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The role of E-selectin in two murine models of acute inflammation

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The role of E-selectin in two murine models of acute inflammation

by

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ABSTRACT

E-selectin, an endothelial cell adhesion molecule, is expressed on the surface of activated endothelial cells. Its role in neutrophil recruitment in acute murine inflammation, a previously undescribed entity, was the focus of this work. The interaction between P-selectin and E-selectin in neutrophil extravasation was also investigated.

Two murine models of acute inflammation were utilized in this work: acute pneumonitis induced by intranasal (IN) instillation of bacterial lipopolysaccharide (LPS) and chemical peritonitis induced by intraperitoneal (IP) injection of thioglycollate broth. Intranasal LPS, a novel model, resulted in a progressive influx of neutrophils into the lungs begining near airways and extending into distant alveoli 4-6 hours later. Intraperitoneal thioglycollate is a model commonly used to examine the role of adhesion molecules in acute inflammation.

E-selectin expression, which peaked 4-6 hours post-treatment, was immunohistochemically detected on the endothelium of scattered vessels in the lungs of mice following IN instillation of LPS. P-selectin was detected in endothelial cells in control mouse lungs and increased post-treatment with IN LPS.

Blocking with anti-E-selectin MAb did not decrease the neutrophil count in the peritoneal cavity following IP injection of thioglycollate. Nor did these antibodies decrease the neutrophil count in the bronchoalveolar lavage of mice 2 hours after IN instillation of LPS; whereas, by 6 hours post-treatment, there was a moderate decrease. However, when anti-P-selectin MAb were combined with the anti-E-selectin MAb, a significant increase in blocking was seen in the 6 hour group, demonstrating a redundancy in the function of these molecules. A

carbohydrate analog of the selectin ligand was unable to block neutrophil influx in either model.

Transgenic mice deficient in the gene for E-selectin had no macro- or microscopic abnormalities when compared to wild-type mice. They did have slightly elevated numbers of neutrophils in circulating in blood. However, neutrophil numbers in the peritoneal lavage of E-selectin deficient mice were not different from wild-type mice after IP thioglycollate. In conclusion, the role of E-selectin in acute murine inflammation is one which overlaps with P-selectin.

GENERAL INTRODUCTION

Preamble

Acute inflammation is characterized by neutrophilic infiltrates (1). While neutrophils are essential components of the inflammatory response, their potent secretory products can cause tissue damage and contribute to the pathogenesis of a variety of diseases (2).

In inflammatory sites, the endothelium plays an active role in the recruitment of neutrophils and other leukocytes by expressing surface adhesion molecules that interact with complementary ligands on leukocytes (3). Leukocyte adhesion to the endothelium involves transient events between endothelial and leukocyte adhesion molecules that result in the extravasation of leukocytes. This process, in the case of neutrophils, involves three steps: initial attachment and rolling of neutrophils along the activated endothelium, firm adhesion of activated neutrophils to the endothelium, and extravasation of neutrophils into the surrounding inflamed tissue (4).

The endothelial-leukocyte adhesion molecules are members of three families: the integrin superfamily, the immunoglobulin superfamily, and the selectins. Integrin and immunoglobulin superfamily members mediate the firm adhesion of activated neutrophils to the endothelium and their subsequent extravasation. The remaining family, the selectins, mediate the rolling phase of neutrophil recruitment.

E-selectin, P-selectin, and L-selectin are the three known members of the selectin family. E- and P-selectin are expressed on the surface of activated endothelial cells in areas of acute inflammation, while P-selectin is also expressed

on activated platelets. L-selectin is constitutively expressed on the surface of a variety of leukocytes including neutrophils (5).

Studies characterizing the expression and function of adhesion molecules have used antibodies recognizing functional epitopes. These "blocking" antibodies bind to the adhesion molecule and block interaction with the ligand. Adhesion molecule antagonists, such as blocking antibodies, can therefore be used to examine the relative importance of a particular adhesion pathway in the extravasation of leukocytes (6). In addition, and perhaps more importantly, adhesion molecule antagonists may represent a novel approach to the treatment of neutrophil-mediated inflammatory diseases.

The adhesion molecule of interest in these studies was E-selectin, because novel, previously unavailable reagents have been generated to examine its role in murine inflammation. Understanding the role of E-selectin in mice is a critical link to full characterization of this molecule in other species. Potentially, adhesion pathways that are shown to be crucial in animal models of inflammation could be blocked in neutrophil-mediated diseases of humans. Studies in animal models of disease have already demonstrated a reduction in tissue injury by blocking a variety of adhesion molecules, and human trials are currently underway using an anti-CD18 (a member of the integrin superfamily) monoclonal antibody.

Statement of objectives

- Develop and characterize murine models of acute inflammation to examine the role of E-selectin.
- Describe the expression of E-selectin and, secondarily, P-selectin in a murine model of acute inflammation.

- Examine the ability of selectin antagonists to block the infiltration of neutrophils into the site of inflammation *in vivo*. A second, related question was examined: is there redundancy in the selectin family such that blocking both E- and P-selectin results in an additive decrease in inflammation?
- Characterize the phenotype of transgenic mice deficient in the gene for E-selectin and utilize these mice to study the role of E-selectin in models of acute inflammation.

Explanation of dissertation format

This dissertation is in a non-traditional format arranged in parts. The style is based on the *American Review of Respiratory Disease*. Each part is meant to stand alone. However, the organization into parts is for the purpose of the dissertation. The material is not intended for publication in the format presented. This work, which took place in the Department of Toxicology and Pathology at Hoffmann-La Roche, inc., Nutley, NJ, was part of the *in vivo* support for a much larger research effort examining the role of adhesion molecules, E-selectin in particular, in inflammation. Several portions of this work will be published in collaboration with these colleagues. A literature review precedes the first paper. All cited literature appears once at the end of the dissertation.

LITERATURE REVIEW

Neutrophils as mediators of inflammation

Infiltration of neutrophils is a hallmark of acute inflammation. These leukocytes accumulate in areas of acute inflammation where they play a crucial role in defense against bacterial infections and removal of damaged or necrotic cells. The necessity for neutrophils in host defense is illustrated by the increased susceptibility to infection and decreased life expectancy of individuals lacking the ability to mount an appropriate acute inflammatory response (7). However, while neutrophils are necessary and protective, they can also cause tissue damage. These destructive activities likely contribute to the pathogenesis of a variety of disease states, including adult respiratory distress syndrome (ARDS) (8), rheumatoid arthritis (9), reperfusion injury (10, 11), and ulcerative colitis (12).

Phagocytosis is the process through which neutrophils attach to, engulf, and kill or degrade invading pathogens and/or particulate matter. Attachment requires recognition of the bacterium as foreign which can occur to a limited degree without serum (1) but is most efficient when opsonins, such as antibodies and complement components, coat the particle. Engulfment proceeds through the extension of pseudopodia around the object being phagocytized thus forming a membrane bound phagosome which fuses with lysosomes creating a phagolysosome. Killing and degradation involve a variety of mechanisms which are by convention divided into two systems based on location within the neutrophil: plasma membrane and intracellular granules (2). These two systems were once thought to operate as distinct entities but are now known to act synergistically in the destruction of both invading pathogens and adjacent host tissues (13).

The membrane-associated system utilizes an NADPH oxidase present in the neutrophil plasma membrane to produce toxic oxygen metabolites via the oxidation of NADPH and reduction of oxygen (14). These reactive oxygen species, including H_2O_2 and the hydroxyl radical, are capable of mediating endothelial damage (15, 16). Perhaps more importantly, hydrogen peroxide combines with a halide in the presence of myeloperoxidase contained in the azurophilic granules of the neutrophil to form a hypohalous acid with enhanced killing and tissue destructive ability (1, 2). The hypohalous acid HOCl, formed from the oxidation of a chloride ion, is a very potent oxidant and is produced in large quantities (17) by activated neutrophils, suggesting it may play an important role in neutrophil mediated-damage.

While the NADPH oxidase system lacks specificity and has products with relatively short half-lives, the enzymes contained within the neutrophil intracellular granules can catalyze multiple reactions and attack specific substrates. More than 20 of these enzymes have been identified, including microbicidal enzymes, neutral proteinases, metalloproteinases, acid hydrolases, and lactoferrin (2). Proteolytic enzymes, such as elastase, coliagenase, and gelatinase normally play a role in the degradation of the extracellular matrix as part of repair processes. Their activity is limited by powerful antiproteinases, such as α_1-proteinase inhibitor and α_2-macroglobulin, present in serum and extracellular fluid (18, 19). When the balance between the proteinases and antiproteinases is lost, the proteinases can cause injury of viable tissues. *In vitro* (20, 21, 22) and *in vivo* (23) studies have demonstrated a role for neutrophil-derived proteinases in neutrophil-mediated injury. Oxidants produced by the NADPH oxidant system inactivate proteinase

inhibitors creating a synergy which enhances the neutrophil tissue destructive capabilities (24, 25).

Endothelial cell-leukocyte interactions

Neutrophil extravasation into sites of inflammation is dependent upon their adherence to the local endothelium (26). Two basic mechanisms which mediate this interaction have been described (27). The first mechanism is direct activation of the leukocytes by inflammatory mediators, such as leukotriene B_4 (LTB_4) (28), f-Met-Leu-Phe (fMLP), and C5a (29). Once activated, neutrophils are able to adhere to unstimulated endothelial cells *in vitro* but only under conditions of flow with wall shear stress lower than would be encountered *in vivo* (30) suggesting that this mechanism may be an *in vitro* artifact. The second proposed mechanism involves cytokine activation of endothelial cells with subsequent leukocyte rolling along the endothelium, activation of the leukocytes, and finally stable adhesion prior to migration of leukocytes into the surrounding tissue (Fig. 1). This dynamic, three-step model of leukocyte interaction with the endothelium (rolling, leukocyte activation, firm adhesion) was first suggested by Kishimoto (31) and shortly thereafter supported by Butcher (32). That leukocytes leave the stream of flowing blood in the center of a post-capillary venule and roll along the endothelium prior to extravasation has been proposed for over a century (33) and was described in detail in the 1970s (34, 35, 36). However, the exact mechanisms which mediate the interaction of leukocytes with endothelial cells has been delineated more clearly only in the last decade during which a vast amount of work has been done to define and clarify leukocyte-endothelial interactions and the involved adhesion molecules. There are three superfamilies of molecules with members

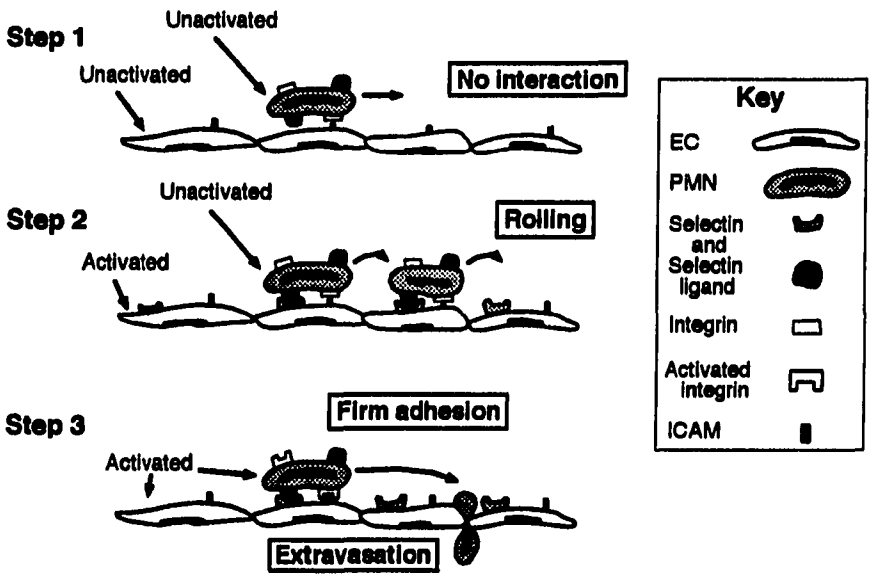

Figure 1. Illustration demonstrating the three step model of leukocyte interaction with endothelial cells (EC). Step 1 represents leukocyte flow through a vessel with no inflammatory stimulus. Step 2 shows the rolling that occurs when the selectins interact with their ligands on activated endothelial cells. Symbols for the selectins and selectin ligands are interchangeable to represent the presence of L-selectin on the leukocyte while E- and P-selectin are expressed on endothelial cells. Step 3 demonstrates the firm adhesion of activated leukocytes with endothelial cells which is followed by extravasation of the leukocytes. From: *Adhesion: Its Role in Inflammatory Disease* by Harlan. Copyright © 1991 by W.H. Freeman and Company. Reprinted with permission (4).

having known roles in the interaction of neutrophils with endothelial cells: integrins, immunoglobins, and selectins.

Integrin superfamily

The β_2 integrins, also known as the leukocyte integrins (37, 38), are the most widely studied of the leukocyte-endothelial cell adhesion molecules. They belong to the much larger integrin superfamily whose members are heterodimeric, cell-surface glycoproteins composed of an α and a β subunit. The integrins have three subfamilies grouped by their β subunits (β_1, β_2, or β_3) with each subfamily

Table 1

The leukocyte integrins

adhesion molecule	*pseudonyms*	*cellular distribution*	*control*	*ligand*
CD11a/CD18	LFA-1, $\alpha_L\beta_2$	all leukocytes	constitutively expressed, avidity controlled	ICAM-1, 2, 3
CD11b/CD18	Mac-1, Mo-1, $\alpha_m\beta_2$, CR3	granulocytes, monocytes, macrophages, large granular lymphocytes	constitutively expressed, up-regulated from granules, avidity controlled	ICAM 1,2, iC3b, fibrinogen, LPS
CD11c/CD18	p150,95, $\alpha_x\beta_2$, CR4	granulocytes, monocytes, macrophages	constitutively expressed, up-regulated from granules, avidity controlled	fibrinogen, C3d, C3bi

having distinct characteristics. The leukocyte integrins have the β_2 subunit, designated CD18, noncovalently bound to one of three different α subunits, CD11a, CD11b, or CD11c, forming what are known as LFA-1, Mo-1/Mac-1/CR3, and p150,95 respectively (Table 1) (39, 40, 41).

As suggested by the name, the leukocyte integrins are found exclusively on leukocytes and hematopoetic progenitor cells (Fig. 2). CD11a/CD18 (LFA-1) is present on all leukocytes and plays an important role in adhesion and cell-mediated cytotoxicity involving lymphocytes in particular. CD11b/CD18 (Mac-1) expression is limited to granulocytes, monocytes and macrophages, and large granular lymphocytes. It has a variety of functions, including binding to iC3b, through which it mediates leukocyte adherence and aggregation, chemotaxis, antibody-dependent cellular toxicity, and phagocytosis and intracellular killing of

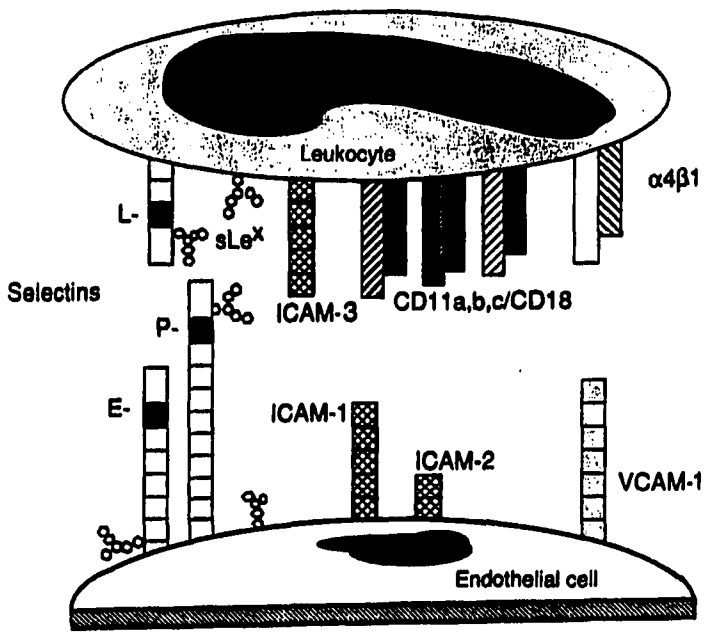

Figure 2. Model of a leukocyte and an endothelial cell demonstrating the location of the various adhesion molecules on each cell type. Not all molecules will be present on a particular type of leukocyte or a given endothelial cell. Modified figure reproduced, with permission, from the Annual Review of Immunology, Volume 11, ©1993, by Annual Reviews of Immunology (3).

iC3b-opsonized microorganisms. The expression of CD11c/CD18 (p150,95) is limited to granulocytes, monocytes, and macrophages, and its main function is an involvement in monocyte adherence and aggregation (42).

LFA-1 and Mac-1 are the integrins responsible for binding stimulated neutrophils to the endothelium and are also involved in their subsequent transendothelial migration (43, 44, 45). Surface expression of both molecules increases upon activation of neutrophils by a wide variety of stimulatory factors including phorbol esters, fMLP, tumor necrosis factor-α (TNF), and C5a (38). However, increased surface expression is not correlated with the ability of the neutrophils to bind to the endothelium (46) suggesting the requirement for a

conformational change resulting in an increased avidity prior to the adhesive event (41, 47). For example, LFA-1 on the surface of phorbol ester-stimulated, adherent T lymphocytes is converted from a low affinity to a high affinity state without a change in surface density (48, 49).

The autosomal, recessive congenital condition known as leukocyte adhesion deficiency I (LAD I) clearly demonstrates the importance of the β_2 integrins. LAD I is a syndrome characterized by severe and recurrent infections, delayed wound healing, and marked peripheral blood granulocytosis which results from a deficiency or lack of surface expression of these integrins due to a defect in the CD18 component of the heterodimer (42). Numerous studies using blocking monoclonal antibodies (MAb) to functional epitopes on CD11/CD18 have confirmed the role β_2 integrins play *in vitro* (28, 37) and *in vivo* (6). In addition, transgenic mice with a hypomorphic (partially deficient) CD18 allele mimic the clinical signs seen in LAD I patients. They represent a useful, albeit somewhat diluted, model for this syndrome and further demonstrate the role of the β_2 integrins (50).

Immunoglobulin superfamily

Members of the immunoglobulin (Ig) superfamily comprise the complementary endothelial ligands for the leukocyte integrins. These adhesion molecules, like all Ig superfamily members, are characterized by Ig homology domains each containing a disulfide loop. To date, four Ig family members which are involved in leukocyte-endothelial cell interactions have been identified and cloned: intercellular adhesion molecule-1 (ICAM-1) (51), intercellular adhesion molecule-2 (ICAM-2) (52), intercellular adhesion molecule-3 (ICAM-3) (53, 54), and vascular cell adhesion molecule-1 (VCAM-1) (55) (Table 2).

Table 2

The immunoglobulin superfamily

adhesion molecule	*structural characteristics*	*cellular distribution*	*control*	*ligand*	*bind PMN to endothelium?*
VCAM-1	7 Ig-like domains	endothelial cells, macrophages, myoblasts, bone marrow fibroblasts	cytokine and LPS induced expression	VLA-4, $\alpha_4\beta_1$ integrin	no
ICAM-1 (CD54)	5 Ig-like domains	ubiquitous	constitutively expressed at low level, induced by LPS and cytokines	LFA-1, Mac-1, CD43; adhesion is Ca^{++}-dependent	yes
ICAM-2	2 Ig-like domains	endothelial cells, lymphocytes, monocytes	constitutively expressed	LFA-1; Ca^{++}-dependent	yes
ICAM-3	5 Ig-like domains	all leukocytes	constitutively expressed	LFA-1; Ca^{++}-dependent	no

VCAM-1 Present on cytokine- and LPS-activated endothelial cells (Fig. 2), tissue macrophages, dendritic cells, myoblasts, and bone marrow fibroblasts (55), VCAM-1 is the ligand for very late antigen-4 (VLA-4) (56) which is not a β_2 integrin but rather a β_1 integrin. The β_1 subfamily, also referred to as the VLA proteins, contains at least six members whose main function is attachment of cells to the extracellular matrix through recognition of the tripeptide RGD (57). The VLA proteins can specifically interact with collagen, fibronectin, and/or laminin (58). In general, VLA-4 is present on lymphocytes, monocytes, and several T- and B-cell leukemic lines but not on neutrophils. Human neutrophils do not bind to VCAM-1 nor is their binding to cytokine-activated human umbilical vein endothelial cells (HUVE) inhibited by a blocking anti-VCAM-1 antibody (41).

ICAM-1 The remaining members of the Ig family involved in leukocyte adhesion, the intercellular adhesion molecules (ICAMs), were identified by their ability to bind LFA-1 (54, 59, 60, 61, 62). ICAM-1 (CD54) has five Ig-like domains (63), contains no RGD motifs, and bears homology to the neural cell adhesion molecule, NCAM (51). Its expression on many cell types including endothelial cells (Fig. 2), lymphocytes, macrophages, and many epithelial cells is cytokine-inducible (64). ICAM-1 has low, constitutive expression on endothelial cells which is significantly increased by interleukin-1 (IL-1), TNF, interferon-γ (IFN-γ) (65), and bacterial lipopolysaccharide (LPS) (66). The protein synthesis-dependent expression is increased rapidly in the first 3 hours and remains elevated for up to 36 hours (40). Mac-1 (CD11b/CD18) is another ligand for ICAM-1 and binds in a manner which is more temperature sensitive and lower in avidity than the LFA-1:ICAM-1 interaction (67). Mac-1 interacts with the third domain of ICAM-1 while LFA-1 interacts with the first domain (63). In addition, ICAM-1 on activated endothelial cells mediates adhesion of unactivated neutrophils via LFA-1, while adhesion in the presence of chemotactic factors, which stimulate neutrophils, is a Mac-1-dependent process (43). The importance of ICAM-1 *in vivo* is demonstrated by transgenic mice deficient in ICAM-1, which have decreased neutrophil migration into the peritoneum following intraperitoneal (IP) instillation of thioglycollate (68). Blocking MAb to ICAM-1 have also been demonstrated to inhibit neutrophil, eosinophil, and lymphocyte emigration in several *in vivo* models of inflammation (69, 70, 71, 72, 73).

ICAM-2 ICAM-2, another member of the Ig superfamily, has two Ig-like domains which share homology with the two amino-terminal domains of ICAM-1 (52). It is present on the surface of resting lymphocytes and unstimulated

endothelial cells (Fig. 2) and is not inducible with cytokines or LPS making it the predominant ligand for LFA-1 on resting endothelial cells (74). LFA-1 is the only known ICAM-2 counter-receptor and has lower affinity for ICAM-2 than ICAM-1. Blocking MAb studies have demonstrated that LFA-1-dependent binding to both resting and activated endothelial cells could be totally accounted for by ICAM-1 and ICAM-2 together (61).

ICAM-3 While ICAM-1 and ICAM-2 play a role in neutrophil adhesion to endothelial cells, ICAM-3 appears to have no involvement in neutrophil localization in inflammatory conditions. It is a recently cloned molecule which was identified by monoclonal antibodies based on its high expression on all leukocytes, ability to bind LFA-1 (54), and its ability to inhibit lymphoblastoid cell adhesion to purified LFA-1 independent of ICAM-1 and ICAM-2 (62). Composed of five Ig-like domains and bearing 52% amino acid identity to ICAM-1, ICAM-3 is expressed only on leukocytes and is completely absent on endothelial cells even following stimulation by LPS or cytokines (53). It is the major LFA-1 ligand on resting lymphocytes (75) and CD1+ epidermal dendritic Langerhans cells (76). Because of its pattern of expression, ICAM-3 is believed to function in the initiation of the immune response.

Selectin family

The remaining family of adhesion molecules involved in leukocyte-endothelial interactions, the selectins, currently has three known members: E-selectin, P-selectin, and L-selectin (Table 3). Originally, the members of this family were studied independently by numerous laboratories in different fields of study as activation-induced endothelial and platelet surface proteins and the lymphocyte homing receptor. However, the cloning and sequencing of these molecules

Table 3

The selectins

adhesion molecule	*pseudonyms*	*cellular distribution*	*control*	*ligand*
L-selectin	LECAM-1, LAM-1, Leu-8, $gp90^{MEL}$, mLHR	granulocytes, some lymphocytes, monocytes	constitutively expressed, shed from surface upon activation, avidity controlled?	sulfated polysaccharides, phoshorylated monosaccharides, sLe^a, sLe^x
P-selectin	GMP-140, CD62, PADGEM	endothelial cells, platelets	constitutively expressed, upregulated from granules by histamine, PAF, thrombin, others; upregulated expression by cytokines and LPS	sulfated glycolipids, sLe^a, sLe^x
E-selectin	ELAM-1, LECAM-2	endothelial cells	upregulated expression by cytokines and LPS	sLe^a, sLe^x

revealed a common structural motif composed of an NH_2-terminal Ca^{2+}-dependent (C-type) lectin-like domain (~120 residues), an epidermal growth factor-like (EGF) domain (~30 residues), between 2 and 9 complement-regulatory homology (CR) repeat sequences (~60 residues each), a transmembrane domain, and a short intracytoplasmic tail (77, 78, 79, 80). The lectin and EGF domains bear the important functional epitopes as has been demonstrated using chimeras with missing or mixed domains, peptide blocking, site specific mutagenesis, and blocking antibodies which bind to these domains (78, 81, 82, 83, 84, 85, 86, 87, 88, 89). The selectins are highly glycosylated with about 30% of their mass composed of carbohydrates (77, 80, 90). To clear confusion created by multiple names for the same family and its members and to indicate the

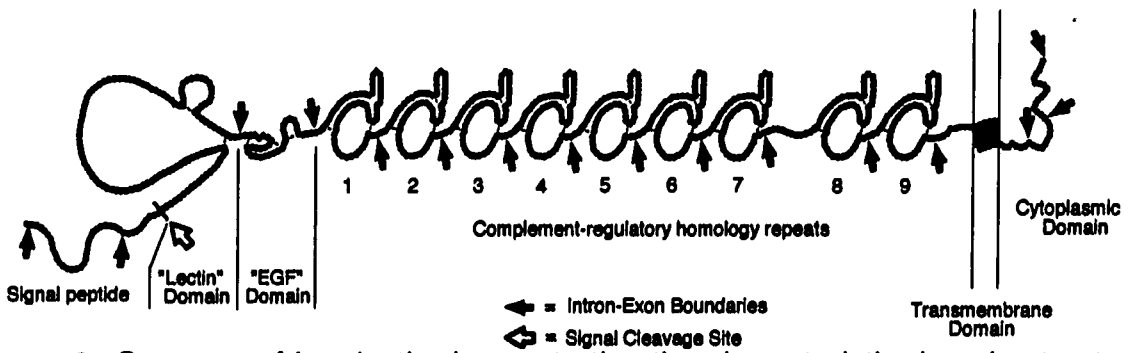

Figure 3. Structure of L-selectin demonstrating the characteristic domain structure of the selectins: amino-terminal lectin-like domain, EGF-like domain, complement regulatory-like repeats, transmembrane domain, and the short cytoplasmic tail. L-selectin contains 9 complement regulatory-like repeats while E-selectin has 6 and P-selectin has 2. The positions of the exon-intron boundaries relative to the domain structure demonstrates the relationship between the two. Similar boundaries are present in E- and P-selectin. Reprinted with permission from The American Society for Biochemistry and Molecular Biology (91).

carbohydrate-recognition imparted by the common lectin-like domain, the term selectin was proposed for this family of adhesion receptors. Using this standard nomenclature, members of the family are designated by a capital letter prefix determined by the cell type on which the molecule was originally identified (E:endothelium, P:platelets, L:lymphocytes) (92).

Analysis of genomic clones of the selectins demonstrated a correlation between the structural domains of the proteins and the exon-intron structure of the genes (Fig. 3) (91, 93, 94, 95). The lectin-like and EGF-like domains of the selectins have 60% amino acid (aa) identity and the CR repeats have 40% aa identity (96). The lectin domain, with possible involvement of the EGF-like domain, is crucial for the adhesive function of the selectins (97). Chromosomal *in situ* hybridization has been used to map the three known members of the selectin

family to the long arm of chromosome 1 (93, 98, 99), while long range restriction site analysis has demonstrated tight clustering of the selectin genes in both the human and mouse genome (100). This cluster is located near the complement-regulatory proteins composed of structurally homologous CR repeats (101). Indirect evidence, including the above, suggests exon shuffling and gene duplication likely played a role in the evolution of the selectin family (97).

L-selectin The first selectin studied as an adhesion molecule, L-selectin, was identified using a MAb (Mel-14) generated against a murine lymphoma. This MAb blocks lymphocyte binding to high endothelial venules of lymph nodes *in vitro* (102) and lymphocyte homing to lymph nodes *in vivo* (103). Relatively recently, it was realized that Leu-8, described shortly before the Mel-14 antigen as a human lymphocyte marker (104), is actually human L-selectin (105).

L-selectin is constitutively expressed on neutrophils, monocytes, some lymphocytes, and other myeloid cells (106, 107, 108, 109, 110) and is rapidly shed from the cell surface by proteolytic cleavage upon cellular activation (38, 111, 112) a process believed to play a role in regulating the activity of this molecule (113). L-selectin has been shown to be at least partially responsible for the adhesion of neutrophils, lymphocytes, and monocytes to activated endothelium in areas of inflammation (114, 115, 116, 117, 118). Like the integrins, it is proposed that leukocyte activation leads to an increase in the activity of L-selectin which in this case is immediately prior to being shed (119).

P-selectin P-selectin was originally identified by a MAb generated to study platelet activation. Stored in alpha and dense granules of platelets and Weibel-Palade bodies of endothelial cells (120, 121, 122), P-selectin is very rapidly expressed through membrane fusion of the intracellular granule with the cell

surface upon stimulation by inflammatory mediators, such as thrombin, complement components, oxygen radicals, and histamine (123, 124, 125). It mediates leukocyte binding to activated endothelial cells or platelets (126, 127, 128, 129). Surface expression of P-selectin is normally transient (5); however, free radicals result in prolonged expression which suggests the presence of activated neutrophils and their products could contribute to prolonged surface expression (4, 125, 130). There is also evidence suggesting P-selectin can be synthesized *de novo* in response to cytokines, such as IL-1 and TNF (131, 132, 133), although this does not fit the original dogma which tied P-selectin to early stages of inflammation only (4). Within minutes after its expression on the surface of endothelial cells, P-selectin begins to be downregulated via endocytosis with routing through the Golgi and recycling back to Weibel-Palade bodies (134).

A strain of transgenic mice deficient in the P-selectin gene fully illustrate the importance of P-selectin in neutrophil extravasation at sites of inflammation. These mice have elevated peripheral neutrophil counts, virtually total absence of neutrophil rolling in exteriorized mesenteric venules, and delayed recruitment of neutrophils into the peritoneal cavity following intraperitoneal thioglycollate instillation (135).

E-selectin E-selectin, unlike P- and L-selectin, is not constitutively expressed but requires *de novo* RNA and protein synthesis (27, 136). *In vitro* exposure to the cytokines IL-1α , IL-1β, and TNF as well as bacterial LPS or substance P result in E-selectin expression on the surface of endothelial cells within 1-2 hours with maximal expression at 4-6 hours and a decline to nearly basal levels by 24 hours (110, 136, 137, 138). The upregulation of E-selectin expression occurs via massive gene transcription (139). While interferon-γ does

not induce E-selectin expression, it can prolong its expression in response to other cytokines (140, 141). E-selectin expression is also regulated by programmed disappearance both by internalization and subsequent lysosomal degradation and by release from the surface of the activated endothelial cells (134, 142, 143, 144, 145).

In vivo, like *in vitro*, E-selectin expression has experimentally been demonstrated by immunohistochemical staining and Northern blotting to begin 1-2 hours after stimulation by LPS, TNF, or IL-1 given intradermally (ID) or intravascularly (IV) to primates or rodents (146, 147, 148). With a single injection of an inflammatory stimulus, this expression peaks at approximately 4-6 hours and returns to very low levels or is absent by 24-48 hours after the stimulus (149, 150). Studies in humans have demonstrated E-selectin expression in a wide variety of conditions including cardiac allograft rejection, atopy, ultraviolet B skin exposure, systemic sclerosis, graft-versus-host disease, and rheumatoid arthritis (151, 152, 153, 154, 155, 156). It is interesting to note the prolonged expression of E-selectin in chronic conditions since *in vitro* data had not suggested the sustained expression of E-selectin for the days and sometimes weeks duration which is seen *in vivo* in natural disease states.

E-selectin mediates binding of neutrophils, eosinophils, basophils, monocytes, and cutaneous lymphocyte associated antigen expressing (CLA^+) $CD4^+$ memory T cells to activated endothelial cells (157, 158, 159, 160, 161). Leukocyte binding to E-selectin is not, unlike many of the other adhesion molecules, augmented by activation of the leukocytes (162). The interaction of human neutrophils with E-selectin on endothelial cells results in the activation of CD18 and subsequent integrin-immunoglobulin mediated binding (163, 164).

The endothelial cell specific expression of E-selectin and induction by cytokines has led to interest in the regulatory 5' flanking region of the gene. Consensus binding sequences for both NF-κB and AP-1 transcription factors are present in this region in the human E-selectin gene, while the NF-κB-like sequence is missing from the murine gene (93, 165). The murine gene retains cytokine inducibility albeit at a lower level than the human homologue (165). In human umbilical vein endothelial cells, NF-κB-like protein activation is necessary but not sufficient for cytokine induction of E-selectin gene transcription (139). Two E-selectin promoter elements and their DNA-binding factors, NF-ELAM1 and 2, which are well conserved in rabbits, mice, and humans, have been identified. These elements are, along with NF-κB, essential for E-selectin transcription (166). The NF-κB-like activation is protein kinase C (PKC)-independent; however, PKC agonists can, alone, stimulate expression of E-selectin (167, 168). Inhibition of E-selectin expression has been achieved by corticosteroids and pharmacologic elevations of cyclic AMP (cAMP) levels (169, 170).

Soluble selectins Soluble isoforms of the selectins have been identified in the circulating blood of normal individuals (144, 171, 172, 173). The soluble form of P-selectin, sP-selectin, represents an alternately spliced variant lacking the transmembrane domain (174), while soluble L- and E-selectin (sL- and sE-selectin) are released from the surfaces of activated cells by an as yet incompletely understood process of proteolytic cleavage . The soluble selectins, when bound to a plastic surface, are capable of mediating the adhesion of target cells (175).

Elevated serum levels of soluble selectins are seen in a variety of disease states including elevated sP-selectin in thrombotic thrombocytopenic purpura and hemolytic uremic syndrome, elevated sE-selectin in septic shock and renal failure,

and elevated sL-selectin in sepsis and AIDS (144, 171, 175, 176). There is some evidence that the soluble selectins may have anti-inflammatory activity. For instance, sP-selectin blocks TNF-mediated activation of the β_2 integrin complex thus interfering with CD11b-mediated binding of PMN to the endothelium (177). While sE-selectin and sL-selectin have been shown *in vitro* to inhibit leukocyte adhesion (173, 178); it has been suggested that soluble selectins, through their anti-inflammatory activity, may serve to limit leukocyte-endothelial cell interactions. Members of the immunoglobulin family, such as ICAM-1 and VCAM-1 have had soluble forms identitified as well (143, 175).

Selectin ligands Cloning of the selectins identified the presence of the common amino-terminal domains which are homologous to the C-type lectins suggesting carbohydrates may be the natural ligands for the selectins. Involvement of carbohydrates in lymphocyte adhesion had been suggested in studies which predate the cloning of the selectins. Both mannose-6-phosphate and certain other phosphorylated monosaccharides and PPME (phosphomannin), a mannose-6-phosphate containing yeast core polysaccharide, blocked lymphocyte adhesion to lymph node high endothelial venules (179). The relationship between the selectins and carbohydrates was first suggested when lymphocyte binding to phosphomannin-coated beads was blocked by Mel-14 anti-L-selectin antibodies (180).

The search for carbohydrate ligands to the selectins involved various techniques, including neutralizing antibodies, soluble carbohydrate inhibitors, transfection by fucosyltransferases, enzymatic modification of carbohydrates, and direct binding of cells/receptors to purified endogenous ligands (181). There is however, much controversy about the exact nature of the selectin ligands. Much of

the confusion has arisen from the assumption that dogma regarding protein-protein interaction is applicable in the context of iectin-carbohydrate interactions. Applying knowledge obtained from previous studies of plant and animal lectins can be helpful when interpreting results from selectin-carbohydrate studies (182). A major difference between protein-carbohydrate and protein-protein interactions is the lower affinity and restriction of the protein-carbohydrate bonds. Experimental conditions such as temperature, time, and shear force can significantly affect lectin-carbohydrate interactions and lead to erroneous conclusions. In addition, apparently unrelated oligosaccharides can mimic one another in free solution thereby inhibiting the same ligands.

Each of the selectins has been demonstrated to bind to various natural and synthetic sugars which can be categorized into three basic types: sialylated Lewis x (sLe^x) and related structures (sLe^a), phosphorylated mono- and polysaccharides (mannose-6-phosphate, PPME), and sulfated polysaccharides (fucoidan, heparin) (5). In 1990 a pentasaccharide termed LNF-III and containing the Lewis x determinant (Le^x: Galβ1-4(Fucα1-3)GlcNAc) was shown to block P-selectin-dependent platelet rosetting (183). The sialylated form of Le^x (sLe^x: Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNaAc) (Fig. 4) was found to be a ligand for E-selectin by several groups of investigators less than 2 years after the cloning of the gene (81, 184, 185, 186, 187). The demonstration that this sialylated, fucosylated lactosaminoglycan is recognized by P-selectin and L-selectin followed (188, 189, 190, 191). The sialic acid and fucose residues are required and must be in specific linkages in order to serve as ligands for the selectins (181, 192, 193, 194). Molecules containing a related molecule, sialyl Lewis a (sLe^a: (Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAc) (Fig. 4), interact with murine L-selectin and human E-

and P-selectin (190, 194, 195, 196). Fucosylated lactosamines including sLe^x are found on the surfaces of neutrophils, monocytes, some lymphocytes and a variety of cancer cells (195, 197, 198, 199, 200); while sLe^a is not usually present on peripheral leukocytes but is prevalent on certain cancer cells suggesting a role for sLe^a in cancer metastasis (81, 201, 202).

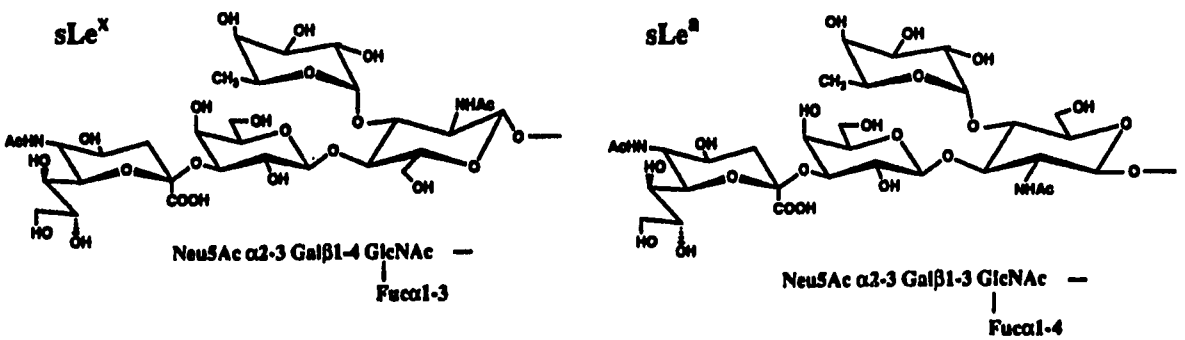

Figure 4. Structure of the sLe^x and sLe^a selectin ligands. These oligosaccharides contain a terminal sialic acid (Neu5Ac) linked a2-3 to galactose (Gal) which is linked to N-acetylglucosamine (GlcNAc) with a fucose attached. The galactose and fucose linkage to the GlcNAc differs between the two sugars. Reproduced from the *Journal of Clinical Investigation*, 1993, vol. 91, pp. 379-387 by copyright permission of the American Society for Clinical Investigation (5).

As discussed above, there is controversy surrounding identification of the selectin ligands. The subject is reviewed thoroughly in Bevilaqua and Nelson, 1993 and Bevilaqua, 1993 (3,5). Binding of selectins to their target cells is a calcium-dependent, saturable, high affinity event (97). There is some consensus about the carbohydrate ligands for the selectins which can be summarized as follows: P-selectin binds certain sulfated glycolipids, such as sulfatide, in addition to sLe^x and sLe^a; while E-selectin binds only to sLe^x, sLe^a, and related structures. L-selectin can bind sulfated polysaccharides, phoshorylated monosaccharides,

and sLe^x and sLe^a although it appears to have a lower affinity than E-selectin for these two sugars (5). Identification of the simplest monosaccharide or oligosaccharide ligand is useful but only tells a partial story, because these simple units are part of a larger more complex molecule with combinations of oligosaccharides from the same or adjacent macromolecules involved in lectin binding. Two children with a defect in fucose metabolism resulting in an inability to fucosylate carbohydrates have been identified. The neutrophils from these patients lack sLe^x and are unable to adhere to activated endothelial cells thus demonstrating the importance of sLe^x in neutrophil adhesion to endothelial cells (203).

Proteins have also been examined for potential involvement in selectin-mediated adhesion. E-, P-, and L-selectin bind with high affinity to a small number of specific cell surface proteins. For example, immunoprecipitation of murine lymph nodes with a MAb (MECA-79) against the peripheral lymph node addressin identified several glycoprotein species between 50 and 200 kDa (204). Two of these molecules appear to correspond to the 50 and 90 kDa sulfated, sialylated, and fucosylated glycoproteins which were affinity purified from murine lymph nodes using an L-selectin-Ig chimera (205). A cDNA encoding the 50 kDa glycoprotein was isolated and found to encode a novel mucin-like cell adhesion molecule with clusters of carbohydrates (206). This molecule was later named GlyCAM-1(97). P-selectin was used to affinity purify a 120 kDa glycoprotein from myeloid cells (207) that has been proposed to be involved in the presention of carbohydrates to P-selectin. Soluble E-selectin identified a 150 kDa glycoprotein as the only species that could be affinity isolated from mouse myeloid cells (208). Interestingly,

protease treated HL60 cells (a myeloid leukemia line) can still bind E-selectin but can no longer bind P-selectin (209).

Selectins and leukocyte rolling Selectins mediate the rolling phase of neutrophil extravasation (Fig. 1) (31, 33, 115, 135, 210, 211) via rapid binding and low detachment rate between the selectins and their carbohydrate ligands located on the ends of flexible protein counter-receptors on neutrophils (212). Bond length and flexibility play a crucial role in the ability of leukocytes to roll at a variety of shear rates (213). Rolling is mediated by adhesive events rather than hemodynamic factors (214) and is the first recognizable interaction between neutrophils and activated endothelium. It is reversible as in cases of mild inflammation in which leukocytes are released and return to the flowing bloodstream or can result in stable adhesion and subsequent extravasation (34). Examination of the venules in intact mouse ears suggests neutrophil rolling may be a normal physiologic process in uninflamed tissues (215).

The rolling phenomenon can be studied *in vitro* using parallel-plate flow chambers with shear stresses equal to those found in post-capillary venules (1-4 dynes/cm^2) (216, 217) or *in vivo* via intravital microscopy (115, 218, 219). Exteriorization of tissues in preparation for intravital microscopy alone has been shown to induce rolling (219). As early as 1967, leukocyte rolling was reported to depend upon divalent cations as evidenced by absence of rolling upon superfusion with EDTA (220) and was later shown to be inhibited by dextran sulfate (221).

Studies provide evidence for selectin mediation of the rolling process. *In vitro*, MAb to L-selectin blocks neutrophil adhesion to activated, cultured endothelial cells under conditions of flow, a state in which MAb to CD18 had no

effect (211, 222). Intravital microscopy has illustrated L-selectin- mediated rolling of rabbit, human, and rat neutrophils in mesenteric venules (115, 210, 218). Flowing neutrophils can roll on surfaces coated with purified P-selectin, histamine-stimulated endothelial cells, or immobilized, activated platelets providing *in vitro* evidence for P-selectin-mediated neutrophil rolling (33, 223, 224) In addition, neutrophils in transgenic knockout mice deficient in the gene for P-selectin do not roll in mesenteric venules. In the case of E-selectin, under conditions of flow, neutrophils roll along immobilized, purified E-selectin and E-selectin transfected L-cell monolayers (225). This rolling is completely inhibited by anti-E-selectin antibodies and is inhibited more than 65% by anti-L-selectin antibodies suggesting a shared adherence pathway for these two selectins (226).

L-selectin may be particularly important in the rolling phenomenon. Picker *et al.* demonstrated that L-selectin-mediated neutrophil adhesion *in vitro* is accounted for, in part, by neutrophil L-selectin presentation of oligosaccharides, including sLex to E-selectin and P-selectin expressed on vascular endothelial cells (227). This was supported by work showing that anti-E-selectin and anti-L-selectin antibodies do not have additive effects although each alone is additive with anti-ICAM-1 antibodies (116). L-selectin is constitutively expressed at high levels and concentrated on the neutrophil surface microvilli (227) making it uniquely suited for a central role in the rolling of unactivated neutrophils along the surface of activated endothelial cells.

Rolling neutrophils are slowed enough to allow them time to become activated by chemical mediators in the local environment (38), or on the surface of the endothelial cells. For example, adhesion to the selectins (163) or interaction with platelet activating factor (PAF) (228) result in neutrophil activation. Upon

activation, neutrophiis undergo dramatic, rapid changes including shedding of L-selectin (38) and an increase in β_2 integrin avidity for their ligands (44, 46). Once activated, the leukocytes are able to interact with the endothelium in a stable manner.

Stable adhesion of neutrophils is required prior to extravasation, and unlike rolling, is mediated by the leukocyte integrins. Work by Arfors et. ai. demonstrated that, *in vivo*, anti-CD18 monoclonal antibodies prevent the firm adhesion and extravasation of neutrophils but not their rolling along post-capillary venular endothelium (231). *In vitro* experiments have elegantly modeled the requirement for each of these three steps in the interaction of neutrophils with endothelial cells prior to extravasation (33, 211). These models involved neutrophil interactions with either purified adhesion molecules or activated endothelial cells and demonstrated the importance of selectins in rolling followed by activation of the neutrophils and subsequent stable adhesion mediated by the integrin-immunoglobulin counterreceptors. All three steps were shown to be essential.

Blocking selectin-mediated inflammation Animal models of inflammation have been utilized to demonstrate the importance of the selectins in mediating inflammation *in vivo*. A variety of antagonists including, MAb, selectin chimeras, and carbohydrates have been shown to reduce neutrophil influx and inflammation in these models which have examined several different tissues and inflammatory stimuli. Table 4 summarizes these studies.

Table 4

In vivo models in which antagonists have blocked selectins

animal model	*antagonist*	*reference*
Decrease reperfusion injury in the ear (rabbits)	MAb against P-selectin	Winn et al. (232)
Inhibit neutrophil influx and injury in cobra venom factor -induced pulmonary injury (rats)	MAb against P-selectin	Mulligan et al. (233)
	L- and P-selectin-IgG chimeras	Mulligan et al. (234)
	oligosaccharides (sLe^x and its analogs)	Mulligan et al. (235)
Protect myocardium and endothelium in cardiac ischemia and reperfusion injury (cats)	MAb against P-selectin	Weyrich et al. (169)
Inhibit neutrophil influx into peritoneum after IP thioglycollate (mice)	MAb against L-selectin	Jutila et al. (236)
	L-selectin-IgG chimera	Watson et al. (114)
	heparin oligosaccharides	Nelson et al. (237)
Prevent neutrophil influx into inflamed skin (mouse)	MAb against L-selectin	Lewinsohn et al. (109)
Inhibit neutrophil influx and injury in IgG immune complex-induced pulmonary injury (rats)	MAb against E-selectin	Mulligan et al. (238)
	L- and E-selectin chimeras	Mulligan et al. (235)
	tetra- and pentasaccharide derivatives of sLe^x	Mulligan et al. (239)
Reduce antigen-induced pulmonary neutrophil influx and late-phase airway obstruction (monkeys)	MAb against E-selectin	Gundel et al. (240)

PART 1

DEVELOPMENT AND CHARACTERIZATION OF MURINE MODELS OF ACUTE INFLAMMATION

ABSTRACT

Two murine models of acute inflammation were developed to evaluate the role of the endothelial adhesion molecule E-selectin in the recruitment of neutrophils to areas of inflammation in the mouse. The first model, intraperitoneal (IP) injection of thioglycollate broth, was used to study the infiltration of neutrophils into the peritoneal cavity of mice (n=2 mice/dose/time point for thioglycollate treated mice and 1 mouse/time point for saline treated mice). Compared to IP injection of 1.0 mL saline, significantly greater numbers of neutrophils were present in the peritoneal lavage fluid of mice injected with 0.5 and 1.0 mL of thioglycollate after 2 and 4 hours. The second model, a novel model of intranasal instillation of 50 μg bacterial lipopolysaccharide (LPS) in 50 μL sterile saline, was examined using a time course study of histologic changes in the lungs and neutrophilic infiltration into the bronchoalveolar lavage (BAL) fluid in 6 mice/time point. To demonstrate that intranasally instilled fluid reached the lungs, gross distribution of inhaled saline was also evaluated. Progressively increasing numbers of neutrophils were seen in the BAL fluid from 1 through 6 hours after stimulation, followed by a slight decrease in neutrophil numbers by 24 hours. Histologic changes included mild edema and inflammatory cell infiltrates beginning multifocally around airways and extendeding into surrounding alveoli. Patchy, multifocal distribution of the inhaled saline was demonstrated by the addition of blue dye to the saline. It was concluded that both models would be potentially useful for studies examining the involvement of E-selectin in neutrophil recruitment into sites of acute inflammation in the mouse.

INTRODUCTION

While a significant number of *in vivo* studies have examined and more clearly defined the role of immunoglobulin and integrin superfamily members in leukocyte adherence to endothelium and emigration into sites of inflammation, very few studies have looked at the role selectins, particularly E- and P-selectin, play *in vivo* (6). Animal models used to evaluate the importance of adhesion molecules in acute inflammation must have quantifiable markers of inflammation such as a neutrophilic infiltrate or edema. When antagonists to various adhesion molecules are given prior to an inflammatory stimulus, it is necessary to measure a decrease in the inflammation in order to substantiate the importance of the molecule.

Monoclonal antibodies are the most commonly used adhesion molecule antagonists and are effective inhibitors of inflammation in a wide variety of inflammatory conditions and tissues. Interestingly, the vasculature of different tissues appears to have distinct adherence mechanisms. Doerschuk et al. (1990) showed that CD-18-dependent neutrophil migration into the abdominal wall differed from that induced in the lungs. In the lungs, inflammation induced by one stimulus was blocked by anti-CD18 monoclonal antibodies but inflammation induced by another stimulus was not. Therefore, in addition to being tissue-specific, adhesion molecules are also stimulus-specific (241). This stimulus specificity was further illustrated by Mulligan et al. (1991 and 1992) in rat lung models (238, 233).

Because of apparent organ specificity in the role of adhesion molecules in acute inflammation, study of two different tissue beds was considered crucial in the design of experiments to examine the role of E-selectin. The lung and peritoneal cavity were selected because they represent sites where collection and

quantification of inflammatory infiltrates is possible via lavage. The mouse was chosen as the study animal, because novel reagents were available for specific murine adhesion molecules.

A well characterized murine model of acute inflammation, the intraperitoneal (IP) injection of 0.5 to 1.0 mL of thioglycollate broth, induces a rapid and predictable influx of neutrophils into the peritoneal cavity (242). L-selectin has been demonstrated to play a role in the influx of neutrophils in this model with the use of anti-L-selectin MAb or an L-selectin-IgG chimera as the antagonist (236, 114). In addition, P-selectin has been shown to be important in neutrophil migration into the peritoneal cavity following IP injection of thioglycollate in transgenic mice deficient in the gene for P-selectin (135). The P-selectin knock-out mice, when compared to genotypically normal mice, had lower numbers of neutrophils in peritoneal lavage fluid at several early time points.

Inhalation of lipopolysaccharide (LPS) into the lungs has been described as a model of acute inflammation in several species. LPS, a proinflammatory component of bacterial cell walls, causes a measurable increase in neutrophils recovered by bronchoalveolar lavage (BAL) of a variety of animals, including guinea pigs and sheep, when it is inhaled following aerosolization (243, 244). In general, neutrophil numbers in the BAL peak at around 6-12 hours and return to baseline levels by 48 hours, while monocytic and lymphocytic infiltrates peak at 24 and 48 hours, respectively, following inhalation of LPS by these species (245, 246). Intratracheal and intrabronchial instillation of LPS provide additional routes that have been used in experimental models to deliver LPS to the air spaces of the lungs. These routes recapitulate the inflammatory infiltrates seen with aerosolized LPS (241, 245). However, administration of inflammatory mediators by the

intratracheal and intrabronchial routes is technically challenging in mice due to the small size of the airways. Another method of LPS delivery is intranasal (IN) instillation of LPS. Although this route is not commonly used in inflammation research, it is utilized by investigators in the fields of immunology and virology (247, 248). Pilot studies were conducted to compare the effects of nebulized LPS to intranasal instillation of LPS on lung histology and the cellular composition of the BAL. These studies demonstrated that intranasal administration of LPS induced a more severe and reliable inflammatory state making it the preferred delivery system for the studies that followed (249).

For these reasons, chemical peritonitis induced by intraperitoneal instillation of thioglycollate and pneumonitis induced by intranasal instillation of LPS were evaluated as models for subsequent studies of E-selectin in acute inflammation. The purpose of the following studies was to characterize these models. The primary goal of the thioglycollate study was to determine whether a reproducible model of peritonitis, with a significant influx of neutrophils, could be induced (as reported in the literature). The goals of the IN LPS study were to characterize neutrophil influx into the BAL, histopathologic changes, and gross distribution of an inhaled aqueous solution.

MATERIALS AND METHODS

Thioglycollate-induced peritoneal inflammation

Mice

The mice were 7 week old, VAF®, males of the Charles River Crl:CD-1 (ICR)BR strain purchased from Charles River Breeding Laboratories (Raleigh, NC). The mice were group housed in suspended stainless steel cages over non-contact bedding. The murine pathogen-free environment was maintained with a 12 hour light/dark cycle at 72 ± 4° F and 50 ± 20% humidity. Purina Certified Rodent Chow 5002 (Purina Mills Inc., Richmond, IN) and reverse osmosis water were provided *ad libidum*. The animals were conditioned for at least one week prior to treatment.

Thioglycollate administration and peritoneal lavage

Two mice were evaluated in each thioglycollate treatment group and at each time point, while the saline treatment groups had one animal per time point. Mice were injected IP with 0.5 or 1.0 mL of thioglycollate broth (Sigma Chemical Co., St. Louis, MO) or 1.0 mL of sterile saline (Abbott Laboratories, North Chicago, IL). At 2 and 4 hours after the IP injections, mice were sacrificed by CO_2 inhalation. After removing the skin overlying the peritoneum, 10 mL of Dulbecco's phosphate buffered saline (PBS) (Gibco Laboratories, Grand Island, NY) containing 10 U/mL of heparin was injected into the peritoneal cavity. The peritoneal wall was gently massaged prior to withdrawal of the lavage fluid through a 22 gauge needle. Yields of lavage fluid were between 7-8 mL/animal. Lavage fluid was kept on ice in polypropylene tubes until evaluated (<30 minutes). The total number of cells in the lavage fluid was manually counted with a Neubauer hemacytometer. Cytocentrifuged cell preparations were made by centrifuging 100 µL of peritoneal lavage fluid at 800 rpm for 5 minutes on a Cytospin 2™ (Shandon Scientific Ltd.,

Runcorn, UK) then staining the cells with a modified Wright's stain on a Hematek™ autostainer (Miles Laboratories, Inc., Elkhart, IN). Two slides were made for each animal, and differential counts were done by counting 100 leukocytes/slide using a light microscope at 630x oil immersion.

Statistics

Data were analyzed using a two-way ANOVA with a factorial design using dose, time and dose/time interaction as factors. Independent variables found to be significant at $p<0.05$ were then analyzed by a Student's t-test to determine if there was an effect due to that variable. Statistical significance was assigned when $p<0.05$.

Intranasal LPS-induced pulmonary inflammation

Mice

The mice used for this study were 9 week old, male, Charles River VAF® Crl:CD-1 (ICR)BR mice purchased from Charles River Breeding Laboratories (Raleigh, NC). The animals were housed and treated as described above.

LPS administration

Mice were anesthetized in a desiccation chamber containing methoxyflurane (Metofane®, Pitman-Moore, Mundelein, IN). Once in a deep plane of anesthesia, mice were removed from the chamber and given an intranasal instillation of 50 µL of sterile saline containing 50 µg (1.0 mg/mL) LPS (from *Escherichia coli* Serotype 055:B5, Sigma Chemical Co., St Louis, MO) by placing the saline on the external nares of the anesthetized mouse until inhalation was observed.

Bronchoalveolar lavage

At 1, 2, 4, 6 and 24 hours after IN instillation of LPS, 6 mice/time point were sacrificed via a lethal injection (0.2 mL IP) of Euthanasia 5 Solution® (Henry Schein Inc., Port Washington, NY). Once moribund following the lethal injection, they were exsanguinated by aortic transection. Bronchoalveolar lavages were performed by inserting an 18.5 gauge nylon catheter into a small tracheostomy opening of the exposed trachea. The trachea was secured to the catheter using 5-0 silk suture material. Lungs were lavaged *in situ* with a total of 4 mL of cold PBS containing 10 U/mL of heparin instilled in 5 repeated lavages of 0.8 mL each. BAL fluid was put into polyethylene tubes and kept on ice until evaluation (<30 minutes). Total leukocyte count in the BAL fluid was quantified with a Neubauer hemacytometer. Cytocentrifuged cell preparations were made by centrifuging 100 µL of BAL fluid at 800 rpm for 5 minutes on a Cytospin 2™ followed by staining the cells with a modified Wright's stain on a Hematek™ autostainer. Two slides were made for each animal, and differential counts were done by counting 100 leukocytes/slide using a light microscope at 630x oil immersion.

Histologic evaluation

Following euthanasia and exsanguination as described above, 1.0 mL of 10% neutral buffered formalin was instilled into the trachea of two mice/time point, and the trachea was ligated with 5-0 silk suture material. The inflated lungs were immersion fixed in 10% neutral buffered formalin. After fixation, each lobe was dissected free and trimmed in a standard manner, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for routine histologic examination.

Analysis of gross distribution of inhaled droplets

Fifty microliters of saline containing monastral blue (4% by volume) (Sigma) was instilled intranasally into 5 mice as described above. Four hours later, the animals were sacrificed via a lethal injection (0.2 mL IP) of Euthanasia 5 Solution®. The gross distribution of the blue dye was visually evaluated and photographed.

RESULTS

Thioglycollate-induced peritoneal inflammation

Neutrophil migration into the peritoneal cavity

Intraperitoneal injection of 0.5 or 1.0 mL of thioglycollate resulted in a significant acute inflammatory response in the peritoneal cavity of mice when compared to the saline treated animals, as measured by the total number of neutrophils (PMN)/mL in the peritoneal lavage fluid (Table 1). In this experiment, there was no effect of time or dose of thioglycollate.

Table 1

Neutrophil migration into the peritoneal cavity following IP thioglycollate

animal #	*IP treatment*	*sacrifice time after dosing*	*PMN x 10^4/mL of peritoneal lavagate*
1	0.5 mL thioglycollate		34.52
2	0.5 mL thioglycollate		30.64
3	1.0 mL thioglycollate	2 hr	25.73
4	1.0 mL thioglycollate		35.41
5	1.0 mL saline		0.37
6	0.5 mL thioglycollate		48.64
7	0.5 mL thioglycollate		11.12
8	1.0 mL thioglycollate	4 hr	30.16
9	1.0 mL thioglycollate		57.15
10	1.0 mL saline		0.26

Intranasal LPS-induced pulmonary inflammation

Neutrophil influx into the airways and air spaces of the lungs

Intranasal instillation of 50 µL of 1.0 mg/mL LPS/saline into the lungs of mice was evaluated at 1, 2, 4, 6, and 24 hrs. The IN LPS resulted in significant increases in the number of neutrophils over the course of the experiment. The number of neutrophils recovered by BAL increased over time until 6 hours but declined by 24 hours (Fig. 1). In agreement with published reports of normal mice, essentially no neutrophils were recovered in BAL fluid of control animals receiving no intranasal instillation (250). The intranasal instillation of 25 µL sterile saline did not result in an increase in neutrophil numbers in the BAL fluid relative to normal control levels (numerical data not shown) (Fig. 2).

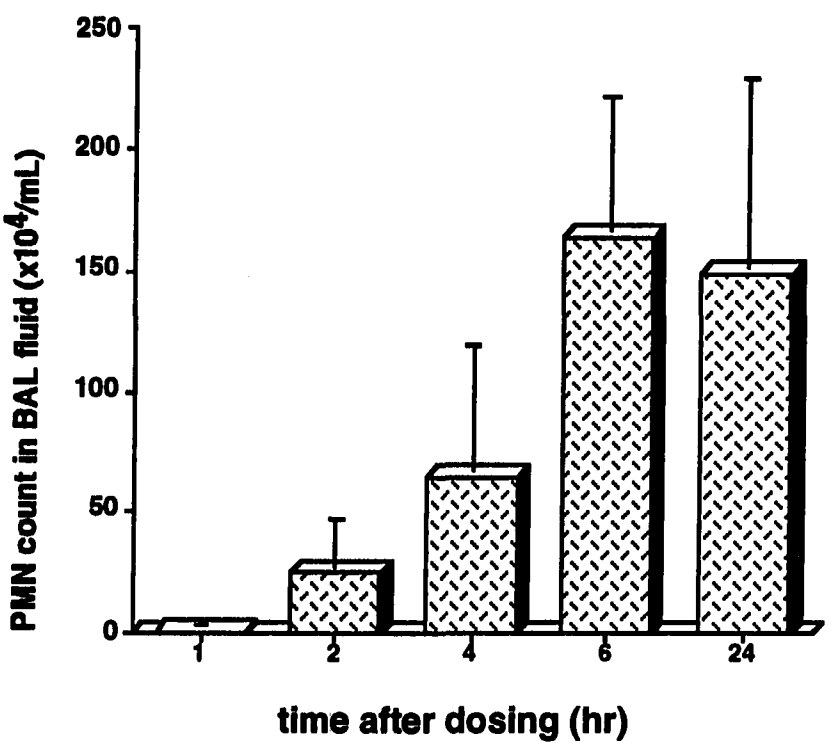

Figure 1. Neutrophil numbers in the BAL fluid of mice given IN LPS

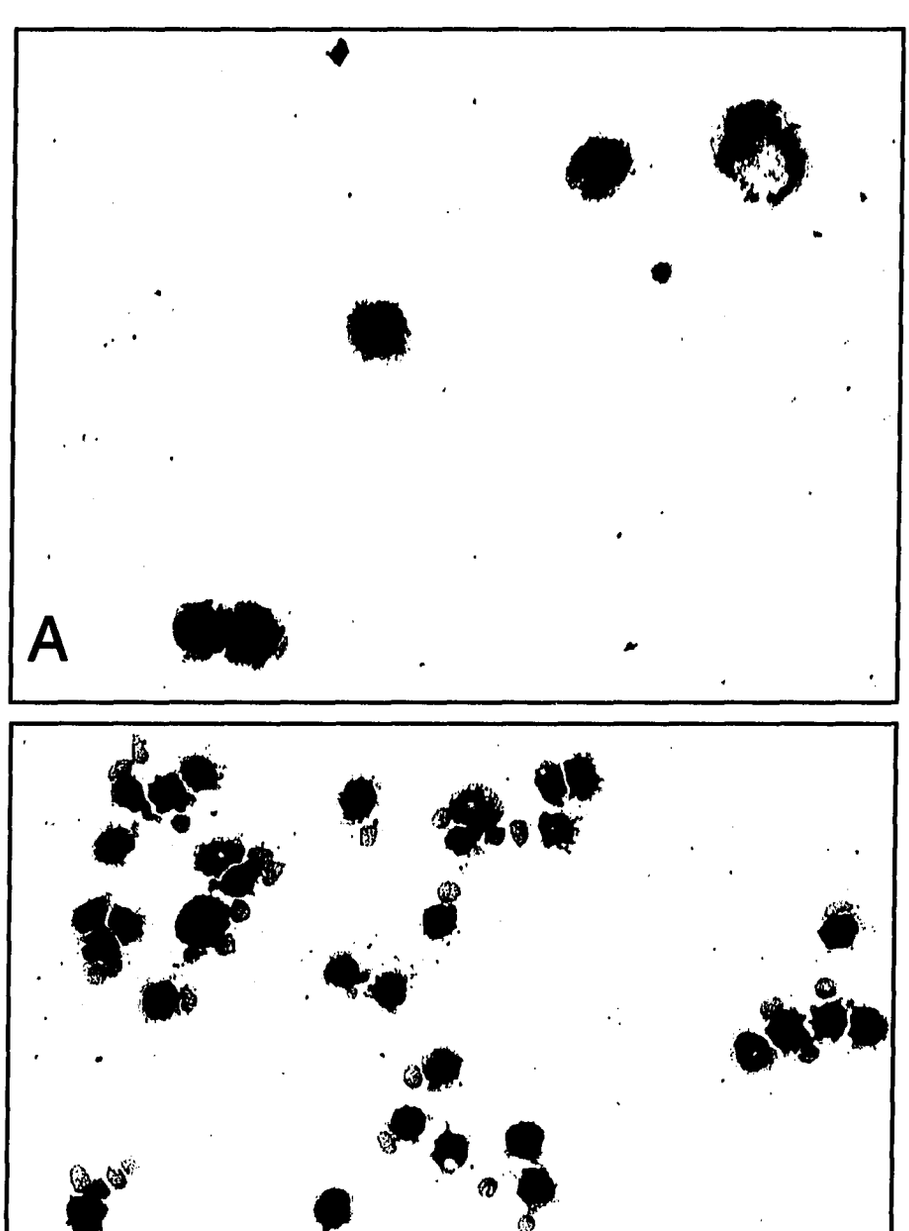

Figure 2. Cytospin preparations of the bronchoalveolar lavage fluid from **A.** a mouse given 50 μL IN saline 4 hours earlier and **B.** a mouse given an IN instillation of 50 μL of 1.0 mg/mL LPS 4 hours earlier. Notice the increased cellularity, neutrophils in particular, in the LPS-treated animal. (200X)

Histopathology

Animals sacrificed at 1 and 2 hours (n=2 animals/time point) had similar histopathologic lesions in the lungs. There were mild infiltrates of neutrophils into and around many airways (especially medium-sized bronchioles) and around vessels associated with airways. Mild edema and margination of neutrophils along the endothelium were also present in these regions. From 4 through 24 hours, the lungs became progressively more infiltrated with leukocytes, predominately neutrophils, and the edema became slightly more pronounced. By 24 hours, the infiltrates around and within airways had progressed multifocally from mild to moderate and extended into the surrounding alveoli (Fig. 3).

Gross distribution of inhaled droplets

The monastral blue dye was visually evident on the pleural surface and scattered throughout the parenchyma on cut surface of the lungs in all mice studied (Fig. 4). The distribution varied somewhat from mouse to mouse, but approximately 40-60% of the lungs were stained blue. The size of the individual areas varied from pinpoint to large (several mM) with irregular patches involving approximately 70% of the left lobe in one mouse. In all mice, blue dye was present in all lung lobes, although the extent of involvement of the separate lobes varied between mice. Overall, the left lobe appeared to be slightly more involved in these mice .

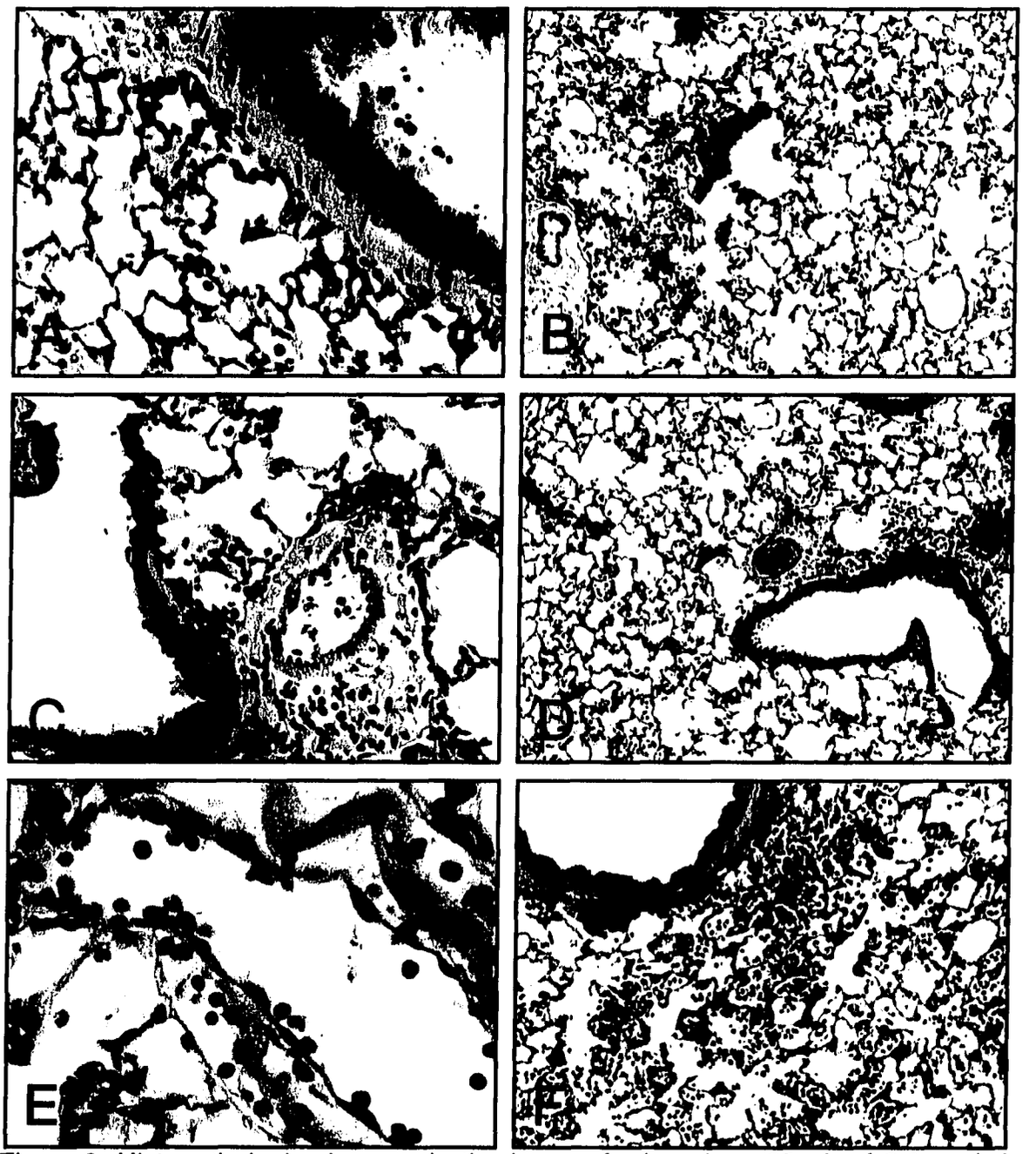

Figure 3. Histopathologic changes in the lungs of mice given 25 µL of 2.0 mg/mL LPS. 1 hr later: **A.** Neutrophils are seen in alveolar walls immediately surrounding the airway.(100X) 4 hr later: **B.** and **C.** There is mild edema and inflammation around the airways. **B.**(50X), **C.**(100X) 6 hr later: **D.**The neutrophils are present in small numbers in alveoli distant from the airways.(50X) **E.** Neutrophil margination along the wall of a small venule.(200x) 24 hr later: **F.** The inflammation in the alveoli is moderate in severity.(50X) Hematoxylin and eosin.

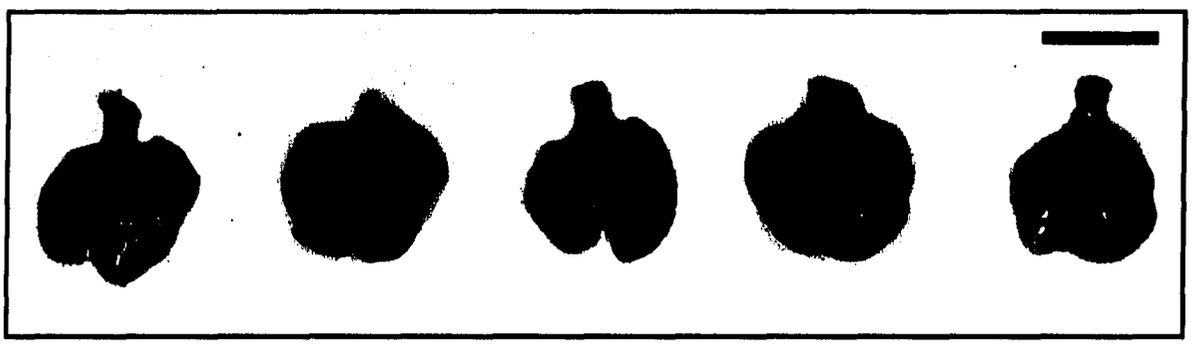

Figure 4. Lungs from mice which inhaled 50 µL of sterile saline containing 4% monastral blue dye to visualize the distribution of saline delivered by intranasal instillation. Bar=1 cm.

DISCUSSION

In these studies, both the IP thioglycollate and IN LPS models had a rapid influx of neutrophils into the lavage fluid. The number of neutrophils recovered in the lavage fluid from the lungs and peritoneal cavity is reliable (see part 3 of this dissertation) and, in the case of the thioglycollate model, consistent with previous reports (114).

The intranasal instillation of LPS resulted in an influx of neutrophils into the air spaces beginning by 1 hour and increasing through the first 6 hours after treatment. By 24 hours, the number of PMN/mL in the BAL fluid had begun to decrease. Time points between 6 and 24 hours were not examined, because the focus of these studies was the neutrophilic influx occurring in the first 6 hours of inflammation. Histologic examination demonstrated an influx of inflammatory cells around airways early in the inflammatory response with extension into alveoli by 6 hours. Twenty-four hours post-treatment, the inflammatory cell infiltrate in the alveoli had progressed from mild to moderate. Distribution of the monastral blue dye clearly showed that intranasal instillation of an aqueous solution is an effective method for delivery of inflammatory mediators to the lungs.

The paradox between the slight decrease in neutrophil numbers in the BAL and increasing inflammation seen histologically has several potential explanations. A portion of the difference can be accounted for by an increase in mononuclear cells relative to neutrophils in the 24 hour post-treatment group. In addition, neutrophils in the alveoli may not be accessible to BAL fluid or may have increased adhesive properties which prevent them from being flushed from the lungs (250).

Several problems arose during the development of the IN model. The most significant problem was the inability to determine whether the delivery of the LPS

had been successful when saline alone was used. To alleviate this problem, monastral blue dye was added to the saline for intranasal instillation. The grossly visualized dye in the lungs was a simple indicator of a successful inhalation exposure.

The magnitude of neutrophilic influx into the peritoneal cavity of the mice receiving IP thioglycollate was similar to that reported in the literature. As reported by Watson et al., mice treated 4 hours earlier with 1.0 mL of thioglycollate had approximately 250 x 10^4 PMN in the peritoneal lavage fluid while mice in this study had 300 x 10^4 (114). Although a greater increase in neutrophil numbers in the peritoneal lavage fluid of mice in the 4 hour group relative to the 2 hour group was expected, the number of animals studied was small. (A larger sample population would likely have given the expected results.) Since the purpose of these experiments was to determine whether significant inflammation could be induced, the sacrifice of animals to duplicate reports in the literature was considered unnecessary.

In conclusion, intranasal instillation of LPS and intraperitoneal injection of thioglycollate were determined to be useful models in which to study the *in vivo* role of E-selectin in acute murine inflammation.

PART 2

EXPRESSION OF E- AND P-SELECTIN IN MICE GIVEN INTRANASAL LIPOPOLYSACCHARIDE

ABSTRACT

E-selectin and P-selectin are inducible endothelial cell adhesion molecules belonging to the selectin family. They are expressed on the surface of activated endothelial cells in areas of acute inflammation where they play a role in the recruitment of leukocytes. In this study, immunohistochemical staining with monoclonal rat anti-mouse E- and P-selectin antibodies was used to characterize the expression profile of these molecules in the lungs of mice following intranasal (IN) instillation of bacterial lipopolysaccharide (LPS). Tissue sections from mice sacrificed at 0 (untreated), 1, 2, 4, 6, and 24 hours post-treatment were evaluated (n=2 mice/time point for 0, 1, 2, 6, and 24 hours, n=8 for 4 hours). No E-selectin staining was seen in the untreated or 1 hour post-treatment groups. Staining was visible in endothelial cells of a small number of arterioles, small veins, and venules in the 2 hour group; staining peaked at 4-6 hours. The positively stained blood vessels tended to be located near bronchioles. Staining in the 24 hour post-treatment group had decreased to near baseline levels. A small number of vessels were involved, even in the peak staining period (4 and 6 hours). In comparison, P-selectin staining was seen at all time points between 0 and 24 hours. Basal expression was present in a few capillaries, small veins, and venules in the untreated group. The number of vessels staining for P-selectin increased in subsequent groups through 6 hours and extended to include larger veins, arterioles, and small arteries. At 24 hours post-treatment, the staining had decreased to a level similar to that seen in the control group. In general, the staining with P-selectin antibodies involved 3-5 times more vessels than staining seen with E-selectin antibodies. These data indicate that E- and P-selectin expression was upregulated in the lungs of mice given IN LPS. At the time points

evaluated, expression of both adhesion molecules peaked at 4-6 hours and involved a small percentage of vessels.

INTRODUCTION

In vitro expression of the endothelial adhesion molecule E-selectin has been well characterized on cultured endothelial cells stimulated by the cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNF) or bacterial lipopolysaccharide (LPS) (77, 136). The expression peaks at 4-6 hours post-stimulation with a gradual decline to baseline levels 24-48 hours after stimulation. By comparison, a related adhesion molecule, P-selectin, is very rapidly expressed on the endothelium. Within minutes, P-selectin appears on the surface of cultured endothelial cells following activation by a variety of mediators, including thrombin, histamine, H_2O_2, and terminal complement components (122, 121, 125, 130, 252). Expression occurs through the redistribution of P-selectin from the membranes of intracellular storage granules to the surface of the endothelial cell. Although the expression has been thought to be short-lived (3, 5), recent *in vitro* and *in vivo* studies have demonstrated a prolongation of P-selectin surface expression that, like E-selectin expression, is cytokine-induced with *de novo* synthesis of new mRNA and protein and is several hours in duration (131, 132, 133). The expression of P-selectin, as measured by Northern blotting, peaks at 4 hours in murine endothelioma cells treated with TNF (253) and in mice given intraperitoneal (IP) LPS (133).

The *in vivo* expression of E-selectin has been described in several animal models, the majority of which are primate models, because (when used for immunohistochemical staining) the available anti-human E-selectin antibodies recognize E-selectin in primates but not in other species. In one study, septic shock was induced in baboons with live *Escherichia coli,* and the animals were sacrificed 6 hours later. E-selectin expression was detected

immunohistochemically in capillaries, venules, small veins, arterioles, and arteries in a wide variety of tissues (254). Another study in baboons examined the time course of E-selectin expression by injecting LPS into the skin and by examining skin biopsies with immunohistochemical staining. In these baboons, E-selectin expression on the endothelium of dermal venules was clearly evident by 2 hours after the injection of LPS and decreased thereafter to virtual absence by 9 hours post-treatment (150). In a similar study, E-selectin expression was compared in an intradermal LPS injection model and in a cutaneous delayed-type hypersensitivity (DTH) model in rhesus monkeys. In this study, E-selectin expression began 0.5 hours after the LPS injection and peaked between 4-8 hours post-treatment but remained slightly elevated for up to 72 hours. In the DTH model, E-selectin expression was seen one day post-stimulation and extended through the 11 day study period (255).

In mice, IP LPS has been shown to cause immunohistochemically detectable expression of E-selectin in the medium and small veins of the lung, heart, and kidney (149) in a study which used rabbit polyclonal antibodies generated against human soluble E-selectin rather than anti-murine-E-selectin antibodies. No other reports of *in vivo* studies examining the expression of E-selectin in mice have been published to date. Novel monoclonal antibodies (MAbs), specific for murine E-selectin (256), were produced at Hoffmann-La Roche and utilized for the following experiment, the goal of which was to evaluate the expression of E-selectin over a 24 hour time course in the lungs of mice treated with intranasal (IN) LPS. A second objective of the study was to characterize P-selectin expression in the model by immunohistochemistry as a prelude to exploring functional activity.

MATERIALS AND METHODS

Mice

The mice were 7-8 week old, VAF®, males of the Charles River Crl:CD-1 (ICR)BR strain purchased from Charles River Breeding Laboratories (Raleigh, NC). The mice were group housed in suspended stainless steel cages over non-contact bedding. The murine pathogen-free environment was maintained with a 12 hour light/dark cycle at 72 ± 4° F and 50 ± 20% humidity. Purina Certified Rodent Chow 5002 (Purina Mills Inc., Richmond, IN) and reverse osmosis water were provided *ad libidum*. The animals were conditioned for at least one week prior to treatment.

LPS administration

Mice were anesthetized in a desiccation chamber containing methoxyflurane (Metofane®, Pitman-Moore, Mundelein, IN). Once in a deep plane of anesthesia, mice were removed from the chamber and given an intranasal instillation of 50 µL of sterile saline containing 50 µg (1.0 mg/mL) LPS (from *Escherichia coli* Serotype 055:B5, Sigma Chemical Co., St Louis, MO) and 4% monastral blue dye (Sigma) by placing the saline on the external nares of the anesthetized mouse until inhalation was observed. The monastral blue dye was added to the saline as a method of determining whether the inhalation was successful.

Tissue collection and handling

At 0 (untreated), 1, 2, 4, 6, and 24 hours after LPS instillation, 2 mice per time point were sacrificed via a lethal injection (0.2 mL IP) of Euthanasia 5 Solution® (Henry Schein Inc., Port Washington, NY). Once moribund following the lethal injection, they were exsanguinated by aortic transection. The lungs were then inflated *in situ* with 0.8 mL of a solution containing 1/3 Tissue Tek OCT (Miles,

Inc., Elkhart, IN) and 2/3 PBS by volume. A ligature was tied around the trachea. The lungs were removed from the thorax and immersed in supercooled isopentane for 10 seconds. The frozen, inflated lungs were quickly sectioned , embedded in OCT compound in a cryomold, and snap frozen in supercooled isopentane to prevent thawing and subsequent collapse of the transected lungs. The OCT blocks were stored at -80°C. E-selectin expression at the 4 hour time point was examined in greater detail. The lungs of an additional 6 treated mice were collected 4 hours after IN LPS by the above method.

Immunohistochemistry

Cryostat sections (6-7 μm) were air dried onto silane-coated slides (Cel-Tek Inc., Glenview, IL), fixed in cold (4°C) acetone for 5 minutes, and air dried again. The sections were stored at -20°C in sealed plastic packages until use. All subsequent steps were carried out at room temperature. Cryosections were rehydrated in phosphate buffered saline (PBS) for 15 minutes prior to a 30 minute incubation in 1% H_2O_2 in methanol to block endogenous peroxidase. The slides were loaded onto an automated immunostainer (Cadenza, Shandon Scientific Ltd., Runcorn, UK) for the remainder of the immunostaining procedure. The sections were allowed to react with 10% normal rabbit serum (diluted 1:10 in PBS) for 30 minutes followed by 15 minutes each in avidin then biotin blocking solutions (Vector Laboratories, Burlingame, CA). Pilot studies revealed that the avidin and biotin blocking steps significantly decreased nonspecific staining. The monoclonal antibodies employed for the immunostaining were anti-E- (MAb 9A9) and P-selectin (MAb 10A10) and were provided by Dr. Barry Wolitzky (Dept. of Inflammation and Autoimmune Disease, Hoffmann-La Roche, Nutley, NJ). Irrelevant, class and isotype matched rat MAbs were used in appropriate

concentrations as negative controls. Dilutions of the primary antibody were optimized in pilot experiments to be 10 μg/mL in PBS containing 0.1% bovine serum albumin (BSA). Sections stained with MAb 9A9 or the irrelevant control were incubated for 2 hours; whereas, 10A10 and its control were incubated for 1 hour. The primary MAbs were followed by a 30 minute incubation in a biotinylated rabbit anti-rat IgG (mouse adsorbed) (Vector Laboratories, Burlingame, CA) using 50 μL of the secondary antibody diluted in 10 mL of PBS containing 150 μL of normal rabbit serum. Then the slides were incubated for 30 minutes in the avidin-biotin-peroxidase conjugate which was prepared as follows: 5 mL PBS mixed with 25 g of non-fat powdered milk and two drops (100 μL) each of bottle A and bottle B from the Vector Elite Kit (ABC Elite Kit, Vector Laboratories, Burlingame, CA). Antibody binding was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (ISOPAC®, Sigma Chemical Co., St. Louis, MO) mixed 1:2 with 35 μL H_2O_2 in 50 mL of distilled water and incubated for 5 minutes. The slides were then rinsed in water, counterstained with hematoxylin, dehydrated, cleared and coverslipped.

RESULTS

E-selectin expression in the lungs of mice following treatment with IN LPS was detected using immunohistochemical staining with an anti-murine E-selectin MAb (9A9) (Fig. 1). Untreated control mice and mice examined at one hour post-LPS treatment had no positive staining in the lungs. Animals in the 2 hour post-treatment group had mild positive staining in endothelial cells lining arterioles and venules adjacent to bronchioles. Compared with staining in the 2 hour group, staining in the 4 and 6 hour groups had increased in terms of number of vessels involved and the intensity of staining (Table 1). The distribution of staining, however, remained the same, involving the arterioles and venules near airways. In the 24 hour post-treatment group, staining was greatly decreased to very low or non-detectable levels. Peak staining, which was seen in the 4 and 6 hour groups, was patchy and involved a relatively small number of vessels (10-15 vessels/ lung section). Specificity of the staining was demonstrated by a lack endothelial staining in serial lung sections stained with an isotype matched rat anti-mouse MAb.

Lungs of the control and IN LPS-treated mice were also stained with MAb 10A10, an anti-murine P-selectin Ab (Fig. 1). Mild, constitutive P-selectin expression was seen in scattered vessels (capillaries, small veins, and venules) in lungs of untreated mice. This staining consisted of punctate foci within the cytoplasm of the endothelial cells. Between 1 and 6 hours post-treatment, staining had progressively increased to involve larger vessels, such as arteries and larger veins. In these groups, individual endothelial cells had a more diffuse surface staining rather than solely punctate, cytoplasmic staining seen in earlier groups. Staining in the 24 hour post-treatment group was very similar in intensity and

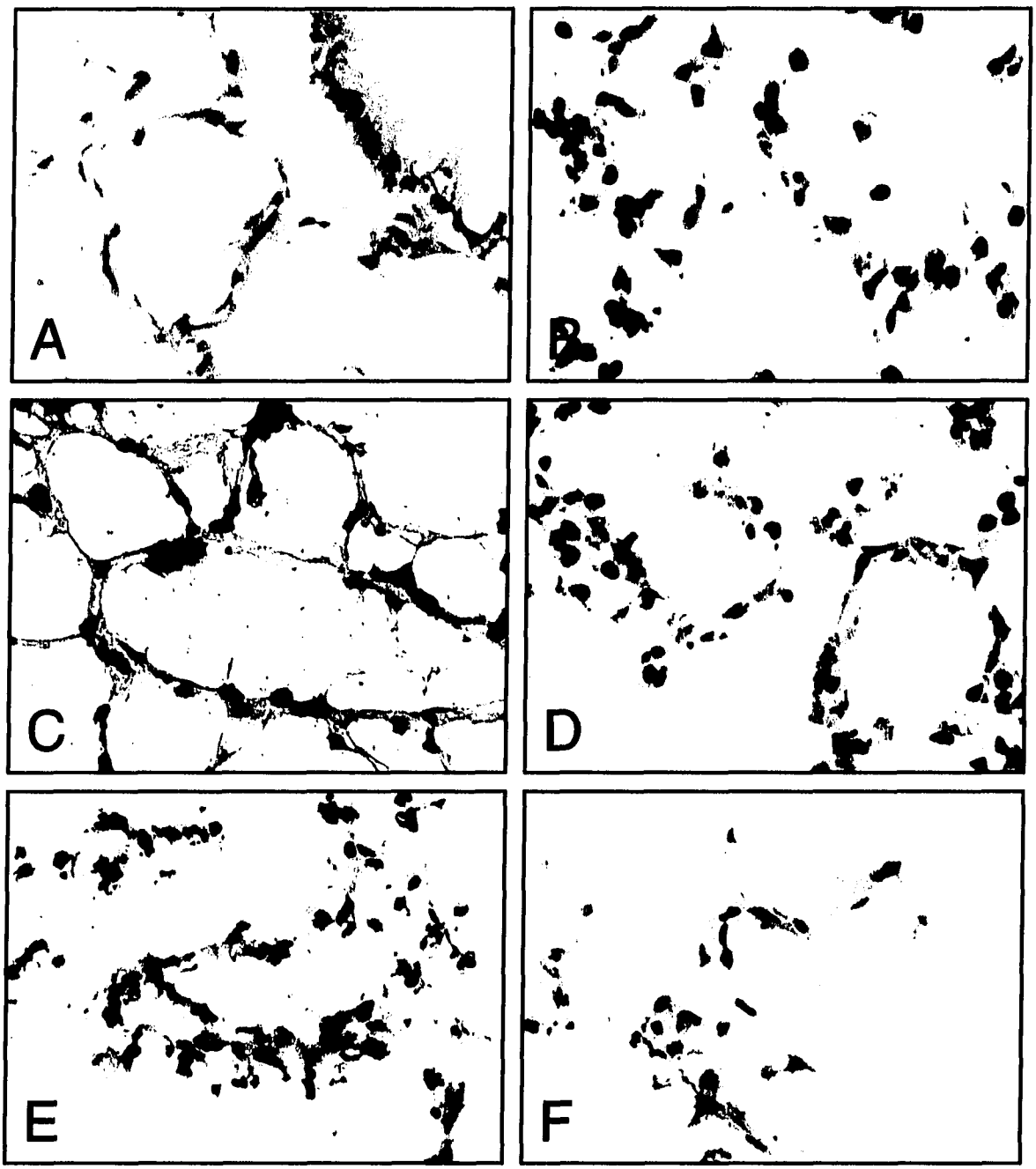

Figure 1. **A, B:** Lungs of untreated mice immunohistochemically (IHC) stained for E-selectin (**A**) and P-selectin (**B**). **C, D, F:** Lungs of mice treated 4 hours earlier with intranasal (IN) LPS IHC stained for E-selectin (**C**), P-selectin (**D**), and with an isotype-matched control antibody (**F**). **E:** Lungs from a mouse 24 hours after IN instillation of LPS IHC stained for E-selectin. IHC staining with monoclonal anti-murine E-selectin (9A9), P-selectin (10A10), and IL12-receptor (control) antibodies using aviding-biotin complex (ABC) method, DAB chromogen, hematoxylin counterstain. (400X)

distribution to the basal staining seen in the untreated group. Overall, 3-5 fold more vessels stained positively for P-selectin than for E-selectin (Table 1). A notable difference between the expression of E- and P-selectin detected in these studies was that capillaries, arteries and large veins were stained by P-selectin MAb but not E-selectin MAb.

Table 1

Comparison of E- and P-selectin expression in mouse lungs following intranasal instillation of bacterial lipopolysaccharide (LPS)

hours post-treatment	*extent of vessel involvement*		*type of vessels staining positively*	
	E-selectin	*P-selectin*	*E-selectin*	*P-selectin*
0 (control)	-	+	none	capillaries, venules, and small veins
1	-	++	none	capillaries, venules, and small veins
2	+	+++	arterioles and venules near bronchioles	capillaries, venules, veins and arterioles
4	++	+++++	arterioles and venules near bronchioles	capillaries, venules, veins arterioles, and small arteries
6	++	++++	arterioles and venules near bronchioles	capillaries, venules, veins and arterioles
24	+	+	arterioles and venules near bronchioles	capillaries, venules, and small veins

The extent of vessel involvement was examined in regions in which blue macrophages were present indicating delivery of LPS to that area.
At least 10 fields were examined in each lung section.
The criteria for extent of vessel involvement was:
- = 0 positively stained vessels in the entire lung section
+ = 0-2 positively stained vessels/200x field
++ = 2-4 positively stained vessels/200x field
+++ = 4-6 positively stained vessels/200x field
++++ = 6-10 positively stained vessels/200x field
+++++ = >10 positively stained vessels/200x field

The fixation method (OCT/PBS inflation followed by snap freezing before sectioning) used in these studies is a novel method of fixation developed in the pilot studies. This method resulted in good morphologic preservation and proper inflation of the lungs compared to other methods that were attempted.

DISCUSSION

This study demonstrated the upregulation of E- and P-selectin expression in the lungs of mice in response to an acute inflammatory stimulus (IN instillation of LPS) as demonstrated by immunohistochemistry and immunoprecipitation. This upregulation was rapid, occurring within 2 hours for E-selectin and 1 hour for P-selectin. Upregulation peaked by 4-6 hours and returned to near baseline levels by 24 hours post-treatment. Immunoprecipitation studies performed in the laboratory of Ms. Christine Norton (Dept. of Inflammation and Autoimmune Disease, Hoffmann-La Roche, Nutley, NJ) confirmed the immunohistochemical finding of approximately 5-fold more P-selectin than E-selectin staining. In these studies 3 mice/group were treated with IN saline alone or saline containing LPS (as described above) and sacrificed 3 hours later. Pooled lungs from each group were metabolically labeled for 2 hours, detergent lysed, extracted, and immunoprecipitated with anti-E- (9A9) and anti-P-selectin (10A10) MAbs.

The results presented here are consistent with the time course of E-selectin expression reported to occur in cultured human and murine endothelial cell lines, in which expression peaks 4-6 hours after cytokine or LPS stimulation and returns to basal levels by 24-48 hours post-stimulation (253, 5). The P-selectin results reported here are consistent with the reports of others. P-selectin is constitutively expressed and stored pre-formed in cytoplasmic granules of endothelial cells and platelets (253, 5). The punctate staining seen in this study is consistent with the basal constitutive presence of P-selectin within intracytoplasmic granules. The peak expression of P-selectin through the 4-6 hour time points supports recent reports of prolonged P-selectin expression in mice and dogs both *in vitro* and *in vivo* (131, 132, 133).

The presence of E- and P-selectin on the surface of endothelial cells in mice given IN LPS suggests that these molecules may play a role in the early recruitment of neutrophils in this model. However, endothelial cell adhesion molecule expression does not always correlate with neutrophil margination and extravasation (257). For example, expression of the adhesion molecules E-selectin and vascular cell adhesion molecule 1 (VCAM-1) did not correlate with leukocyte adhesion in mice injected intraperitoneally with LPS (149). Therefore, the strategy of interfering with or blocking selectin-dependent interactions was used to study the functional role of selectins in neutrophil recruitment. These experiments will be discussed in Part 3.

PART 3

SELECTIN ANTAGONISTS IN MURINE MODELS OF ACUTE INFLAMMATION

ABSTRACT

Adhesion molecules expressed on the surface of endothelial cells and leukocytes play a role in the recruitment of leukocytes to areas of inflammation. The relative importance of a particular adhesion molecule in leukocyte adherence to the endothelium can be examined *in vivo* with antagonists that block the adhesion pathway of interest *in vitro*. This study utilized antagonists to two members of the selectin family of adhesion molecules (E- and P-selectin) to examine their roles in the infiltration of neutrophils into sites of acute inflammation in two murine models of inflammation. Mice (n=4/group) were pretreated with intravenous injection of 200 μg (4 hour group) or 500 μg (3 and 6 hour groups) of monoclonal antibody (MAb) 9A9, an anti-E-selectin MAb. Monoclonal Ab 9A9 did not block neutrophil influx into the peritoneal cavity of mice injected intraperitoneally (IP) with 1.0 mL thioglycollate as measured by the number of neutrophils lavaged from the peritoneal cavity of mice 3, 4, and 6 hours post-treatment (as compared to pretreatment with IV saline or control MAb). An analog of the selectin ligand, acid Lewis X (acid Le^x), was also used as an antagonist in this model using 8 mice/group and 4 equally divided IV doses of 50 μg each. Two and 4 hours after the IP injection of 1.0 mL of thioglycollate, the number of neutrophils in the peritoneal lavage fluid was not reduced by acid Le^x treatment.

The role of E- and P-selectin was examined in a second model of acute inflammation using intranasal (IN) instillation of bacterial lipopolysaccharide (LPS). Neutrophil numbers in the bronchoalveolar lavage (BAL) fluid were used as the endpoint. Eight mice/group were pretreated with anti-E-selectin MAb (10E6) (200 μg), anti-P-selectin MAb (10E10) (100 μg), a combination of 10E6 and 10E10 (respectively, 200 and 100 μg each), sterile saline (0.2 mL), or control MAbs

(isotype and dose matched to 10E10 and 10E6) and sacrificed at 2 and 6 hours after the IN LPS. Two hours post-treatment, the MAb combination (10E6 and 10E10) and anti-P-selectin (10E10) MAb alone significantly decreased the number of neutrophils in the BAL fluid. However, at 6 hours post-treatment, blocking of neutrophil recruitment was significant in the combination group and in the anti-E-selectin (10E6 alone) group, while the P-selectin alone group did not show any significant blocking. The combination treatment was a significantly better blocker than the anti-E-selectin antibody alone. When acid Le^x was used as the antagonist in the same model, no blocking was seen at 2 or 6 hours post-treatment. In conclusion, these data indicate that anti-E-selectin MAb did not block thioglycollate-induced peritoneal inflammation. Furthermore, P-selectin (but not E-selectin) played a role in neutrophil influx into lungs in the IN LPS model at 2 hours. However, by 6 hours post-treatment, E-selectin and P-selectin were both involved in this influx. The carbohydrate acid Le^x is not an effective antagonist of neutrophil influx at early time points in the IP thioglycollate model and IN LPS model in mice.

INTRODUCTION

Neutrophils participate in the pathogenesis of a variety of acute inflammatory diseases by the release of non-specific activation products such as proteases and oxygen radicals, that can damage host tissues (2). Examples of these neutrophil-mediated inflammatory conditions include ischemia-reperfusion injury (182) and adult respiratory distress syndrome (8). The recruitment of neutrophils to sites of inflammation involves several sequential steps: neutrophil rolling along the activated endothelium, neutrophil activation and firm adhesion to the vessel wall, and migration of neutrophils into the surrounding tissue (32). These events require the interaction of complementary adhesion molecules expressed on the surfaces of neutrophils and endothelial cells in inflammatory sites.

Several adhesion molecules have been shown to have a role in neutrophil-endothelial interaction. For example, endothelial cells can be activated to express E-selectin, a member of the selectin family of adhesion molecules, in response to interleukin-1 (IL-1), tumor necrosis factor-α (TNF), and bacterial lipopolysaccharide (LPS). In cultured endothelial cells, this expression begins within 1-2 hours after an inflammatory stimulus and peaks in approximately 6-8 hours (5). On the other hand, thrombin and histamine result in rapid expression (within a few minutes) of P-selectin (another member of the selectin family) on the surface of endothelial cells. E- and P-selectin bind to oligosaccharides, sialyl Lewis X (sLe^x) and related carbohydrates, present on the surface of neutrophils. This interaction between the selectins and their carbohydrate (CHO) ligands is believed to mediate the rolling stage of neutrophil extravasation (5).

Selectin-mediated rolling is followed by adhesive events between members of two other families of adhesion molecules, both of which are members of larger

protein superfamilies: the immunoglobulins and integrins (3). More specifically, the β_2 integrins on neutrophils bind to members of the immunoglobulin superfamily, ICAM-1 and 2, on the surface of activated endothelial cells. This integrin-immunoglobulin interaction mediates the firm adhesion and transendothelial migration phases of neutrophil extravasation at sites of acute inflammation.

A deficit in either the integrin/immunoglobulin or selectin/CHO system will result in impairmed emigration of neutrophils as demonstrated by human leukocyte adhesion deficiency I (LAD I), the congenital deficiency of functional leukocyte integrins, and leukocyte adhesion deficiency II (LAD II), the congenital absence of sLe^x (42, 203). Individuals with either of these syndromes lack the ability to recruit normal numbers of neutrophils into areas of inflammation; therefore, they suffer from chronic infections without pus formation, despite elevated circulating neutrophil counts in the peripheral blood (203, 42)

The clinical syndromes of individuals with LAD I and LAD II suggest that interference with the adhesion cascade, at any point, could result in decreased recruitment of neutrophils and consequently in attenuation of neutrophil-mediated tissue damage. Numerous studies involving animal models of inflammation have demonstrated both a decrease in neutrophil emigration into sites of inflammation and a decrease in tissue damage when monoclonal antibodies (MAb) that bind to functional epitopes of adhesion molecules ("blocking" antibodies) are given systemically prior to the inflammatory insult (6). In addition to MAb, other antagonists, such as prokaryotic peptides, CHO mimetics of the selectin ligands, and adhesion molecule-IgG chimeras, have been used to block neutrophil-endothelial interactions and neutrophil-mediated injury (235, 239, 258).

While the β_2 integrins and ICAM-1 and -2 have been extensively evaluated in animal models, relatively few *in vivo* studies have examined the role of E-selectin in animal models of inflammation (6). Those published to date include rat models of IgG immune complex-induced pulmonary inflammation and glycogen-induced chemical peritonitis and a primate asthma model. A significant reduction in the number of neutrophils in the peritoneal lavage fluid has been reported to occur when $F(ab')_2$ fragments of an anti-E-selectin blocking MAb were injected intravenously (IV) into the rats several times during the course of the intraperitoneal (IP) glycogen experiment (238). Details of this experiment however, were not published. In addition, both neutrophil influx into the lungs and subsequent tissue injury were decreased by a variety of antagonists in a rat model of IgG immune complex-induced lung inflammation. Effective antagonists included MAb, E-selectin-IgG chimeras, and derivatives of sLe^x given systemically (238, 235, 239). MAbs to E-selectin have also been shown to block both the neutrophil influx and late airway obstruction in a primate asthma model (173).

Transgenic mice deficient in the gene for P-selectin were generated to examine the role of P-selectin in murine inflammation. These mice have a defective influx of neutrophils into the peritoneum following IP thioglycollate. In this study, P-selectin appeared to mediate the influx of neutrophils only during the early time points (first few hours), after which, another adhesion mechanism appeared to take over as P-selectin became less important (135). Because P-selectin is expressed early in inflammation and E-selectin is expressed slightly later, E-selectin is a potential candidate for the phase of inflammation which occurs after P-selectin is apparently no longer a significant participant in neutrophil recruitment.

The primary goal of these studies was to examine the role of E-selectin in two murine models of inflammation: intranasal (IN) instillation of LPS and chemical peritonitis induced by IP injection of thioglycollate broth. Monoclonal antibodies that have been demonstrated to block E-selectin-mediated binding of neutrophils *in vitro* (256) were used for the studies. A second objective was to answer the question: If P-selectin and E-selectin are sequentially important in neutrophil adhesive events, does blocking both adhesion molecules result in an additive reduction in the inflammatory infiltrate? Monoclonal antibodies against functional epitopes on P- and E-selectin were used alone and in combination to evaluate this hypothesis in the IN LPS model. A third objective was to study the effects of a CHO mimetic known as acid Lewis X (acid Le^x) which was used as an antagonist in both the IP thioglycollate and IN LPS models of inflammation. The criterion used to evaluate the effectiveness of the antagonists was neutrophil infiltration into lavage fluid.

Several technical details were important in these experiments. Intravenous injection of MAb can induce neutropenia by a variety of mechanisms, including activating leukocytes, inducing neutrophil aggregation, and activating the complement cascade. Therefore, if blocking is observed, it is important to demonstrate a lack of peripheral neutropenia which itself could cause a decrease in neutrophil emigration. In addition, endotoxin-free, isotype matched non-blocking MAbs were always used as negative controls in these experiments.

MATERIALS AND METHODS

Anti-E-selectin MAb and acid Le^x blocking studies in intraperitoneal thioglycollate model

Mice

The mice used for this study were 7-10 week old, VAF®, males of the Charles River Crl:CD-1 (ICR)BR strain purchased from Charles River Breeding Laboratories (Raleigh, NC). The mice were group housed in suspended stainless steel cages over non-contact bedding. The murine pathogen-free environment was maintained with a 12 hour light/dark cycle at 72 ± 4° F and 50 ± 20% humidity. Purina Certified Rodent Chow 5002 (Purina Mills Inc., Richmond, IN) and reverse osmosis water were provided *ad libidum*. The animals were conditioned for at least one week prior to treatment.

Anti-E-selectin monoclonal antibodies

Monoclonal antibodies, provided by Dr. B. Wolitzky (Dept. of Inflammation and Autoimmune Diseases, Hoffmann-La Roche), were generated as described in Norton *et al.*, 1993 (256). Briefly, a recombinant phage containing the structural exons for mouse E-selectin was isolated and characterized. The polymerase chain reaction (PCR) technique was used to fuse the lectin and epidermal growth factor-like (EGF) domains of murine E-selectin to form an artificial cDNA which was expressed in eukaryotic cells. Transient expression in COS cells demonstrated that the lectin and EGF domains were sufficient to mediate the binding of mouse and human neutrophils as well as HL60 cells (a human myeloma cell line) to soluble E-selectin bound to a plate. Recombinant soluble murine E-selectin was purified and used to immunize rats to generate MAb specific for mouse E-selectin. A panel of MAbs directed against mouse E-selectin was characterized These included MAbs 9A9 and 10E10, which inhibit the adhesion of HL60 cells or mouse

neutrophils to COS cells expressing mouse lectin/EGF domains. While MAbs 2B11 and 14D4 recognize murine E-selectin, but they do not block E-selectin-mediated binding. A Limulus amebocyte lysate Pyrotell® test kit (Associates of Cape Cod, Inc., Wood's Hole, MA) was used to establish that endotoxin levels in the MAb were <0.5 EU/mL.

In the first experiment, mice were pretreated with either saline, blocking anti-E-selectin MAb 9A9, or control MAb 2B11 prior to IP injection of 1.0 mL of thioglycollate. Antibody treatment was as follows: 0.2 mL of sterile saline alone or saline containing 200 mg of MAb was injected intravenously (IV) into the tail vein 5 minutes prior to the IP injection of 1.0 mL of thioglycollate (n=4 mice/group). Four hours later, the animals were sacrificed. Subsequent experiments used a lower dose of IP thioglycollate (0.25 mL thioglycollate in 0.25 mL sterile saline). Control animals were pretreated (5 minutes prior to IP thioglycollate) with tail vein injections of 0.2 mL sterile saline alone or saline containing 500 mg of either 9A9 or 14D4 (n=3). Mice were sacrificed 3 and 6 hours later.

Acid Lex

Acid Le^x, a carbohydrate analog of sLe^x was provided by Dr. Donna Huryn (Dept. of Inflammation and Autoimmune Diseases, Hoffmann-La Roche). A manuscript detailing the synthesis of this compound is in preparation. Mice were injected with 1.0 mL of thioglycollate and sacrificed 2 and 4 hours later. Because of the short half-life of carbohydrates in the blood, mice were treated with 200 mg of acid Le^x in four equally divided doses throughout the experiment at the following intervals: 0 (immediately prior to the thioglycollate), 0.5, 1, and 1.5 hours for the group sacrificed at 2 hours post-treatment and at 0, 1, 2, and 3 hours for the group sacrificed at 4 hours post-treatment (n=4). The acid Le^x was diluted in sterile saline

(50 mg acid Le^x in 0.1 mL saline) and injected into the tail vein. Sterile saline (0.1 mL) was injected alone, as a control, using the same regime as the acid Le^x (n=4).

Intraperitoneal thioglycollate and evaluation of peritoneal lavage fluid

Mice were injected IP with 0.25 (with 0.25 mL sterile saline) or 1.0 mL of thioglycollate broth (Sigma Chemical Co., St. Louis, MO). Three, 4, or 6 hours later, mice were sacrificed via CO_2 inhalation. After removing the skin overlying the peritoneum, 10 mL of Dulbecco's phosphate buffered saline (PBS) (Gibco Laboratories, Grand Island, NY) containing 10 U/mL of heparin was injected into the peritoneal cavity. The peritoneal wall was gently massaged prior to withdrawal of the lavage fluid through a 22 gauge needle. The yield of lavage fluid was 7-8 mL/animal. The fluid was kept on ice in polypropylene tubes until evaluation (within 30 minutes). The total number of leukocytes in the lavage fluid was manually counted with a Neubauer hemacytometer. Cytocentrifuged cell preparations were made by centrifuging 100 µL of peritoneal lavage fluid at 800 rpm for 5 minutes on a Cytospin 2™ (Shandon Scientific Ltd., Runcorn, UK), then staining the cells with a modified Wright's stain on a Hematek™ autostainer (Miles Laboratories, Inc., Elkhart, IN). Two slides were made for each animal, and differential counts were done by counting 100 leukocytes/slide using a light microscope at 630x oil immersion.

Statistics

Data were analyzed by a one-way analysis of variance (ANOVA) for each time point. If significant differences were detected among group means, a Student's t-test was applied for comparison of group means. Statistical significance was applied when $p<0.05$.

Anti-E- and P-selectin MAb and acid Le^x blocking studies in intranasal LPS model

Mice

The mice used for this study were 7-8 week old, male, Charles River VAF® Crl:CD-1 (ICR)BR mice purchased from Charles River Breeding Laboratories (Raleigh, NC). The animals were housed and treated as described above.

Monoclonal antibodies

The blocking anti-E-selectin antibody (10E6, an IgG_{2b} MAb) used for this study was generated as described (256). A panel of anti-murine-P-selectin antibodies was generated and screened in the same manner as the anti-E-selectin antibodies. A MAb, 5H1 (an IgG_1 MAb), which recognizes murine P-selectin and blocks murine neutrophil binding to activated endothelial cells, was used as the anti-P-selectin blocker. Control antibodies were irrelevant, isotype matched MAb (8G9 and 24A1). A Limulus amebocyte lysate Pyrotell® test kit was used to establish that endotoxin levels in the MAb preparations were <0.5 EU/mL.

Experimental design

There were 8 mice per group sacrificed at 2 and 6 hours after IN instillation of LPS. Five to 10 minutes prior to intranasal instillation of LPS, one of the following was injected intravenously into the retro-orbital sinus: (1) 0.2 mL sterile saline, (2) a combination of two control MAbs (100 mg MAb 8G9 and 200 mg MAb 24A1 in 0.2 mL sterile saline), (3) a combination of anti-E- and P-selectin MAbs (100 mg MAb 5H1 and 200 mg MAb 10E6 in 0.2 mL sterile saline), (4) anti-E-selectin MAb (200 mg MAb 10E6 in 0.2 mL sterile saline), or (5) anti-P-selectin MAb (100 mg MAb 5H1 in 0.2 mL sterile saline). Pilot studies conducted by Dr. Kim McIntyre (Dept. of Inflammation and Autoimmune Disease, Hoffmann-La Roche) established these antibody doses as 10x the amount required for *in vivo* blocking.

Acid Lex

Acid Le^x was provided by Dr. Donna Huryn (Dept. of Inflammation and Autoimmune Disease, Hoffmann-La Roche). Mice were sacrificed 2 and 4 hours after intranasal instillation of 10 or 100 μg LPS in 50 mL saline. Five animals per group/time point were treated with intravenous injections (in the lateral tail vein) of 200 μg of acid Le^x in four equally divided doses at the following intervals: 0 (immediately prior to the thioglycollate), 0.5, 1, and 1.5 hours for the group sacrificed at 2 hours post-treatment and at 0, 1, 2, and 3 hours for the group sacrificed at 4 hours post-treatment. The acid Le^x was diluted in sterile saline (50 mg acid Le^x in 0.1 mL saline) and injected into the tail vein. Sterile saline (0.1 mL) was injected alone, as a control, using the same regime as the acid Le^x (n=5).

Intranasal instillation of LPS and evaluation of bronchoalveolar lavage fluid

Mice were anesthetized in a desiccation chamber containing methoxyflurane (Metofane®, Pitman-Moore, Mundelein, IN). Once in a deep plane of anesthesia, mice were removed from the chamber and administered 50 mL of sterile saline containing 10, 50, or 100 μg LPS (from *Escherichia coli* Serotype 055:B5, Sigma Chemical Co., St Louis, MO) and 4% monastral blue dye (Sigma) by placing the saline on the external nares of the anesthetized mouse until inhalation was observed. Two and 6 hours later, mice were sacrificed via a lethal dose (0.2 mL IP) of Euthanasia 5 Solution® (Henry Schein Inc., Port Washington, NY). When deeply anesthetized, mice were exanguinated by aortic transection. Bronchoalveolar lavage (BAL) was performed by inserting an 18.5 gauge nylon catheter into a small tracheostomy opening of the exposed trachea. The trachea was secured to the catheter using 5-0 silk suture material. Lungs were lavaged *in situ* with a total of 4 mL of cold PBS containing 10 U/mL of heparin instilled in 5

repeated lavages of 0.8 mL each. BAL fluid was put into polyethylene tubes and kept on ice until evaluation (within 30 minutes). Total leukocyte count in the BAL fluid was quantified with a Neubauer hemacytometer. Cytocentrifuged cell preparations were made by centrifuging 100 mL of BAL fluid at 800 rpm for 5 minutes (Cytospin 2™) followed by staining the cells with a modified Wright's stain on a Hematek™ autostainer. Two slides were made for each animal, and differential counts were done by counting 100 leukocytes/slide using a light microscope at 630x oil immersion.

Hematology

Peripheral blood samples were collected from animals very shortly after the IP Euthanasia 5 Solution® was administered, at which point mice were mildly sedated. Retro-orbital sinus puncture with a capillary tube was used to collect blood into a tube containing EDTA which was shaken vigorously to distribute the EDTA and prevent coagulation. Clotted samples were discarded. An automated hematology analyzer (Coulter Counter Model S-Plus IV, Coulter Electronics, Hialeah, FL) was used to determine a peripheral white blood cell count on the anticoagulated whole blood which was also used to make peripheral blood smears. The smears were stained with a modified Wright's stain on a Hematek™ autostainer and differential white blood cell counts were done by counting 100 leukocytes per smear. Abnormal cellular morphology was noted.

Statistics

Data were analyzed by a one or two-way analysis of variance (ANOVA) for each time point. Since experiments often spanned more than one day, a block design was employed to rule out the effect of date on the statistical model. If significant differences were detected among group means, a Student's t-test was

applied for comparison of group means. Statistical significance was applied when $p<0.05$.

RESULTS

Intraperitoneal thioglycollate

Blocking with anti-E-selectin MAb

Four hours after IP injection of 1.0 mL of thioglycollate, there was no statistically difference between absolute numbers of neutrophils in the peritoneal lavage fluid of mice pretreated with saline, control MAb 2B11, or blocking MAb 9A9 (Table 1) To further examine whether 9A9 could block neutrophil influx in this model, a lower dose of IP thioglycollate was used (0.25 mL thioglycollate in 0.25 mL sterile saline) with the hypothesis that a lower grade inflammation may be easier to block. This study looked at time points 3 and 6 hours after the IP thioglycollate and used the control MAb 14D4. The results of this experiment, as summarized in Table 2, demonstrate that at both doses of thioglycollate, there was no difference between the treatment groups. The results of the MAb blocking studies in the IP thioglycollate model demonstrated that blocking E-selectin alone at these early time points has no effect on the influx of neutrophils into the peritoneum, as measured by absolute neutrophil numbers in the peritoneal lavage fluid.

Blocking with acid Lex

To examine the potential ability of a carbohydrate analog of the selectin ligand sLe^x known as acid Le^x, four divided doses of acid Le^x were given over the course of an IP thioglycollate experiment with a 2 and 4 hour time of sacrifice. Table 3 summarizes the results of these experiments which demonstrate that, at both 2 and 4 hours after treatment with IP thioglycollate, acid Le^x did not decrease the number of neutrophils in the peritoneal lavage fluid. There were four mice/treatment group/time point; however, one animal in the 4 hour acid Le^x-

Table 1

Effect of MAb 9A9 on the high-dose IP thioglycollate-Induced peritoneal inflammation at 4 hours post-treatment

animal #	*treatment*	*total wbc ($x10^4$/mL) in peritoneal lavage*	*% PMN in peritoneal lavage*	*PMN ($x10^4$/mL) in peritoneal lavage*	*mean PMN $x10^4$/mL (± s.d.) for each treatment group*
1	saline	80.0	78.0	62.40	
2	saline	93.0	77.5	72.07	59.3 (11.6)
3	saline	77.0	76.0	58.52	
4	saline	71.0	62.0	44.02	
5	9A9	60.3	62.0	37.39	
6	9A9	58.0	59.0	34.22	53.7 (24.1)
7	9A9	124.0	70.0	86.80	
8	9A9	72.0	78.5	56.52	
9	2B11	127.0	70.0	88.90	
10	2B11	118.0	78.0	92.04	71.2 (25.3)
11	2B11	55.5	67.0	37.19	
12	2B11	98.5	67.5	66.49	

9A9 is a rat anti-mouse E-selectin monoclonal antibody which blocks neutrophil binding *in vitro*

2B11 is an isotype-matched rat anti-mouse E-selectin monoclonal antibody

Table 2

Effect of MAb 9A9 on the low dose IP thioglycollate-induced peritoneal inflammation at 3 and 6 hours post-treatment

treatment	*time of sacrifice*	*PMN x10^4/mL (± s.d.)*
saline		47.6 (29.9)
9A9	3 hours	52.2 (8.1)
14D4		39.9 (7.8)
saline		63.1 (11.0)
9A9	6 hours	69.4 (13.9)
14D4		77.9 (28.9)

9A9 is a rat anti-mouse E-selectin monoclonal antibody which blocks neutrophil binding *in vitro*

14D4 is an isotype-matched rat anti-mouse E-selectin monoclonal antibody

n=3 mice/group

Table 3

Effect of treatment with acid Le^x on IP thioglycollate-induced peritoneal inflammation at 2 and 4 hour time points

treatment (n=8/group)	*time of sacrifice*	*PMN $x10^4$/mL (± s.d.)*
saline	2 hours	21.7 (4.5)
acid Le^x	2 hours	21.6 (7.8)
saline	4 hours	66.8 (8.5)
acid Le^x	4 hours	64.9 (29.3)

treated group suffocated during the IV injection, a rare complication of the restraint procedure. This made n=3 for that group.

Intranasal LPS

Blocking with anti-E- and P-selectin MAb

Two hours after the intranasal instillation of LPS, both the combination of anti-E- and anti-P-selectin MAbs and anti-P-selectin MAb alone resulted in a significant decrease in the neutrophil numbers in the BAL fluid (Fig. 1). By 6 hours post-treatment, the combination of anti-E- and anti-P-selectin MAbs and anti-E-selectin MAb alone resulted in a significant decrease in this parameter. Interestingly, at 6 hours post-treatment, the combination antibody treatment was significantly different from the anti-E-selectin MAb alone (Fig. 1). These results suggest that P-selectin alone is important for the neutrophil influx into the lungs in the early phases of inflammation in this model; while in later time phases, both E- and P-selectin are involved in the recruitment of neutrophils.

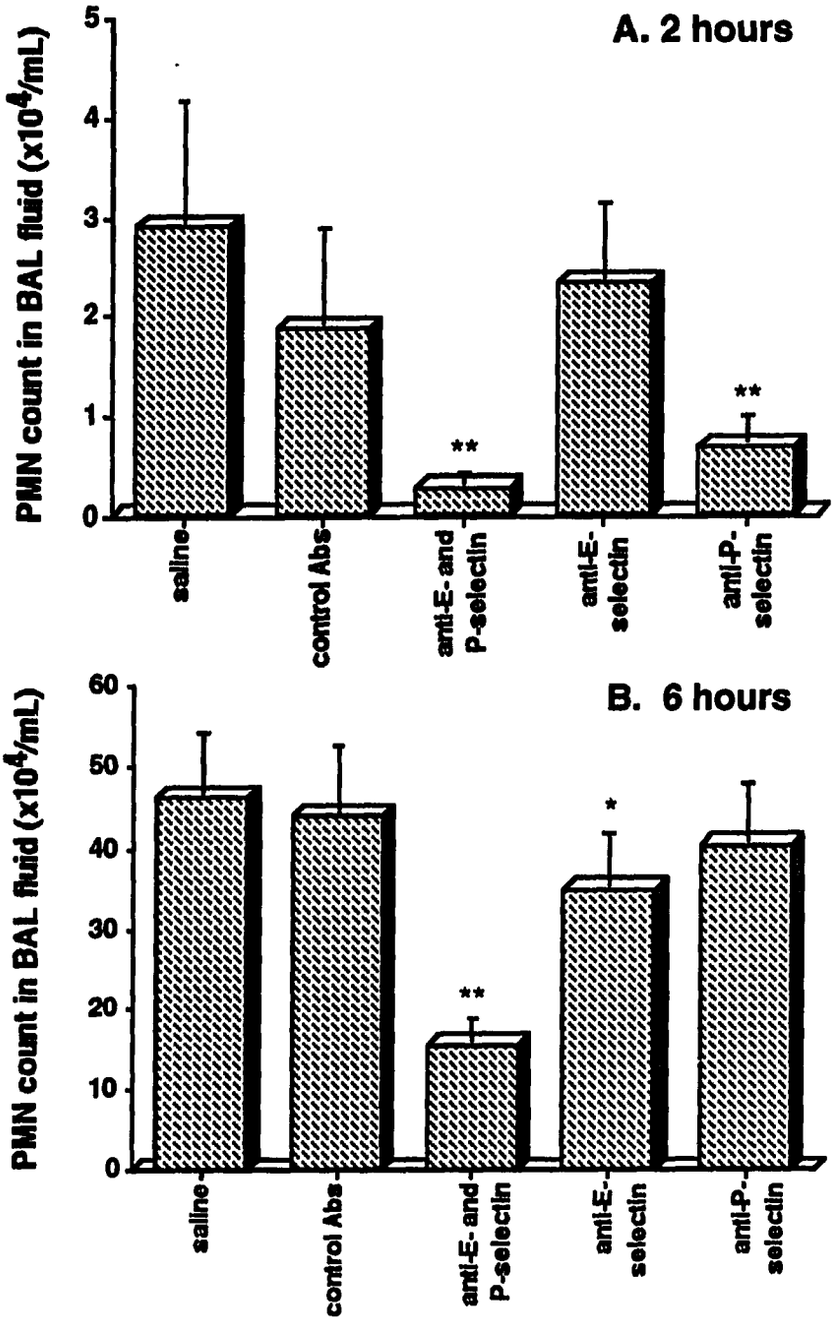

Figure 1. Effect of pre-treatment with blocking MAb on the number of neutrophils in BAL fluid of mice receiving IN LPS 2 hours (A) and 6 hours (B) earlier. Statistically significant groups are indicated by ** ($p<0.01$) and * ($p<0.05$). n=8 mice per group.

Hematology

Treatment with the MAb alone or in the various combinations did not result in peripheral neutropenia at either time point (Fig. 2). It is interesting to note, however, that the mice receiving both anti-E- and anti-P-selectin MAb had an elevation in the numbers of circulating neutrophils in blood collected from the infra-orbital sinus at the time of sacrifice. This elevation was statistically significant at $p<0.05$ in the 6 hour group but not in the 2 hour group. This increase in circulating neutrophils likely reflects a change in neutrophil pools as a consequence of the blockade of the neutrophil influx into the inflamed lungs.

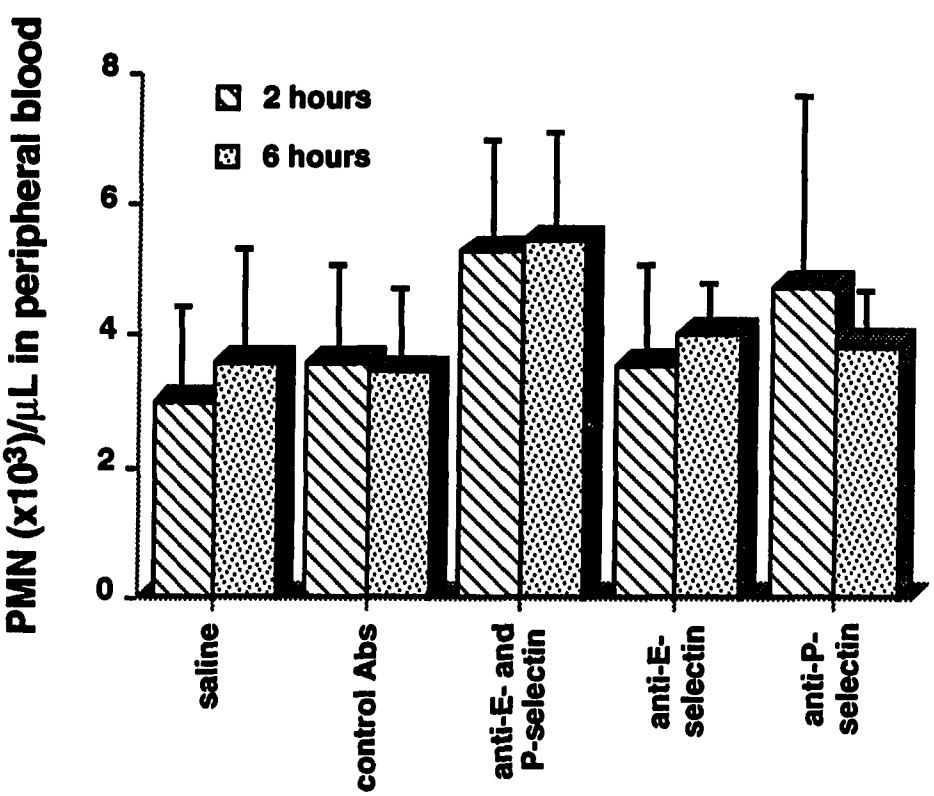

Figure 2. Neutrophil numbers in the peripheral blood collected at the time of sacrifice. Mice were pretreated with MAb or saline, given an IN instillation of LPS, then sacrificed 2 and 6 hours later. n=8 mice/group.

Blocking with acid Lex

There was no significant difference between the numbers of neutrophils in the BAL fluid of the acid Le^x or saline groups at 2 or 4 hours post-treatment with either dose of IN LPS (Fig. 3). These results indicate that acid Le^x does not reduce the early neutrophil influx into the lungs of mice treated with IN LPS.

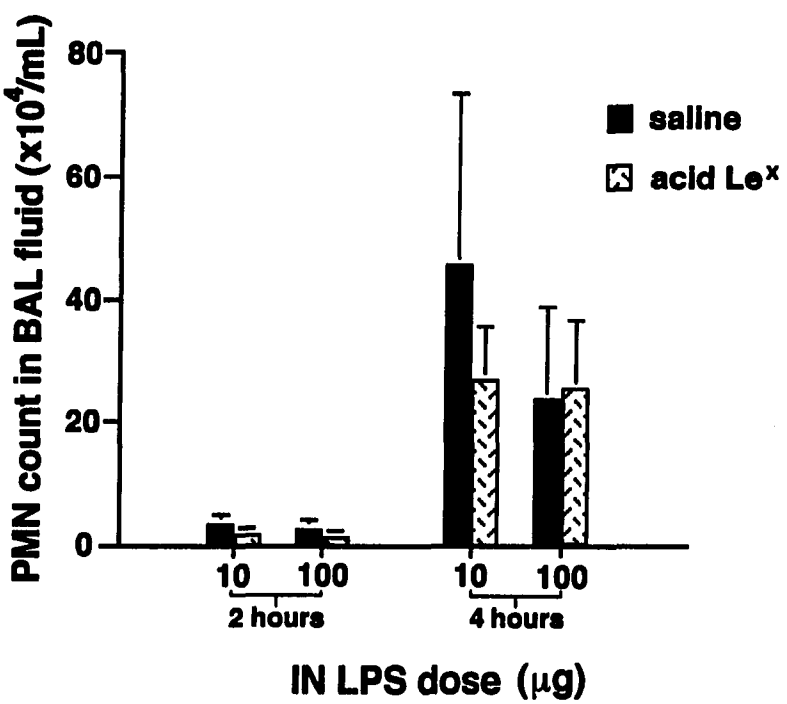

Figure 3. Effect of IV acid Le^x or saline treatment on neutrophil count in the BAL fluid of mice receiving two doses (10 and 100 μg/mouse) of IN LPS at 2 and 4 hours post-treatment. n=5 mice/group.

DISCUSSION

These studies demonstrate the overlapping functions of E- and P-selectin in recruitment of neutrophils into sites of inflammation in the mouse. Blocking E-selectin alone failed to significantly reduce the influx of neutrophils into the peritoneal cavity in the IP thioglycollate model; while in the IN LPS model, blocking E-selectin reduced neutrophil infiltrates at 6 hours post-treatment but not at 2 hours. In comparison, significant blocking was seen 2 hours post-treatment with the anti-P-selectin antibodies. Similar blocking was observed in the double antibody blocking studies at the 2 hour time point suggesting P-selectin alone is responsible for the selectin-mediated recruitment of neutrophils by the activated endothelium at early stages of inflammation. This finding is in complete agreement with those in transgenic mice deficient in the gene for P-selectin, which demonstrated a role for P-selectin in leukocyte interactions with endothelium in the early phase of leukocyte recruitment into inflammatory sites (135). Interestingly, 6 hours post-treatment, both E- and P-selectin were important in neutrophil influx into the BAL fluid of mice treated with IN LPS. Anti-E-selectin MAb blocked approximately 20% while the combination of anti- E- and P-selectin MAb blocked 65% of the neutrophilic infiltrate in the IN model 6 hours post-treatment. These data suggest that P-selectin alone mediates the influx at 2 hours post-treatment. By 6 hours post-treatment, E-selectin is more important than P-selectin although P-selectin still plays an additive role in selectin-mediated binding of neutrophils to the endothelium in sites of inflammation as reflected by the effectiveness of the combined E- and P-selectin MAb blocking.

In these studies, acid Le^x, the carbohydrate analog of the selectin ligand, sLe^x, failed to block infiltration of neutrophils into either the peritoneum of mice

following IP thioglycollate or the lungs following IN instillation of LPS. *In vitro* studies have demonstrated that acid Le^x is equivalent to sLe^x in its ability to inhibit binding of human PMN or HL60 cells (a myeloid leukemia cell line) to recombinant soluble E-selectin (Dr. Bob Campbell, Dept. of Inflammation and Autoimmune Disease, Hoffmann-La Roche, unpublished results). However, the IC_{50} (concentration at which 50% of the binding is inhibited) of these carbohydrates is in the low mM range which is orders of magnitude lower than that for blocking MAbs. Moreover, carbohydrates usually have a very short half-life in the serum. The blood levels of acid Le^x could not be measured in these studies due to the lack of an assay to evaluate them. Nevertheless, the recent publication of the structure of crystallized E-selectin demonstrates that the amino acid side chains involved with the carbohydrate binding are on the surface of the molecule (259). The low affinity of the soluble carbohydrates for these surface residues may explain the lack of blocking ability due to the high shear forces encountered within blood vessels. *In vivo*, the carbohydrate ligands for the selectins are clustered on protein scaffolds which increases the avidity of the selectin-carbohydrate interactions (261). In conclusion, the inability of acid Le^x to block the neutrophil influx in the IP thioglycollate and IN LPS models is thought to demonstrate a lack of *in vivo* efficacy for this soluble, monomeric carbohydrate analog of sLe^x rather than a lack of E- or P-selectin involvement in the recruitment of the neutrophils.

The increase in the circulating neutrophils in the peripheral blood of animals given IN LPS in the double blocking antibody groups is interpreted to result from the inability of these cells to leave the blood stream because of the antibody blockade. Similarly, at 2 hours post-treatment, the anti-P-selectin MAb could significantly block neutrophil extravasation and also caused an increase in

peripheral blood neutrophils. Work by Dr. Kim McIntyre (Dept. of Inflammation and Autoimmune Diseases, Hoffmann-La Roche) has established the link between blocking neutrophil extravasation and the increase in circulating PMN (unpublished data). Following the same antibody dosing regime established by the studies presented here but without an inflammatory stimulus, blood was collected at 2 and 6 hours after the antibody treatment. This work showed that in the absence of inflammation, there was no increase in circulating numbers of neutrophils in the antibody treatment groups relative to the saline group at either time point. Further work by others at Hoffmann-La Roche support the results seen in the IN LPS double antibody blocking study (unpublished results). These experiments used the blocking regime of E- and P-selectin MAb alone and in combination in the IP thioglycollate model and demonstrated that P-selectin was important at early time points in mice given IP thioglycollate but both E-selectin and P-selectin were important at later time points.

Adhesion molecule pathways have a great deal of redundancy leading to overlap of function between the various adhesion molecules. "Knocking out" the function of one selectin can be compensated for by one or more of the other selectins. This study demonstrates the overlap in function between the endothelial cell selectins, E- and P-selectin, in the recruitment of neutrophils to the lung in the IN LPS model in the mouse. This overlap may be peculiar to mice since blocking of E-selectin alone has been demonstrated to block neutrophil influx into sites of acute inflammation in other species (238, 240). However, few *in vivo* studies have addressed the overlapping roles of E- and P-selectin, so this principle of redundancy may not be unique to the mouse. The findings reported here suggest that therapies directed at the selectins in neutrophil-mediated diseases should be

aimed at blocking the common ligand rather than at each of the selectins alone. However, carbohydrate analogs with higher avidities will need to be synthesized for *in vivo* efficacy.

PART 4

E-SELECTIN DEFICIENT TRANSGENIC MICE

ABSTRACT

E-selectin is an adhesion molecule expressed on the surface of activated endothelial cells in areas of inflammation. *In vitro* studies and studies in rats and primates have demonstrated that E-selectin plays a role in the recruitment of leukocytes, including neutrophils, to areas of acute inflammation. The goal of these studies was to investigate the phenotype of transgenic mice deficient in the gene for E-selectin. Macro- and microscopic examination of tissues and organs revealed no difference between 3 transgenic mice deficient in the gene for E-selectin (generated by gene targeting in embryonic stem cells) and 3 wild-type, line-matched mice. Immunohistochemical staining confirmed the absence of protein on endothelial cells in the heart and lungs of E-selectin deficient (knockout) mice following systemic treatment with bacterial lipopolysaccharide. Circulating levels of neutrophils in the peripheral blood of knockout mice were higher than, but not statistically different from, neutrophil counts in the circulation of the wild-type mice. E-selectin knockout mice had a decreased ability to recruit neutrophils to an area of acute inflammation as indicated by decreased numbers of neutrophils in the peritoneal lavage following intraperitoneal (IP) injection of thioglycollate. This decrease was small, however, and was not statistically significant. These knockout mice represent a novel animal system in which to evaluate the role of E-selectin in murine inflammatory responses. The studies presented here demonstrate that the knockout mice develop normally and may have an only mildly impaired inflammatory response in the IP thioglycollate model of inflammation.

INTRODUCTION

E-selectin, a member of the selectin family of leukocyte-endothelial cell adhesion molecules, is expressed on the surface of activated endothelial cells in areas of acute inflammation. *In vitro*, its expression on cultured endothelial cells peaks within 4-6 hours of stimulation by interleukin-1 (IL-1), tumor necrosis factor-α (TNF), or bacterial lipopolysaccharide (LPS) where it can support binding of neutrophils, monocytes, eosinophils, basophils, and a subpopulation of memory T cells (3, 5). Little work has been done to define the role of this adhesion molecule *in vivo* (6).

Transendothelial migration of leukocytes begins with leukocyte rolling, followed by firm attachment of activated leukocytes to the endothelium and finally migration across the endothelium (31). There are three families of adhesion molecules involved in these three steps: the selectins, members of the immunoglobulin superfamily, and the leukocyte integrins. The selectins and their carbohydrate ligands mediate the rolling stage of transendothelial migration, while the immunoglobin superfamily members and their ligands, the leukocyte integrins, mediate the firm attachment and transendothelial migration stages (3). Disruption of any of these steps can block the extravasation of leukocytes into an inflammatory site (6).

Two interesting conditions exist in humans which underscore the importance of the endothelial-leukocyte adhesion molecules in normal inflammatory processes. These conditions, known as leukocyte adhesion deficiency I (LAD I) and leukocyte adhesion deficiency II (LAD II), are characterized by an inability to recruit neutrophils into sites of inflammation with a resultant clinical syndrome of chronic infections without pus formation despite elevated circulating neutrophil

counts in the peripheral blood (203, 42). A different mechanism underlies the altered inflammatory response in these two syndromes. LAD I is the result of a defect in the synthesis of the CD18 subunit of the leukocyte integrins such that the stable adherence and transendothelial migration phases of leukocyte extravasation are unable to occur. On the other hand, LAD II affects the rolling phase because it results from a defect in the synthesis of the carbohydrate ligand for the selectins. Both conditions illustrate that interference with any phase of neutrophil extravasation can block neutrophils from entering an area of inflammation.

Transgenic mice deficient in the gene for intercellular adhesion molecule-1 (ICAM-1, a member of the immunoglobulin superfamily) and P-selectin (a member of the selectin family) have been used to study the role of these two adhesion molecules in *in vivo* models of inflammation. They represent novel test systems in which to do these studies. Mice deficient in ICAM-1 exhibited impaired neutrophil emigration in response to IP thioglycollate and decreased contact hypersensitivity to 2,4-dinitrofluorobenzene (68). P-selectin knockout mice have decreased rolling of neutrophils in the venules of exteriorized mesentery and diminished neutrophil influx into the peritoneal cavity in the early phases of inflammation induced by IP thioglycollate (135).

Mice deficient in the gene for E-selectin were generated with the goal of using them as an *in vivo* test system in which to evaluate the role of E-selectin in inflammation. The following studies were designed to characterize the phenotype of these mice by a histologic survey of organs, immunohistochemical staining of hearts and lungs following systemic lipopolysaccharide (LPS) to confirm the lack of E-selectin expression, and by peripheral leukocyte counts. In addition, the role of

E-selectin in the recruitment of neutrophils into the peritoneal cavity following IP injection of thioglycollate broth was evaluated using these novel mice.

MATERIALS AND METHODS

Mice

Transgenic mice deficient in the gene for E-selectin (knockout) were generated and provided by Dr. Mark Labow (Dept. of Molecular Sciences, Hoffmann-La Roche). These mice were produced by gene targeting using homologous recombination in embryonic stem cells, a widely used technique for altering the murine genome (262). The null allele was verified by RNA analysis [Using Northern blots and reverse transcriptase-polymerase chain reaction (RT-PCR)]. The only E-selectin message detected by RT-PCR was a fusion RNA with the *neo* insert, as expected. Mice were housed in polycarbonate shoe-box caging with filter tops on certified 1/4" Bed-O-Cob (Anderson Industrial Products, Maumee, OH) bedding in a rodent pathogen-free environment. Picolab Mouse Diet 20 #5058 (Purina Mills Inc., Richmond, IN) and reverse osmosis water via a water bottle were provided *ad libidum*.

Histopathologic survey

Complete necropsies were performed on 4 week old, male and female, knockout (KO, -/-) and line-matched control mice (wild type, wt, +/+) (n=3/group) which were sacrificed by CO_2 inhalation and immediately exsanguinated by aortic transection. The following tissues were immersion fixed in 10% neutral buffered formalin: kidney, liver, spleen, brain, thymus, lungs (inflated with 0.6 mL formalin), heart, stomach, intestines, and pancreas. After fixation, tissues were trimmed in a standard manner, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for routine histologic examination.

Peripheral blood leukocyte counts

Peripheral blood samples were collected from 5 male and 5 female mice, 6-8 weeks old, of both KO and wt genotype following sedation by CO_2 inhalation. Retro-orbital sinus puncture with a capillary tube was used to collect blood into a tube containing EDTA (which was shaken vigorously to distribute the EDTA and prevent coagulation). Clotted samples were discarded. An automated hematology analyzer (Coulter Counter Model S-Plus IV, Coulter Electronics, Hialeah, FL) was used to determine a peripheral white blood cell count on the anticoagulated whole blood which was also used to make peripheral blood smears. The smears were stained with a modified Wright's stain on a Hematek™ autostainer (Miles Laboratories, Inc., Elkhart, IN) and differential white blood cell counts were done by counting 100 leukocytes per smear. Cellular morphology was evaluated.

Immunohistochemistry

Four hours after IP injection of 1 mL of sterile saline containing 100 μg (0.1 mg/mL) LPS (from *Escherichia coii* Serotype 055:B5, Sigma Chemical Co., St. Louis, MO), three KO and three wt mice (7-8 week old, males) were sacrificed via a lethal injection (0.2 mL IP) of Euthanasia 5 Solution® (Henry Schein Inc., Port Washington, NY). Once moribund, they were exsanguinated by aortic transection. The lungs were then inflated *in situ* with 0.8 mL of a solution containing 1/3 Tissue Tek OCT (Miles, Inc., Elkhart, IN) and 2/3 PBS by volume. A ligature was tied around the trachea. The lungs were removed from the thorax and immersed in supercooled isopentane for 10 seconds. The frozen, inflated lungs were quickly sectioned, embedded in OCT compound in a cryomold, and snap frozen in supercooled isopentane to prevent thawing and subsequent collapse of the

transected lungs. Sections of the heart, and kidney were also embedded in OCT in cryomolds and snap frozen. The OCT blocks were stored at -80°C.

Cryostat sections (6-7 μm), air dried onto silane coated slides (Cel-Tek Inc., Glenview, IL), were fixed in cold (4°C) acetone for 5 minutes and air dried again. The sections were stored at -20°C in sealed plastic packages until use. All subsequent steps were carried out at room temperature. Cryosections were rehydrated in phosphate buffered saline (PBS) for 15 minutes prior to a 30 minute incubation in 1% H_2O_2 in methanol to block endogenous peroxidase. The slides were loaded onto an automated immunostainer (Cadenza, Shandon Scientific Ltd., Runcorn, UK) for the remainder of the immunostaining procedure. The sections were allowed to react with 10% normal rabbit serum (diluted 1:10 in PBS) for 30 minutes followed by 15 minutes each in avidin then biotin blocking solutions (Vector Laboratories, Burlingame, CA). Pilot studies revealed that the avidin and biotin blocking steps significantly reduced nonspecific staining. The monoclonal antibody (MAb) employed for the immunostaining was an anti-E-selectin (MAb 9A9) antibody provided by Dr. Barry Wolitzky (Dept. of Inflammation and Autoimmune Disease, Hoffmann-La Roche). An irrelevant, isotype matched rat MAb was used at the same concentration as a negative control. Dilutions of the primary antibody were optimized in pilot experiments to be 10 μg/mL in PBS containing 0.1% bovine serum albumin (BSA). Sections were incubated with MAb 9A9 or the irrelevant control for 2 hours. The primary MAbs were followed by a 30 minute incubation in a biotinylated rabbit anti-rat IgG (mouse adsorbed) (Vector Laboratories, Burlingame, CA) using 50 μL of the secondary antibody diluted in 10 mL of PBS containing 150 μL of normal rabbit serum. Then the slides were incubated for 30 minutes in the avidin-biotin-peroxidase conjugate which was

prepared as follows: 5 mL PBS mixed with 25 g of non-fat powdered milk and two drops (100 µL) each of bottle A and bottle B from the Vector Elite Kit (ABC Elite Kit, Vector Laboratories, Burlingame, CA). Antibody binding was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (ISOPAC®, Sigma Chemical Co., St. Louis, MO) mixed 1:2 with 35 µL H_2O_2 in 50 mL of distilled water and incubated for 5 minutes. The slides were then rinsed in water, counterstained with hematoxylin, dehydrated, cleared and coverslipped.

Thioglycollate-Induced peritoneal inflammation

The following experiment was performed on two different days. Mice in trial 1 were 6 weeks old and included randomly mixed numbers of males and females. Trial 2 had two males and two females per treatment group and were 7-9 weeks old. Knockout and line-matched control mice were injected IP with 1.0 mL of thioglycollate broth (Sigma Chemical Co., St. Louis, MO). At 2, 4, 6, and 8 hours post-treatment, 8 control and 8 KO mice were sacrificed by CO_2 inhalation. After removing the skin overlying the peritoneum, 8 mL of Dulbecco's phosphate buffered saline (PBS) (Gibco Laboratories, Grand Island, NY) containing 10 U/mL of heparin was injected into the peritoneal cavity. The peritoneal wall was gently massaged prior to withdrawal of the lavage fluid through a 23 gauge needle. Yields of lavage fluid were between 5-6 mL/animal. Lavage fluid was kept on ice in polypropylene tubes until evaluated (<30 minutes). The total number of cells in the lavage fluid was manually counted with a Neubauer hemacytometer. Cytocentrifuged cell preparations were made by centrifuging 100 µL of peritoneal lavage fluid at 800 rpm for 5 minutes on a Cytospin 2™ (Shandon Scientific Ltd., Runcorn, UK) then staining the cells with a modified Wright's stain on a Hematek™ autostainer (Miles Laboratories, Inc., Elkhart, IN). Two slides were made for each

animal, and differential counts were done by counting 100 leukocytes/slide using a light microscope at 630x oil immersion.

Statistics

Data were analyzed by a two-way analysis of variance (ANOVA). Since experiments spanned two days, a block design was employed to include the effect of date on the statistical model. A log transformation was done to determine if statistical significance could be applied to the transformed numbers. Treatment groups (ie: KO vs. line-matched controls) were compared at each time point using the Student's t-test. Statistical significance was applied when $p<0.05$.

RESULTS

Histopathologic survey

There was no difference histologically between the 3 transgenic mice deficient in the gene for E-selectin and the 3 line-matched controls in the following tissues: kidney, liver, spleen, brain, thymus, lungs, heart, stomach, intestines, and pancreas. At the time of necropsy, all organs were of normal size, appearance, and position. Therefore, the mice appeared to develop normally.

Peripheral blood leukocyte counts

Within each sex and overall, the mice deficient in the gene for E-selectin appeared to have higher numbers of neutrophils circulating in the peripheral blood relative to the line-matched control mice (Fig. 1), although the difference between these groups was not statistically significant ($p<0.5$). However, the small number of animals examined ($n=5$) may have precluded proper evaluation of this parameter in this study.

Immunohistochemical confirmation of the E-selectin-deficient phenotype

Three each of E-selectin-deficient and line-matched control mice were injected IP with 100 μg of LPS four hours earlier, sacrificed, and necropsied to collect and snap-freeze sections of the lungs, heart, and kidney. Frozen sections were immunohistochemically stained with MAb 9A9 (rat anti-murine E-selectin MAb). The knockout mice had no staining of the heart, lung, or kidney with anti-E-selectin MAb 9A9. In contrast, a significant amount of staining was present in the tissue of line-matched control mice. The positive staining was observed in the endothelium lining the atria and on the heart valves, in capillaries of the

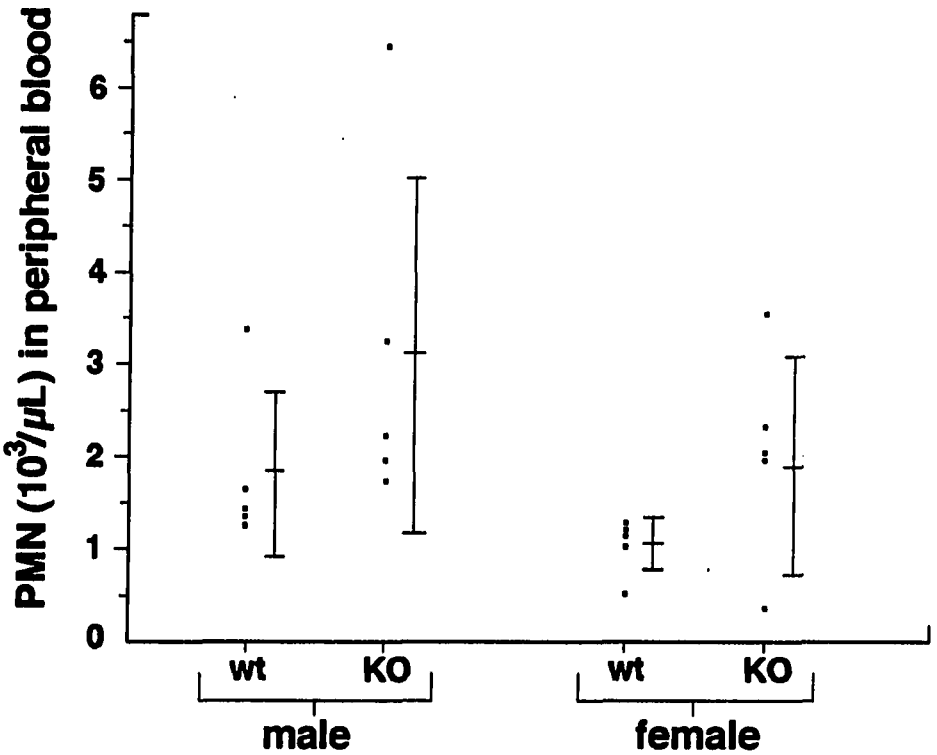

Figure 1. Comparison of absolute neutrophil counts in the peripheral blood of mice deficient in the gene for E-selectin (KO) and line-matched control (wt) mice.

myocardium, and in scattered small veins and venules in the lungs (Fig. 2). It is interesting to note that this expression was not associated with neutrophil margination or emigration. Kidneys failed to stain with the MAb in both KO and wt mice. The specificity of staining was demonstrated by the lack of staining by an isotype matched rat anti-mouse MAb (negative control MAb) in serial sections of heart, lung, and kidney.

Effect of E-selectin deficiency on neutrophil influx into inflamed peritoneal cavity

Thioglycollate broth was injected into the peritoneal cavity of wild-type and E-selectin deficient mice. Two, 4, 6, and 8 hours later, the mice were sacrificed and peritoneal lavages were performed to assess whether the absence of E-selectin would result in an inability of the KO mice to recruit neutrophils to an acute inflammatory site. At the 2 hour time point, KO and wt mice had essentially the same number of neutrophils in the peritoneal lavage fluid (Fig. 3). Although there was an apparent decrease in the mean neutrophil counts in the peritoneal lavage fluid of KO mice at the other time points, the difference was not statistically significant at any time point (Fig. 4). There was a significant difference between the KO and the wt mice at $p<0.05$ when all timepoints were pooled for analysis.

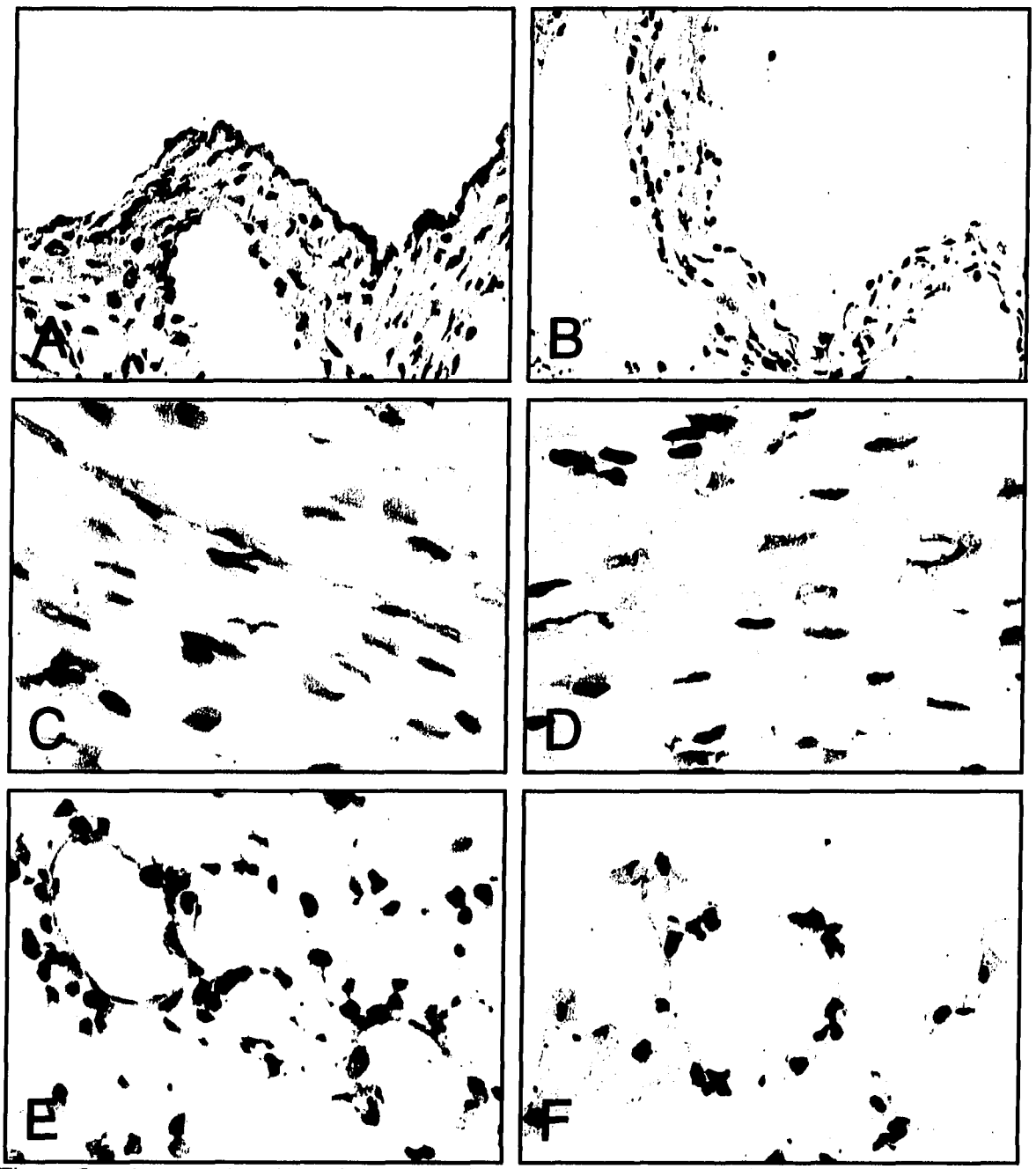

Figure 3. Heart valve (**A, B**), myocardium (**C, D**), and lung (**E, F**) from mice given 100 µg IP LPS 4 hours earlier. **B, D,** and **F** are from transgenic mice deficient in the gene for E-selectin (knockout); while **A, C,** and **E** are from control mice. Immunostaining with monoclonal antibody 9A9 (anti-murine E-selectin) using avidin-biotin peroxidase complex (ABC) method (DAB chromogen, hematoxylin countersatin). The brown staining demonstrates the presence of E-selectin on the endothelium of the control mice. (200X: **A, B**; 400X: **C, D, E, F**)

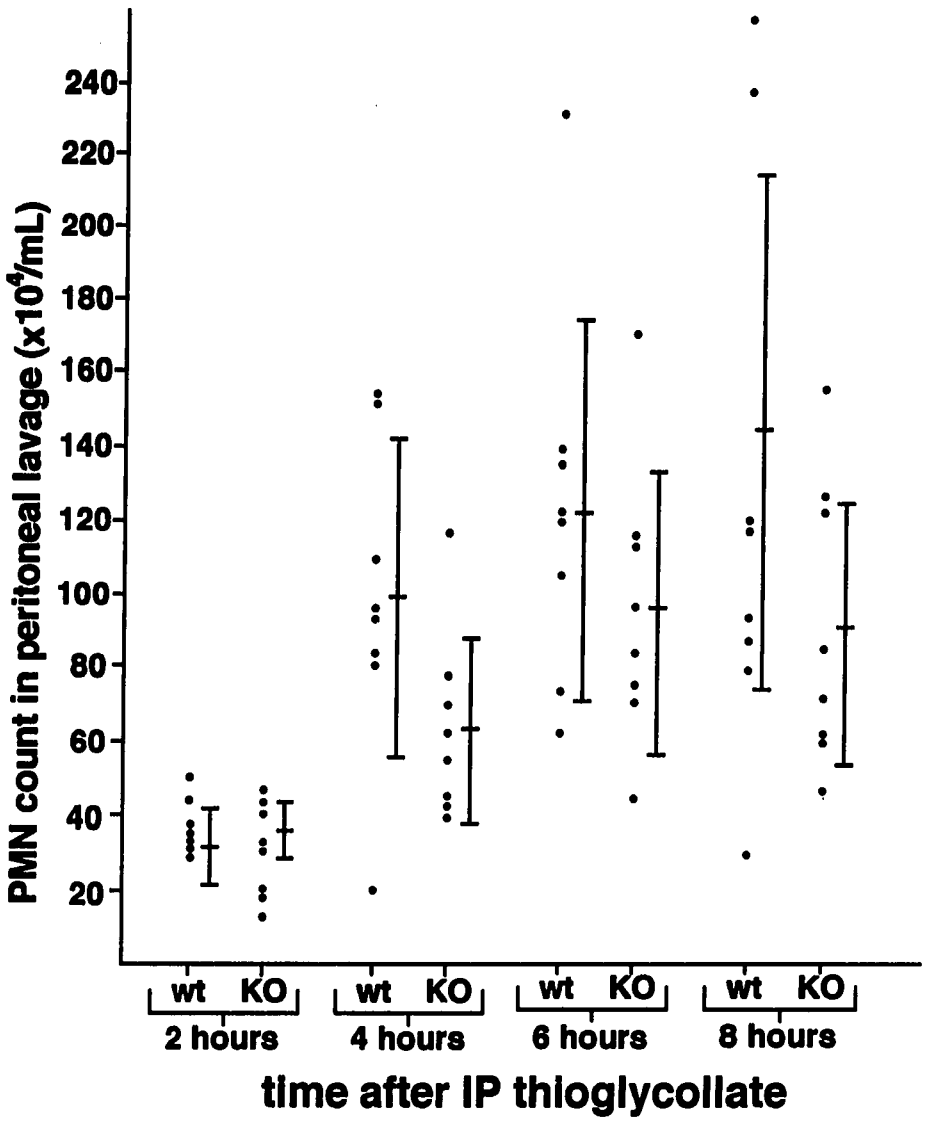

Figure 4 Comparison of neutrophil counts in the peritoneal lavage of mice at 2, 4, 6, and 8 hours after thioglycollate administration. Bar represents the mean ± standard deviation for each group. (wt=wild type; KO=knockout mice).

DISCUSSION

These studies demonstrate that transgenic mice deficient in the gene for E-selectin did not exhibit statistically significant phenotypic alterations in tissue morphology, circulating leukocyte counts, or neutrophil recruitment to sites of acute inflammation. This is in contrast to the increased numbers of circulating leukocytes and impaired neutrophil emigration in response to IP thioglycollate reported in transgenic mice deficient in the genes for both P-selectin and ICAM-1 (135, 68). However, the lack of a significant defect in the inflammatory response agrees with monoclonal antibody (MAb) blocking studies in which an anti-E-selectin MAb with *in vitro* blocking activity failed to decrease the influx of neutrophils into the peritoneal cavity following IP thioglycollate (see Part 3 of this dissertation).

Immunohistochemical staining verified the lack of E-selectin protein expression on the surface of activated endothelial cells in the KO mice indicating that the lack of observable phenotypic changes was not due to a lack of fully null alleles (-/-). An absence of E-selectin protein expression was also confirmed by immunoprecipitation studies of heart and lung extracts from mice injected 4 hours earlier with IP LPS (C. Norton, personal communication).

The E-selectin KO mice were fertile, viable, of normal size, and had macro- and microscopically normal tissue and organ development demonstrating that E-selectin is not required for normal development of the tissues examined. While E-selectin potentially could play a role in development, it clearly is not essential.

Although there was no statistical difference in neutrophil counts in the circulating blood and the neutrophil influx into the peritoneal cavity following IP thioglycollate between the groups in this study, there was an apparent trend as measured by these parameters. Circulating neutrophils were mildly increased in

the KO mice of both sexes relative to the wt mice of the same sex. In addition, the 4, 6, and 8 hour post-IP thioglycollate groups had a modest decrease in the influx of neutrophils into the peritoneal cavity. A difficulty encountered in these studies was related to the extreme novelty of these mice which resulted in a lack of sex- and age-matched animals and the availability of few animals. Poor matching of animal age and sex may have resulted in high standard deviations, which would necessitate the use of high numbers of animals (which were not available) to attain statistical significance. Perhaps with a larger number of animals in the study groups, these trends would have had statistical significance. Studies by Dr. Kim McIntyre (personal communication) using anti-P-selectin MAbs in wt vs. KO mice have demonstrated a decrease in neutrophil numbers in the peritoneal lavage of KO mice treated with the blocking MAb following IP injection of thioglycollate. Similar results, including decreased neutrophil influx and edema, were found in a model of delayed-type hypersensitivity in the ears of mice in which an anti-P-selectin MAb was given to KO mice. Further studies are planned as animals become available, including using blocking anti-P-selectin MAb in the transgenic mice in a model of intranasal instillation of bacterial lipopolysaccharide. In addition, circulating neutrophil numbers will be measured in additional, older animals that are of interest because transgenic mice deficient in the gene for CD18 did not have consistent elevations in peripheral neutrophil counts until the mice were 10-14 weeks old (50). In conclusion, although these studies had limitations, they suggest a mild deficit in recruitment of neutrophils into the peritoneum of E-selectin knockout mice following IP instillation of thioglycollate. Also, these E-seiectin deficient mice appear to develop normally but may have mildly elevated numbers of neutrophils in the peripheral circulation.

GENERAL DISCUSSION AND SUMMARY

This work represents a portion of a comprehensive evaluation of the role of E-selectin in murine inflammation which was conducted by the adhesion molecule team at Hoffmann-La Roche, Inc., Nutley, NJ using novel and unique reagents and animal systems.

The results obtained from these studies were somewhat surprising in that work in rats and primates has demonstrated a clear role for E-selectin in acute inflammation (238, 240). In contrast, the present studies were unable to demonstrate a role for E-selectin that could be separated from that of another member of the selectin family, P-selectin. Blocking E-selectin did not decrease the neutrophil count in the peritoneal lavage of mice following IP thioglycollate at 3, 4, or 6 hours post-treatment nor did it block neutrophil influx into the lungs following IN LPS at 2 hours post-treatment. However, in the IN LPS model at 6 hours post-treatment, E-selectin blocking was only highly significant in the presence of blocking antibodies to P-selectin. Work by colleagues at Hoffmann-La Roche have supported these findings in mice. It has been demonstrated that IV administration of the combination of anti-E- and -P-selectin MAb resulted in a highly significant blockade of neutrophils into the peritoneal cavity at 4, 6, and 8 hours after IP injection of thioglycollate broth or Sephadex beads (K. McIntyre, unpublished data); however, neither of these models was blocked by anti-E-selectin alone. Similarly, in delayed-type hypersensitivity studies in mice, it was shown that anti-E- and P-selectin MAbs together (but neither MAb alone) were able to block edema and neutrophil infiltration in the ears of mice (P. Will, unpublished data).

The general conclusion from these experiments is that in several models of inflammation involving multiple tissues, murine E-selectin and P-selectin appear to

have overlapping or redundant roles. P-selectin, alone, is important in the first 1-2 hours of an inflammatory response as demonstrated in this work and others (135). However, the P-selectin-dependent phase is followed by a phase in which neutrophil influx involves both E- and P-selectin. Thus, both molecules can mediate the influx of neutrophils into the area of inflammation, suggesting that blocking of both molecules is necessary to decrease neutrophil influx. In light of this work, antagonists should be directed at the common carbohydrate ligand of the selectins rather than either of the selectins present on activated endothelial cells. However, this work has also demonstrated that a soluble carbohydrate, acid Le^x, was not effective *in vivo* as an antagonist of the selectin-mediated extravasation of neutrophils. Synthetic sLe^x analogs, or mimetics having higher avidity, will be needed if the selectins are to be a realistic target for anti-inflammatory therapies.

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