the PMN is approximately "...1000 times less deformable than the red blood cell and requires about 1 s to deform and enter a narrow capillary compared with 1/500 s for a red blood cell." (reviewed by Doerschuk et al., 1989). The pulmonary capillary bed consists of a dense network of short tubular segments (reviewed by Hogg, 1987). In a single pass through the lung, it has been estimated that a PMN negotiates approximately 100 tubular segments to travel from the pulmonary artery to the pulmonary vein (Ibid). Because of the large number of capillary segments and the size discrepancy between the PMN and lumen of the capillary, it has been suggested that PMN transit through the normal lung may be delayed, relative to the red blood cell, not because of the engagement of adhesion molecules, but because of the extra time required for the cell to deform and pass through the narrow capillary segments (Hogg, 1987; Doerschuk et al., 1987; Downey and Worthen, 1988). This hypothesis is supported by the recent observation that in the dog, the pulmonary capillary transit times of CD18-deficient PMNs are similar to those of normal PMNs (Yoder et al., 1990).
Previous work in our laboratory established that the normal marginated pool of PMNs in the rabbit lung is located primarily within the alveolar capillaries (Doerschuk et al., 1987). The mean diameter of the rabbit PMN is 6-7 µm (Schmid-Schonbein et al., 1980b; Beyers et al., 1989) and this is larger than the mean pulmonary capillary diameter of 5.8 ± 0.4 (SD) µm (Beyers et al., 1989). In fact, only 45% of the measured capillary diameters were greater than the mean PMN diameter (Ibid). As early as 1894, it was recognized that PMN sequestration in the rabbit lung could be enhanced by an intravenous injection of peptone (Bruce, 1894). Since this report, numerous investigators have shown, in other animal species and humans, that PMN sequestration in the lung is also enhanced by infusion of various chemotactic stimuli (reviewed by Harlan et al., 1992). In all cases, there is a rapid fall in the circulating PMN count with a return to normal after several hours (Ibid). Several investigators have suggested that the enhanced sequestration of activated PMNs in the lung is due to a decrease in PMN deformability (Hogg, 1987; Doerschuk et al., 1989; Worthen et al., 1989). Experimental data supporting this hypothesis include: the observation that rabbit PMNs are less deformable (stiffer) following activation with formyl peptide and this correlates with their increased retention in the lung (Worthen et al., 1989); in the rabbit lung, the transient PMN sequestration produced by an intravenous injection of formyl peptide was not blocked by pretreatment with an anti-CD18 MAb and is therefore, CD18-independent (Lundberg and Wright, 1990); the greatest number of PMNs sequester in the alveolar capillaries following an infusion of zymosan activated plasma (ZAP) or C5f (a fragment of the fifth component of complement) in rabbits (Doerschuk et al., 1989) and dogs (Lien et al., 1991), respectively.

However, decreased PMN deformability may not be the only mechanism of enhanced PMN sequestration. Systemic activation of complement in humans is associated with increased
(5-fold) expression of CD11b/CD18 on PMNs in vivo and it has been suggested that this increased CD18 expression may be related to PMN sequestration in the lung (Arnaout et al., 1985). Direct evidence for CD18 involvement in this process was provided by an in vivo videofluorescence microscopy study of the effect of ZAP infusion on PMN sequestration in dog lung (Yoder et al., 1990). This study showed that the pulmonary capillary transit time of normal dog PMNs is increased by ZAP infusion, but more importantly, that ZAP infusion had no effect on the transit time of CD18-deficient dog PMNs (Ibid). Additional studies in the rabbit suggest that ZAP induced PMN sequestration in the lung is a two-step process that probably depends on both decreased PMN deformability and increased PMN adhesion (Doerschuk, 1992). The data supporting this conclusion are that administration of the anti-CD18 antibody, MAb 60.3, did not prevent the initial fall in the circulating PMN count that occurs within 1 minute following ZAP infusion. Significantly, MAb 60.3 did prevent the sustained accumulation of PMNs in the lung that occurred between 7 and 15 minutes (Ibid). The data show that the initial fall in the circulating PMN count is CD18-independent and likely mediated by a decrease in PMN deformability. In contrast, sustained PMN sequestration in the lung may depend upon a transient increase in PMN adhesiveness that is CD18-dependent (Doerschuk et al., 1992). However, this study does not entirely exclude the possibility that the initial CD18-independent sequestration produced by ZAP may be due to the rapid and reversible induction of a CD18-independent adhesive process (Ibid). In vitro and in vivo data show that the CD18-independent adhesive processes are largely mediated by the selectin family of adhesion molecules (i.e. L-selectin, E-selectin, and P-selectin; see above). A role for L-selectin in normal or enhanced PMN margination in the lung has not yet been determined.

Transmission electron microscopic studies have clearly demonstrated that PMNs can
emigrate directly from alveolar capillaries during acute inflammation in rabbits (Walker et al., 1992), mice (Loosli and Baker, 1962), and dogs (Lien et al., 1991). Significantly, in response to an airway administration of C5f in the dog, 90% of PMN emigration in the lung was from capillaries rather than venules (Lien et al., 1991). This implies that, during acute inflammation, PMN emigration in the pulmonary circulation is fundamentally different from that in the systemic circulation (Ibid) where the major site of emigration is in the veins (Allison, Jr. et al. 1955; Harlan et al., 1992). Interestingly, PMN emigration in the pulmonary and systemic circulations may not just differ in the preferred site of emigration, but also in the preferred mechanism of emigration.

In the systemic circulation, PMN emigration towards all inflammatory stimuli examined to date is mediated by CD18 (Harlan et al., 1992). However, previous work in our laboratory established that PMN emigration in the lung is different and can occur through either a CD18-independent or CD18-dependent mechanism, depending on the nature of the inflammatory stimulus (Doerschuk et al., 1990a, 1990b). Specifically, the data showed that systemic administration of the anti-CD18 monoclonal antibody MAb 60.3 did not inhibit PMN emigration in response to an intrabronchial instillation of Streptococcus pneumoniae or hydrochloric acid, but it completely inhibited PMN emigration when these stimuli were instilled into the peritoneal cavity. Conversely, MAb60.3 largely inhibited PMN emigration into the rabbit lung in response to an intrabronchial instillation of phorbol ester, Escherichia coli organisms and E. coli endotoxin. The mechanism of CD18-independent PMN emigration in the rabbit is unknown, but it has been suggested that it may be due to the large number of alveolar macrophages within the lung (Mileski et al., 1990); S. pneumoniae may induce the release of a macrophage product that is chemotactic or proadhesive for PMNs (Ibid). The
evidence is that in the rabbit peritoneum, PMN emigration towards *S. pneumoniae* is normally CD18-dependent, but when the number of peritoneal macrophages was experimentally increased, a significant number of PMNs emigrated by a CD18-independent mechanism (Ibid). Importantly, PMN emigration towards *E. coli* organisms was CD18-dependent, even when the number of macrophages was increased (Ibid). This observation established that the presence of the macrophage is essential to the induction of CD18-independent emigration (Ibid). Moreover, it was proposed that macrophages might ingest streptococcal bacteria and release chemotactic or proadhesive bacterial degradation products (Ibid).

1.4 SUMMARY AND OBJECTIVES

In summary, there is a large body of evidence to suggest that PMN adherence to and emigration across endothelial cells is largely effected by three distinct classes of adhesion molecules, the selectins (P- and E-selectin on the endothelium and L-selectin on the PMN), the leukocyte integrins (CD11a/CD18 and CD11b/CD18) on the PMNs, and a member of the immunoglobulin super family (ICAM-1) expressed on the endothelium. In the systemic circulation, the PMNs preferentially adhere to and emigrate from post-capillary and collecting venules rather than arteries or arterioles. The initial adhesion of the PMN to the inflamed venular endothelium is thought to be mediated primarily by L-selectin on the PMN and P- and E-selectin on the endothelium; L-selectin functions to allow PMNs to leave the axial flow of the blood stream and marginate along the blood vessel wall. Although the endothelial ligand for PMN L-selectin at sites of acute inflammation has not been identified, *in vitro* data suggest that the PMN L-selectin contains an sLex oligosaccharide that can be "presented" to either P- or E-selectin. Once the PMN makes contact with the endothelium, local cytokine activation
promotes the shedding of L-selectin and the firm adhesion of the PMN; firm adhesion is necessary for PMN emigration, a process mediated by the CD18 glycoprotein complex. ICAM-1 functions as an endothelial ligand for both CD11a/CD18 and CD11b/CD18. In vivo, in the systemic circulation, PMN emigration towards all inflammatory stimuli examined to date is mediated by CD18.

In the pulmonary circulation, PMNs sequester (marginate) in the normal lung, but this margination appears to be determined by physical factors rather than leukocyte-endothelial adhesion molecules. During an inflammatory response, enhanced and sustained sequestration of PMNs in the lung is mediated by the CD18 glycoprotein complex. Whether other leukocyte-endothelial adhesion molecules, like L-selectin or ICAM-1, participate in this process is unknown. Depending upon the nature of the inflammatory stimulus, PMNs emigrate by either a CD18-dependent or CD18-independent mechanism. The mechanism of CD18-independent PMN emigration in the lung has not yet been determined, nor has a role for ICAM-1 in CD18-dependent or -independent PMN emigration been established.

The main objective of this thesis is to quantitate and compare the surface expression of L-selectin, CD18, and ICAM-1 during CD18-independent and CD18-dependent PMN emigration in the lung.

My working hypothesis is that, during an acute inflammatory response, the adherence of PMNs to the pulmonary microvasculature and their subsequent emigration into the alveolar airspace are dependent upon the complex interaction of cell surface adhesion molecules expressed on both the PMN and the endothelium. The principal site of PMN emigration during acute inflammation in the lung is the alveolar capillaries. PMNs that sequester in the capillary bed near the injured site are activated by the release of local inflammatory mediators
and cytokines. Some of these stimuli will also activate the capillary endothelial cells. Therefore, PMN emigration in response to inflammatory stimuli that evoke either a CD18-independent or CD18-dependent PMN emigration is associated with a downmodulation of L-selectin, an upmodulation of CD18, and an upmodulation of ICAM-1.

The method of approach used in this study entailed the successful application of monoclonal antibodies and colloidal gold markers to the ultrastructural localization and quantitation of L-selectin, CD18 and ICAM-1 in normal and acutely inflamed rabbit and mouse lungs. In addition, for comparative purposes, this method was used to study the in vitro expression of L-selectin on unstimulated human PMNs and on human PMNs engaged in transmigration of an IL-1-stimulated endothelial monolayer.

The Specific Aims of this thesis are:

1. To determine, in the rabbit lung, whether L-selectin expression is downmodulated and whether CD18 expression is upmodulated in response to stimuli that induce CD18-independent and CD18-dependent PMN emigration.

2. To determine, in the mouse lung, whether ICAM-1 expression is upmodulated in response to stimuli that are known to induce CD18-independent and CD18-dependent PMN emigration in the rabbit lung.

3. To determine, in vitro, whether L-selectin is topographically positioned on the human PMN surface such that it renders L-selectin-associated sLex more "bioavailable" for the vascular selectins P- and E-selectin.

4. To determine, in vitro, whether the process of human PMN transendothelial migration is associated with L-selectin downmodulation.
CHAPTER TWO
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2.1 SOURCES OF MATERIALS

2.1.1 Animals

New Zealand White (NZW) female rabbits (2-3 kg) were purchased from R & R Rabbitry (Stanwood, WA, USA) and BALB/c female white mice (18-20 g) were purchased from Charles River Canada Ltd. (St. Constant, Que., Canada).

2.1.2 Monoclonal antibodies

The four monoclonal antibodies used in the studies described in this thesis were received as generous gifts. The DREG-200 mouse monoclonal IgG antibody, against human L-selectin (Jutila et al., 1990; Kishimoto et al., 1990) and against rabbit L-selectin (von Andrian et al., 1991), was provided by Dr. E.C. Butcher (Stanford University School of Medicine, Stanford, CA, USA). The 60.3 mouse monoclonal IgG antibody, against human CD18 (Beatty et al., 1983; Wallis et al., 1986) and against rabbit CD18 (Lindbom et al., 1990), was provided by Dr. J.M. Harlan (University of Washington, Seattle, WA, USA). The YN1/1.7.4 rat monoclonal IgG antibody, against mouse ICAM-1 (Takei, 1985; Prieto et al., 1989), was provided by Dr. F. Takei (University of British Columbia, Terry Fox Laboratory, Vancouver, B.C., Canada). The CSLEX-1 mouse monoclonal IgM antibody, against human sLex (Picker et al., 1991), was provided by Dr. L. Picker (University of Texas Southwestern Medical Center, Dallas, TX, USA).
2.2 RABBIT PNEUMONIA EXPERIMENTS

2.2.1 Induction of pneumonia in the rabbit

Streptococcal or endotoxin pneumonias were induced in 2-3 kg NZW female rabbits (Doerschuk et al., 1990a; 1990b). Briefly, the rabbits were anesthetized with an intra-muscular injection of ketamine hydrochloride (Ketalean, MTC Pharmaceuticals, Cambridge, Ontario, Canada, 25-40 mg/kg) and acepromazine maleate (Atravet, Ayerst Laboratories, Montreal, Quebec, Canada, 2-3 mg/kg). The trachea was surgically exposed and a small incision was made just below the larynx. A narrow, flexible infant feeding tube (Argyle Feeding Tube, 3 1/2 FR X 12", X-ray opaque, Argyle, Division of Sherwood Medical, St. Louis, MO, USA) was inserted through the tracheostomy and manoeuvred into a distal bronchus of the right lung using fluoroscopy. A unilateral pneumonia was induced by instilling intrabronchially 0.15 ml/kg of either *Streptococcus pneumoniae* (10^9 organisms/ml saline, a clinical isolate provided by Microbiology, St. Paul’s Hospital, Vancouver, B.C., Canada, (n=3)) or endotoxin (*Escherichia coli* serotype 055:B5, Sigma, St. Louis, MO, USA, 10 μg/ml saline, (n=3)). Both inflammatory instillates contained 5% colloidal carbon (Pelikan, Fount India, Hannover, Germany) to mark the distribution of the instillate in the lung. Following instillation, all incisions were sutured and the pneumonias were allowed to develop for 4h. Control rabbits (n=3) received no anesthesia and no instillates.

2.2.2 Preparation of rabbit lung tissue for cryoultramicrotomy

At the end of the 4h treatment period, each rabbit received an intravenous overdose of pentobarbital. The chest and pericardial sac were opened and the base of the heart was ligated
to keep the pulmonary blood volume. The thoracic organs were removed and the right hilum was clamped with a hemostat. The left lung was then inflated at 20 cm H₂O pressure with 0.025% glutaraldehyde in ice cold phosphate buffered saline (PBS), which consisted (in mM) of 138 NaCl, 13.2 KH₂PO₄, and 53.2 Na₂PO₄ (pH 7.2), and the left hilum was clamped. Because previous studies in this laboratory have determined that approximately half of the alveolar space within the pneumonic region is filled with edematous fluid (Doerschuk, unpublished observation), a fixative instilled into this edematous region would be diluted by approximately 50%. To compensate for this dilutional effect, the right hilum was unclamped and inflated with ice cold PBS containing double strength (0.05%) glutaraldehyde and then clamped once more. Control rabbits were similarly treated except that the lungs were not clamped prior to instillation of fixative and both lungs were inflated with ice cold PBS containing 0.025% glutaraldehyde.

The lungs were fixed for 1h at 4°C and then cut into 2 mm³ tissue blocks. The pneumonic regions were identified by colloidal carbon staining and tissue blocks were taken from these "blackened" regions and fixed for an additional 1h at 4°C in 0.05% buffered glutaraldehyde. Comparable tissue blocks were also taken from control rabbit lungs and from the uninvolved, contralateral "non-blackened" lungs of each rabbit that had received an inflammatory instillate. These tissue pieces were fixed for an additional 1h at 4°C in 0.025% buffered glutaraldehyde. Following the fixation period, all lung tissue blocks were rinsed in ice cold PBS and then cryoprotected by immersion in PBS containing 2.3M sucrose overnight at 4°C.

The next day, cryoprotected lung tissue blocks (n=16 blocks from each control, pneumonic, and contralateral lung) were mounted on brass specimen pins and frozen in liquid
nitrogen (Section 2.6.2).

2.3 MOUSE PNEUMONIA EXPERIMENTS

2.3.1 Induction of pneumonia in the mouse

Streptococcal or endotoxin pneumonias were induced in BALB/c female mice (18-20 g, n=13) using a modification of the rabbit pneumonia protocol (Section 2.2.1). This modification was necessary for two reasons. The first is that the small size of the mouse, relative to the rabbit, precluded the possibility of catheterizing a distal bronchus and inducing a unilateral pneumonia. The second reason is that mice are approximately 1000 times more resistant to the effects of endotoxin than rabbits (Beutler et al., 1985). Therefore, three changes were introduced to the protocol as follows: 1) bilateral, rather than unilateral, pneumonias were induced by tracheal instillation, 2) the dose of *E. coli* endotoxin delivered to each mouse was increased approximately 1000X relative to the rabbit and, 3) the endotoxin pneumonias were allowed to develop over 24h instead of 4h because, even with the elevated dose of endotoxin, an appreciable pneumonia did not develop until 24h.

Mouse pneumonias were induced using the following protocol: BALB/c female mice (18-20 g, n=13) were anesthetised with halothane (Fluothane, Ayerst Laboratories, Montreal, Que., Canada), the trachea surgically exposed and a 25 gauge needle attached to a 1 c.c. syringe containing the instillate was inserted just below the larynx. A bilateral pneumonia was induced by instilling *S. pneumoniae* (0.1 ml, $10^9$ organisms/ml, n = 3) or *E. coli* endotoxin (0.1 ml, 2 mg/ml, n=3) mixed with 5% colloidal carbon in saline intratracheally. The neck incision was closed, but not sutured, and the mice were allowed to recover for 4h and 24h,
respectively. Control mice were similarly anesthetised and received sterile tracheal instillates containing 5% colloidal carbon in saline and were sacrificed 4h (n=2) and 24h (n=2) later. Additional controls included mice (n=3) that received no anesthesia and no instillates.

2.3.2 Preparation of mouse lung tissue for cryoultramicrotomy

At the end of the treatment period (4h or 24h), each mouse was killed with an overdose of halothane, the chest and pericardial sac were opened, and the thoracic organs were removed. The lungs were fixed by instilling ice cold PBS containing 0.4% glutaraldehyde down the trachea.

Fixation was allowed to proceed for 1h at 4°C, after which time the lungs were cut into 2 mm³ tissue blocks. The pneumonic lung regions were identified by colloidal carbon staining and tissue blocks were taken from these "blackened" regions and fixed for an additional 1h at 4°C in 0.4% buffered glutaraldehyde. In those control mice that received colloidal carbon instillates or no instillates at all, tissue blocks from "blackened" or "pink" regions of lung respectively, were taken and also fixed for an additional 1h at 4°C in 0.4% glutaraldehyde. Following the fixation period, all lung tissue blocks were rinsed in ice cold PBS and then cryoprotected overnight by immersion at 4°C in PBS containing 2.3M sucrose.

The next day, lung tissue blocks (n=20 blocks/mouse) were placed on filter paper strips (20 mm X 7 mm) cut from Whatman #1 (Whatman International Limited, Maidstone, Eng. UK) that had been pre-moistened in cryoprotectant (PBS containing 2.3M sucrose). Excess cryoprotectant was removed and the filter paper strips carrying the lung tissue blocks were immersed in liquid N₂ and rapidly frozen. The frozen filter strips were then transferred to Nalgene cryovials and stored under liquid nitrogen for up to two years prior to cryosectioning.
In order to prepare the lung tissue blocks for cryosectioning, they were retrieved from liquid nitrogen and rapidly thawed by immersion (with constant swirling) in room temperature cryoprotectant (PBS containing 2.3M sucrose). The thawed lung tissue blocks were then mounted on brass specimen pins as described in Section 2.6.2.

**2.3.3 Preparation of mouse lung tissue for light microscopy**

To verify that *S. pneumoniae* and *E. coli* endotoxin instillation caused PMN emigration into the alveolar airspace, five to ten lung tissue blocks fixed in 0.4% glutaraldehyde from each control and treated mouse were re-fixed in fresh PBS containing 2% glutaraldehyde for 1h. These tissue blocks were dehydrated through a graded alcohol series (30%, 50%, 70%, 90%, and 100% ethanol), infiltrated and embedded in glycol methacrylate (JB4 Embedding Kit, Polysciences, Warrington, PA, USA), sectioned at 2 μm on a Sorvall JB4 microtome (Porter-Blum) using a glass knife and stained with Toluidine blue O. Photomicrographs were made on a Zeiss Universal light microscope using Fujichrome Tungsten 64 ASA film.

**2.4 HUMAN LEUKOCYTE-RICH PLASMA EXPERIMENTS**

**2.4.1 Collection of human LRP**

Leukocyte-rich plasma (LRP) was obtained from adult human volunteers (n=3) using a modification (Doerschuk, 1987) of a method originally described by Boyum (1974). Briefly, to prevent coagulation, venous blood (24 ml) was drawn into acid citrate dextrose (ACD, 6 ml). Erythrocyte (RBC) sedimentation was induced by the addition of 25 ml of 4% dextran (100-200 kD) in PMN buffer, which consisted (in mM) of 138 NaCl, 27 KCl, 8.1 Na₂HPO₄,
and 5.5 glucose (pH 7.4). A sharp interface appeared between the sedimented RBC and the LRP within 30-40 min. The RBC fraction was discarded, and the LRP fraction was centrifuged at 1000 rpm (200 g) (Beckman Model TJ-6 centrifuge) for 8 min, the supernatant discarded and the leukocytes were resuspended in 1 ml of PBS. Because the LRP suspensions contained large numbers of PMNs that were easily identified by transmission electron microscopy, no further purification of the leukocytes was required.

2.4.2 Preparation of human LRP for cryoultramicrotomy

The resuspended LRP fraction was fixed in 0.05% buffered glutaraldehyde for 5 min at room temperature, washed in PBS, and resuspended in warm (37°C) PBS containing 2% agarose (Ultrapure, low melting point (<30°C), Bethesda Research Laboratories, Gaithersburg, MD, USA). After cooling to room temperature, the LRP-agarose gel was cut into 2 mm³ blocks and these blocks were then immersed in cryoprotectant (PBS containing 2.3M sucrose) overnight at 4°C.

The next day, cryoprotected LRP-agarose blocks (n=16 blocks/human subject) were mounted on brass specimen pins and frozen in liquid N₂ as described in Section 2.6.2.

2.5 HUMAN PMN TRANSENDOTHELIAL MIGRATION EXPERIMENTS

2.5.1 Preparation of acellular amniotic tissue for cell culture

Human amniotic tissue was prepared by a well established protocol in Dr. C.W. Smith’s laboratory at Baylor College of Medicine, Houston Texas (Furie et al., 1984; Furie and McHugh, 1989). In brief, placentas were obtained from vaginal and Cesarean deliveries. The
amnion was separated from the chorion by blunt dissection and fastened to Teflon rings (16 mm I.D., 22 mm O.D., 9.5 mm high) with Viton (vinylidene fluoride-hexafluoropropylene) O-rings (C.E. Conover, Fairfield, NJ, USA). The amniotic epithelium was removed by lysis with 0.25N NH$_4$OH for 1h at room temperature and then stored for up to one month at 4°C in HEPES-buffered saline (137 mM NaCl, 4mM KCl, 11 mM glucose, 10 mM HEPES, 500 U/ml penicillin, and 200 μg/ml streptomycin, pH 7.4). Just prior to seeding with human umbilical vein endothelial cells (HUVECs), the stored amniotic preparation was incubated in M199 (GIBCO Laboratories, New York, NY, USA) containing 20% fetal calf serum (FCS).

2.5.2 Culturing human umbilical vein endothelial cell (HUVEC) monolayers on prepared amniotic tissue

The endothelial cells were harvested from 5 to 10 human umbilical veins by collagenase perfusion according to Huang et al. (1988), and characterized as endothelial cells by the ability to bind acetylated low-density lipoprotein (Voyata et al., 1984), and to express factor VIII (Jaffe et al., 1973). Pooled cells were resuspended in M199 supplemented with 20% heat-inactivated fetal bovine serum (HyClone Laboratories Inc., Logan UT), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 μg/ml amphotericin B. Four or five days after plating, cells from confluent cultures were detached with 0.125% trypsin (ICN Biomedicals, Cleveland, OH, USA) and 1 mM EDTA in Ca$^{2+}$- and Mg$^{2+}$-free PBS, pooled and seeded on the stromal surface of the human amniotic tissue at a density of 1.5 X 10^4 cells; each ring contained 2 cm$^2$ of exposed tissue. The HUVEC cultures generally reached confluence in 2 days, and were used for PMN migration experiments from 7 to 10 days post-seeding. Confluence was determined by silver nitrate staining according to Furie et al. (1984).
2.5.3 Isolation of PMNs

On two separate occasions, PMNs were isolated from adult human volunteers (n=2) using established protocols (English and Andersen, 1974). Briefly, PMNs were isolated from heparinized (10U/ml) venous blood that was sedimented and centrifuged at 1000g for 30 min over Ficoll-Hypaque gradients. Contaminating erythrocytes were removed from the preparation by hypotonic lysis in 0.2% NaCl for 45 s. The PMNs were resuspended in cold Dulbecco’s PBS (Gibco Laboratories, Grand Island, NY) containing 0.2% dextrose at 4°C, pH 7.4 and added to the IL-1 activated HUVEC monolayer (Section 2.5.4) within 2h. The PMN suspensions were 95% pure and trypan blue exclusion showed greater than 97% viability.

2.5.4 Induction of PMN transmigration across HUVEC monolayers

HUVEC monolayers were treated with human monocyte derived interleukin-1 (IL-1, Genzyme Inc., Boston, MA, USA) for 3-4h, a time previously shown to be optimal for the enhancement of PMN adherence (Smith et al., 1988). The IL-1 was added directly to the media in which the monolayers were cultured at a final concentration of 3 U/ml. The monolayers (n=9 per experiment) were then rinsed with serum-free M199 at 37°C for 20 to 30 minutes (Lawrence et al., 1990) and the PMNs were added to the upper chamber (a total of 1 X 10^6 cells/2-cm² ring). This yields an expected ratio of 5 PMNs/endothelial cell at confluence (Huang et al. 1988). The PMNs were allowed to settle onto and migrate across each HUVEC monolayer for 5 min.

2.5.5 Preparation of transmigrated HUVEC monolayers for cryoultramicrotomy

At the end of the 5 min PMN transmigration period, the monolayer preparations were
fixed at 4°C with PBS buffer (pH 7.2) containing 0.1% glutaraldehyde for 15 min and then rinsed in ice cold PBS for 10 min. The HUVEC monolayer preparations were immersed in cryoprotectant (PBS buffer containing 2.3M sucrose) at 4°C overnight and then, on the next day, each HUVEC monolayer was detached from its Teflon ring using a pair of fine forceps, re-immersed in cryoprotectant and shipped on ice from Houston, Texas to Vancouver, British Columbia.

Upon arrival in Vancouver, the HUVEC monolayers were rapidly frozen by plunging them in liquid nitrogen. The frozen monolayers were stored in Nalgene cryovials under liquid nitrogen for up to one year. In order to prepare the monolayers for cryosectioning, they were retrieved from liquid nitrogen and rapidly thawed by immersion (with constant swirling) in room temperature cryoprotectant (PBS containing 2.3M sucrose). The thawed monolayers were then mounted on brass specimen pins as described in Section 2.6.2.

2.6 CRYOULTRAMICROTOMY

2.6.1 Fabrication of brass specimen pins for cryosectioning

In the rabbit study, all of the cryoprotected lung tissue samples were mounted on individual specimen pins (n=240), frozen in liquid N₂, and stored until they were retrieved for cryosectioning (Section 2.2.2). Additional specimen pins were also required for the mounting of mouse and human cryoprotected samples (Sections 2.3.2, 2.4.2, and 2.5.5). Since only 10 specimen pins were supplied with the cryoultramicrotome (RMC, Tuscon, AZ, USA), and because the cost of purchasing additional specimen pins was prohibitive ($12.00 each, US funds), another source of specimen pins was required. After much trial and error, the final
solution to the problem was to develop a "Specimen Pin Jig" (Figure 2) that could be used in the laboratory to produce inexpensive, brass specimen pins.

One-half inch (12.5 mm) long brass specimen pins were cut from 1/8 inch (3.2 mm) brazing rod using a metal hacksaw with 32 teeth/inch (13 teeth/cm) and a custom built aluminium "Specimen Pin Jig" (Figure 2). The jig was mounted in a table-top vice and the brazing rod was inserted into the jig up to the "swing-stop". The hacksaw blade was then inserted into the guide slot and the rod was cut. The newly cut pin was retrieved from the block by moving the swing-stop out of the way and pushing the pin forward with the remaining portion of the brazing rod. The swing-stop was then lowered and the operation repeated. Thirty specimen pins could be made in 15 minutes at a material cost of just under $1.00.

2.6.2 Mounting of cell and tissue samples to the brass specimen pins

Brass specimen pins were placed vertically into holes (3.3 mm diameter X 5 mm deep) that had been drilled into a modified nylon kitchen cutting board (70mm X 70mm X 10mm). Each cryoprotected specimen (lung tissue, agarose blocks, HUVEC monolayers) was trimmed under cryoprotectant into the shape of a pyramid on the nylon cutting board using a sharp razor blade. The trimmed specimen was then carefully positioned onto the specimen pin with its apex pointing away from the newly cut surface of the pin. Excess cryoprotectant was withdrawn with the ragged edge of a torn piece of filter paper (Whatman #1) leaving a thin film of cryoprotectant behind. The prepared specimen was then rapidly frozen at -196°C by plunging it into liquid nitrogen. The hacksaw blade, that was used to cut the specimen pins, imparted a serrated pattern to the mounting surface of the pin and these serrations strengthened the adhesive bond that formed between the specimen and the pin during freezing. Once frozen,
the mounted specimens were either cryosectioned immediately, or catalogued and maintained under liquid nitrogen in Nalgene cryovials (Nalge Company, Rochester, NY, USA) for up to three years before being retrieved for cryosectioning.

2.6.3 Cryosectioning and section retrieval

All cryoprotected, frozen specimens mounted on brass specimen pins were cryosectioned according to established procedures (Tokuyasu, 1973, 1978, 1983; Griffiths et al., 1984). Ultrathin (80-100 nm) cryosections were cut at -85°C (using a diamond knife on an RMC MT6000-XL ultramicrotome equipped with a CR2000 cryochamber (RMC, Tuscon, AZ, USA)), collected on cryoprotectant droplets (PBS containing 2.3M sucrose) and transferred to Formvar coated grids. The grids containing the cryosections were stored (30 min to 5 h, section side down) on PBS containing 5% fetal calf serum (PBS-FCS, 4°C) prior to immunolabeling. This procedure removes the sucrose and blocks potential non-specific antibody binding sites.

2.7 IMMUNOGOLD LABELING OF CRYOSECTIONS

2.7.1 Immunogold labeling of PMNs in rabbit lung

To determine the effect of *S. pneumoniae* and *E. coli* endotoxin instillation on L-selectin and CD18 immunoreactivity on rabbit PMNs, lung cryosections were immunogold labeled at room temperature with monoclonal antibodies (MAbs) against L-selectin and CD18. Cryosections that had been incubated in PBS-FCS (Section 2.6.3) were immunolabeled with either the primary mouse MAb DREG-200 (against L-selectin) or MAb 60.3 (against CD18)
at a concentration of 0.01 mg/ml in PBS-FCS for 30 min. Additional cryosections were cut and immunolabeled with non-immune mouse IgG (0.01 mg/ml in PBS-FCS) obtained from the Sigma Chemical Company (St. Louis, MO, USA) in place of the primary MAb for estimation of non-specific gold label. Cryosections were then washed in PBS-FCS (15 min) and immunolabeled with a secondary polyclonal goat-anti-mouse IgG (whole molecule) conjugated to 10 nm colloidal gold (Sigma Chemical Company, St. Louis, MO, USA) diluted in PBS-FCS ($A_{520} = 0.1$) for 30 min.

2.7.2 Immunogold labeling of mouse lung

To determine the effect of *S. pneumoniae* and *E. coli* endotoxin instillation on ICAM-1 immunoreactivity on endothelial, Type I, and Type II cells in the mouse lung, cryosections were immunogold labeled at room temperature with a MAb against ICAM-1. Cryosections that had been incubated in PBS-FCS (Section 2.6.3) were immunolabeled with the primary rat monoclonal antibody YN1/1.7.4 (against mouse ICAM-1) at a 1:100 dilution of the hybridoma supernatant in PBS-FCS for 30 min. To assess non-specific gold labeling, additional cryosections were incubated with non-immune rat IgG (0.01 mg/ml in PBS-FCS) obtained from the Sigma Chemical Company (St. Louis, MO, USA) in place of the MAb. Cryosections were then washed in PBS-FCS (15 min) and immunolabeled with a secondary polyclonal goat-anti-rat IgG (H + L) conjugated to 10 nm colloidal gold (Amersham Canada Limited, Oakville, Ont., Canada) diluted in PBS-FCS ($A_{520} = 0.1$) for 30 min.

2.7.3 Immunogold labeling of human LRP

To determine whether the topographical distribution of L-selectin is different than that
of CD18 and total sLex, ultrathin cryosections of glutaraldehyde fixed human LRP were
immunogold labeled at room temperature with MAbs against L-selectin, CD18 and sLex.
Cryosections of human LRP that had been incubated in PBS-FCS (Section 2.6.3) were
immunolabeled with mouse monoclonal antibodies MAb DREG-200 (against L-selectin, mouse
IgG), MAb 60.3 (against CD18, mouse IgG), or MAb CSLEX-1 (against sLex, mouse IgM)
at a concentration of 0.01 mg/ml in PBS-FCS for 30 minutes. To assess non-specific gold
labeling, additional cryosections were cut and immunolabeled with non-immune mouse IgG
(0.01 mg/ml in PBS-FCS) or the non-immune murine IgM myeloma protein TEPC-183 (Sigma
Chemical Company, St. Louis, MO, USA) in place of the primary MAb. Cryosections were
then washed in PBS-FCS (15 min) and immunolabeled with a secondary polyclonal goat-anti-
mouse IgG conjugated (whole molecule) to 10 nm colloidal gold (A<sub>s20</sub> = 0.1, Sigma Chemical
Company (St. Louis, MO)) in PBS-FCS for 30 min.

2.7.4 Immunogold labeling of transmigrating human PMNs

To investigate the possibility that L-selectin downmodulation is coincidental with PMN
adherence to an activated endothelium, cryosections of PMNs transmigrating an IL-1 stimulated
HUVEC monolayer were immunolabeled at room temperature with mouse MAb DREG-200
(against L-selectin) at a concentration of 0.01 mg/ml in PBS-FCS for 30 min. To assess non-
specific gold labeling, additional cryosections were cut and immunolabeled with non-immune
mouse serum (0.01 mg/ml in PBS-FCS) in place of the primary MAb. Cryosections were then
washed in PBS-FCS (15 min) and immunolabeled with a secondary polyclonal goat-anti-mouse
IgG (whole molecule) conjugated to 10 nm colloidal gold (A<sub>s20</sub> = 0.1, Sigma Chemical
Company (St. Louis, MO)) in PBS-FCS for 30 min.
2.7.5 Post-fixation and contrast enhancement of labeled cryosections

Thirty minutes after the incubation with the secondary polyclonal antibody-gold conjugate (Sections 2.7.2 to 2.7.4), all cryosections were washed in PBS (10 min), fixed in 1% glutaraldehyde in PBS (10 min) to immobilize the immunogold complexes (G. Griffiths, personal communication), washed in distilled water (20 min) and finally embedded and contrasted with 1.8% methylcellulose (25 cps, Sigma Chemical Company, St. Louis, MO, USA) containing 0.3% uranyl acetate (Polysciences, Warrington, PA, USA) in distilled water (10 min, 4°C).

2.8 IMMUNOELECTRON MICROSCOPY

2.8.1 Immunogold quantitation of L-selectin and CD18 on rabbit PMNs

An analysis of the surface immunoreactivity of L-selectin and CD18 on rabbit PMNs was carried out using a systematic, random sampling procedure. A minimum of two and a maximum of eight lung tissue blocks were randomly selected from the sixteen blocks that were available for each rabbit. These blocks were cryosectioned (Section 2.6), immunolabeled (Section 2.7), and viewed at a primary magnification of 1,650X on a Philips EM400 transmission electron microscope. At this magnification, gold particles were not visible but it was possible to identify colloidal carbon blackened areas and to recognize PMNs within the blood microvasculature, interstitium, and airspace of pneumonic lung tissue. Thus, without any knowledge of the gold labeling density on the cells, the first ten PMNs that were encountered in each lung region (microvasculature, interstitium, and airspace) were photographed at a magnification of 10,000-12,500X.
Gold particles were counted directly from the photographic negatives (magnification 10,000-12,500X). Each negative was placed on a light table and gold particles were viewed through a photographer's loupe (8X, Agfa) and manually counted. Gold particles were only counted on lengths of free plasma membrane; PMN plasma membrane surfaces in close contact with other cells or tissue components were not counted because of the potential for physical exclusion of the immunogold reagents (i.e. MAbs and colloidal gold). The plasma membrane lengths were measured on photographically enlarged (27,500X) prints using a digitizing tablet (SummaSketch II, Summagraphics, Seymour, CT, USA) connected to an IBM-compatible personal computer (Packard Bell, Model PB686, Korea) running Bioquant System IV software (R & M Biometrics, Nashville, TN, USA). The digitizing tablet was exactly calibrated on a daily basis using a similarly enlarged photographic image of an electron microscope diffraction grating. As an additional check for measurement accuracy and reproducibility, a randomly selected cell profile that had been previously measured was measured again at the beginning of each session. The difference between the two measurements was always less than 1%.

The gold label density on each PMN profile examined was calculated by dividing the number of gold particles counted by the length of plasma membrane examined. The amount of gold that was specific for L-selectin and CD18 was calculated by subtracting the amount of non-specific gold label on rabbit PMNs labeled with non-immune mouse IgG (0.3 gold particles/μm in the L-selectin study, for all PMNs; 0.1 gold particles/μm in the CD18 study, for all PMNs) from the amount of gold label on rabbit PMNs labeled with MAbs DREG-200 and 60.3. The change in L-selectin and CD18 immunoreactivity on the PMN plasma membrane, relative to the amount of specific gold label on control intravascular PMNs, was determined using a least squares estimate for the mean specific gold label in a given group.
The fold difference was taken to be the ratio of a treatment group's least squares estimate to the control group's least squares estimate (Mood et al., 1974).

2.8.2 Immunogold quantitation of ICAM-1 in mouse lung

An analysis of the surface immunoreactivity of ICAM-1 on mouse pulmonary endothelia and epithelia was carried out using a systematic, random sampling procedure. Two to three lung tissue blocks were randomly selected from the twenty blocks that were available for each mouse. The blocks were cryosectioned (Section 2.6), immunolabeled (Section 2.7), and viewed at a primary magnification of 1,650X on a Philips EM400 transmission electron microscope. At this magnification, gold particles were not visible but it was possible to identify regions of lung tissue in which the alveolar wall was not twisted or obscured by overlapping sections. In normal mice (no tracheal instillations), this process allowed random selection of alveolar wall segments without apriori knowledge of the gold labeling density. In mice that had received tracheal instillates, the lung regions were considered for gold quantitation only if colloidal carbon were detected in the alveolar airspace or within alveolar macrophages.

Gold particles were manually counted at 125,000X magnification using the electron microscope. At this magnification, the microvascular endothelial and alveolar type I and II epithelial cell surfaces were identified and gold particles were only counted on the plasma membrane lengths that were unobstructed and not in close contact with other cells or tissue components. This ensured that the cell surface being counted had been fully exposed to the immunogold reagents (i.e. MAbs and colloidal gold).

Enlarged (27,500X) photographic prints were made from the negatives and taped
together to construct photographic montages of each alveolar wall segment. Four to twelve different alveolar wall segments were photographed from each mouse. The plasma membrane lengths that had been gold counted in the electron microscope were measured on a calibrated digitizer connected to a personal computer (Section 2.8.1). Within each alveolar wall segment, the total length of plasma membrane was recorded for both the pulmonary capillary endothelium and the alveolar epithelium (Type I and II pneumocytes).

Data from the photographic montages were combined and the gold label density on each cell type was calculated by dividing the total number of gold particles counted by the total length of plasma membrane examined. The amount of gold label that was specific for ICAM-1 was calculated by subtracting the non-specific gold label obtained with non-immune rat IgG (0.2 gold particles/µm plasma membrane, for all endothelial and epithelial cells) from the gold label obtained with MAb YN1/1.7.4. The change in ICAM-1 immunoreactivity on endothelial and epithelial cell surfaces, relative to the amount of specific gold label on control mouse endothelial and epithelial cell surfaces, was determined using a least squares estimate for the mean specific gold label in a given group. The fold difference was taken to be the ratio of a treatment group’s least squares estimate to the control group’s least squares estimate (Mood et al., 1974).

2.8.3 Immunogold localization of L-selectin in human LRP

Two to three LRP-agarose blocks were randomly selected, out of a possible sixteen, from each of the three human subjects. These blocks were cryosectioned (Section 2.6), immunolabeled (Section 2.7), and viewed on a Philips EM400 transmission electron microscope. Approximately twenty PMNs were examined from each individual subject for
each MAb studied. Representative photomicrographs were taken at a primary magnification of 10,000-12,500X.

2.8.4 Immunogold localization of L-selectin on transmigrating human PMNs

Six tissue blocks were randomly selected from each of the two PMN transmigration experiments. These blocks were cryosectioned (Section 2.6), immunolabeled (Section 2.7), and viewed on a Philips EM400 transmission electron microscope. Every PMN that was encountered was photographed at a primary magnification of 10,000- to 12,500X.

2.9 STATISTICAL ANALYSIS

2.9.1 Determination of the minimum number of PMN profiles required to accurately estimate the mean gold label density within an animal

To determine whether a sample of ten PMNs from each lung region accurately represents an animal’s response to the treatment, it was necessary to perform a theoretical calculation. It was assumed that the ten PMNs examined within a particular lung region (e.g. intravascular, interstitial, or airspace) represent a random sample of the total number (population) of PMNs within that lung region. An important concept in statistics (Glantz, 1987; Zar, 1984) is that the standard error (SE) associated with the sample mean decreases, relative to the standard deviation (SD) of the population, as the sample size (n) increases according to the equation

\[ SE = \frac{SD}{\sqrt{n}} \]
This equation can be re-arranged to yield

\[
\frac{SE}{SD} = \frac{1}{\sqrt{n}}
\]

The magnitude of SE/SD decreases as a function of the square root of \( n \). Therefore, an increase in the sample size causes a decrease in the magnitude of SE/SD which is indicative of an increase in the accuracy with which the sample mean reflects the population mean. This is graphically illustrated below:

The ratio of SE/SD decreases (increasing accuracy of the mean) rapidly from 1.0 to 0.3 as the sample size increases from 1 to 10. Because the standard error is influenced by the square root of the sample size, doubling the sample size to 20 would only marginally improve the accuracy of the mean (SE/SD ratio falls from 0.3 to 0.2). Actual gold labeling data values were used to confirm this prediction (data obtained from the L-selectin study of intravascular
PMNs within the pneumonic lung of one rabbit 4h after a bronchial instillate of *E. coli* endotoxin) and the results are shown below:

<table>
<thead>
<tr>
<th># PMN (n)</th>
<th>mean (gold particles/μm)</th>
<th>SE</th>
<th>SD</th>
<th>Predicted SE/SD</th>
<th>Actual SE/SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.2</td>
<td>0.4</td>
<td>1.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>20</td>
<td>1.1</td>
<td>0.3</td>
<td>1.3</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Notice that the mean, SE, and SD change very little (or not at all) as a result of increasing the number of PMNs (n) from 10 to 20. Importantly, the actual and predicted SE/SD ratios are in agreement. Based on these findings, an examination of 10 PMNs from each lung region was considered sufficient and appropriate for estimating each animal’s response to the treatment.

2.9.2 Determination of intra- and interobserver error associated with gold particle counts

To determine the intraobserver error associated with the counting of gold particles, 20 photographic negatives of L-selectin and CD18 immunogold labeled rabbit PMNs were randomly selected. The gold particles on the cell profiles were re-counted and these new counts were compared with the original counts using a least squares linear regression. Correlation analysis of these data resulted in an *r* value of 0.98 (*P* < 0.0001). This result showed that gold particle counts obtained by this observer (AB) were highly reproducible.

To determine the interobserver error associated with the counting of gold particles,
a separate set of 20 photographic negatives of L-selectin and CD18 immunogold rabbit PMNs were randomly selected. The second observer (MK) was asked to count the gold particles on the cell profiles and then these counts were compared with those of the first observer (AB) using a least squares linear regression. Correlation analysis of these data resulted in an $r$ value of 0.91 ($P < 0.0001$) that showed that the gold particle counts between observers were highly correlated.

2.9.3 Statistical analysis of L-selectin and CD18 immunoreactivity on rabbit PMNs

Parametric statistical methods were used to analyse the data; the data are summarized as mean $\pm$ SD. There are two underlying assumptions for the use of parametric statistics. The first assumption is that the variance between the groups must be approximately equal (Glantz, 1987; Zar, 1984) and therefore, in this study, a mathematical transformation was performed on each data set as recommended by Zar (1984). For the L-selectin study, each data point ($x$) was transformed by the mathematical expression

$$\frac{1}{(x+0.5)}$$

whereas, a log transformation was performed on the CD18 data set. The second assumption is that the data are normally distributed. In this study, The transformed data sets were checked for normality using a quantile plot. A quantile plot displays the relative fraction of data versus the response values. By way of illustration, the quantile plot of the transformed CD18 data is shown on the next page:
In this graphical analysis, the exposure group mean was subtracted from each animal's response. This forces the data to consist of a set of responses all centred about a zero mean value. If the entire data set, from all of the groups, is normally distributed then a quantile plot will display an "S-shaped" or "ogive" curve, and this quantile plot is clearly S-shaped (Wilkinson, L. 1990).

In both the L-selectin study and the CD18 study, a one-way ANOVA was used to detect differences in specific gold label on PMNs in the various regions (intravascular, interstitial, airspace, contralateral) of the lungs of rabbits that received no instillate, S. pneumoniae, or E. coli endotoxin. Statistical significance for differences between groups was accepted at $P < 0.05$, with corrections made for multiple comparisons by the Duncan Multiple Range test. Calculations were done using SAS version 6.03, (SAS Institute, Inc., Cary, NC) on a 386 computer with a numeric coprocessor.
2.9.4 Statistical analysis of ICAM-1 immunoreactivity in the mouse lung

The data are summarized as mean ± SD. To make use of parametric statistical tests, the variance between the groups was made approximately equal by a mathematical transformation of each data set as recommended by Zar (1984). To compare ICAM-1 immunoreactivity on different cell types (endothelial, Type I, and Type II) within the control mice that received no instillate, each data point \( x \) was transformed using the mathematical expression

\[
\frac{1}{(x + 1.0)}
\]

whereas, the equation used to compare ICAM-1 immunoreactivity on Type I pneumocytes after different exposures was

\[
\sqrt{x}
\]

Finally, the mathematical transformation used to compare ICAM-1 immunoreactivity on endothelial cells, as well as ICAM-1 immunoreactivity on Type II pneumocytes, after different instillate exposures was

\[
\frac{1}{(x + 0.5)}
\]

A quantile plot (see Section 2.9.3) of each transformed data set confirmed that each set was normally distributed (Wilkinson, L. 1990).

Four different one-way ANOVAs were used to detect overall differences in specific gold particles/\( \mu \)m plasma membrane. The first ANOVA tested for differences between endothelial, Type I and Type II pneumocytes in control mice. The second ANOVA tested for differences between endothelial cells exposed to different instillates. The third ANOVA tested for differences between Type I pneumocytes exposed to different instillates. Finally, the fourth ANOVA tested for differences between Type II pneumocytes exposed to different
instillates. Because there is an increased chance of a Type I error when using "multiple" ANOVAs, a Bonferroni-like correction was employed to minimize this possibility. As a result, the overall statistical significance for differences between groups was accepted at $P \leq 0.05/4$ (i.e. $P \leq 0.0125$) with corrections made for multiple comparisons by the Duncan Multiple Range test. Calculations were done using SAS version 6.03, (SAS Institute, Inc., Cary, NC) on a 386 computer with a numeric coprocessor.
CHAPTER THREE
RESULTS

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3.1 ELECTRON MICROSCOPIC OBSERVATIONS OF PMNs IN RABBIT LUNG

3.1.1 Morphology of intravascular PMNs

The general ultrastructural appearance of intravascular PMNs in the lungs of control rabbits was not different from that of intravascular PMNs in rabbit lungs exposed to colloidal carbon mixed with *S. pneumoniae* or *E. coli* endotoxin. The PMNs were easily distinguished from the other circulating blood leukocytes (i.e. mononuclear cells, eosinophils and basophils) by their large segmented nucleus that contained characteristic accumulations of heterochromatin associated with the inner nuclear membrane (Figure 3). The cytoplasm of the PMN, in comparison to the other blood leukocytes, was very electron dense and contained large numbers of electron translucent, membrane-bound granules. Few mitochondrial profiles were present and the Golgi apparatus was small and rarely encountered.

The shape of the intravascular PMNs was influenced by the size of the blood vessel. When the diameter of the blood vessel lumen was large, relative to the size of the PMN, the PMN was generally spherical in shape (Figures 3). The PMN was usually positioned at some distance from the endothelium and its plasma membrane was frequently ruffled due to the presence of numerous small microvillar projections. However, when the diameter of blood vessel lumen was small, relative to the size of the PMN, the PMN had an elongated shape and the contour of its plasma membrane was smooth and closely apposed to the endothelium lining the vessel (Figure 4).

3.1.2 Morphology of interstitial PMNs

Within the alveolar wall, an interstitial region separated the endothelium of the
microvasculature from the alveolar epithelium (Type I and II cells) that lines the alveolar space. In the control rabbit lung, this interstitium consisted of a loose array of collagen fibres, elastin and connective tissue cells. Four hours after the instillation of *S. pneumoniae* or *E. coli* endotoxin mixed with colloidal carbon, PMNs were found within the interstitium of the pneumonic lung, but never within the interstitium of the contralateral lung. Interstitial PMNs were irregular in shape (Figure 5) and often had cytoplasmic projections (pseudopods) that extended out into the connective tissue matrix. The cells were usually surrounded by an electron translucent region in which no interstitial matrix components (collagen or elastin) could be detected.

3.1.3 Morphology of airspace PMNs

Polymorphonuclear leukocytes (PMNs) were never observed within the alveolar airspace of control rabbits. However, airspace PMNs were frequently encountered in the pneumonic lungs, but not the contralateral lungs, of rabbits exposed to *S. pneumoniae* (Figure 6) or *E. coli* endotoxin (Figure 7) mixed with colloidal carbon for 4h. In both instances, the airspace PMNs were generally spherical in shape and showed extensive ruffling of the plasma membrane. In some cases, colloidal carbon particles were associated with the plasma membrane surface, but only rarely were these particles ingested. Unlike the endotoxin instillate, the streptococcal instillate contained live bacteria. These bacteria were frequently ingested by the airspace PMNs and, in some cases, the PMNs contained so many phagocytosed bacteria that the PMN cytoplasm was almost entirely obscured (Figure 8). This contrasts sharply with the behaviour of the resident alveolar macrophages. Alveolar macrophages ingested more colloidal carbon than the airspace PMNs and, in the case of the streptococcal instillate, they showed no
evidence of bacterial phagocytosis (Figure 9).

3.2 IMMUNOELECTRON MICROSCOPY OF PMNs IN RABBIT LUNG

3.2.1 Summary of morphometric data on rabbit PMNs

The total number of gold particles counted was 12,617 in the L-selectin study and 69,578 in the CD18 study (n=9 rabbits). The length of PMN plasma membrane examined was 5.0 mm in the L-selectin study and 3.4 mm in the CD18 study (n=9 rabbits). For both studies, the mean length of plasma membrane examined on intravascular PMNs tended to be less than that examined on interstitial and airspace PMNs (Tables 1 and 2). This finding may relate to the observation that intravascular PMNs were occasionally in close contact with the endothelium (Figure 4). As mentioned earlier (Section 2.8.1), those portions of the PMN membrane in close contact with the endothelium were not measured and thus, shorter lengths of plasma membrane were recorded for these PMNs. Alternatively, if degranulation accompanies PMN emigration then this may increase the surface area and the surface:volume ratio of interstitial and airspace PMNs.

3.2.2 Localization and quantitation of L-selectin & CD18 immunoreactivity in control rabbit PMNs

L-selectin immunoreactivity was detected only on the plasma membrane of PMNs; there was no detectable intracellular pool of L-selectin. Two distinct topographical distributions of gold particles were observed on the cell surface of L-selectin positive PMNs. When the PMN surface displayed microvillar processes, L-selectin immunoreactivity was consistently localized
to these microvilli and not to the "flat" regions of the plasma membrane between the microvilli (Figure 10). When the PMN surface lacked microvillar processes and was relatively smooth, the gold particles appeared to be grouped into small clusters and these clusters were detected over the entire surface of the PMN (Figure 11).

By comparison, only one topographical distribution of gold particles was observed on CD18 positive rabbit PMNs. The gold particles were found along the entire length of the plasma membrane and showed no preference for microvillar processes. In addition, unlike the immunolocalization of L-selectin, CD18 immunoreactivity was frequently detected in many of the cytoplasmic granules within the PMN (Figure 12).

On control rabbit PMNs, L-selectin immunoreactivity was much more variable than CD18 immunoreactivity (5.6 ± 3.2 (SD) vs 8.6 ± 1.8 gold particles/µm, in three rabbits); the coefficient of variation for L-selectin and CD18 immunoreactivity was 57% and 21% respectively.

3.2.3 Effect of pneumonia on L-selectin cell surface immunoreactivity on rabbit PMNs

Four hours after the instillation of *S. pneumoniae*, L-selectin immunoreactivity on intravascular (Figures 13 and 14) and airspace PMNs (Figure 15) was reduced by 78% and 99%, respectively, when compared to control intravascular PMNs (Table 3, each P < 0.05). The L-selectin immunoreactivity on the pneumonic intravascular PMNs was extremely variable; the coefficient of variation was much greater compared to control PMNs (167% vs. 57%). L-selectin positive intravascular PMNs were detected in only one of the three pneumonic rabbit lungs examined. From this lung, two tissue blocks were examined and all of the L-selectin positive cells came from one tissue block (Figure 13); the other tissue block was L-selectin
negative. Of the 30 PMNs examined from the three pneumonic lungs, 24 of them showed negligible ($\leq 0.3$ gold particles/μm) or no L-selectin gold label (Figure 14). Because L-selectin immunoreactivity was negligible on intravascular PMNs from two of the three rabbits, L-selectin immunoreactivity was only determined on interstitial PMNs from the pneumonic lung of the one rabbit which had L-selectin positive intravascular PMNs. Nine of the 10 interstitial PMNs examined came from the one tissue block that had L-selectin positive intravascular PMNs. Despite a high coefficient of variation (367%) L-selectin immunoreactivity on these interstitial PMNs (Figure 16) was significantly less than that on intravascular PMNs from the same tissue blocks (0.3 ± 1.1 vs. 2.5 ± 2.7, in 10 PMNs, unpaired $t$ test, $P < 0.05$). Streptococcal pneumonia in the right lung had no effect on L-selectin immunoreactivity on intravascular PMNs in the contralateral left lung (Figure 17); L-selectin immunoreactivity on these contralateral PMNs was not different than that on control intravascular PMNs (Table 3).

Four hours after the instillation of *E. coli* endotoxin, L-selectin immunoreactivity on intravascular PMNs within the pneumonic right lung or contralateral left lung (Figure 18) was not different from that on control intravascular PMNs (Table 3). However, L-selectin immunoreactivity on PMNs that had emigrated into the interstitium (Figure 19) and alveolar airspace (Figure 20) was reduced by 86% and 98%, respectively, compared to intravascular control PMNs (Table 3, each $P < 0.05$).

3.2.4 Effect of pneumonia on CD18 cell surface immunoreactivity on rabbit PMNs

Four hours after the instillation of *S. pneumoniae*, CD18 immunoreactivity on PMNs in each region of the pneumonic lung was significantly greater than that on control
intravascular PMNs (Table 4). Within the pneumonic lung, CD18 immunoreactivity on intravascular (Figure 21) and interstitial PMNs (Figure 22) was virtually identical and 2.6 times greater than that on control intravascular (Figure 12) PMNs (each P < 0.05). The maximum increase in CD18 immunoreactivity was observed on those PMNs that had emigrated into the alveolar airspace (Figure 23). The level of CD18 immunoreactivity on these PMNs was 5.5 times greater than that on the control intravascular PMNs (Table 4, P < 0.05). In addition, the gold label density on these airspace PMNs was significantly greater than that on the intravascular and interstitial PMNs (each P < 0.05). CD18 immunoreactivity on intravascular PMNs in the contralateral lung (Figure 24) was not different from that on control intravascular PMNs (Table 4; compare Figures 12 and 24).

Instillation of *E. coli* endotoxin, in contrast to *S. pneumoniae*, did not result in upmodulation of CD18 immunoreactivity on the intravascular PMNs compared to control intravascular PMNs (Table 4, P = 1.0). However, CD18 immunoreactivity was increased 2-fold on interstitial PMNs (Table 4, P < 0.05; Figure 25) and 3.2-fold on airspace PMNs (Table 4, P < 0.05; Figure 26) when compared to that on control intravascular PMNs. Because CD18 immunoreactivity was similar on intravascular PMNs in the pneumonic lung region and in the control lung region, CD18 immunoreactivity on PMNs in the contralateral lung was not quantitated.

### 3.3 LIGHT MICROSCOPY OF MOUSE LUNG TISSUE

Light microscopy showed that PMNs did not emigrate into the alveolar airspace during the 4 or 24h period following intratracheal instillation of a sterile saline solution of colloidal carbon. Lung tissue sections from mice that received colloidal carbon in saline for 4h (Figures
27 and 28) or 24h (Figures 29 and 30) did not differ from those of control mice that received no instillates except for the presence of ingested carbon particles within the cytoplasm of the alveolar macrophages.

In contrast, large numbers of PMNs were found in the alveolar airspace 4h after intratracheal instillation of *S. pneumoniae* mixed with colloidal carbon (Figures 31 and 32). In some alveoli, an equally large number of extravasated red blood cells could also be seen (Figure 32). The presence of red blood cells in the airspace was unique to streptococcal pneumonia as it was never observed in the lungs of mice instilled with *E. coli* endotoxin. Streptococcal bacteria were frequently ingested by airspace PMNs, but it was only the alveolar macrophages that ingested the colloidal carbon. Interstitial PMNs were frequently observed in the connective tissue surrounding the bronchial arteries (Figure 31), but it could not be determined by light microscopy whether PMNs had also emigrated into the interstitium of the alveolar walls.

By comparison, PMNs were found in the alveolar airspace 24h after intratracheal instillation of *E. coli* endotoxin and colloidal carbon (Figures 33 and 34). In general, unlike the airspace PMNs, the alveolar macrophages ingested large amounts of colloidal carbon. Extravascular PMNs were observed in the bronchovascular interstitium (Figure 33), but once again, the resolution of the microscope was insufficient to determine whether interstitial PMNs were also present within the alveolar walls.
3.4 IMMUNOELECTRON MICROSCOPY OF MOUSE LUNG TISSUE

3.4.1 Summary of morphometric data on mouse lung tissue

In the ICAM-1 study of mouse lung, 75% of the total number of gold particles (41,166) counted were localized to the Type I pneumocytes (Table 5). In total, 13 mice were examined and the cumulative length of plasma membrane examined in this study for the endothelial, Type I, and Type II cells was 2.5 mm, 3.6 mm, and 2.0 mm, respectively. The mean length of plasma membrane examined on each cell type in this study was: endothelial, $192 \pm 38$ (SD) $\mu$m; Type I, $263 \pm 116$ $\mu$m; Type II, $158 \pm 36$ $\mu$m.

3.4.2 Localization and quantitation of ICAM-1 immunoreactivity in control mouse lung

ICAM-1 was constitutively expressed on the luminal surfaces of both pulmonary capillary endothelial (Figures 35 and 36) and alveolar epithelial cells (Figure 37); there was negligible ICAM-1 immunoreactivity on the ablumenal and lateral surfaces of these cells and there were no detectable intracellular pools of ICAM-1. Epithelial ICAM-1 immunoreactivity was primarily limited to the Type I pneumocytes (Figure 37). By comparison, very few gold particles were detected on the surface of Type II pneumocytes (Figures 37 and 38). There was no obvious pattern to the distribution of these gold particles on either endothelial or Type I cells. Quantitation of the gold label density showed that ICAM-1 immunoreactivity on Type I pneumocytes was 22 times greater than that found on the endothelial cells ($10.6 \pm 3.0$ (SD) vs. $0.5 \pm 0.1$ gold particles/µm, Bonferroni-adjusted $P < 0.0125$) and 212 times greater than that found on the Type II pneumocytes (Table 6, Bonferroni-adjusted $P < 0.0125$). The large difference in Type I and Type II pneumocyte ICAM-1 immunoreactivity was best appreciated
at the cellular junction between these cells, where the rarity of gold particles on the Type II pneumocyte contrasted sharply with the large numbers of gold particles on the Type I pneumocyte (Figure 38).

3.4.3 Effect of colloidal carbon instillation on ICAM-1 immunoreactivity in mouse lung

An examination of lungs 4h and 24h after the intratracheal instillation of colloidal carbon showed, that in the absence of an inflammatory stimulus (S. pneumoniae or E. coli endotoxin), there was no significant change in ICAM-1 expression on pulmonary endothelial cells or Type I pneumocytes (Table 6). By comparison, 24h after, but not 4h after, colloidal carbon instillation, ICAM-1 immunoreactivity on Type II pneumocytes was 52 times greater than that found on control Type II pneumocytes (Table 6, Bonferroni-adjusted P < 0.0125).

3.4.4 Effect of pneumonia on pulmonary endothelial ICAM-1 immunoreactivity

Four hours after intratracheal instillation of S. pneumoniae and colloidal carbon, ICAM-1 immunoreactivity on pulmonary capillary endothelial cells was not significantly different from that on control pulmonary endothelium (Table 6). Twenty four hours after intratracheal instillation of E. coli endotoxin and colloidal carbon into the lungs of mice, ICAM-1 immunoreactivity on pulmonary capillary endothelial cells (Table 6, Figure 39) was estimated to be 4.2 times greater than that on pulmonary endothelial cells of control mice and this increase was statistically significant using the Duncan Multiple Range test. However, the overall Bonferroni-adjusted P value for the ANOVA was 0.017 and this exceeded the acceptable level of significance (P < 0.0125). Zar (1984) points out that it is not an actual requirement that multiple comparison testing be performed only if the ANOVA rejects a
multisample hypothesis of equal means. On the advise of a professional statistician (Dr. G.E.I. Smith, Consulting Statistician for the Canadian Wildlife Service, Delta, B.C.) it is best to report that the 4.2-fold increase in ICAM-1 expression borders on statistical significance, rather than simply dismissing the observation as statistically insignificant.

3.4.5 Effect of pneumonia on alveolar epithelial ICAM-1 immunoreactivity

Neither streptococcal nor endotoxin pneumonias had any significant effect on ICAM-1 immunoreactivity on Type I pneumocytes. However, there was a downward trend towards a decrease in gold particle density on the surface of the Type I pneumocytes following the tracheal instillation of *S. pneumoniae* or *E. coli* endotoxin and colloidal carbon (Table 6).

Four hours after intratracheal instillation of *S. pneumoniae* mixed with colloidal carbon, ICAM-1 immunoreactivity on Type II pneumocytes (Figure 40) was 42 times greater than that on Type II pneumocytes in the control mouse lung (Table 6, Bonferroni-adjusted P < 0.0125). Similarly, but more striking, 24h after tracheal instillation of colloidal carbon mixed with *E. coli* endotoxin, ICAM-1 immunoreactivity on Type II pneumocytes (Figure 39) was 178 times greater than that on Type II pneumocytes in the control mouse lung (8.9 ± 4.8 (SD) vs. 0.1 ± 0.1 gold particles/μm, in 3 mice, Bonferroni-adjusted P < 0.0125).

3.5 IMMUNOELECTRON MICROSCOPY OF HUMAN PMNs

3.5.1 Localization of L-selectin, CD18, and sLex on human PMNs in LRP

An electron microscopic examination of fixed and cryosectioned human LRP showed that most of the leukocytes were PMNs. These PMNs were generally spherical in shape, but
their surface was frequently ruffled due to the presence of numerous small microvillar projections. Immunogold labeled cryosections showed that L-selectin immunoreactivity on the PMN cell surface was consistently localized to microvillous processes (Figure 41). Only a small fraction of L-selectin labeling was identified in "flat" regions of the PMN surface, and no appreciable labeling was observed in cytoplasmic granules. This preferential distribution of L-selectin was apparent in all human PMNs examined (60 from three different individuals). In contrast, the immunoreactivity of total sLex and CD18 appeared randomly distributed over the plasma and granule membranes of the PMNs from all three donors (Figures 42 and 43, respectively).

3.5.2 Localization of L-selectin immunoreactivity on transmigrating human PMNs

An electron microscopic examination of fixed and cryosectioned IL-1 stimulated endothelial monolayers showed PMNs in various stages of transmigration. L-selectin immunoreactivity on human PMNs that had not yet contacted the IL-1-stimulated endothelial surface (Figures 44 and 45) was similar to that observed in the human LRP preparations (Figure 41). As before, the L-selectin immunoreactivity was preferentially localized to the microvillar processes and no appreciable gold label was observed in the cytoplasmic granules. Adherent PMNs, those that had not yet penetrated the endothelial monolayer but were in close contact with its surface, were positive for L-selectin immunoreactivity. However, L-selectin immunoreactivity was restricted to the portion of the PMN surface that was not in contact with the endothelium (Figure 46); L-selectin immunoreactivity could not be demonstrated on the PMN surface that was in close contact with the endothelium.

Several PMNs were also observed in the process of transmigrating the endothelium
(Figures 47 to 49) where the advancing portion (pseudopod) of the PMN had just penetrated the endothelial monolayer. Qualitatively, fewer gold particles were present on the surface of the advancing pseudopod (Figure 49) relative to that on the rest of the cell (Figure 48). L-selectin immunoreactivity was still detected on PMNs that had completely transmigrated the endothelium (Figures 50 and 51). These PMNs accumulated between the endothelium and its basal lamina (Figure 51). L-selectin immunoreactivity on these cells was qualitatively less than that on non-migrated PMNs, but greater than that on PMNs labeled with non-specific mouse IgG (Figure 52).
CHAPTER FOUR
DISCUSSION

4.1 EXPRESSION OF PMN, ENDOTHELIAL, AND EPITHELIAL ADHESION MOLECULES DURING PNEUMONIA

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4.1 EXPRESSION OF PMN, ENDOTHELIAL, AND EPITHELIAL ADHESION MOLECULES DURING PNEUMONIA

4.1.1 Alterations in the expression of PMN L-selectin and CD18 during pneumonia

The first specific aim of this thesis was to determine whether L-selectin expression is downmodulated and whether CD18 expression is upmodulated in response to stimuli that induce CD18-independent and CD18-dependent PMN emigration in the rabbit lung. The results clearly show that CD18-independent PMN emigration towards a bronchial instillate of \textit{S. pneumoniae} is associated with L-selectin downmodulation and CD18 upmodulation on intravascular, interstitial, and airspace PMNs. A similar alteration in the expression of L-selectin and CD18 is observed during CD18-dependent PMN emigration towards a bronchial instillate of \textit{E. coli} endotoxin, but only after PMNs emigrated into the interstitium. Regardless of the mechanism of PMN emigration, the change in L-selectin and CD18 expression occurs only on PMNs within the pneumonic site. The evidence for this finding is that L-selectin and CD18 expression on PMNs within the pulmonary microvasculature of the non-pneumonic contralateral lung is not different from control. This finding implies that PMN activation is a focal phenomenon and restricted to the site of acute inflammation.

The DREG-200 and 60.3 MAbs were made against human L-selectin (Kishimoto et al., 1990) and human CD18 (Beatty et al., 1983; Wallis et al., 1986), respectively. On rabbit PMNs, the DREG-200 MAb (von Andrian et al., 1991) and the 60.3 MAb (Arfors et al., 1987, Price et al., 1987; Doerschuk et al., 1990a, 1990b) recognize a functional L-selectin equivalent and a functional CD18 equivalent, respectively. The rabbit glycoprotein recognized by MAb 60.3 is GP150/GP85 and this glycoprotein is the functional equivalent of human
CD11/CD18 (Lindbom et al., 1990). Ultrastructural (Bainton et al., 1987; Jones et al., 1990a) and biochemical (Arnaout et al., 1984; O'Shea et al., 1984; Todd et al., 1985; Springer et al., 1986; Jones et al., 1988, 1990a, 1990b) studies conducted on unstimulated human PMN preparations have shown that the majority of CD18 is stored as a latent intracellular granular pool (largely CD11b/CD18 and some CD11c/CD18) with lesser amounts being expressed on the cell surface (CD11a/CD18, CD11b/CD18, and CD11c/CD18). During PMN activation, CD18 surface expression can increase 2-10 fold and fusion of the CD18-rich cytoplasmic granules with the plasma membrane provides the mechanism for this increase (Arnaout et al., 1984; Bainton et al., 1987; Jones et al., 1988, 1990a, 1990b). In the present study, the majority of the CD18 in normal rabbit PMNs is localized within the intracellular granules, supporting the presence of a latent pool that can be mobilized to the surface during PMN activation. Direct evidence of increased CD18 surface expression during PMN activation is shown by the increased numbers of CD18-specific gold particles on the cell surface of emigrating PMNs in both streptococcal and endotoxin pneumonias. Human PMNs show decreased L-selectin expression during activation (Smith et al., 1991) and this is most likely due to shedding of L-selectin from the cell surface (Kishimoto et al., 1989a). Importantly, during streptococcal and endotoxin pneumonias, the rabbit antigen recognized by the DREG-200 MAb is also downmodulated as PMNs are activated and CD18 expression increases. Collectively, these observations are consistent with the concept that the rabbit antigens recognized by the DREG-200 and 60.3 MAbs are the functional equivalents of human L-selectin and CD18, respectively.

The present ultrastructural finding that the shape of intravascular capillary PMNs is sometimes elongated agrees with the idea that PMNs must deform in order to pass through
narrow capillary segments (Hogg, 1987; Martin et al., 1987; Doerschuk et al., 1987; Downey and Worthen, 1988). However, in capillaries with diameters greater than that of the PMN, the PMNs are generally spherical in shape and display microvillar projections. A striking observation regarding the microvilli is that L-selectin immunoreactivity is consistently associated with these surface processes, rather than the flatter regions of the cell. Importantly, this finding may be common to other animal species as L-selectin expression on human PMNs is also associated with microvillar processes (Section 4.2). In the systemic circulation, spherically-shaped PMNs "roll" (marginate) along the vessel wall of inflamed post-capillary venules (Atherton and Born, 1972, 1973; Arfors et al., 1987; von Andrian et al., 1991; Ley et al., 1991; Tangelder and Arfors, 1991). The association of L-selectin with surface microvilli may facilitate this rolling phenomenon (discussed in Section 4.2). However, the physical constraints of narrow capillary segments probably inhibit PMN rolling and the present finding that elongated PMNs show very few microvillar processes supports this hypothesis. Of particular interest is the additional finding that L-selectin-specific gold particles are detected in small clusters at discrete sites along the flattened PMN surface. This observation implies that L-selectin is not redistributed on the surface during PMN elongation, because if it were, then a more even distribution of gold particles would be expected. Whether microvilli reform at these L-selectin clusters once the PMN passes into a larger vessel (i.e. post-capillary venule) is unknown.

During streptococcal pneumonia, L-selectin expression is downmodulated and CD18 expression is upmodulated on intravascular PMNs and this confirms that these PMNs are activated (Kishimoto et al., 1989a; Jutila et al., 1989, 1990, 1991; Smith et al., 1991; von Andrian et al., 1991). Importantly, these activated PMNs do not appear to circulate in
appreciable numbers as evidenced by the observation that PMNs within the contralateral lung express normal amounts of L-selectin and CD18. These observations imply that L-selectin is not involved in the retention of activated PMNs within the pulmonary microvasculature of the pneumonic region. Although CD18 expression is enhanced on these intravascular PMNs, it is unlikely to play a role in PMN retention based on the finding that PMN emigration is not inhibited by the anti-CD18 MAb 60.3 (Doerschuk et al., 1990a, 1990b). Retention of activated PMNs in response to *S. pneumoniae* within the lung must involve CD18-independent mechanisms of adhesion and/or a decrease in PMN deformability that prevents the PMN from passing through narrow capillary segments (Hogg, 1987; Doerschuk et al., 1989; Worthen et al., 1989).

The activation of intravascular PMNs within the pulmonary microvasculature implies that PMNs within the capillary lumen of the pneumonic lung are being stimulated by the local release of soluble inflammatory mediators and cytokines (e.g. IL-8, LTB₄, PAF, TNF-α, C3a, and C5a). This postulate assumes that the soluble stimuli are not washed away and diluted by capillary blood flow. In support of this is the recent finding that capillary blood flow within the pneumonic region is reduced by 80% at 4h (Coxson et al., 1990; Doerschuk, Markos, Coxson, English, and Hogg. (Submitted)). Reduced capillary blood flow would potentially allow the soluble mediators to accumulate and activate the intravascular PMNs. Moreover, because blood flow is not entirely obliterated, it may continue to deliver unstimulated PMNs to the pneumonic region. This could explain the present finding that although most (80%) of the intravascular PMNs were L-selectin negative, 20% (6/30 cells) of these PMNs expressed normal levels of L-selectin. The mechanism of blood flow reduction is unknown, observations made in the present study suggest that it may be caused, in part, by a loss of microvascular
integrity. The evidence is that red blood cells and fibrin were frequently detected within the alveolar airspace and this suggests that there has been a gross disruption of the alveolar capillary wall. Importantly, activated PMNs are less deformable (more "stiff") than unstimulated PMNs (Worthen et al., 1989). The combined effects of no or low blood flow and decreased PMN deformability may make it very difficult for activated PMNs to pass through narrow capillary segments and rejoin the circulation.

In response to *S. pneumoniae*, emigrated airspace PMNs show increased CD18 surface expression (5.5-fold greater than control intravascular PMNs) and this may contribute to the present finding that airspace PMNs ingest large numbers of bacteria. This increase in CD18 expression is likely due to upregulation of CD11b/CD18 adhesion proteins onto the cell surface by specific granule fusion with the plasma membrane (Arnaout et al., 1984; Bainton et al., 1987; Jones et al., 1988, 1990a, 1990b). During the inflammatory response, streptococcal bacteria are likely to activate both the alternative (Winkelstein and Tomasz, 1978; Hummel et al., 1981, 1985) and classical (Loos et al., 1986) complement pathways. Because CD11b/CD18, in addition to binding ICAM-1, binds to the complement split product C3bi (reviewed by Wright et al., 1990), then binding of CD11b/CD18 to C3bi-opsonized bacteria should greatly facilitate PMN phagocytosis (Wright and Meyer, 1986). Consistent with this hypothesis is the recently published *in vitro* finding that PMN phagocytosis of serum-opsonized *Streptococcus agalactiae* is largely inhibited by the anti-CD18 MAb R15.7 (Sherman et al., 1992). Significantly, PMN emigration in the rabbit lung towards *S. agalactiae* is also CD18-independent (Ibid). Collectively, these data imply that although CD18 expression is not necessary for PMN emigration during streptococcal pneumonia, it may be important to bacterial phagocytosis. It is curious that although the phagosomal membrane surrounding the
ingested bacteria is derived (presumably) from an invagination of the plasma membrane, it does not show CD18 immunoreactivity. This observation implies that CD18 is either excluded from the phagosomal membrane during its formation or, that fusion of the phagosome with a primary lysosome results in enzymatic degradation of phagosomal-associated CD18.

Endotoxin pneumonia, in contrast to streptococcal pneumonia, is not associated with a significant change in L-selectin expression on intravascular PMNs. However, PMNs that emigrate into the interstitium show a significant decrease (P < 0.05) in L-selectin expression. These findings imply that L-selectin downmodulation is associated with transendothelial migration and that it occurs only after the PMN adheres to the capillary endothelium. A recent study demonstrated that IL-1-stimulated endothelial cells express a surface membrane-associated form of platelet activating factor (PAF) (Kuijpers et al., 1992a). Importantly, in vitro, this membrane-associated PAF induces L-selectin downmodulation on transmigrating human PMNs (Ibid). It would be of great interest to determine whether the pulmonary capillary endothelium expresses membrane-associated PAF during endotoxin pneumonia in rabbits. This question could be addressed by immunogold labeling lung cryosections with a MAb directed against PAF. However, such a study would require raising an anti-PAF MAb, because at present, none are commercially available.

In the pulmonary circulation, PMN adherence to the endothelium may occur by either a CD18-dependent or CD18-independent mechanism that is specific to the inflammatory stimulus (Doerschuk et al., 1990a, 1990b). *S. pneumoniae* elicits CD18-independent PMN emigration into the alveolar space (Ibid). Yet, our results indicate that CD18 expression on the PMN surface increases prior to emigration and increases further once the PMNs have reached the airspace in response to *S. pneumoniae*. In contrast, CD18 expression is not
increased on intravascular PMNs prior to CD18-dependent emigration in response to *E. coli* endotoxin. Increased CD18 expression is only observed once the PMNs have left the pulmonary microvasculature and are present within the interstitium and alveolar airspace. Thus, the levels of CD18 expression on PMNs do not correlate with the function of this molecule during PMN emigration in response to CD18-independent and CD18-dependent stimuli. This finding is not really surprising, because there have been several independent *in vitro* studies showing that increased CD18 expression on the PMN surface is not a prerequisite for adherence to endothelial cells (Vedder and Harlan, 1988; Lo et al., 1989a, 1989b; Schleiffenbaum 1989). Furthermore, there is increasing evidence that the existing CD18 on the surface of the PMN is sufficient for adhesion and migration. Although both CD11a/CD18 and CD11b/CD18 contribute to this process (reviewed by Smith, 1992), only CD11b/CD18 shows increased surface expression following PMN activation (Arnaout et al., 1984). Many *in vitro* studies have shown that newly expressed CD11b/CD18 on the surface of the neutrophil appears to be non-active (Wright and Meyer, 1986; Buyon et al., 1988; Philips et al., 1988, Nourshargh et al., 1989; Lo et al., 1989b). Activation of CD11b/CD18 into a competent adhesion receptor is thought to require a conformational change (Altieri and Edgington, 1988; Robinson et al., 1992). The mechanism(s) responsible for this conformational change is largely unknown, but it may involve a phosphorylation event (Wright and Meyer, 1986; Buyon et al., 1990). Alternatively, it may be induced by a lipid known as integrin modulating factor-1 (IMF-1) (Hermanowski-Vosatka et al., 1992). IMF-1 is transiently expressed on activated PMNs and it induces an equally transient increase in binding avidity on CD11b/CD18 for the complement fragment C3bi (ibid).

The present finding that interstitial PMNs in both streptococcal- and endotoxin-induced
pneumonias express very little L-selectin implies that L-selectin is not important for PMN migration through the interstitial matrix. This agrees with the generally held concept that L-selectin functions in the initial contact between the PMN and the inflamed vascular endothelium (Lewinsohn et al., 1987; Jutila et al., 1989, 1991; von Andrian et al., 1991; Ley et al., 1991). Interestingly, CD18 can serve as a receptor for Type I collagen (Monboisse et al., 1991) and in the present study, interstitial PMNs were often in close contact with fibrillar (Type I) collagen. Because PMN emigration towards *S. pneumoniae* is CD18-independent (Doerschuk et al., 1990a, 1990b), CD18-mediated adherence to collagen can not be essential for PMN migration through the interstitium. Conversely, because PMN emigration towards *E. coli* endotoxin is CD18-dependent, the possibility is raised that part of this CD18-dependency may reside in a requirement for PMN adhesion to collagen during interstitial migration.

In the systemic circulation, PMN emigration towards all acute inflammatory stimuli examined to date is dependent upon the expression of CD18 adhesion molecules (Harlan et al., 1992). The reason why PMN emigration towards *S. pneumoniae* in the lung is CD18-independent is unknown, but it has been argued that the presence of macrophages is essential to the development of the CD18-independent response (Mileski et al., 1990). In response to a peritoneal instillate of *S. pneumoniae*, PMN emigration is normally CD18-dependent; however, if peritoneal macrophages are activated and their numbers increased prior to the delivery of the instillate, then PMN emigration can proceed by a CD18-independent mechanism (Ibid). This observation suggests that macrophage ingestion of streptococcal bacteria results in the release of chemotactic or proadhesive bacterial degradation products (Ibid). However, in the present study of the lung, this postulate is not supported because alveolar macrophages rarely ingest streptococcal bacteria. Instead, it is the emigrated PMNs that ingest large
numbers of bacteria. It must be pointed out that these observations are only valid up to the first 4h following the instillation of *S. pneumoniae*. Whether macrophages ingest more bacteria as the pneumonia develops was not determined in this study, however, the present findings are consistent with Metchnikoff’s (1905) early observations on acute inflammation. He showed that PMNs in general are far more efficient at bacterial phagocytosis than macrophages. In fact, he remarked:

"Macrophages can also ingest the bacteria of acute diseases, but, save in exceptional cases, their intervention is of little moment."[Metchnikoff, 1905].

These observations suggest that *S. pneumoniae* activates alveolar macrophages by some other mechanism that does not involve bacterial phagocytosis. Although the present study offers no insight into the mechanism of streptococcal activation of alveolar macrophages, it seems likely macrophage activation is accompanied by the release of a monokine(s) that is either chemotactic or proadhesive for PMNs (Mileski et al., 1990). This hypothesis could be tested using the present rabbit model of streptococcal pneumonia. Rabbit lungs infected with streptococcal organisms could be lavaged, and the recovered lavage fluid examined for chemotactic or proadhesive macrophage products that promote CD18-independent transendothelial migration *in vitro*.

4.1.2 Alterations in the expression of ICAM-1 during pneumonia

The second specific aim of this thesis was to determine, in the mouse lung, whether ICAM-1 expression is upmodulated in response to stimuli that are known to induce CD18-independent and CD18-dependent PMN emigration in the rabbit lung. The results clearly show that, in response to the CD18-independent stimulus, *S. pneumoniae*, capillary endothelial ICAM-1 expression is not upmodulated during PMN emigration. Conversely, endothelial
ICAM-1 expression is upmodulated (a 4.2-fold increase over control levels) in response to the CD18-dependent stimulus, *E. coli* endotoxin; however, this finding must be interpreted with caution because the increase only borders on statistical significance. An additional new finding in the present study is that in normal lung, constitutive ICAM-1 expression is 22-fold greater on the alveolar epithelium compared to the capillary endothelium and the epithelial expression is mainly restricted to the Type I pneumocytes. Interestingly, epithelial ICAM-1 expression is not increased on Type I pneumocytes during streptococcal and endotoxin induced pneumonias, but a significant increase in ICAM-1 expression is observed on Type II pneumocytes.

In response to *S. pneumoniae*, PMN emigration in the rabbit lung is known to be CD18-independent (Doerschuk et al., 1990a, 1990b); however, it is not known if emigration is also a CD18-independent phenomenon in the mouse lung. Constitutive pulmonary capillary endothelial ICAM-1 expression, as determined by the immunogold method, is very low and barely above background. Moreover, capillary endothelial ICAM-1 expression is not increased at 4h when significant numbers of PMNs are in the alveolar airspace. These observations imply that constitutive endothelial ICAM-1 expression is very low and that increased ICAM-1 expression is not required for PMN emigration. This is consistent with the concept of CD18-independent emigration, because ICAM-1 is an adhesive ligand for CD11a/CD18 and CD11b/CD18 (Rothlein et al., 1986; Smith et al., 1989; Diamond et al., 1990, 1991). Following cytokine stimulation, both *in vitro* and *in vivo*, endothelial cells become more adhesive for PMNs and a significant portion of this increased adhesiveness is due to an increase in ICAM-1 expression (Smith et al., 1988, 1989). However, regardless of whether PMNs emigrate by a CD18-independent mechanism, the results clearly demonstrate that increased
ICAM-1 expression is not required for PMN diapedesis from the pulmonary microvasculature during streptococcal pneumonia in the mouse.

In response to lipopolysaccharide (endotoxin), PMN emigration in the mouse lung is known to be dependent upon the expression of CD18 (Rosen and Gordon, 1990). The present study suggests, but does not statistically confirm, that ICAM-1 expression is increased 4.2-fold on pulmonary capillary endothelial cells (see Section 3.4.4). An increase in ICAM-1 expression would be consistent with the concept of CD18-dependent PMN emigration and, importantly, this increase is coincidental with the presence of PMNs in the alveolar space 24h after the instillation of E. coli endotoxin. In vitro, ICAM-1 expression on endothelial monolayers typically increases 6-fold over baseline following exposure to IL-1 or endotoxin and is nearly maximal by 4h (Smith et al., 1988, 1990); it remains elevated for at least 48h following exposure to IL-1 (Luscinskas et al., 1991). Four hours after endothelial stimulation, PMN adhesion and transendothelial migration in vitro is maximal (Smith et al., 1988; Luscinskas et al., 1991). By 8h, although PMNs continue to adhere by an ICAM-1/CD18-dependent mechanism, they seldom migrate (Smith et al., 1988; Luscinskas et al., 1991). In the present study, PMN emigration was negligible at 4h following endotoxin instillation. Although the present study did not examine ICAM-1 expression at 4h, the lack of PMN emigration implies that the level of ICAM-1 expression was insufficient to support CD18-dependent PMN emigration. Interestingly, rabbits are approximately 1000 times more sensitive to the effects of endotoxin than mice (Beutler et al., 1985). The present study confirms this resistance by showing that BALB/c mice required a higher dose of endotoxin than that used in the rabbit (10 mg/kg versus 1.5 μg/kg, respectively) to elicit PMN emigration into the alveolar airspace. Moreover, CD18-dependent PMN emigration towards endotoxin in the
rabbit occurs within 4h of instillation (this study; Doerschuk et al., 1990a, 1990b). These observations predict that the time course for increased ICAM-1 expression on rabbit alveolar capillaries will be much more rapid than that observed in the mouse.

The alveolar macrophage is probably an important and major target for endotoxin (Christman et al., 1989; Sylvester et al., 1990; Rankin et al., 1990; Rietschel et al., 1991; Issekutz et al., 1991). Significantly, BALB/c mouse peritoneal macrophages respond poorly to several different stimuli including IFN-γ and endotoxin (Oswald et al., 1992). Presumably, BALB/c mouse alveolar macrophages also respond poorly to endotoxin. The binding of endotoxin to monocytes is known to result in the synthesis and release of lymphokines such as IL-1, TNF-α, and IL-6 (reviewed by Rietschel et al., 1991). IL-1 and TNF-α are both capable of inducing endothelial ICAM-1 expression (reviewed by Lobb, 1992). Moreover, endotoxin-stimulated alveolar macrophages can also release products that act directly on PMNs. For example, human alveolar macrophages stimulated with endotoxin release potent neutrophil chemoattractants such as LTB₄ and IL-8 (Sylvester et al., 1990; Rankin et al., 1990). More recently, it was shown that 3h after endotoxin-stimulation, rabbit alveolar macrophages also release a novel protein factor with an apparent molecular mass of 22 to 32 kD, which under gel filtration conditions probably exists as a dimer with an apparent molecular mass of 45 to 60 kD (Issekutz et al., 1991). When this protein is injected intradermally into the rabbit, it induces PMN infiltration (Ibid). Interestingly, this protein factor does not induce PMN migration in vitro, suggesting that it may induce endothelial cells to elaborate chemotactic factors (Ibid). If CD18-dependent PMN emigration were dependent upon the release of alveolar macrophage-derived chemotactic factors for PMNs and/or cytokines that induce ICAM-1 upregulation, then sub-optimal production of these macrophage products in BALB/c
mice could account for the observed hyporesponsiveness towards endotoxin.

To our knowledge, this is the first study to quantitate and compare the expression of ICAM-1 on specific cell types in peripheral lung tissue. ICAM-1 has been detected on tracheal epithelial cells during allergic airway inflammation in cynomolgus monkeys, but its constitutive expression appears to be negligible, or very low at best (Wegner et al., 1990). In contrast, the high level of constitutive epithelial ICAM-1 expression on the Type I pneumocyte implies that it is required for normal lung function. One intriguing possibility is that epithelial ICAM-1 participates in alveolar macrophage locomotion over the epithelial cell surface and thereby, assists the macrophage in scavenging inhaled particulates. The data from this study show that ICAM-1 is constitutively expressed on alveolar Type I pneumocytes and not Type II pneumocytes. Although the alveolar surface is comprised of nearly equal numbers of Type I and II pneumocytes, the squamous Type I pneumocytes make up approximately 95% of the alveolar surface (Thurlbeck and Miller, 1988). Collectively, these observations predict that the majority of the alveolar surface expresses ICAM-1. It is well established that ICAM-1 is an adhesive ligand for the leukocyte integrins CD11a/CD18 and CD11b/CD18 (Rothlein et al., 1986; Smith et al., 1989; Diamond et al., 1990, 1991). Alveolar macrophages obtained by lung lavage from humans (Albert et al., 1992), monkeys (Ibid), and mice (Rosen and Gordon, 1990) express high surface levels of CD11a/CD18 and low or insignificant levels of CD11b/CD18 and CD11c/CD18. The basal adherence of human alveolar macrophages to an unstimulated alveolar epithelial cell monolayer is CD18-independent (Albert et al., 1992). However, stimulation of the monolayer with TNF-α increased epithelial ICAM-1 expression 9-fold and increased numbers (5-10% more) of macrophages were found to adhere to this stimulated monolayer. Significantly, this increased adherence was CD18-dependent and
completely inhibitable by MAb 60.3 (Ibid). The contribution of ICAM-1 to this CD18-dependent adherence was not determined, but a role for ICAM-1 is implied.

In the present study, normal Type II pneumocytes express little or no ICAM-1 on their cell surfaces. However, acute inflammation in the lung is accompanied by a significant increase (48-fold in response to *S. pneumoniae* and 178-fold in response to *E. coli* endotoxin) in Type II pneumocyte ICAM-1 expression. Twenty-four hours after endotoxin instillation, the levels of ICAM-1 expression are similar to those on normal Type I pneumocytes. Increased surface expression of ICAM-1 is considered to be a time dependent process requiring mRNA and protein synthesis (Dustin et al., 1986). Induction of mRNA and protein synthesis reflects a change in cellular activity. In the Type II pneumocyte, this may also reflect a change in cellular function.

In normal lung, Type II pneumocytes have two very important functions. First, they synthesize and secrete pulmonary surfactant which forms a continuous lining layer that covers the alveolar epithelial surface (Kuhn, III and Finke, 1972; Junqueira et al., 1989; Uhal et al., 1991); surfactant functions to decrease the surface tension in the lung and prevent alveolar collapse during expiration (Deavers, 1984; Junqueira et al., 1989; Uhal et al., 1991). Second, they are considered to be the progenitor cells that give rise to both Type I and II pneumocytes during normal epithelial cell turnover (Adamson and Bowden, 1975; Uhal et al., 1991). Importantly, when the alveolar epithelium is injured during an inflammatory response, Type II pneumocyte proliferation increases to provide new sister cells for epithelial cell replacement. This conclusion is based on the observation that the lung injury produced by inhalation of 90% oxygen (Adamson and Bowden, 1974) or 15-17 ppm nitrogen dioxide (Evans et al., 1973) in mice and rats, respectively, resulted in increased mitotic activity and increased incorporation
of tritiated thymidine in Type II pneumocytes. Of particular relevance to the present study is the published finding that in the rat, Type I pneumocytes are injured during streptococcal pneumonia (Rhodes et al., 1989). Moreover, the injured Type I pneumocytes are replaced by proliferating Type II pneumocytes (Ibid). In the present study, Type II pneumocyte ICAM-1 expression increases during both streptococcal and endotoxin pneumonias and this implies that increased ICAM-1 expression may be an early differentiation event that precedes Type II pneumocyte proliferation.

It has been reported that tracheal instillation of colloidal carbon (4-fold more than was used in the present study) into the lungs of mice typically results in acute inflammation as evidenced by PMN emigration into the alveolar space (Adamson and Bowden, 1978, 1980, 1982; Bowden and Adamson, 1978). In the present study, smaller amounts of colloidal carbon were instilled and did not induce PMN emigration, but did result in a 52-fold increase in ICAM-1 surface expression on Type II pneumocytes. Interestingly, significant epithelial injury does not seem to be associated with colloidal carbon instillation (this study; Adamson and Bowden, 1978, 1980, 1982) and yet ICAM-1 expression is increased. If increased ICAM-1 expression is associated with Type II pneumocyte proliferation, then instillation of colloidal carbon should result in increased Type II pneumocyte proliferation as measured by the tritiated thymidine method (Evans et al., 1973; Adamson and Bowden, 1974). To date there are no studies to confirm or reject this hypothesis.

Assuming, for the moment, that increased ICAM-1 expression is an early differentiation event that is linked to and precedes Type II pneumocyte mitosis, then the stimulus for increased Type II pneumocyte proliferation may also be the stimulus for increased ICAM-1 expression. In vitro, alveolar macrophages stimulated with opsonized zymosan particles produce a Type
II pneumocyte growth-factor that increases cell proliferation (Brandes and Finkelstein, 1989). The apparent molecular mass of this growth factor is $\geq 25$ kD and its ability to promote Type II pneumocyte proliferation \textit{in vitro} is unique when compared with other known growth promoting macrophage products (Ibid). In the present study, alveolar macrophages ingest large amounts of carbon particles. Ingestion of carbon can induce macrophages to produce soluble activation factors for PMNs (Adamson and Bowden, 1982) and perhaps for Type II pneumocytes. In \textit{vitro}, a particulate stimulus (opsonized zymosan) induces alveolar macrophage production of a Type II pneumocyte growth factor (Brandes and Finkelstein, 1989). The amount of growth factor produced is dependent upon the concentration of the stimulus and the length of time that the macrophages are exposed to the particles (Ibid). If colloidal carbon, in the absence of \textit{S. pneumoniae} or \textit{E. coli} endotoxin, also stimulates macrophages to produce this growth factor, then the observation that the Type II pneumocyte ICAM-1 expression is increased at 24h, but not at 4h, following colloidal carbon instillation may simply relate to the length of time that the macrophages are exposed to the carbon particles. It remains to be determined whether colloidal carbon particles induce macrophages to produce Type II pneumocyte growth factor and whether Type II pneumocyte exposure to this growth factor results in increased ICAM-1 expression.

4.2 OBSERVATIONS ON L-SELECTIN

4.2.1 The topographical distribution of L-selectin on the PMN surface

The third specific aim of this thesis was to determine, \textit{in vitro}, whether L-selectin is topographically positioned on the PMN surface such that it renders L-selectin-associated
sLex more "bioavailable" for the vascular selectins P- and E-selectin. The results on human PMNs clearly show that L-selectin is topographically positioned on the PMN surface microvilli. This observation is consistent with the hypothesis that L-selectin-associated sLex can be preferentially "presented" to the vascular selectins, even though it accounts for only 5% of protein associated surface sLex (Picker et al., 1991), because its concentration on the microvilli renders it more "bioavailable." Consistent with this proposal is the in vitro finding that microvilli mediate the initial contact between the PMN and the endothelium (Beesley et al., 1979). During acute inflammation in the systemic circulation, PMNs make transient contacts with the endothelium and they "roll" along the vessel wall. Intravital microscopic studies in the systemic circulation have demonstrated that the "rolling" of PMNs along the inflamed venular endothelium is largely mediated by L-selectin (von Andrian et al., 1991; Ley et al., 1991). Rolling PMNs are essentially spherical (Tangelder and Arfors, 1991) and the present study suggests that this rolling behaviour and its dependence on L-selectin is explained by the unique association of L-selectin with the microvillar processes. The uniqueness of this association is suggested by the present finding that CD18 and total sLex are not preferentially expressed by surface microvilli.

Although PMN L-selectin-associated sLex is an important presenter of sLex to the vascular selectins P- and E-selectin (Kishimoto et al., 1991; Picker et al., 1991), it may not be the only PMN carbohydrate ligand for these adhesion molecules. Both glycolipids and glycoproteins on the leukocyte surface express the sLex epitope (reviewed by Paulson, 1992) and several studies have examined the possibility that these other sLex-bearing molecules also bind to P- and E-selectin. Protease treatment of PMNs cleaves L-selectin from the surface and abolishes soluble $^{125}$I-P-selectin binding (Moore et al., 1991). This observation suggests that
the PMN ligand for P-selectin is a surface protein or glycoprotein and this is consistent with L-selectin presentation of sLex to P-selectin. However, the binding of soluble P-selectin to PMNs is not affected by PMN activation (Moore et al., 1991) and this implies that PMN L-selectin can not be the major ligand for P-selectin, because PMN activation is associated with L-selectin downmodulation (this study; Kishimoto et al., 1989a; Jutila et al., 1989, 1990, 1991). This contradicts an independent collaborative finding that the anti-L-selectin MAb DREG-56 inhibited PMN binding to P-selectin-transfected COS (monkey kidney cell line) cells by 70% (Picker et al., 1991). The reason for this discrepancy is unclear.

Several other investigators have used the myeloid/monocytic cell line HL60 to study the leukocyte ligand for E-selectin. Interestingly, HL60 cells bind to E-selectin-transfected CHO (chinese hamster ovary) cells and this binding is unaffected by protease digestion of HL60 cells (Larsen et al., 1992). Furthermore, when HL60 cells are grown in the presence of inhibitors that block the synthesis of N-linked glycosylated glycoproteins, binding to E-selectin is not diminished (Leeuwenberg et al., 1991). Collectively, these findings imply that the HL60 ligand for E-selectin is a glycolipid. This conclusion appears to contradict two other studies demonstrating that the PMN L-selectin glycoprotein functions as an important ligand for E-selectin (Kishimoto et al., 1991; Picker et al., 1991). However, HL60 cells are not directly comparable to PMNs since they do not express L-selectin (Tedder et al., 1989). Furthermore, PMNs bind E-selectin-transfected cells more avidly than HL60 cells (Kishimoto et al., 1991) and this strengthens the concept that L-selectin is the major PMN "presenter" of sLex to E-selectin (Picker et al., 1991).
4.2.2 Downmodulation of L-selectin during PMN transmigration

The fourth, and last, specific aim of this thesis addressed whether the process of PMN transendothelial migration is associated with L-selectin downmodulation. The results show that, *in vitro*, L-selectin downmodulation is associated with PMN transendothelial migration and the evidence supporting this conclusion is that there is a qualitative reduction in L-selectin immunoreactivity on PMNs as they transmigrate an IL-1-stimulated endothelial monolayer. This observation implies that L-selectin downmodulation is initiated by PMN contact with and migration through an activated endothelium. Interestingly, complete downmodulation of L-selectin was never observed over the 5 minute period of adhesion and migration; PMNs continue to express low levels of L-selectin even after they transmigrate the endothelium.

The application of large numbers of PMNs (10⁶) to an IL-1-stimulated endothelial monolayer is an ideal way to induce synchronous transendothelial migration. In theory, random sections through this monolayer should show many PMN profiles in the process of adhesion and migration. In practice, the endothelial monolayers were very difficult to cryosection and the ultrastructural preservation was less than optimal. However, L-selectin antigenicity was maintained and the ultrastructural preservation was adequate for identifying PMNs in various stages of transendothelial migration. Surprisingly few adherent PMNs were observed as most had already transmigrated the endothelium by 5 minutes. However, in the few cases where an adherent PMN was observed, the portion of the PMN surface in contact with the endothelium was L-selectin negative. One interpretation of this observation is that L-selectin is downmodulated at the point of contact with the endothelium. Indeed, prolonged (30 minutes) adherence of PMNs to an IL-1-stimulated endothelium results in L-selectin
downmodulation (Smith et al., 1991). Alternatively, it is just as likely that L-selectin molecules are expressed on the PMN surface in contact with the endothelium, but because they are engaged with their endothelial ligands, they are not accessible to immunodetection with the DREG-200 MAb. Moreover, the surface of the PMN appears to be tightly apposed to the endothelium at the point of contact and this may preclude the penetration of immunogold reagents at the contact site. By comparison, the free-surface of the adherent PMN was L-selectin positive and the intensity and distribution of gold label was not appreciably different from that on the surface of non-adherent PMNs. If L-selectin were downmodulated on the PMN surface only at the point of contact with the endothelium, then L-selectin downmodulation on adherent PMNs is focal and contact dependent. One possible mechanism of L-selectin downmodulation is suggested by an independent study which showed that IL-1 stimulates endothelial monolayers to express cell surface-associated platelet activating factor (PAF). Importantly, this membrane-associated form of PAF contributes to L-selectin downmodulation on transmigrating PMNs (Kuijpers et al., 1992a). Although the present study does not confirm that L-selectin is downmodulated on the PMN surface that is in contact with the IL-1-stimulated endothelium, the lack of L-selectin immunoreactivity on the contact surface is consistent with a model of endothelial-associated PAF-induced L-selectin downmodulation.

Within 5 minutes, adherent PMNs quickly become activated as evidenced by their ability to transmigrate the endothelium and by the large numbers of PMNs that accumulate beneath the endothelium. Transmigration begins when the PMN extends a pseudopod through the endothelial monolayer. Importantly, the pseudopod is less immunoreactive for L-selectin than the portion of the PMN that has not yet passed through, or contacted, the endothelium. This observation is again consistent with the idea that L-selectin downmodulation is initiated
on the PMN surface in contact with the endothelium. The mechanism of L-selectin downmodulation on the PMN pseudopod is likely explained by L-selectin shedding during PMN activation (Kishimoto et al., 1989a). *In vitro* studies have demonstrated that following cell activation, PMNs shed \( \geq 95\% \) of their L-selectin within 5 minutes (Kishimoto et al., 1989a; Jutila et al., 1990). This time course for L-selectin shedding is compatible with the time course of the present study in which PMN transmigration was terminated by glutaraldehyde fixation 5 minutes after the addition of PMNs to the IL-1-stimulated endothelial monolayer. Furthermore, in the present study, there was no evidence of L-selectin internalization during PMN transmigration, supporting the concept that L-selectin is shed from the surface during PMN transendothelial migration.

### 4.3 Sensitivity and Limitations of the Immunogold Technique

Very little information is available for comparing the sensitivity of the immunogold technique to conventional methods of surface antigen detection. One FACS (fluorescence-activated cell sorter) study has compared the labeling of cell surface antigens with primary monoclonal antibodies followed by secondary polyclonal antibodies that are tagged with either a gold particle or a fluorescent marker. Importantly, when the secondary antibody is tagged with a colloidal gold particle (40 nm in diameter), it can be detected in the side scatter (90\(^\circ\)) channel in logarithmic amplification mode (Totterman and Festin, 1989). The results clearly showed that the colloidal gold method "...recognizes a population identical to that detected by the fluorochromes..." (Ibid). An independent study has shown that the immunogold labeling of cell surface antigens can be used to successfully study lymphocyte subsets by transmission electron microscopy (Hoogeveen et al., 1988).
In the present study, the immunogold technique was coupled to cryoultramicrotomy. The sensitivity of the cryotechnique is far superior to that obtained with epoxy or methacrylate embedded sections (personal experience; reviewed by van Bergen en Henegouwen, 1989). Moreover, surface detection of antigens on both PMNs and endothelial cells was enhanced in the present study because plasma proteins within the pulmonary microvasculature were not preserved during fixation. Gold labeling is enhanced, in the absence of plasma proteins, because the immunogold reagents had complete access to the endothelial or PMN cell surface throughout the depth (80-100 nm) of the cryosection. This was verified by tipping the goniometer stage in the electron microscope and observing the plasma membrane profiles en face. Importantly, gold particle counts were made on plasma membrane surfaces that had both length and depth; therefore, this is really a surface area count rather than a two-dimensional linear count. However, because the section thickness was held constant throughout the study, it was not a confounding variable in this study.

Two limitations of the immunogold technique were encountered in the present study. One was that it was not possible to detect colloidal gold particles at the site of close contact or adhesion between the PMN and the endothelium. Immunogold reagents are likely excluded from these regions because the adhesion molecule epitope recognized by the MAb is already engaged in cell-cell adhesion. Alternatively, the immunogold reagents may not be able to physically penetrate the close contact region between the cells. Obviously, an understanding of the expression of adhesion molecules at the contact region between the PMN and the endothelium is of great interest and importance. Receptors and ligands may be clustered at these sites as has been described for CD11b/CD18 during PMN adhesion to C3bi-coated erythrocytes (Detmers et al., 1987). Future prospects for studying the contact region with the
immunogold technique will require raising specific MAbs against cytoplasmic epitopes on the adhesion molecules.

A second limitation in this study relates to the small numbers of animals that were examined (9 rabbits; 13 mice); the labour intensive nature of these immunogold studies precluded the possibility of studying more animals. This limitation is best illustrated by the finding that it was not possible to determine with statistical certainty whether ICAM-1 expression on the pulmonary capillary endothelium is significantly increased during CD18-dependent PMN emigration in the mouse lung. This is an important question and one that can only be answered by studying larger numbers of mice.
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5.1 SUMMARY AND CONCLUSIONS

The main objective of this thesis has been to quantitate and compare the in vivo surface expression of three leukocyte-endothelial adhesion molecules (L-selectin and CD18 on PMNs; ICAM-1 on endothelial and epithelial cells) during CD18-independent and CD18-dependent PMN emigration in the lungs of rabbits and mice.

The working hypothesis of this thesis was that during an acute inflammatory response, the adherence of PMNs to the pulmonary microvasculature and their subsequent emigration into the alveolar airspace are dependent upon the complex interaction of cell surface adhesion molecules expressed on both the PMN and the endothelium. The principal site of PMN emigration during acute inflammation in the lung is the alveolar capillaries. PMNs that sequester in the capillary bed near the injured site are activated by the release of local inflammatory mediators and cytokines. Some of these stimuli will also activate the capillary endothelial cells. Therefore, PMN emigration in response to inflammatory stimuli that evoke either CD18-independent or CD18-dependent emigration, is associated with a downmodulation of L-selectin, an upmodulation of CD18, and an upmodulation of ICAM-1.

In this study, rabbits and mice received airway instillates of Streptococcus pneumoniae or Escherichia coli endotoxin to induce CD18-independent or CD18-dependent PMN emigration, respectively. Following the development of pneumonia, the lungs were fixed in glutaraldehyde, cryoprotected in 2.3M sucrose, frozen in liquid nitrogen, and sectioned on a cryoultramicrotome. Ultrathin cryosections were immunogold labeled with MAbs DREG-200 (against L-selectin) and 60.3 (against CD18) in the rabbit, or YN1/1.7.4 (against ICAM-1) in the mouse. Gold particles on the plasma membrane surfaces of PMNs, endothelial cells, and epithelial cells were quantitated by transmission electron microscopy and expressed as gold
The results clearly show that CD18-independent PMN emigration towards a bronchial instillate of *S. pneumoniae* is associated with L-selectin downmodulation and CD18 upmodulation on intravascular PMNs, prior to emigration. A similar alteration in the expression of L-selectin and CD18 is observed during CD18-dependent PMN emigration towards a bronchial instillate of *E. coli* endotoxin, but only on PMNs that have emigrated into the interstitium and airspace. It is concluded that increased CD18 expression on PMNs is not required for CD18-dependent emigration in the lung and this finding supports the concept that qualitative (conformational) changes rather than quantitative changes may be important to CD18-dependent adhesion and migration.

Interestingly, CD18 expression is increased 5.5-fold on PMNs within the alveolar airspace during streptococcal pneumonia and yet, CD18 is not required for CD18-independent PMN emigration. Because CD11b/CD18 is also capable of binding the complement split product C3bi, increased expression of CD11b/CD18 may facilitate PMN phagocytosis of C3bi-opsonized bacteria. In evidence is the observation that large numbers of streptococcal bacteria were ingested by airspace PMNs.

An important new finding is that regardless of the mechanism of PMN emigration, the change in PMN L-selectin and CD18 expression occurs only on PMNs within the pneumonic region. The evidence supporting this conclusion is that within the pulmonary microvasculature of the non-pneumonic contralateral rabbit lung, L-selectin and CD18 expression on intravascular PMNs is not different from control. Because downmodulation of L-selectin and upmodulation of CD18 occurs during PMN activation, these observations imply, in the case of streptococcal pneumonia, that PMNs become activated as they transit through the
inflammatory lesion and that once activated, they do not rejoin the circulation in appreciable numbers. In the case of endotoxin pneumonia, PMN activation occurs during transendothelial migration, as evidenced by an 86% reduction in L-selectin expression and a 100% increase in CD18 expression on interstitial PMNs. This observation is consistent with a role for L-selectin in PMN adhesion to the vascular endothelium and argues that L-selectin is not involved in PMN migration through the interstitial matrix.

PMN emigration towards lipopolysaccharide (endotoxin) in the mouse lung is known to be CD18-dependent. ICAM-1 expression increases 4.2 fold in response to \textit{E. coli} endotoxin and this finding borders on statistical significance, suggesting that ICAM-1 may be an important adhesive ligand for CD18 adhesion molecules during CD18-dependent emigration in the lung. Although it remains to be determined whether PMN emigration towards \textit{S. pneumoniae} in the mouse lung is CD18-independent, the observation that PMN emigration occurred in the absence of an increase in capillary endothelial ICAM-1 expression is consistent with the concept of a CD18-independent emigration mechanism.

A potentially important new observation is that in normal mouse lung, constitutive ICAM-1 expression is 22-fold greater on the alveolar epithelium compared to the capillary endothelium and epithelial expression is mainly restricted to the Type I pneumocytes. The function of this epithelial ICAM-1 expression is unknown. However, alveolar macrophages express high surface levels of CD11a/CD18 and it is conceivable that macrophages adhere to epithelial ICAM-1 as they travel over the epithelial surface scavenging inhaled particulates. Interestingly, Type II pneumocytes constitutively express very little ICAM-1, yet during pneumonia, ICAM-1 expression increased as much as 178-fold. The reason for this increased expression is unknown, but it may be an early differentiation event that precedes Type II
pneumocyte proliferation. When the epithelium is injured, Type II pneumocytes proliferate and replace the damaged epithelium (Adamson and Bowden, 1974; Evans et al., 1973). It is conceivable that during pneumonia, Type II pneumocytes are stimulated by inflammatory mediators, possibly released by the alveolar macrophage. However, epithelial injury, as measured by morphologic criteria, is not a prerequisite for Type II pneumocyte ICAM-1 induction since the epithelium appeared to be undamaged at 24h following the instillation of colloidal carbon alone, yet the Type II pneumocytes showed a 52-fold increase in ICAM-1 expression.

For comparative purposes, the immunogold method was used to study the in vitro expression of L-selectin on unstimulated human PMNs and on human PMNs engaged in transmigration of an IL-1-stimulated endothelial monolayer. There were two specific aims to this part of the study and the question addressed by the first was whether L-selectin is topographically positioned on the PMN surface such that it renders L-selectin-associated sLex more "bioavailable" for the vascular selectins P- and E-selectin. In vitro results on human PMNs demonstrate that L-selectin is topographically positioned on the PMN surface microvilli. Remarkably, the same observation was made for PMNs in the rabbit and this suggests that it may be a common finding in other animal species as well. Although L-selectin-associated sLex accounts for only 5% of protein associated surface sLex (Picker et al., 1991), its concentration on the microvilli may render it more "bioavailable" for the vascular selectins, P- and E-selectin. The other specific aim addressed whether L-selectin downmodulation is coincidental with PMN transendothelial migration. The results show that L-selectin downmodulation is coincidental with PMN transendothelial migration and it appears to be induced by contact with the endothelium. Interestingly, L-selectin was also downmodulated on rabbit PMNs during
transendothelial migration towards *E. coli* endotoxin and, as mentioned above, L-selectin downmodulation is an indication of PMN activation. The mechanism of PMN activation was not determined in this thesis, but it may involve an endothelial-associated form of PAF that has been shown to contribute to L-selectin downmodulation (Kuijpers et al., 1992a).

**5.2 FUTURE PROSPECTS**

The findings reported in this thesis have suggested several potential avenues of future research. As already mentioned, it remains to be determined whether ICAM-1 expression is significantly increased on pulmonary capillary endothelial cells during CD18-dependent PMN emigration in the mouse lung. This question could be best addressed by repeating the present study, but with larger numbers of mice. Five control mice and 5 endotoxin-treated mice would provide sufficient statistical power to determine whether the 4.2-fold increase in endothelial ICAM-1 expression during endotoxin pneumonia is significant. If ICAM-1 expression is significantly increased at 24h, it would be worth examining earlier time points to determine whether PMN emigration can occur prior to an increase in endothelial ICAM-1 expression. Finally, a group of mice could be pretreated with the blocking anti-ICAM-1 MAb YN1/1.7.4 (Doerschuk et al., 1992) to determine whether ICAM-1 expression is essential for CD18-dependent PMN emigration in the lung.

A second avenue of research would be to determine whether the presence of ICAM-1 on the Type I pneumocytes facilitates alveolar macrophage motility and thereby enhances the macrophages ability to scavenge and ingest inhaled particulates. The working hypothesis is that alveolar macrophages crawl over the epithelial surface in order to phagocytose inhaled particulates and that the adhesive interaction between the macrophage and the epithelium is
both CD18- and ICAM-1-dependent. This hypothesis predicts that macrophages will ingest fewer particulates if their motility is inhibited by an anti-ICAM-1 blocking MAb, like YN1/17.4 (Doerschuk et al., 1992). An aerosol mixture of colloidal carbon particles in saline could be delivered into the lungs of mice and the numbers of macrophages with ingested carbon particles could be determined by light microscopic histology at various time intervals (e.g. 0, 10, 30, and 60 minutes). The experiment could be repeated, but this time the colloidal carbon particles would be mixed with the YN1/1.7.4 MAb. In the presence of this MAb, fewer macrophages should show signs of colloidal carbon ingestion and those that ingest carbon, will contain only minor amounts because they will only have access to carbon particles that have been deposited on or adjacent to the macrophage. If this experiment proved successful, the study could be expanded to determine which member of the CD18-family was interacting with the ICAM-1. This question could be addressed by substituting anti-CD11a, anti-CD11b, or anti-CD11c MAbs for the anti-ICAM-1 MAb.

A third avenue of research would be to determine whether the induction of ICAM-1 expression on Type II pneumocytes is an early differentiation event that precedes proliferation (mitosis). The working hypothesis is that Type II pneumocytes show enhanced proliferation following injury to the alveolar epithelial cell surface and this enhanced proliferation will be preceded by increased ICAM-1 expression. To test this hypothesis, Type II pneumocytes could be induced to proliferate by exposing the lungs of mice to 90% oxygen (Adamson and Bowden, 1974). The rate of Type II pneumocyte proliferation could be monitored by tritiated thymidine incorporation (Ibid) and lung tissue could be simultaneously processed for immunogold labeling of ICAM-1 as described in this thesis.

A fourth, but by no means final, avenue of research would be to determine whether L-
selectin and ICAM-1 are required for CD18-independent emigration in the lung. The morphological data presented in this thesis suggest that ICAM-1 and L-selectin are unlikely to be involved in CD18-independent PMN emigration because endothelial ICAM-1 expression is not increased and L-selectin is downmodulated on intravascular PMNs that have not emigrated. The working hypothesis is that neither L-selectin nor ICAM-1 is required for CD18-independent PMN emigration. This hypothesis could be tested by pretreating rabbits with a mixture of the blocking MAbs 60.3 (anti-CD18) and DREG-200 (anti-L-selectin) or 60.3 and R6.5 (anti-ICAM-1) (Barton et al., 1989; Argenbright et al., 1991) and then instilling *S. pneumoniae* to induce CD18-independent PMN emigration, as described in this thesis. The 60.3 MAb would inhibit any possible contribution of CD18 to the PMN emigration process. PMN emigration could be quantitated by either bronchial lavage or by light microscopic histology. The prediction is that neither the DREG-200 or the R6.5 MAb will have any effect on CD18-independent PMN emigration.

5.3 SIGNIFICANCE OF THIS THESIS

The adherence and emigration of leukocytes at sites of injury or infection is an early and essential step in the defense and repair of the host tissues. However, there are several clinical disorders that arise as complications of an unwanted inflammatory or immune response, including: rheumatoid arthritis, adult respiratory distress syndrome, ischemia-reperfusion injury and allograft rejection (reviewed by Harlan et al., 1992). Current research in this area is focused on the development of anti-adhesion therapies to prevent leukocyte-mediated vascular and tissue injuries (Ibid). The study of leukocyte-endothelial interactions *in vivo* using animal models is a necessary prerequisite to understanding "...the cell biology, physiology, and
clinical relevance of leukocyte-endothelial interactions in humans." (Ibid). The findings presented in this thesis provide new information that confirm and extend our present understanding of leukocyte-endothelial (and epithelial) adhesive interactions. Moreover, these findings offer the possibility of new avenues of research that in themselves will lead to a more complete understanding of the role of adhesion molecules in health and disease.
TABLE 1. *L*-selectin study: Summary of morphometric data on rabbit neutrophils (PMNs) in control lungs and lungs instilled with an inflammatory stimulus (*S. pneumoniae* or *E. coli* endotoxin) and colloidal carbon

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lung region examined</th>
<th>Total # gold particles</th>
<th>Total memb. length (µm)</th>
<th>Mean memb. length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No instillate (n=3 rabbits)</td>
<td>intravascular</td>
<td>3,189&lt;sup&gt;a&lt;/sup&gt;</td>
<td>581&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.4 ± 3.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4h <em>S. pneumoniae</em> (n=3 rabbits)</td>
<td>intravascular</td>
<td>561</td>
<td>515</td>
<td>17.2 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>interstitial&lt;sup&gt;d&lt;/sup&gt;</td>
<td>124</td>
<td>207</td>
<td>20.7</td>
</tr>
<tr>
<td></td>
<td>airspace</td>
<td>93</td>
<td>691</td>
<td>23.0 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>contralateral intravascular</td>
<td>3,700</td>
<td>446</td>
<td>14.9 ± 4.4</td>
</tr>
<tr>
<td>4h <em>E. coli</em> endotoxin (n=3 rabbits)</td>
<td>intravascular</td>
<td>1,569</td>
<td>606</td>
<td>20.2 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>interstitial</td>
<td>563</td>
<td>733</td>
<td>24.4 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>airspace</td>
<td>161</td>
<td>722</td>
<td>24.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>contralateral intravascular</td>
<td>2,657</td>
<td>486</td>
<td>16.2 ± 2.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> total number of gold particles counted on 30 PMN profiles.
<sup>b</sup> total plasma membrane length examined from 30 PMN profiles.
<sup>c</sup> mean ± (SD) PMN plasma membrane length examined/rabbit.
<sup>d</sup> only 10 PMN profiles were examined in one rabbit; all other regions had 30 PMN profiles from three rabbits.
TABLE 2. CD18 study: Summary of morphometric data on rabbit neutrophils (PMNs) in control lungs and lungs instilled with an inflammatory stimulus (S. pneumoniae or E. coli endotoxin) and colloidal carbon

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lung region examined</th>
<th>Total # gold particles</th>
<th>Total memb. length (µm)</th>
<th>Mean memb. length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No instillate (n=3 rabbits)</td>
<td>intravascular⁴</td>
<td>3,619⁺</td>
<td>397⁻</td>
<td>13.7 ± 3.1⁴</td>
</tr>
<tr>
<td>4h S. pneumoniae (n=3 rabbits)</td>
<td>intravascular</td>
<td>6,312</td>
<td>281</td>
<td>9.4 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>interstitial</td>
<td>10,183</td>
<td>460</td>
<td>15.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>airspace</td>
<td>18,053</td>
<td>396</td>
<td>13.0 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>contralateral intravascular</td>
<td>4,274</td>
<td>434</td>
<td>14.5 ± 4.2</td>
</tr>
<tr>
<td>4h E. coli endotoxin (n=3 rabbits)</td>
<td>intravascular⁴</td>
<td>3,813</td>
<td>340</td>
<td>11.7 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>interstitial</td>
<td>9,704</td>
<td>578</td>
<td>19.3 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>airspace</td>
<td>13,620</td>
<td>538</td>
<td>17.9 ± 1.3</td>
</tr>
</tbody>
</table>

¹ only 29 PMN profiles examined from three rabbits; all other regions had 30 PMN profiles from three rabbits.
⁺ total number of gold particles counted on 30 PMN profiles.
⁻ total plasma membrane length examined from 30 PMN profiles.
⁴ mean ± (SD) PMN plasma membrane length examined/rabbit.
TABLE 3. *L*-selectin immunoreactivity on rabbit neutrophils (PMNs) in control lungs and lungs instilled with an inflammatory stimulus (*S. pneumoniae* or *E. coli* endotoxin) and colloidal carbon

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lung region examined</th>
<th>intravascular</th>
<th>interstitial</th>
<th>airspace</th>
<th>contralateral intravascular</th>
</tr>
</thead>
<tbody>
<tr>
<td>No instillate (Control)</td>
<td></td>
<td>5.6 ± 3.2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>4h <em>S. pneumoniae</em></td>
<td></td>
<td>0.9 ± 1.5</td>
<td>0.04 ± 0.02</td>
<td>8.1 ± 5.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(S. pneumoniae)</td>
<td>(0.22)</td>
<td>(0.01)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4h <em>E. coli</em> endotoxin</td>
<td></td>
<td>2.7 ± 3.7</td>
<td>0.6 ± 1.0</td>
<td>5.6 ± 2.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(E. coli endotoxin)</td>
<td>(0.14)</td>
<td>(0.02)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Three rabbits were studied for each treatment. Values outside of closed brackets are the mean ± (SD) gold particles/µm plasma membrane. Values within closed brackets are the estimated fold difference in L-selectin immunoreactivity relative to that on the control PMNs. NA, not applicable; ND, not determined.

*a* P < 0.05 vs. intravascular (Control).

*b* P < 0.05 vs. intravascular (*E. coli* endotoxin)
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lung region examined</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>intravascular</td>
<td>interstitial</td>
<td>airspace</td>
<td>contralateral</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>intravascular</td>
</tr>
<tr>
<td>No instillate (Control)</td>
<td>8.6 ± 1.8</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4h S. pneumoniae</td>
<td>21.7 ± 6.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.5 ± 4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.4 ± 7.6&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>9.1 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>(2.6)</td>
<td>(2.6)</td>
<td>(5.5)</td>
<td></td>
</tr>
<tr>
<td>4h E. coli endotoxin</td>
<td>11.0 ± 4.3</td>
<td>16.7 ± 2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.4 ± 13.2&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.0)</td>
<td>(3.2)</td>
<td></td>
</tr>
</tbody>
</table>

Three rabbits were studied for each treatment. Values outside of closed brackets are the mean ± (SD) gold particles/μm plasma membrane. Values within closed brackets are the estimated fold difference in CD18 immunoreactivity relative to that on the control PMNs. NA, not applicable; ND, not determined.

<sup>a</sup> P < 0.05 vs. intravascular (Control).
<sup>b</sup> P < 0.05 vs. interstitial (S. pneumoniae).
<sup>c</sup> P < 0.05 vs. intravascular (S. pneumoniae).
<sup>d</sup> P < 0.05 vs. intravascular (E. coli endotoxin).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lung cell type</th>
<th>Total # gold particles</th>
<th>Total memb. length (μm)</th>
<th>Mean memb. length (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No instillate (n=3 mice)</td>
<td>endothelial</td>
<td>502&lt;sup&gt;a&lt;/sup&gt;</td>
<td>666&lt;sup&gt;b&lt;/sup&gt;</td>
<td>222 ± 74&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Type I</td>
<td>15,355</td>
<td>1326</td>
<td>442 ± 147</td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>110</td>
<td>337</td>
<td>112 ± 55</td>
</tr>
<tr>
<td>4h colloidal carbon (n=2 mice)</td>
<td>endothelial</td>
<td>282</td>
<td>343</td>
<td>172 ± 65</td>
</tr>
<tr>
<td></td>
<td>Type I</td>
<td>5,058</td>
<td>522</td>
<td>261 ± 69</td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>101</td>
<td>392</td>
<td>196 ± 51</td>
</tr>
<tr>
<td>24h colloidal carbon (n=2 mice)</td>
<td>endothelial</td>
<td>220</td>
<td>268</td>
<td>140 ± 63</td>
</tr>
<tr>
<td></td>
<td>Type I</td>
<td>1,990</td>
<td>281</td>
<td>134 ± 28</td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>718</td>
<td>270</td>
<td>135 ± 23</td>
</tr>
<tr>
<td>4h S. pneumoniae (n=3 mice)</td>
<td>endothelial</td>
<td>799</td>
<td>699</td>
<td>233 ± 57</td>
</tr>
<tr>
<td></td>
<td>Type I</td>
<td>5,298</td>
<td>847</td>
<td>282 ± 91</td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>1,140</td>
<td>468</td>
<td>156 ± 30</td>
</tr>
<tr>
<td>24h E. coli endotoxin (n=3 mice)</td>
<td>endothelial</td>
<td>1,201</td>
<td>586</td>
<td>195 ± 28</td>
</tr>
<tr>
<td></td>
<td>Type I</td>
<td>3,258</td>
<td>586</td>
<td>195 ± 38</td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>5,134</td>
<td>572</td>
<td>191 ± 37</td>
</tr>
</tbody>
</table>

<sup>a</sup> total number of gold particles counted on the electron microscope.
<sup>b</sup> total plasma membrane length examined on the electron micrographs.
<sup>c</sup> mean ± (SD) plasma membrane length examined in each mouse.
TABLE 6. ICAM-1 immunoreactivity in control mouse lungs and lungs instilled with colloidal carbon ± an inflammatory stimulus (*S. pneumoniae* or *E. coli* endotoxin)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lung cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>endothelial</td>
</tr>
<tr>
<td>No instillate (Control)</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>4h colloidal carbon</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>24h colloidal carbon</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>4h <em>S. pneumoniae</em></td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>24h <em>E. coli</em> endotoxin</td>
<td>2.0 ± 1.2&lt;sup&gt;c&lt;/sup&gt; (4.2)</td>
</tr>
</tbody>
</table>

Three mice were studied for each treatment, except for 4h and 24h colloidal carbon where two mice were studied for each treatment. Values outside of closed brackets are the mean ± (SD) gold particles/μm plasma membrane. Values within closed brackets are the estimated fold difference in ICAM-1 immunoreactivity relative to that on the corresponding control cell type.

<sup>a</sup> Bonferroni-adjusted P < 0.0125 vs. endothelial (Control).
<sup>b</sup> Bonferroni-adjusted P < 0.0125 vs. Type II (Control).
<sup>c</sup> Bonferroni-adjusted P = 0.017 vs. endothelial (Control).
Figure 1: Figure showing the leukocyte-endothelial adhesion molecules that have been characterized at the molecular level and shown to be important for PMN adhesion and migration during acute inflammation. Note that the endothelial adhesion molecules E- and P-selectin recognize PMN molecules bearing the sialyl Lewis X antigen (sLex*); however, the identity of these PMN molecules awaits characterization. Interestingly, L-selectin on the PMN also bears an sLex epitope and there is some evidence that it can bind to endothelial E-selectin and P-selectin. L-selectin also has its own lectin-binding domain and can potentially interact with additional, and as yet uncharacterized, endothelial carbohydrate-bearing ligands. The leukocyte integrins CD11a/CD18 and CD11b/CD18 can both bind to the endothelial ligand ICAM-1 (intercellular adhesion molecule-1) and CD11a/CD18 can also bind to ICAM-2. The endothelial ligand recognized by CD11c/CD18 has not been characterized, nor has its role in the inflammatory process been clearly defined. See Introduction for more information on each adhesion molecule.
LEUKOCYTE-ENDOTHELIAL ADHESION MOLECULES
IMPORTANT TO
PMN ADHESION AND EMIGRATION

CD11c/CD18
CD11b/CD18
ICAM-1
ICAM-2
E-selectin
P-selectin

© A. Burns 1991

INFLAMED TISSUE
Figure 2: Figure showing the "Specimen Pin Jig" that was specially constructed to manufacture large numbers of inexpensive brass specimen pins for cryosectioning. The jig was made from a rectangular-shaped block of aluminium. A central guide hole (1/8 inch diameter) was drilled through the long axis of the jig. In addition, two hacksaw blade guide slots were cut into the top surface of the jig at right angles to the guide hole axis. A moveable "swing stop" was mounted at either end of the jig using a nut and bolt. In this way, the jig could be used by either a left-handed or right-handed operator. To fabricate the specimen pins, the jig was secured in a table-top vice and a 2-3 foot long piece of brass brazing rod (1/8 inch diameter) was inserted into the guide hole until it butted up against the aluminum "swing stop." Holding the rod firmly in one hand, a metal hacksaw (32 teeth/inch) was then used to cut through the brazing rod. To retrieve the newly cut pin, the swing stop was moved aside and the length of brazing rod pushed forward until the pin fell out onto the table. With the brazing rod in position, the swing stop was lowered once more and another pin was cut. Numerous specimen pins were produced in only a few minutes using this method.
Figure 2

Specimen Pin Jig

- Swing Stop
- Guide Hole 1/8" I.D.
- Hacksaw Blade Guide Slots

1 1/2"

2 1/4"

1 1/4"

1/8"

© A. Burns
1990
Figure 3: Electron micrograph of an intravascular PMN in a rabbit that received a bronchial instillate of *E. coli* endotoxin mixed with colloidal carbon. Three nuclear lobes (N) are visible within this PMN and electron dense accumulations of heterochromatin are evident. Note that the diameter of the blood vessel is greater than that of the PMN and that the PMN is approximately spherical in shape. The surface of the PMN is convoluted and it possesses numerous short microvilli (arrowheads). The cytoplasmic granules of this PMN appeared empty (electron translucent) suggesting that the granule contents have been lost during tissue processing. Scale = 1.0 μm.

Figure 4: Electron micrograph of an elongated intravascular PMN in a control rabbit. Because the diameter of the capillary is very small (3.0 μm), the PMN is elongated in shape and it has a flattened surface. This surface is tightly apposed (black arrows) to the endothelium lining the pulmonary capillary. This particular piece of lung tissue was not subjected to immunogold labeling because it was fixed with a relatively high concentration of glutaraldehyde (0.1%, rather than 0.025%); at 0.1% glutaraldehyde, the affinity of MAb 60.3 for CD18 is significantly reduced. However, this micrograph illustrates the important point that, in cryosectioning, a small change in the concentration of the fixative can dramatically affect the ultrastructural preservation of the tissue. In evidence is the improved preservation of the PMN cytoplasmic granule contents which are no longer electron translucent (see Figure 3). A single nuclear lobe (N) and a single centriole (white arrow) can also be appreciated in the cytoplasm of this PMN. Scale = 1.0 μm.
Figure 5: Electron micrograph of an interstitial PMN in a rabbit that received a bronchial instillate of *E. coli* endotoxin mixed with colloidal carbon. Note the elongated shape of this PMN and the filipodial extension (arrow). Collagen fibres (arrowheads) confirm the interstitial location of the PMN. N = nuclear lobe. Scale = 1.0 μm.

Figure 6: Electron micrograph of an airspace PMN in a rabbit that received a bronchial instillate of *S. pneumoniae* mixed with colloidal carbon. The shape of this PMN is essentially spherical and the cell surface is highly ruffled. Note that, although there were colloidal carbon particles (arrowhead) on the cell surface of the PMN, there was no evidence of carbon ingestion. However, there was evidence of bacterial ingestion (arrows). N = nuclear lobe. Scale = 1.0 μm.
Figure 7: Electron micrograph of an airspace PMN in a rabbit that received a bronchial instillate of *E. coli* endotoxin mixed with colloidal carbon. The shape of this PMN is essentially spherical and the cell surface is quite ruffled. Note that, a large amount of colloidal carbon was associated with the cell surface (arrows), but there was no evidence of carbon ingestion. N = nuclear lobe. Scale = 1.0 μm.

Figure 8: Electron micrograph of an airspace PMN in a rabbit that received a bronchial instillate of *S. pneumoniae* mixed with colloidal carbon. Note the large numbers of bacteria that have been ingested by this PMN (arrows) and the lack of carbon ingestion. N = nuclear lobe. Scale = 1.0 μm.
Figure 9: Electron micrograph of a pneumonic lung in a rabbit that received a bronchial instillate of *S. pneumoniae* mixed with colloidal carbon. Four PMNs (P), an alveolar macrophage (M), a hemolysed red blood cell (R), and colloidal carbon (*) can be seen within the alveolar airspace. In contrast to the PMNs, the alveolar macrophage ingested more colloidal carbon (arrowheads) than it did bacteria (arrow). Scale = 2.0 μm.
Figure 10: Immunoelectron microscopic detection of L-selectin on a spherically-shaped intravascular PMN in control rabbit lung. Note that the majority of immunogold particles are localized to the surface microvilli (arrowheads). No intracellular pool of L-selectin was associated with the cytoplasmic granules (*). N = nuclear lobe. Scale = 0.5 μm.

Figure 11: Immunoelectron microscopic detection of L-selectin on an elongated intravascular PMN in control rabbit lung. Note the generally smooth and flattened appearance of the cell surface. Despite the lack of surface microvilli, immunogold particles appeared to be grouped into small clusters (arrowheads), rather than being distributed as single particles over the cell surface. The cytoplasmic vesicles (*) did not show evidence of gold labeling. N = nuclear lobe. Scale = 0.5 μm.
Figure 12: Immunoelectron microscopic detection of CD18 on an intravascular PMN in control rabbit lung. Immunogold particles were detected on the microvilli (arrowheads) and flatter regions (arrows) of the cell surface. A large intracellular pool of CD18 was present as evidenced by the intense gold labeling over the cytoplasmic granules. The majority of these gold particles were associated with the inner surface of the granule membrane. N = nuclear lobe. Scale = 0.25 μm.
Figure 13: Immunoelectron microscopic detection of L-selectin on an intravascular PMN in rabbit lung following a bronchial instillate of *S. pneumoniae* mixed with colloidal carbon. Clusters of immunogold particles (arrowheads) are seen over the entire free surface of the cell. Six out of ten PMNs examined in this rabbit were L-selectin positive, the other four PMNs showed no evidence of gold labeling. Arrows = colloidal carbon particles associated with the alveolar epithelium. N = nuclear lobe. Scale = 0.5 μm.

Figure 14: Immunoelectron microscopic detection of L-selectin on an intravascular PMN in rabbit lung following a bronchial instillate of *S. pneumoniae* mixed with colloidal carbon. Note the absence of gold labeling on the surface microvilli (arrowheads), the flatter regions of the cell surface (arrows), and the cytoplasmic granules (*). In contrast to the findings reported in Figure 14, of the ten PMNs examined in this rabbit, all of them were L-selectin negative. N = nuclear lobe. Scale = 0.25 μm.
Figure 15: Immunoelectron microscopic detection of L-selectin on an airspace PMN in rabbit lung following a bronchial instillate of *S. pneumoniae* mixed with colloidal carbon. Note the lack of gold particles on the microvilli (arrowheads) and the flatter regions of the cell surface (arrows). There is evidence of bacterial ingestion (star) in preference to colloidal carbon (*). N = nuclear lobe. Scale = 0.5 μm.
Figure 16: Immunoelectron microscopic detection of L-selectin on an interstitial PMN in rabbit lung following a bronchial instillate of *S. pneumoniae* mixed with colloidal carbon. This is the same rabbit in which L-selectin positive intravascular PMNs (Figure 14) were found. The interstitial location of this PMN is confirmed by the presence of collagen fibres (arrowheads) and elastin (star). Note the scarcity of gold particles on the cell surface (arrows) and complete lack of gold label within the cytoplasmic vesicles (*). N = nuclear lobe. Scale = 0.5 μm.
**Figure 17:** Immunoelectron microscopic detection of L-selectin on an intravascular PMN from the contralateral, non-pneumonic lung of a rabbit that received a bronchial instillate of *S. pneumoniae* mixed with colloidal carbon. Note the abundant gold labeling on the cell surface and its preference for the microvilli (arrows). Gold label was not detected within the cytoplasmic granules (*). N = nuclear lobe. Scale = 0.5 μm.

**Figure 18:** Immunoelectron microscopic detection of L-selectin on an intravascular PMN from the contralateral, non-pneumonic lung of a rabbit that received a bronchial instillate of *E. coli* endotoxin mixed with colloidal carbon. Large numbers of gold particles are present on the cell surface (arrows) but none are located over the cytoplasmic granules (*). N = nuclear lobe. Scale = 0.25 μm.
Figure 19: Immunoelectron microscopic detection of L-selectin on an interstitial PMN in rabbit lung following a bronchial instillate of *E. coli* endotoxin mixed with colloidal carbon. No gold particles are located on the surface of this PMN or within the cytoplasmic granules (*). Collagen fibres (arrows) adjacent to the surface of the PMN clearly establish the interstitial location of this PMN. N = nuclear lobe. Scale = 0.5 μm.

Figure 20: Immunoelectron microscopic detection of L-selectin on an airspace PMN in rabbit lung following a bronchial instillate of *E. coli* endotoxin mixed with colloidal carbon. No gold particles are present on the surface of this PMN or within the cytoplasmic granules (*). Interestingly, this PMN appears to have formed an adhesive contact (arrows) with the alveolar epithelial surface. N = nuclear lobe. Scale = 0.5 μm.
**Figure 21:** Immunoelectron microscopic detection of CD18 on an intravascular PMN in rabbit lung following a bronchial instillate of *S. pneumoniae* mixed with colloidal carbon. Large numbers of gold particles are evenly distributed all over the entire cell surface (arrows), as well as in the cytoplasmic granules (*). The labeling density on the cell surface is greater than that on control intravascular PMNs (see Figure 12). N = nuclear lobe. Scale = 0.25 μm.
Figure 22: Immunoelectron microscopic detection of CD18 on an interstitial PMN in rabbit lung following a bronchial instillate of \textit{S. pneumoniae} mixed with colloidal carbon. The gold label density on the cell surface is similar to that on intravascular PMNs within the pneumonic region (see Figure 21). Many of the cytoplasmic granules (*) show gold particle labeling along the inner membrane surface. Some portions of the PMN cell surface appear to be in contact with the interstitial collagen fibres (Type I collagen, arrows). This observation is interesting because CD18 can serve as a receptor for Type I collagen. Although CD18-mediated adherence to collagen can not be required for CD18-independent PMN emigration, it may augment PMN migration through the interstitium. $N = \text{nuclear lobe}$. Scale = 0.25 $\mu$m.
Figure 23: Immunoelectron microscopic detection of CD18 on an airspace PMN in rabbit lung following a bronchial instillate of *S. pneumoniae* mixed with colloidal carbon. The gold label density on the cell surface is much greater than that observed on intravascular (Figure 21) or interstitial (Figure 22) PMNs within the pneumonic region. The gold particles are widely distributed over the entire cell surface of this airspace PMN, except where the surface is in contact with colloidal carbon (arrow). A few cytoplasmic granules (*) show gold labeling along their inner membrane surface. Note the absence of gold particles on the phagosomal membrane surrounding an ingested bacterium (star). N = nuclear lobe. Scale = 0.25 μm.
Figure 24: Immunoelectron microscopic detection of CD18 on an intravascular PMN in the contralateral, non-pneumonic lung of a rabbit that received a bronchial instillate of *S. pneumoniae* mixed with colloidal carbon. Few gold particles are present on the cell surface (arrowheads) and the density of gold label is similar to that of control intravascular PMNs (Figure 12). Remarkably, the vast majority of gold particles are located over the cytoplasmic granules (*) and most of these particles are associated with the inner membrane surface of the granule. N = nuclear lobe. Scale = 0.25 μm.
**Figure 25:** Immunoelectron microscopic detection of CD18 on an interstitial PMN in rabbit lung following a bronchial instillate of *E. coli* endotoxin mixed with colloidal carbon. Along the entire free surface of this PMN, numerous CD18-specific gold particles can be seen (arrowheads). The labeling density appears to be somewhat higher than that on control intravascular PMNs (Figure 12). Note that the cell surface of the PMN is in contact with another interstitial cell (arrowheads). The identity of this second cell is unknown, but it is probably another leukocyte as evidenced by the surface gold labeling of its plasma membrane. Interestingly, in spite of the observation that both of these cells are CD18 positive, no gold particles are found along the contact region (white arrows). N = nuclear lobe. Black arrows = collagen fibres. Scale = 0.5 μm.
Figure 26: Immunoelectron microscopic detection of CD18 on an airspace PMN in rabbit lung following a bronchial instillate of *E. coli* endotoxin mixed with colloidal carbon. Large numbers of gold particles are present along the entire cell surface (arrowheads). The gold label density is much greater than that on control intravascular PMNs (Figure 12). In spite of this increase in surface CD18 immunoreactivity, large numbers of gold particles are still detected within the cytoplasmic granules (*) and these particles are clearly associated with the inner membrane surface of the granules. N = nuclear lobe. Scale = 0.5 μm.
Figure 27: Light micrograph of mouse lung tissue 4h after the tracheal instillation of a saline solution of colloidal carbon. A few alveolar macrophages (arrows) are present, but the alveolar airspace shows no evidence of PMN infiltration and these lungs are not different from that of normal mice (not shown). Ar = bronchial artery. Br = bronchiole. Stain = Toluidine blue O. Magnification = 200X.

Figure 28: Light micrograph of alveolar macrophages in mouse lung tissue 4h after the tracheal instillation of a saline solution of colloidal carbon. Each macrophage (arrows) ingested colloidal carbon as evidenced by the golden-brown cytoplasmic accumulations. Br = bronchiole. Stain = Toluidine blue O. Magnification = 500X.
Figure 29: Light micrograph of mouse lung tissue 24h after the tracheal instillation of a saline solution of colloidal carbon. The appearance of the lung is similar to that of the lung at 4h after colloidal carbon instillation (Figures 27 and 28). Again, a few alveolar macrophages are seen within the airspace (arrows) and there is no evidence of PMN infiltration. Ar = bronchial artery. Br = bronchiole. Stain = Toluidine blue O. Magnification = 200X.

Figure 30: Light micrograph of alveolar macrophages in mouse lung tissue 24h after the tracheal instillation of a saline solution of colloidal carbon. Note the cytoplasmic accumulations of golden-brown colloidal carbon particles within each of the alveolar macrophages (arrows). Stain = Toluidine blue O. Magnification = 500X.
Figure 31: Light micrograph of mouse lung tissue 4h after the tracheal instillation of a \textit{S. pneumoniae} mixed with colloidal carbon. Alveolar macrophages ingested colloidal carbon (large arrowhead) and this confirmed the delivery of \textit{S. pneumoniae} to the lung region shown. Large numbers of interstitial (small arrowheads) and airspace (arrows) PMNs can be seen. Airspace PMNs show little evidence of colloidal carbon ingestion. The dotted-line denotes the boundary between the bronchovascular interstitium and the alveolar airspace. \textit{Ar} = bronchial artery. \textit{Br} = bronchiole. \textit{Stain} = Toluidine blue O. Magnification = 500X.

Figure 32: Light micrograph of an alveolar PMN infiltrate in mouse lung 4h after the tracheal instillation of a \textit{S. pneumoniae} mixed with colloidal carbon. Numerous PMN aggregates can be seen within the alveolar airspace (arrows) and these PMNs again show no evidence of colloidal carbon ingestion. The alveolar walls are poorly defined amongst the extravasated red blood cells (*). \textit{Stain} = Toluidine blue O. Magnification = 800X.
Figure 33: Light micrograph of mouse lung tissue 24h after the tracheal instillation of a *E. coli* endotoxin mixed with colloidal carbon. The dotted-line denotes the boundary between the bronchovascular interstitium and the alveolar airspace. Note the presence of PMNs within the bronchovascular interstitium (arrowheads). Alveolar macrophages containing ingested colloidal carbon are present and this confirms the delivery of *E. coli* endotoxin to this region of the lung. Ar = bronchial artery. Br = bronchiole. Stain = Toluidine blue O. Magnification = 500X.

Figure 34: Light micrograph of an alveolar PMN infiltrate in mouse lung tissue 4h after the tracheal instillation of a *E. coli* endotoxin mixed with colloidal carbon. The airspace contains both PMNs (arrows) and alveolar macrophages (arrowheads). Unlike the streptococcal pneumonias (Figures 31 and 32), no extravasated red blood cells are present. The presence of colloidal carbon within the alveolar macrophages confirms the delivery of *E. coli* endotoxin to the lung region. Note the minimal amount of carbon ingestion by the airspace PMNs. Magnification = 500X.
Figure 35: Electron micrograph showing a cross-section of a pulmonary capillary in normal mouse lung tissue. This section has been immunogold labeled for ICAM-1, but at this magnification, gold particles can not be distinguished. A small region of this pulmonary capillary has been enlarged in Figure 36 for the purpose of illustrating ICAM-1 expression on the endothelium. R = red blood cell. Scale = 1.0 µm.

Figure 36: Immunoelectron microscopic detection of ICAM-1 on the pulmonary capillary endothelium in normal mouse lung. Note that this micrograph is an enlargement of the pulmonary capillary shown in Figure 35. Very few gold particles (arrows) are found on the lumenal surface of the capillary endothelium. R = red blood cell. Scale = 0.25 µm.
Figure 37: Immunoelectron microscopic detection of ICAM-1 on the alveolar epithelial cell surface in normal mouse lung. The expression of ICAM-1 is largely restricted to the Type I pneumocytes (arrows). Very few gold particles are present on the surface of the Type II pneumocyte (II). Scale = 0.5 μm.
Figure 38: Electron micrograph contrasting the difference in ICAM-1 immunoreactivity between a Type I and II pneumocyte in normal mouse lung. Large numbers of immunogold particles are located on the surface of the Type I pneumocyte (black arrows) but not on the Type II pneumocyte (II). Note how the immunogold labeling abruptly ceases at the cellular junction (white arrows) between the two cells. A lamellar body (*) containing surfactant can be appreciated within the cytoplasm of the Type II pneumocyte. Scale =0.25 μm.
Figure 39: Immunoelectron microscopic detection of ICAM-1 in mouse lung 24h after a tracheal instillation of *E. coli* endotoxin mixed with colloidal carbon. Immunogold labeling is strictly extracellular, and the gold particles are exclusively detected on lumenal cell surfaces. Significantly, in contrast to normal mouse lung (Figures 37 and 38), large numbers of immunogold particles are present on the surface (arrows) of the Type II pneumocyte (II). ICAM-1 immunoreactivity is also increased on the pulmonary capillary endothelium (small arrowheads) when compared to normal mouse lung (Figure 36). The gold label density on the surface of the Type I pneumocyte appears to be less than that observed in normal mouse lung (Figures 37 and 38). Scale = 0.5 μm.
Figure 40: Immunoelectron microscopic detection of ICAM-1 on a Type II pneumocyte in mouse lung 4h after a tracheal instillation of *S. pneumoniae* mixed with colloidal carbon. In contrast to normal mouse lung (Figures 37 and 38), numerous gold particles are found (arrows) on the surface of the Type II pneumocyte (II). This increase in ICAM-1 immunoreactivity is confined to the lumenal cell surface. No immunogold labeling is seen over the cytoplasm or intracellular organelles.
Figure 41: Immunelectron microscopic detection of L-selectin on normal human PMNs in a suspension of leukocyte-rich plasma. L-selectin immunoreactivity is concentrated on microvilli (arrows) of the PMN surface and is absent from granule membranes (*). Scale = 0.25 μm.

Figures 42: Immunelectron microscopic detection of total sLex immunoreactivity on normal human PMNs in a suspension of leukocyte-rich plasma. In contrast to L-selectin (Figure 41), total sLex immunoreactivity is found on both the plasma membrane and granule membranes (*). Gold particles do not show a preference for surface microvilli (arrows) and are often associated with the flatter regions of the cell surface (arrowheads). Scale = 0.25 μm.

Figure 43: Immunelectron microscopic detection of CD18 immunoreactivity on normal human PMNs in a suspension of leukocyte-rich plasma. In contrast to L-selectin (Figure 41), CD18 immunoreactivity is found on both the plasma membrane and granule membranes (*). Gold particles do not show a preference for surface microvilli (arrow) and are often associated with the flatter regions of the cell surface (arrowheads). Scale = 0.25 μm.
Figure 44: Electron micrograph of an IL-1 stimulated human endothelial monolayer 5 minutes after the addition of unstimulated human PMNs. The endothelial monolayer (arrows) is resting on a layer of collagen fibres (*). Two PMNs are in the field of view. One is non-adherent (A), while the other (B) has adhered to the endothelial surface. This cryosection was immunogold labeled for L-selectin, but gold particles can not be appreciated at this magnification. For the purpose of viewing the L-selectin immunoreactivity, magnified views of cell "A" and cell "B" are shown in Figures 45 and 46, respectively. Scale = 2.0 μm.
Figure 45: Immunoelectron microscopic detection of L-selectin on a human PMN that has not yet adhered to an IL-1-stimulated endothelial monolayer. Note that this micrograph is an enlargement of cell "A" in Figure 44. L-selectin immunoreactivity is restricted to the cell surface. Gold particles are largely concentrated on the surface microvilli (arrows), with fewer gold particles being detected on the flatter regions of the cell surface (arrowheads). Scale = 0.5 μm.
Figure 46: Immunoelectron microscopic detection of L-selectin on a human PMN that has adhered to an IL-1-stimulated endothelial monolayer. Note that this micrograph is an enlargement of cell "B" in Figure 44. L-selectin immunoreactivity (arrowheads) is restricted to that portion of the cell surface that is not in contact with the endothelium. The adherent portion of the PMN (arrows) is flattened and tightly apposed to the endothelium. No gold particles can be seen along this contact region. N = nuclear lobe. Scale = 0.5 μm.
Figure 47: Electron micrograph of a human PMN that was engaged in transmigrating the IL-1 stimulated endothelial monolayer (E). The PMN has extended a pseudopod through the endothelial monolayer (arrows). A gap exists between the monolayer and the layer of collagen fibres (*). Note that this cryosection was immunogold labeled for L-selectin and magnified views of these immunogold particles are provided in Figures 48 and 49. Scale = 2.0 μm.

Figure 48: Immunelectron microscopic detection of L-selectin on a portion of a transmigrating human PMN that has not yet penetrated the IL-1-stimulated endothelial monolayer. Note that this micrograph is an enlargement of a portion of the transmigrating PMN in Figure 47. L-selectin immunoreactivity is conspicuously present on the surface microvilli (arrows), as well as flatter regions of the cell surface (arrowheads). Gold particles are not found within the cytoplasmic granules (*). N = nuclear lobe. Scale = 0.25 μm.

Figure 49: Immunelectron microscopic detection of L-selectin on a portion of a transmigrating human PMN that has penetrated an IL-1-stimulated endothelial monolayer. Note that this micrograph is an enlargement of a portion of the transmigrating PMN in Figure 47. L-selectin immunoreactivity is not detected within the cytoplasmic granules (*). The gold label density on the cell surface appears to be reduced compared to that portion of the cell that had not yet penetrated the endothelium (Figure 48). Importantly, L-selectin immunoreactivity is still detected on the most distal tip of the pseudopod that has penetrated the endothelium (arrows). Scale = 0.25 μm.
**Figure 50:** Electron micrograph of several human PMNs that have penetrated the IL-1 stimulated endothelial monolayer (E) within 5 minutes. Three PMN profiles (A, B, and C) can be seen lying within a large space between the endothelial monolayer and the layer of collagen fibres (*). This cryosection was immunogold labeled for L-selectin and a magnified view of the immunogold particles is provided in Figure 51. Scale = 3.0 μm.

**Figure 51:** Immunoelectron microscopic detection of L-selectin on PMNs that have completely penetrated the IL-1-stimulated endothelial monolayer. Note that this micrograph is an enlargement of cells "A" and "B" in Figure 50. At this magnification, it is apparent that these PMNs occupy a space that is between the endothelium and the basal lamina (arrowheads). Small amounts of L-selectin immunoreactivity are still detected on the cell surface (arrows) even though these PMNs have completely transmigrated the endothelium. No gold particles are located over the cytoplasmic granules (*). Scale 1.0 μm.
Figure 52: Immunoelectron microscopic detection of non-immune mouse IgG labeling of a non-adherent human PMN as a control for non-specific gold labeling. Note the lack of gold particle labeling on the surface microvilli (arrows) and flatter regions of the cell surface (arrowheads). In addition, no gold particles can be seen over the cytoplasmic granules (*). These observations confirm the specificity and sensitivity of the immunogold labeling technique for the detection of L-selectin immunoreactivity on human PMNs. Scale = 0.5 μm.
REFERENCES


