STUDIES ON THE MOLECULAR BASIS OF EOSINOPHIL ADHESION TO ENDOTHELIUM

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by

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Addendum consisting of 1 video, VHS format, 4 out of 5 clips (see Appendix 6 for running time)

ABSTRACT

Studies on the molecular basis of eosinophil adhesion to endothelium

Clare A. McNulty

Selective adhesion may be important for the preferential accumulation of eosinophils in asthma and allergic diseases. The Stamper–Woodruff frozen section assay (FSA) was used to define the adhesion of human peripheral blood eosinophils and neutrophils to endothelium in a model of chronic airway inflammation, the nasal polyp. Eosinophil and neutrophil adhesion to nasal polyp endothelium (NPE) was β2 integrin-dependent. Eosinophil adhesion was inhibited to a lesser extent by mAbs against β1 integrins and VCAM-1. Adhesion of both eosinophils and neutrophils to NPE was activation-dependent, as shown by inhibition with azide. Neutrophil adhesion was mediated by PAF and IL-8 signalling through pertussis toxin (PTX)-sensitive G-protein coupled receptors (GPCR). In contrast, eosinophil adhesion was PTX-insensitive, and the chemokine receptor CCR3 was not involved. The eosinophil activation step was further explored under shear flow conditions. Neutrophils firmly arrested on TNF-α-stimulated human umbilical vein endothelial cells (HUVEC) under flow. In contrast, eosinophils rolled unless exogenous chemoattractants such as PAF, eotaxin, and RANTES were added. Priming eosinophils with IL-5 before addition to HUVEC caused arrest of the entire population. The flow assay was also used to investigate the receptors involved in leukocyte adhesion to IL-13-stimulated HUVEC. Eosinophils but not neutrophils showed enhanced binding to IL-13-stimulated HUVEC compared to medium-cultured cells. This adhesion was mediated by P-selectin/ PSGL-1, and to a lesser extent VLA-4/ VCAM-1. These findings were consistent with the pattern of adhesion receptor expression in nasal mucosa, with weak expression of VCAM-1 compared with P-selectin. In summary, I have demonstrated a difference in integrin usage and the mechanism of integrin activation between eosinophils and neutrophils. An additional priming step in the adhesion cascade appears to be required for eosinophils, but not neutrophils, to respond to an activating stimulus and arrest on endothelium. P-selectin/ PSGL-1 interactions were pivotal to eosinophil arrest on Th2-cytokine-stimulated endothelium and are potential targets for inhibition of eosinophil migration.

ABBREVIATIONS

7TM	seven transmembrane
aFGF	acidic fibroblast growth factor
BAL	bronchoalveolar lavage
BBE	bovine brain extract
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular calcium
cAMP	cyclic adenosine monophosphate
CLA	cutaneous lymphocyte antigen
DG	diacylglycerol
EBM	endothelial cell basal medium
EDTA	diaminoethane-tetraacetic acid
EGTA	ethyleneglycol-bis (2-aminoethyl)-N, N, N`, N`-tetraacetic acid
ELR	glutamic acid, leucine, arginine
ERK	extracellularly regulated kinase
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
fMLP	n-formylmethionylleucylphenylalanine
FSA	frozen section assay
g	rcf, relative centrifugal force
G	gauge
GDP	guanosine diphosphate
GFFKR	glycine, phenylalanine, phenylalanine, lysine, arginine
GlyCAM	glycosylation-dependent cell adhesion molecule
GPCR	G-protein-coupled receptor
GTP	guanosine triphosphate
h	hours
HBSS	Hanks' balanced salt solution
hEGF	human epidermal growth factor
HEPES	N-[2-hydroyxyetyl]piperazine-N'-[2-ethanesulphonic acid]
hpf	high power fields
HSA	human serum albumin
HUVEC	human umbilical vein endothelial cells
ICAM	intercellular adhesion molecule
IFN	interferon

IP_3	inositol 1,4,5-triphosphate
IMS	industrial methylated spirits
Jak/ Stat	Janus kinase/ signal transducers and activators of transcription
kDa	kiloDalton
LAD	leukocyte adhesion deficiency
LFA	leukocyte function-associated antigen
mAb	monoclonal antibody
mCi	milli Curie
min	minutes
M199	medium 199
MAdCAM	mucosal addressin cell adhesion molecule
MAP kinase	mitogen-activated protein kinase
MIDAS	metal ion dependent adhesion site
NPE	nasal polyp endothelium
pen/ strep	penicillin/ streptomycin
PBS	phosphate buffered saline
PECAM	platelet endothelial cell adhesion molecule
PKC	protein kinase C
PIP_2	phosphatidylinositol 4, 5-bisphosphate
PLA_2	phospholipase A_2
PLC	phospholipase C
PMA	phorbol 13-myristate 13-acetate
PSGL	P-selectin glycoprotein ligand
RANTES	regulated upon activation, normal T cell expressed and secreted
rh	recombinant human
rpm	revolutions per minute
RT	room temperature
SEM	standard error of the mean
$sLe^{x/a}$	sialyl $Lewis^{x/a}$
S. mansoni	*Schistosoma mansoni*
SMF	specific median fluorescence
TBS	TRIS-buffered saline
T. spiralis	*Trichinella spiralis*
TRIS	hydroxymethyl-methylamine
U	units

VCAM	vascular cell adhesion molecule
VLA	very late antigen

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CHAPTER ONE

Introduction

INTRODUCTION

Allergic diseases, such as asthma, atopic dermatitis, allergic rhinitis, and related conditions such as nasal polyposis, affect approximately one third of the general population and are among the most common diseases encountered by physicians in their clinical practice. The overall prevalence of allergic diseases, and morbidity related to them, has risen progressively in industrialised societies during the past 20 years. Taken together, these diseases constitute one of the major problems of modern day medicine, as a considerable portion of the healthcare budget is expended in their treatment. It has been noted that the global economic costs of bronchial asthma are estimated to exceed the combined cost of the AIDS/ HIV infection and tuberculosis (Gleich, 2000). Due to the enormity of the problem, there has been a worldwide effort to identify factors that contribute to the aetiology of allergic diseases. In order to develop more effective therapies for the management of this group of diseases, it will be critical to fully characterise their pathophysiology.

Allergic diseases are intrinsically linked with inflammation. The classical symptoms of inflammation (redness, swelling, heat, and pain) are the result of underlying biochemical events triggered by tissue damage, infection, or foreign bodies. One of the hallmarks of inflammation is the infiltration of specific leukocyte subsets from the blood into the affected tissue. Leukocytes are bone marrow-derived cells of diverse form and function. They circulate in the blood in a quiescent state of low adhesiveness, before migrating into the tissues to function as a primary line of host defence in the destruction of microorganisms, and to participate in immune functions and tissue repair.

Eosinophils are a characteristic feature of the immunopathology of asthma, but are not found in the normal lung. There is considerable evidence that these leukocytes are critical effector cells in asthma pathogenesis, by virtue of their basic cytotoxic proteins, which have been shown to be released upon activation *in vivo*, and be toxic *in vitro*. Although eosinophils have not been shown to cause disease directly, there is a close association between eosinophils and tissue damage in a variety of allergic diseases. Thus, understanding the mechanisms by which eosinophils migrate into tissue should help define selective targets for the development of novel drug therapies.

Part I Eosinophil Biology

1.1 History

Eosinophils are a morphologically and pharmacologically distinct population of non-dividing granulocytes that differentiate from myeloid precursor cells in the bone marrow. Credit for their discovery is given to Paul Ehrlich, who in 1879 coined the term "eosinophil" to describe a population of white blood cells containing cytoplasmic granules with a high affinity for acid aniline dyes such as eosin (Gleich, 2000).

Since the early part of this century, the eosinophil has been associated with helminthic parasitic infection, certain malignancies and especially with allergy and asthma (Brown, 1898; Wardlaw *et al.*, 1997). The role of the eosinophil in allergic disease has been in continual debate throughout the 20th century. Thirty years ago, researchers were drawn to the idea that eosinophils had an anti-allergic effect, due to their ability to degrade mast cell-derived mediators of anaphylaxis such as histamine (Goetzl *et al.*, 1975). This hypothesis remained the dominant explanation for eosinophil function until the late 1970s, when the observation that eosinophils were able to kill the larvae of *Schistosoma mansoni* led to the current belief that the teleological role of eosinophils is in host defence against helminthic parasites (Butterworth, 1984). In recent years, investigators have highlighted the capacity of the eosinophil as an effector cell with an important source of pro-inflammatory cytokines and cytotoxic proteins that have the potential to induce the pathologic changes characteristic of asthma (Gleich, 2000).

1.1.1 Eosinopoiesis

Eosinophils, along with all immature haemopoietic progenitors, are contained within a population expressing CD34, a cell surface sialomucin-like glycophosphoprotein. When CD34+ cells are exposed to the pluripotent cytokine growth factors interleukin-3 (IL-3) and granulocyte macrophage colony-stimulating factor (GM-CSF) *in vitro*, either alone or in combination, they become progressively committed to the myeloid lineage (Saeland *et al.*, 1989; Ottmann *et al.*, 1990). Another cytokine, IL-5, is the terminal differentiation factor for eosinophils, and is active only on eosinophils and basophils in humans (Denburg, 1998). IL-5 induces expression of the IL-5 receptor α subunit (IL-5Rα) on CD34+ progenitors (Tavernier *et al.*, 2000). IL-5Rα signals commitment to eosinophilic differentiation and has been shown to be upregulated in the bone marrow of allergen-challenged mild asthmatics (Sehmi *et al.*, 1997).

The critical role of IL-5 in eosinopoiesis has been shown by genetic manipulation in mice. There is much evidence to demonstrate that over-production of IL-5 in transgenic mice results in a profound eosinophilia, with increased numbers of eosinophil precursors in the bone marrow (Dent *et al.*, 1990; Tominaga *et al.*, 1991; Sanderson, 1992). In addition, IL-5 gene-deleted mice show a marked reduction of eosinophils in the blood and lungs after allergen challenge (Foster *et al.*, 1996). Antibodies against IL-5 in animal models have prevented antigen-dependent peripheral blood and airway eosinophilia, and airway hyperresponsiveness (Garlisi *et al.*, 1999; Hamelmann *et al.*, 1999). Recently, a similar response was seen using an IL-5 antisense oligonucelotide approach to inhibit IL-5 gene expression in mice (Karras *et al.*, 2000). It has been demonstrated that IL-5 production is increased in asthmatic airways (Hamid *et al.*, 1991), and that IL-5 expression inversely correlates with pulmonary function (Robinson *et al.*, 1993b).

Interestingly, mice lacking receptors for IL-5, IL-3, and GM-CSF still produced morphologically normal peripheral blood eosinophils, although they showed an impaired defence mechanism against helminthic infection (Nishinakamura *et al.*, 1996). These findings suggest that eosinopoiesis may occur independently of IL-5, IL-3, and GM-CSF, via an undefined mechanism. The role of IL-5 in the selective accumulation of eosinophils is discussed further in section 1.4.3. Eosinophils circulate in the peripheral blood with a half-life of approximately 18-20 hours before migrating into tissues, where they predominantly reside, accumulating in sites that include the lung, gastrointestinal tract, and skin.

1.1.2 Morphology and heterogeneity

The ultrastructure of mature human eosinophil morphology (Figure 1–1) has been defined by numerous investigators and is reviewed by Dvorak and Weller (Dvorak and Weller, 2000). Giembycz and Lindsay have recently published a thorough review on the pharmacology of the eosinophil (Giembycz and Lindsay, 1999). Eosinophils possess bilobed nuclei and are approximately 8 μm in diameter. The most distinguishing morphological feature of eosinophils is their numerous cytoplasmic, membrane-bound 'secondary' granules. These granules are spherical or ovoid, are between 0.5 and 1 μm in diameter, and contain an electron dense crystalline core, which is surrounded by a less electron dense matrix (Giembycz and Lindsay, 1999). The crystalloid core of the granule is composed of major basic protein (MBP) and the non-core matrix contains three other basic

Figure 1–1 The principle internal structures of a human eosinophil

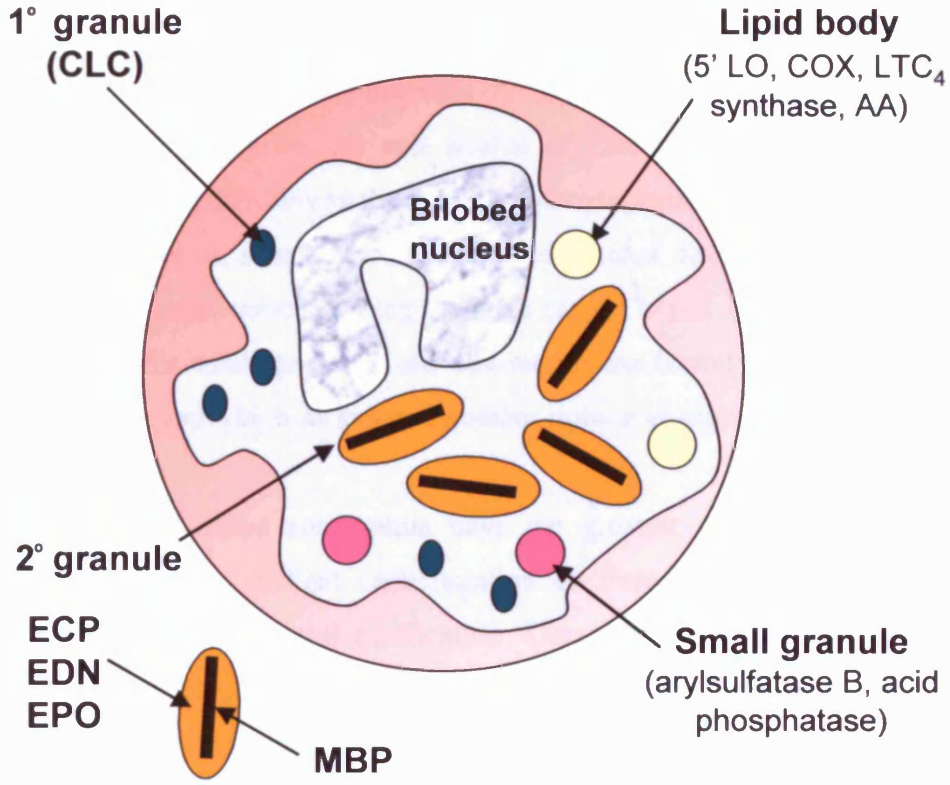


Figure 1–1 The typical bilobed nucleus is shown along with the four main granules. The primary (1°) granule is the main site of Charcot-Leyden crystals (CLC). The secondary (2°) granule consists of a crystalloid core of MBP surrounded by a matrix containing ECP, EDN, and EPO. Lipid bodies represent a site of mediator biosynthesis (COX = cyclooxygenase, 5' LO = 5-lipoxygenase, AA = arachidonic acid). Small granules store enzymes. (Structures are not shown in correct numerical proportions or to scale)

granule proteins, eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN or EPX) (Egesten *et al.*, 1986).

Eosinophils also contain primary granules of variable size, which lack a crystalloid core. In resting eosinophils, they are the sole source of Charcot-Leyden crystal (CLC) protein, which is often found diffusely in the nucleus and cytoplasm of activated eosinophils in sputum and tissues as a hallmark of eosinophil-related disease (Dvorak *et al.*, 1988). In addition, smaller, enzyme-containing granules can be found. Other cytoplasmic structures, often mistaken for small granules, are non-membrane bound lipid bodies, which contain various enzymes and which increase in number upon eosinophil activation (Weller, 1991).

Normal peripheral blood eosinophils have the greatest density of all leukocytes, and consequently, density gradient centrifugation on Percoll or metrizamide was once the standard method of eosinophil purification. Using this method, it became apparent that peripheral blood eosinophils sediment with a range of densities. A proportion of eosinophils from individuals with a raised eosinophil count was less dense than eosinophils from normal subjects (Bass *et al.*, 1980). These "hypodense" eosinophils contain fewer and smaller specific granules, which may explain their less dense phenotype. The relationship between density and the functional capacity of the eosinophil remains inconclusive despite numerous studies, although the weight of evidence suggests that hypodensity represents a primed or partially activated phenotype (Wardlaw, 1995). Density gradient centrifugation has now largely been superseded by immunomagnetic selection, based on the difference in expression of the CD16 IgG receptor between eosinophils and neutrophils (Hansel *et al.*, 1991). This technique does not distinguish between eosinophils of different densities.

1.1.3 Eosinophil receptors

Eosinophils express a large number of membrane receptors, which enable them to interact with the extracellular environment, summarised in Table 1–1. Through these receptors, they can recognise both soluble mediators and insoluble structures such as other leukocytes, vascular endothelium, and parasitic helminths. The expression of eosinophil receptors may be modulated by the synthesis of new receptors, recruitment of receptors from intracellular stores, or from changes in the activation state of the receptor.

Table 1–1 Selected receptors expressed by eosinophils

Immunoglobulin	G-protein-coupled	Haematopoietin/ IFN/ TNF family	Miscellaneous
FcαRIα.1-5	PAF R	IL-3 R	IL-1 R
FcαRIβ	BLT R	IL-5 R	IL-2 R
FcεRI	fMLP R	GM-CSF R	IL-4 R
FcεRIIα	CCR1	IFN-γ R	IL-9 R
FcεRIIβ	CCR3	IL-10 R	IL-13 R
FcγRII	CXCR1/ 2	TNF-α RI	TGF-β R
	C3a R	TNF-α RII	PDGF R
Inducible:	C5a R	CD30	c-kit
FcγRI	β2 adrenoceptor	CD40	CD4
FcγRIII	NK1 R	CD44	CD9
FcμR	Adenosine	CD69	CD52
	Histamine	CD95	CR1
		NGF R	CR3
			CR4
			SCR
			HLA

Table 1–1 Summary of the main categories of receptors expressed by eosinophils. Adapted from Giembycz and Lindsay, 1999.

Figure 1–2 Adhesion receptors expressed on the surface of eosinophils and their counterligands

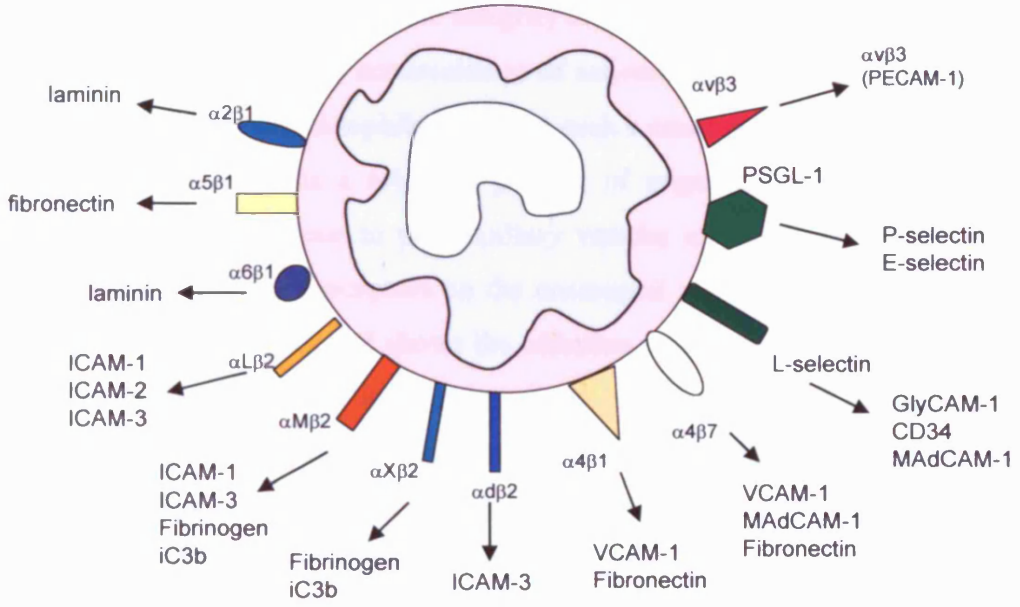


Figure 1–2 Eosinophils express many receptors on their surface, which enable them to participate in the normal immune response and which also aid their infiltration into disease sites. The corresponding endothelial- and extracellular matrix-expressed receptor ligands are also depicted. Adapted from Giembycz and Lindsay, 1999.

1.1.3.1 Adhesion receptors

Migration from the vascular system to the extracellular tissues is a critical aspect of leukocyte function and is essential to the integrity of the immune system. A striking feature of inflammation in asthma is the accumulation of activated eosinophils, without increased numbers of neutrophils. As eosinophils represent such a small proportion of the total blood leukocyte count, this suggests a selective process of migration. The initial step in the migratory pathway is adherence to postcapillary venular endothelium. This occurs as a result of interactions between receptors on the eosinophil surface and their ligands on the endothelial cell surface. Figure 1–2 shows the adhesion receptors expressed on eosinophils and their counter-ligands. There is an increasingly complex array of adhesion receptors involved in this migration pathway. They are grouped into several gene superfamilies and include the selectins, the integrins, and members of the immunoglobulin superfamily (Springer, 1990; Hynes, 1992). Integrins bind to members of the immunoglobulin family, while selectins bind to carbohydrate structures that include the moiety sialyl Lewis X (sLe^x) (Springer and Lasky, 1991). These superfamilies and their relation to eosinophil migration in particular will be discussed in section 1.3. It has become clear that one potential mechanism for preferential localisation of eosinophils at the site of tissue inflammation is a selective adhesion pathway.

1.1.3.2 Immunoglobulin receptors

The eosinophil expresses receptors for IgA (FcαR), IgD (FcδR), and IgG (FcγR). The expression of FcαR (CD89) on eosinophils shows a large donor variability, although expression has been shown to be upregulated on allergic individuals (Monteiro *et al.*, 1993). Only one IgG receptor, FcγRII (CD32), is constitutively expressed on eosinophils to any significant degree (Hartnell *et al.*, 1990). CD32 recognises multivalent IgG and mediates eosinophil functions such as phagocytosis, granule protein secretion, schistosomula killing, and the generation of membrane-derived lipid mediators such as platelet-activating factor (PAF) (Hartnell *et al.*, 1990). Eosinophils will express FcγRIII (CD16) and FcγRI (CD64), as well as CD32 after 2 days' stimulation with IFN-γ *in vitro* (Hartnell *et al.*, 1992). CD16 is not expressed on eosinophils *in vivo*, which provides the basis for the negative immunomagnetic selection process used to purify eosinophils from peripheral blood. The eosinophil also binds IgE and can undertake IgE-dependent functions, such as the killing of schistosomes opsonised with specific IgE (Capron *et al.*, 1981). However, the exact nature of eosinophil IgE binding has yet to be clarified. Resting eosinophils have been shown to bind IgE via the low affinity FcεRII (CD23). Engagement

of FcεRI, a receptor very weakly expressed by eosinophils, does not appear to result in significant mediator release (Kita *et al.*, 1999).

1.1.3.3 Additional receptors

Eosinophils express the complement receptors CR1 and CR3 (Mac-1, CD11b), which is a member of the β2 integrin family. Mac-1 recognises a number of ligands such as C3bi, intercellular adhesion molecule-1 (ICAM-1), fibrinogen, and polysaccharides (Diamond *et al.*, 1990). It is involved in IgE- and IgG-dependent schistosomula killing and adhesion to endothelial cells, amongst other important eosinophil functions. When eosinophils are cultured with cytokines for a prolonged period (> 48 h) they have the ability to express receptors *de novo*. For example, eosinophils express HLA-DR antigens and increased amounts of ICAM-1 after culture in GM-CSF (Weller *et al.*, 1993), both of which are associated with an *in vitro* capacity to present antigen to T cells. CD69, an early activation marker for lymphocytes, can be induced on human peripheral blood eosinophils by GM-CSF stimulation (Hartnell *et al.*, 1993), and has been found on lung eosinophils obtained from patients with eosinophilic pneumonia (Nishikawa *et al.*, 1992).

The C-C chemokines eotaxin, RANTES (regulated on activation, normal T cell expressed and secreted), monocyte chemotactic protein-3 (MCP-3), and MCP-4 are extremely important in recruiting eosinophils to sites of allergic inflammation (refer to section 1.3.2.1.1). The G-protein-coupled receptor (GPCR) for RANTES, eotaxin and MCP-3 has been termed CCR3 and appears to be expressed mainly on eosinophils (Daugherty *et al.*, 1996), also a small subset of Th2-cells (Sallusto *et al.*, 1997), and basophils. Eosinophils also possess a number of specific receptors for soluble inflammatory mediators such as LTB_4, PAF, C3a, and C5a, which belong to the family of seven transmembrane (7TM) region GPCRs (Giembycz and Lindsay, 1999). Eosinophils respond to soluble mediators such as the cytokines GM-CSF, IL-3, and IL-5 via high-affinity cytokine receptors (Tavernier *et al.*, 2000).

1.1.4 Eosinophil mediators

Eosinophils are capable of secreting numerous potent inflammatory mediators, as shown in Table 1–2. These include eosinophil granule proteins, newly formed membrane-derived lipids, cytokines, various proteases, and components of oxidative metabolism.

Table 1–2 Eosinophil-derived mediators

Granule proteins and enzymes	Lipid mediators and oxygen metabolites	Cytokines and chemokines	Growth factors
Non-enzymatic granule proteins	*Lipid mediators*	IL-1α	TNF-α
MBP	PAF	IL-2	TNF-β
	LTB_4	IL-3	EGF
Enzymatic granule proteins	LTC_4	IL-4	PDGF
ECP	TXA_2	IL-5	NGF
EDN	PGE_2	IL-6	VEGF
EPO	5-HETE	IL-8	endothelin
	15-HETE	IL-10	
Other enzymes	5,15-diHETE	IL-11	
collagenase	8,15-diHETE	IL-12	
arylsulphatase B	14,15-diHETE	IL-16	
β-glucuronidase	LXA_4	IFN-γ	
β-hexosaminidase	*Oxygen metabolites*	TNF-α	
catalase	singlet oxygen	GM-CSF	
esterase	superoxide anion	MIP-1α	
histaminase	hydrogen peroxide	RANTES	
acid phosphatase		MIF	

Table 1–2 Eosinophils are a rich source of different varieties of mediators, metabolites, cytokines, and growth factors, as illustrated above. Adapted from Giembycz and Lindsay, 1999.

1.1.4.1 Granule proteins

MBP makes up half of the granule protein, has a molecular weight (mw) of 13.8 kD, and is found in the granule core. It contains 17 arginine residues, which accounts for its basicity (Wardlaw, 1994). MBP is cytotoxic for the schistosomulae of *S. mansoni* (Butterworth, 1984) and for guinea pig and human respiratory epithelial cells (Gleich, 2000). In antigen-challenged guinea pigs, MBP is an antagonist for neuronal M_2 muscarinic receptor function, resulting in airway hyperresponsiveness (Evans *et al.*, 1997). The mechanism of action of MBP is likely to be related to its strong negative charge and hydrophobicity.

ECP is also an arginine rich protein, which displays heterogeneity in its mw, with bands ranging between 16 and 22 kDa. A recent review by Venge *et al.* summarises its molecular and biological properties (Venge *et al.*, 1999). ECP shows 67% amino acid homology with EDN and 31% homology with human pancreatic ribonuclease (Rosenberg *et al.*, 1989). ECP is only expressed in eosinophils or eosinophilic cell lines and is a potent cytotoxic molecule with the capacity to kill mammalian as well as non-mammalian cells, for example, parasites, bacteria, and viruses (Venge *et al.*, 1999). The cytotoxic effect of ECP is thought to be due to its capacity to form channels in cell membranes (Young *et al.*, 1986). Such pores allow the passage of small molecules into the cell, killing it by osmotic lysis. The secreted form of ECP differs structurally and antigenically from the stored form. Thus monoclonal antibodies (mAb) can distinguish between a form of ECP found in the granules of resting eosinophils (mAb EG1) and a secreted form that recognises the activated state (mAb EG2) (Tai *et al.*, 1984). Some studies have disputed the reliability of these antibodies, in one, cellular EG2 reactivity depended on the method of sample preparation (Jahnsen *et al.*, 1994), and in another, EG2 was not specific for eosinophils (Sur *et al.*, 1998). Therefore, caution should be taken when interpreting data using these Abs.

EDN, also known as EPX, is a 15.5 kDa, glycosylated protein possessing much more ribonuclease activity than ECP. EDN expression is not restricted to eosinophils, is not toxic to parasites or mammalian cells, and its only known function other than its ribonuclease activity is the Gordon phenomenon, a neurotoxic effect produced when injected into the cerebrospinal fluid of experimental animals. ECP also exhibits this phenomenon, but to a greater extent (Wardlaw, 1994).

EPO is a haem-containing protein comprising a light (14 kDa) and a heavy (58 kDa) subunit. EPO is toxic for parasites and respiratory epithelium, either alone or when combined with H_2O_2 and halide ions, particularly bromide (Wu *et al.*, 2000). These granule proteins can be found at raised levels in asthmatic sputum and are elevated after experimental allergen challenge in bronchoalveolar lavage (BAL) fluid (Wardlaw *et al.*, 1988). Increased amounts of these proteins have also been found in nasal lavage fluid following allergen challenge (Andersson *et al.*, 1989; Bascom *et al.*, 1989).

1.1.4.2 Lipid mediators

The principle lipid mediators generated by eosinophils are eicosanoids and PAF. Eicosanoids are oxidation products of arachidonic acid, an essential fatty acid found in the membrane of all cells. There are two main pathways for arachidonic acid metabolism in eosinophils, via cyclooxygenase resulting in prostaglandin production, and via 5- and 15-lipoxygenase, which leads to leukotriene generation.

Eosinophils have been shown to generate large amounts of leukotriene C_4 (LTC_4) after stimulation with calcium ionophore (Weller *et al.*, 1983). LTC_4 has also been generated from eosinophils following stimulation by IgG-coated Sepharose beads and opsonised zymosan. The three sulphidopeptide leukotrienes LTC_4, LTD_4 and LTE_4 are able to produce smooth muscle contraction, mucus hypersecretion, and increased vascular permeability, properties all relevant to asthma. On the other hand, LTB_4 stimulates the migration and aggregation of leukocytes, the formation of superoxide anions, and degranulation, and is the predominant product of the 5-lipoxygenase pathway in neutrophils, which produce very little, if any, LTC_4 (Lee and Austen, 1986).

The chemical formula for PAF is 1-o-alkyl-2-acetyl-1-sn-3-phosphorylcholine, and PAF can be potentially synthesised by two pathways. The first, and main, pathway is by acetylation of the inactive precursor lyso-PAF by acetyltransferase; the second is by the action of the enzyme cholinephosphotransferase on ether-linked phospholipids (Kuitert and Barnes, 1995). Numerous inflammatory cells can synthesise PAF, including neutrophils, eosinophils, platelets, vascular endothelial cells, and alveolar macrophages. Although named because of its biological effects on platelets, PAF has a variety of actions on many cells, which are reviewed by Chung (Chung, 1992).

PAF is a chemotactic factor, and having attracted inflammatory cells, causes their activation. PAF is potently chemotactic for eosinophils and is comparable in both potency and efficacy to chemokines. Eosinophils are one of the richest cellular sources of PAF and hypodense eosinophils of asthmatic patients have been shown to release large amounts of PAF *in vitro* (Lee *et al.*, 1984). Eosinophils also generate large amounts of PAF after stimulation with calcium ionophore, zymosan and IgG-coated Sepharose beads (Lee *et al.*, 1984; Cromwell *et al.*, 1990). PAF possesses many biological properties that are relevant to asthma. It indirectly causes bronchoconstriction and is a potent cause of airway microvascular leakage in guinea pigs (O'Donnell and Barnett, 1987). PAF causes a sustained increase in bronchial hyperreactivity in several animal species, including humans (Cuss *et al.*, 1986). However, the role of PAF in the pathogenesis of asthma is questionable. There has been a limited and/ or negative efficacy of PAF antagonists as shown in randomised controlled trials in patients with mild to moderate asthma (Spence *et al.*, 1994; Kuitert *et al.*, 1995). However, a potent PAF receptor antagonist Y-21480 was shown to prevent bronchial hyperresponsiveness in patients with asthma (Hozawa *et al.*, 1995), and inhibit eosinophil activation in BAL from atopic asthmatics (Mizuki *et al.*, 1999).

1.1.4.3 Cytokines

Eosinophils are terminally differentiated end stage leukocytes; therefore, it was once considered that their capacity to transcribe and translate new proteins was very limited. In more recent years, studies have suggested that eosinophils can synthesise and secrete an array of several important inflammatory and regulatory cytokines. Cytokines play an integral role in the initiation, co-ordination, and persistence of the inflammatory process in chronic inflammation of the airways in asthma. Reviews of recent literature (Moqbel *et al.*, 1994b; Wardlaw *et al.*, 1995) suggest that eosinophils can transcribe and/ or translate cytokines and growth factors including IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, TNF-α, TGF-α, TGF-β, MIP-1α and GM-CSF. Many cytokines appear to be stored in specific granules (Moqbel *et al.*, 1994a). The cytokines generated are purported to play a significant role in the development of the Th2 response characteristic of allergic inflammation and helminth infection. However, as most of the studies on cytokines are *in vitro*, uncertainties remain regarding the stimuli *in vivo* that could lead to cytokine generation, and the physiological relevance of the generation of some cytokines (Kita, 1996). Eosinophils can also synthesise the Th1-type cytokines IL-2 and IFN-γ (Woerly *et al.*, 1999), hence could potentially play a role in the polarisation of the immune response.

1.1.4.4 Other mediators

Eosinophils contain a variety of granule-stored enzymes whose role in eosinophil function remains unclear, including acid phosphatase, collagenase, arylsulphatase B, histaminase, catalase and non-specific esterases (Giembycz and Lindsay, 1999). Eosinophils are capable of undergoing a respiratory burst in response to stimulation with opsonised zymosan and phorbol myristate acetate (PMA), resulting in the release of H_2O_2 and superoxide ions, which have the potential to cause tissue damage.

1.1.5 Degranulation and mediator release

The release of proteins can be constitutive, where newly synthesised proteins are continuously produced, e.g. cytokines. Or, following the receptor-mediated stimulation of eosinophils, a regulated release of intracellular components occurs via one of two mechanisms, so-called "piecemeal" degranulation, and compound exocytosis. In piecemeal degranulation, which is also seen in mast cells and basophils, vesicles containing granule proteins bud off from the secondary granules and are transported to the plasma membrane (Dvorak *et al.*, 1991). The granule structures remain, but appear electron-translucent due to depletion of their contents. On the other hand, compound exocytosis involves fusion of the granules themselves with the plasma membrane, whereupon exocytosis of the contents occurs, resulting in eosinophils with a hypogranular or agranular appearance. Such exocytosis is observed during granule deposition following adhesion to the opsonised surfaces of parasitic helminths (McLaren *et al.*, 1977). Necrotic eosinophils undergo cytolysis, which is not strictly a secretory mechanism, but which also results in the release of toxic mediators. This phenomenon is in contrast to apoptosis (see section 1.4.2), where eosinophils are cleared without mediator release. Cytolytic degranulation seems to be present in allergic inflammation of the upper airways (Erjefalt *et al.*, 1999).

1.1.6 Eosinophil signal transduction

The interaction of agonists with cell surface receptors on leukocytes leads to the initiation of a biochemical cascade that can result in a range of cellular responses depending on the cell type and nature of the stimulus. In healthy individuals, circulating leukocytes are in a resting state and unresponsive to stimuli. In contrast, leukocytes at inflammatory sites are fully activated effector cells. An intermediate state exists, where leukocytes pre-exposed to sub-activating doses of certain agonists, results in a "primed" or enhanced functional response to a second activator.

Figure 1–3 Signal transduction pathways in eosinophils

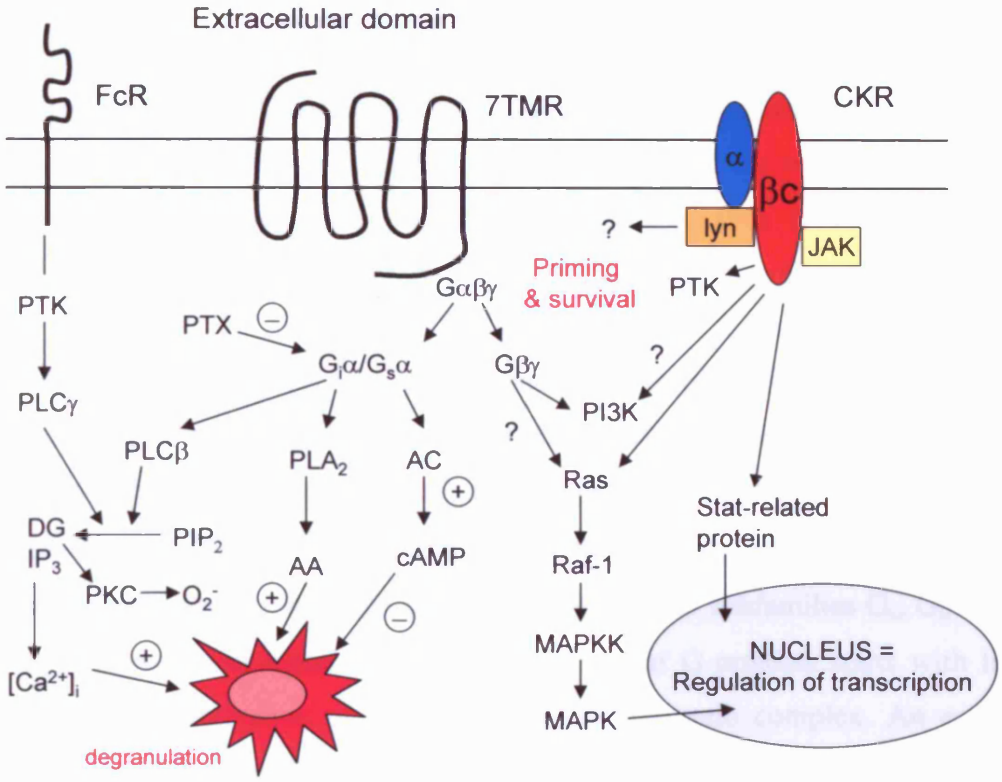


Figure 1–3 The main signal transduction pathways in eosinophils. Abbreviations: AA, arachidonic acid; AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; CKR, cytokine receptor; DG, diacylglycerol; IP_3, inositol 1,4,5-triphosphate; FcR, Fc receptor; JAK, janus kinase; MAPK, mitogen-activated protein kinase; PIP_2, phosphatidyl inositol diphosphate; PKC, protein kinase C; PLC, phospholipase C; PTK, protein tyrosine kinase; PTX, pertussis toxin; 7TMR, seven transmembrane receptor. Adapted from van der Bruggen and Koenderman, 1996.

1.1.6.1 Serpentine receptor signalling

Eosinophils chemotactically respond to a broad variety of agonists such as PAF, fMLP, C5a, CC chemokines such as eotaxin, RANTES, macrophage inhibitory protein-1α (MIP-1α, and MCP-3, and the CXC chemokine IL-8. Some of these mediators have other effects on eosinophils such as priming, degranulation, and activation of the respiratory burst. The receptors for the mediators listed contain 7TM domains, thus they are known as 'serpentine' receptors. In general, serpentine receptors are coupled to G-proteins.

Many membrane receptors involved in cell activation are coupled within the membrane to proteins that bind guanosine diphosphate (GDP) and guanosine triphosphate (GTP), which are collectively known as G-proteins. The G-proteins consist of a large gene family coding for at least sixteen α, four β, and multiple γ subunits (Murphy, 1994). Several different subunits for Gα have been cloned that can be divided into the subfamilies G_s, G_i, G_q, and G_{12}. The best-characterised signal transduction pathway of G-proteins starts with ligand binding, followed by activation of the heterotrimeric G-protein complex. An exchange occurs in the α subunit of the G-protein from a GDP- to a GTP-bound state, resulting in a dissociation of the α subunit from the βγ subunits (Figure 1–3). Liberated Gα and Gβγ subunits can activate many effector molecules, including phospholipase C (PLC) isoforms, PLA_2, adenylate cyclase, guanylate cyclase and several ion channels (Clapham and Neer, 1993; Neer, 1995). Activation of PLC results in hydrolysis of the membrane to generate two second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DG).

IP_3 mobilises Ca^{2+} from intracellular stores leading to a transient rise in $[Ca^{2+}]_i$, whereas DG stimulates protein kinase C (PKC), which catalyses the phosphorylation of serine and threonine residues in many proteins. In a second pathway, activation of adenylate cyclase leads to the formation of cellular adenosine monoposphate (cAMP) and activation of PKA. The specificity of signalling is partially dictated by the combination of the different subunits coupling to a specific 7TM receptor. There is a redundancy in signalling, which may be explained by the fact that different 7TM receptors can couple to a common pool of G-proteins (Murphy, 1994). Human eosinophils express both G_i and G_s class G-proteins. G_i proteins inhibit adenylate cyclase activation and are inhibited by PTX, which catalyses the ADP ribosylation of the $G_i\alpha$-subunits, resulting in their uncoupling from cell surface receptors. G_s protein is the stimulatory regulator of adenylate cyclase and is inhibited by cholera toxin (Sedgwick, 1995).

1.1.6.2 Fc receptor-mediated signalling

The best-characterised triggers for the generation and release of eosinophil mediators are engagement of FcαR and FcγRII receptors, especially when they are presented to the cell bound to a non-phagocytosable adhesive surface such as Sepharose beads (Kita *et al.*, 1994). Cross-linking FcγRII on many cells elicits a variety of intracellular signals, such as a rise in $[Ca^{2+}]_i$ and tyrosine phosphorylation of multiple proteins, which lead to degranulation (Figure 1–3). FcγR- and FcαR-induced degranulation of human eosinophils is inhibited by PTX and the tyrosine kinase inhibitor genistein, suggesting the involvement of tyrosine kinase and G_i class G-proteins (Kita *et al.*, 1994).

1.1.6.3 Cytokine-induced signal transduction

Eosinophil functions are regulated by a multitude of cytokines, including IL-3, IL-4, IL-5, IL-9, and IL-13. Human eosinophils express high affinity receptors for IL-3, IL-5, and GM-CSF (Tavernier *et al.*, 2000). These are heterodimers composed of a 60 kDa cytokine-specific α chain and a 120 kDa common β chain. The α chains bind with low affinity but form a high affinity receptor together with the β chain. The β chain is essential for signal transduction, although unlike the α subunits, it does not possess intrinsic kinase activity. Thus, there are overlapping biological activities of IL-3, IL-5, and GM-CSF. Ligation of receptors for these cytokines induces tyrosine phosphorylation of several substrates, including proteins that belong to the ras and the Jak/ Stat pathways (Figure 1–3). These pathways are complex and have been reviewed elsewhere (Satoh *et al.*, 1992; Darnell, Jr. *et al.*, 1994; Adachi and Alam, 1998). The Jak/ Stat pathway forms an important nuclear signalling route. Jak kinases are essential for activation of Stat proteins, which are in a latent state in the cytoplasm. Upon phosphorylation, Stat proteins form homo- or heterodimers and translocate to the nucleus, whereupon they bind to defined DNA sequences to regulate transcription (Darnell, Jr. *et al.*, 1994).

1.1.6.4 Integrin-mediated signalling

Integrin-mediated signal transduction is discussed in section 1.3.3.1.2.

To summarise, the eosinophil has considerable cytotoxic potential, and expresses many receptors, which allow it to interact with its surrounding environment. The next section reviews the role that eosinophils have to play in disease.

Part II The role of the eosinophil in disease

1.2 Introduction

As effector cells, it is clear that eosinophils play roles that are both beneficial and detrimental to their hosts. Increased numbers of eosinophils in the circulation and in the tissue are characteristic features of several pathologies, including allergic diseases (e.g., asthma, rhinitis, nasal polyposis and atopic dermatitis), other inflammatory disorders (e.g., inflammatory bowel disease, eosinophilic gastroenteritis and pneumonia) and some malignancies (e.g., Hodgkin's disease). In these conditions, it is thought that the inappropriate accumulation and activation of eosinophils can result in direct damage to healthy tissues by a variety of mechanisms, including the release of the pre-formed toxic mediators and oxygen radicals described earlier. The studies in this thesis concentrate on the role that the eosinophil plays in allergic disease.

1.2.1 Eosinophilia

The peripheral blood eosinophil count is a representation of the balance between the rate of eosinophil migration from the bone marrow and entry into the tissues. A normal eosinophil count is generally defined as 0.4×10^9/ l or less. This may vary according to the time of day, exercise, and environmental stimuli (e.g. allergen exposure). An eosinophilia is defined as an abnormal accumulation of eosinophils in blood or tissue. The commonest cause of an eosinophilia worldwide is helminthic infections, with the most common cause in industrialised nations being atopic disease (Rothenberg, 1998). Apart from allergic disease and helminthic parasites, a raised eosinophil count is unusual and may often be the result of drug reactions (Rothenberg, 1998). Hypereosinophilic syndrome is a condition in which there is a high eosinophil count of unknown aetiology.

1.2.2 The role of the eosinophil in host defence

Eosinophils do not play a major role in host defence against microbial pathogens *in vivo*, although they are capable of phagocytosis. For instance, they cannot effectively defend against bacterial infections when neutrophil function is deficient, or absent, such as in the leukocyte adhesion deficiency syndrome (LAD). Rather, eosinophils are believed to have a protective role against larger, non-phagocytosable organisms such as helminthic parasites, which they are particularly effective at killing in the larval stages (Butterworth, 1984). The initial binding of eosinophils to parasitic targets can be mediated by anti-parasitic IgG or IgE Abs, or by C3b on the surface of parasites. Eosinophil-derived factors such as MBP, ECP, and leukotrienes, released by eosinophils after binding, are potent helminthotoxins

and have been shown to kill newborn *Trichinella spiralis* larvae (Lee, 1991) and *Nippostrongylus brasiliensis* (Coffman *et al.*, 1989) *in vitro*. Several groups have investigated the association of IL-5 and eosinophilia in host defence against parasites. Mice deficient in IL-5 are incapable of mounting an eosinophilic response (Coffman *et al.*, 1989). IL-5-deficient mice were also found to be susceptible to infection with *Angiostrongylus cantonensis*, in terms of cranial worm burden and length (Yoshida *et al.*, 1996).

However, doubt has been cast on the host defence role of eosinophils in helminthic disease. Depletion of IL-5 with antibody, hence ablation of the eosinophil response, failed to have any effect on infection with *T. spiralis* or *S. mansoni* (Sher *et al.*, 1990; Herndon and Kayes, 1992). In one study, IL-5-deficient and wild-type mice expelled a primary *T. spiralis* infection with equal efficacy, indicating that immunity to this nematode was both eosinophil- and IL-5-dependent. However, upon challenge infection, the IL-5-deficient mice suffered a higher gastrointestinal worm burden and impaired expulsion rate than the wild-type strain (Vallance *et al.*, 1999). Hence, the importance of IL-5 and eosinophils in enteric host defence may be in subsequent, rather than initial, infections. Overall, eosinophil-mediated protection against parasites seems to be very model-dependent, varying with the parasite species, maturation stage, and whether or not there has been prior exposure to the parasite.

1.2.3 The role of the eosinophil in allergic disease

Eosinophils may be major effector cells in allergic diseases. Eosinophil infiltration and/ or deposition of eosinophil granule proteins are implicated in the pathology of allergic rhinitis, eosinophilic gastroenteritis, coeliac disease, diseases of the eye, and atopic dermatitis (Martin *et al.*, 1996). Atopic dermatitis (AD) is a common, chronic skin disease that is often accompanied by peripheral blood eosinophilia and increased serum IgE levels. Despite a paucity of intact eosinophils in the skin lesions, immunohistochemical studies show extensive extracellular MBP staining, indicative of eosinophil infiltration and degranulation. A correlation has been shown between levels of eosinophil granule proteins in the peripheral blood of AD patients and severity of disease (Kapp, 1993). AD and asthma have many similarities. Both diseases show intrinsic and extrinsic phenotypes (see below), both deviate towards a Th2-like response, and both are characterised by altered physiologic responses (Martin *et al.*, 1996).

1.2.3.1 Asthma: definition, and clinical categories

Asthma is a chronic, multi-factorial disease affecting approximately 10% of the population in developed countries. The prevalence of asthma is rising; levels of hay fever and allergic asthma in the UK have increased significantly over the last two decades, more than doubling amongst adults (Upton *et al.*, 2000). There have been several definitions of asthma since the one given in the first Ciba Symposium in 1959, but it is accepted that asthma is characterised by three main features:

- reversible airway obstruction that is variable in severity and pattern
- airway hyperresponsiveness (AHR), which is an exaggerated airway narrowing in response to many different non-specific stimuli, such as allergen, exercise, and cold air
- infiltration of the bronchial mucosa by inflammatory cells, of which eosinophils are a major component

Asthma is usually associated with atopy, which is the genetic predisposition of about 30% of the population to synthesise IgE for 'external' antigens, particularly inhaled aeroallergens, such as grass pollen (Wardlaw, 1993a). Allergen-specific IgE sensitises mast cells with high affinity IgE receptors to cause an energy-dependent release of both pre-formed, granule-derived and newly-formed, membrane-derived pharmacological substances upon further allergen exposure. Some atopic asthmatics experience an exacerbation of disease on exposure to allergens to which they have been sensitised, and are referred to as "extrinsic" asthmatics. Non-atopic asthmatics, where there is no relationship of disease with IgE-mediated mechanisms and external environmental factors, have been labelled "intrinsic" asthmatics. "Occupational" asthmatics suffer asthma from triggers encountered in the workplace, which may be non-specific irritants that exacerbate existing asthma, or specific agents that may cause asthma. Occupational asthma may persist long after the person has ceased being exposed to the trigger (Wardlaw, 1993a). However, there appear to be no distinguishing histopathological features when biopsy samples within these three categories are compared (Bentley *et al.*, 1992a; Bentley *et al.*, 1992b).

Allergen provocation of allergic asthmatics divides the asthmatic response into two bronchorestrictive responses, illustrated in Figure 1–4. The early-phase reaction (EPR) sees maximal airway narrowing occurring within 15-30 minutes and a return to baseline within 1-2 hours. More than half of the subjects who experience an EPR also develop a

second late-phase reaction (LPR) that commences after 3-5 hours, is maximal at 6-12 hours, and may persist for up to 24 hours. The EPR appears to depend largely on the release of pro-inflammatory mediators from airway mast cells and basophils, leading to airway obstruction and bronchospasm (White, 1999). The development of the LPR and the accompanying AHR are associated with an infiltration of inflammatory cells, predominantly eosinophils and CD4+ Th2 cells, into the bronchial mucosa (White, 1999).

1.2.3.2 The Th2 response in inflammation

T cells play a key role in orchestrating the nature and magnitude of inflammatory responses through the secretion of a specific profile of cytokines. Previous studies characterised the population of lymphocytes in the airways of human asthmatics as activated CD4+ T cells (Wilson *et al.*, 1992; Robinson *et al.*, 1993a). Evidence supporting a pathogenic role for the T cell includes animal studies showing that depletion of CD4+ T cells inhibits antigen-induced airway inflammation (Nakajima *et al.*, 1992). Antigen-activated CD4+ T cells undergo differentiation to either T-helper 1 (Th1) or T-helper 2 (Th2) cells depending on the presence of specific mediators from other leukocytes. These subsets differ functionally. Th1 cells secrete primarily IL-12 and IFN-γ, and are vital regulators of cell-mediated immunity to infection. In contrast, Th2 cells secrete cytokines including IL-4, IL-5, IL-9, IL-13, and GM-CSF (Mosmann *et al.*, 1986), and are instrumental in the development of allergic inflammation. The pattern of cytokine production in atopic asthma has been shown to be of the Th2-type (Robinson *et al.*, 1993a). Recently, a causal relationship was established when Th2, but not Th1 cells, were responsible for airway mucus secretion in mice (Cohn *et al.*, 1997). Studies have suggested that immunotherapies for asthma should direct the immune response towards a Th1-type, to counterbalance the effects of Th2 cells. However, in a recent murine model of allergic asthma, Th1 cells did not attenuate Th2-cell induced AHR and inflammation, but rather caused severe airway inflammation (Hansen *et al.*, 1999), raising concerns about the protective effect of Th1 cells regarding therapies for asthma. It has been suggested that the short time between the EPR and LPR is insufficient for Th2 cytokine synthesis, and that the initial recruitment of inflammatory cells may instead be in response to mast cell mediator release during the EPR (Bradding *et al.*, 1994). Subsequent Th2 cytokine production would then maintain the responses associated with chronic inflammation (Figure 1–4).

Figure 1–4 The cellular basis of the early- and late-phase reactions in asthma

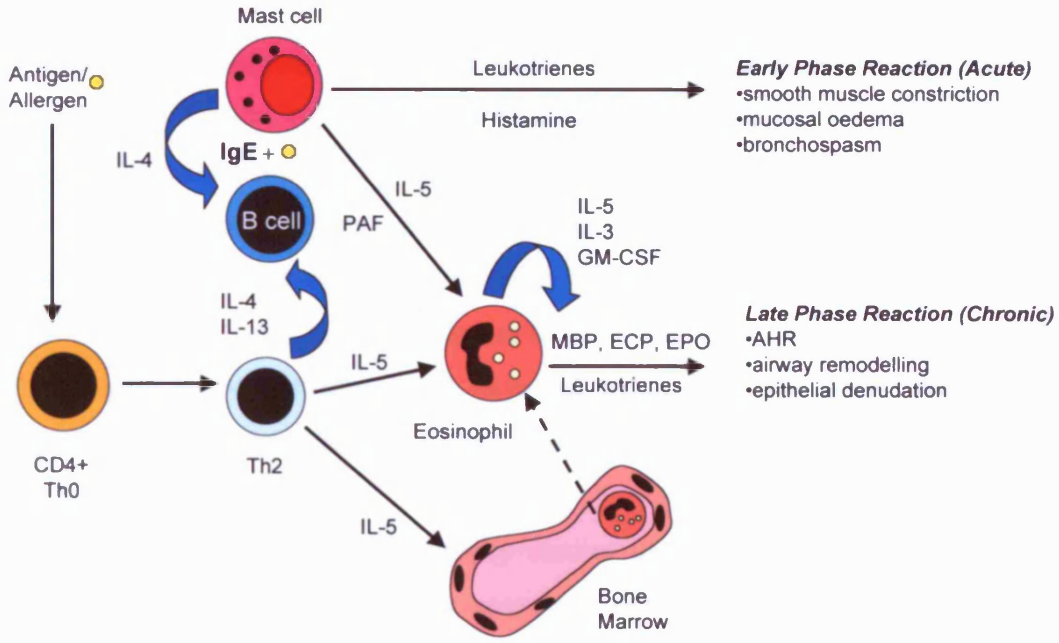


Figure 1–4 The EPR and LPR in bronchial asthma are multi-factorial processes. Briefly, soluble mediators and allergens direct naïve CD4+ T cells to differentiate into a Th2 phenotype, eventually resulting in production of specific IgE by B cells. Re-exposure to allergen elicits an acute reaction (EPR) that is triggered through cross-linking of high-affinity IgE receptors on mast cells (and basophils). This is followed after several hours by a chronic reaction involving inflammatory mediator production by Th2 cells and eosinophils mobilised from the bone marrow.

1.2.3.3 Pathology of asthma

Most of our present knowledge of the basic pathological changes characteristic of asthma accrues from the pathology of asthma deaths; however, it is unclear as to what extent these findings reflect the varied clinical picture of asthma in life. These studies were often poorly controlled, and involved small numbers of patients whose clinical features were atypical of the majority of asthmatics, being at the severe end of the disease spectrum (Wardlaw, 1993b). The advent of fibre-optic bronchoscopy has meant that samples can be obtained from the airways of mild to moderate asthmatics, allowing a number of carefully controlled studies to be performed that have led to an increased understanding of the pathological changes in the asthmatic airway.

The early published data based on autopsy studies identifies the features particular to asthma (Dunnill, 1960) which in many cases can now be attributed to the underlying inflammatory processes. There is an extensive infiltration of the airway wall by inflammatory cells, particularly by eosinophils and lymphocytes. A hypertrophy and hyperplasia of the submucosal glands contributes to excessive mucus production, leading to occlusion of the bronchial lumen. There is an extensive loss of the respiratory epithelium from the surface of the bronchial wall. The best-recognised factor leading to this disruption is epithelial damage due to eosinophil-derived mediators, such as MBP. Other characteristic pathological features are a thickening of the epithelial basement membrane as a result of excess collagen deposition, an increase in thickness of the smooth muscle in the bronchial wall, dilatation of capillaries and postcapillary venules leading to increased vascular permeability, and oedema of the airway wall leading to increased microvascular leakage (Dunnill, 1960).

1.2.3.4 Eosinophils in asthma

Eosinophils may be involved in effecting tissue damage and lung dysfunction in asthma. The importance of the eosinophil in lung damage is supported by considerable clinical and experimental observations. It is well established that a blood and sputum eosinophilia is often observed in association with asthma. Studies of asthmatic patients suggest that eosinophil numbers correlate with the degree of bronchial hyperresponsiveness (Azzawi *et al.*, 1990). Post-mortem immunostaining of the bronchial mucosa of severe asthmatics revealed the presence of large numbers of activated eosinophils (Azzawi *et al.*, 1992) and a high quantity of MBP deposited in the airways (Filley *et al.*, 1982). A study by Bousquet and co-workers showed that the numbers of eosinophils in peripheral blood, BAL fluid,

and bronchial biopsies in a group of asthmatics were elevated compared to normal controls (Bousquet *et al.*, 1990). In addition, an increasing degree of eosinophilia with increasing disease severity was present.

Wardlaw *et al.* demonstrated an increase in concentrations of the eosinophil toxic mediator MBP in BAL fluid from atopic asthmatics compared to normal controls. In this study, correlations were observed between the concentrations of MBP, the numbers of desquamated epithelial cells in the BAL, and the degree of bronchial hyperresponsiveness (Wardlaw *et al.*, 1988). In an animal model of asthma, neutralisation of MBP led to an inhibition of allergen-induced airways hyperreactivity (Lefort *et al.*, 1996). Eosinophil granule proteins have also been shown to potently increase vascular permeability at concentrations in the nanomolar range (Minnicozzi *et al.*, 1994), these concentrations are exceeded by those estimated in tissues (Filley *et al.*, 1982). When eosinophils are prevented from accumulating in asthma with steroids, an improvement is seen in the clinical course of the disease (Diaz *et al.*, 1984).

However, despite these close associations, the evidence of a cause-and-effect relationship between eosinophil products and tissue damage remains circumstantial. Asthma is not universally associated with eosinophilia. Crucially, a recent clinical trial study by Leckie and colleagues examined the effect of a humanised IL-5 antibody on blood and sputum eosinophils, AHR, and the LPR to inhaled allergen in patients with mild asthma (Leckie *et al.*, 2000). However, while anti-IL-5 lowered the mean blood eosinophil and sputum count, there was no significant effect of the antibody on the LPR or on the AHR to histamine. Definitive data for the eosinophil as a causative factor in asthma remains to be discovered.

1.2.3.5 Nasal polyposis

The nose provides the first line of defence against inhaled allergens and other foreign substances. The nasal mucus layer forms a barrier between foreign particles and the mucosal surface, epithelial ciliary activity aids the elimination of trapped particles, antimicrobial substances are secreted from nasal glands to kill susceptible pathogens, and phagocytic cells are present to ingest foreign matter. In addition, immunologic responses such as cell-mediated cytotoxicity and antibody production are involved in nasal defence mechanisms. The nasal mucosa is involved in several inflammatory disorders and as such, is a good model of mucosal immunity and has the advantage of being easily accessible for study.

Nasal polyps are "fluid-filled sacs" of hyperplastic, swollen mucosa that originate from the upper airways and cause nasal obstruction. The aetiology of nasal polyps has not been fully elucidated and no definite predisposing disease seems to be implicated in their formation. However, their pathogenesis appears to be similar in several ways to that of allergic and non-allergic rhinitis and asthma (Kakoi and Hiraide, 1987). Nasal polyps often develop with a history of chronic rhinitis and there is a high incidence of asthma in patients with polyps (Moloney and Collins, 1977). Allergy was considered an important component of the pathogenesis of nasal polyposis for many years, however this has recently been disputed (Slavin, 1992).

A central feature of nasal polyposis is an inflammatory infiltrate where eosinophils represent by far the most prevalent cell type. Lymphocytes are also present in large numbers (Kakoi and Hiraide, 1987). Eosinophils in nasal polyps have been shown to express cytokines that are thought to prolong their survival in tissue (Ohno *et al.*, 1991). Nasal polyps are also rich in blood vessels. They are large pieces of tissue, which are easily obtainable from patients undergoing routine surgery for nasal obstruction. In summary, nasal polyps represent a paradigm of chronic inflammation of the upper airways with a substantial number of features similar to those described in asthma, and provide easy access to abundant human tissue for research into this disease.

In summary, eosinophils may be major effector cells in allergic inflammatory diseases such as asthma, playing both protective and detrimental roles. The final part of the introduction details the mechanisms via which eosinophils accumulate in tissue.

Part III Leukocyte Trafficking

1.3 The multi-step paradigm of leukocyte adhesion to endothelium

Eosinophils are primarily tissue dwelling cells that remain intact in normal tissue but often undergo massive degranulation in disease states. In allergic inflammation, eosinophils are seen to accumulate in places where they are not normally present, such as in the airways and skin. In order to understand the role of the eosinophil in disease, we therefore need to question the mechanisms by which eosinophils migrate into tissues, and once there, how they become activated to release their mediators. A key feature of allergic inflammation is an eosinophil accumulation without an increase in neutrophil numbers; selectivity in migration is likely to be involved. Understanding of the rapid binding events between leukocytes and the endothelium has given rise to a paradigm that views the interactions as a multi-stage process (Butcher, 1991), as depicted in Figure 1–5 for eosinophils. Each step occurs in series, so that each is essential for transmigration to occur. This paradigm allows selectivity to be introduced at any one of the steps, resulting in diversity in the pattern of signals at a particular inflammatory site. Theoretically, migration can be modulated at each step, offering a wide range of targets for pharmacological inhibition. The receptors and mediators involved are complex but have mostly been characterised and will now be discussed in turn.

1.3.1 Tethering

In the first step of the paradigm, cells become lightly tethered to the endothelium, then roll along its surface under physiological blood shear conditions. This interaction is transient and reversible; cells may detach and re-join the bloodstream if they do not receive an activation signal, discussed in section 1.3.2.

1.3.1.1 Selectins

The selectin family of adhesion molecules mediates the tethering of leukocytes to venular endothelial cells, allowing leukocytes to 'roll' along the venular wall (Figure 1–5). Selectin function is uniquely restricted to the vascular system. The individual members of the selectins are designated by prefixes, which were chosen according to the cell type where the molecules were first identified: L- (leukocyte) selectin is expressed on most types of leukocyte, E- (endothelial) selectin is expressed on activated endothelium, and P- (platelet) selectin was first found in the storage granules of platelets, and is also expressed by endothelial cells.

Figure 1–5 Schematic representation of the steps mediating eosinophil adhesion to vascular endothelium

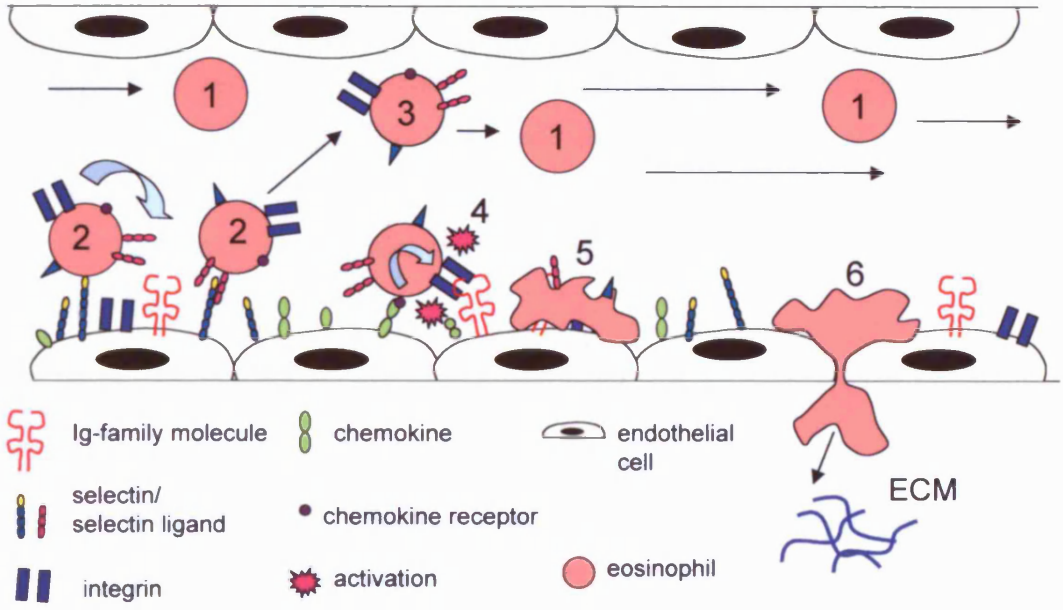


Figure 1–5 The multi-step paradigm of eosinophil adhesion to endothelium. Eosinophils enter the post-capillary endothelium under flow conditions (1). They become tethered to the endothelium (2) through the combined effects of the selectins and their ligands (P-selectin/ PSGL-1), and members of the immunoglobulin superfamily and integrins (VCAM-1/ VLA-4). These interactions allow the eosinophil to roll along the endothelial surface (2). Unless the eosinophil receives an activation signal (4), it will detach from the endothelium and re-join the circulation (3). Integrin activation through chemokines bound to the surface of the endothelium and chemokine receptors on the eosinophil, amongst other signals (4), allows firm adhesion (5). Firm adhesion is mediated by the cellular CD18 integrins LFA-1 and Mac-1 binding to ICAM-1 on the endothelium. VLA-4 and VCAM-1 also contribute at this stage. Following firm adhesion, eosinophils spread and transmigrate through the blood vessel endothelial cell junctions into tissues (6). ECM = extracellular matrix.

Each selectin has a unique, characteristic extracellular region composed of a 120 amino acid N-terminal calcium-dependent lectin domain, a 35-40 amino acid epidermal growth factor (EGF)-like domain, and 2-9 short consensus repeat (SCR) units homologous to domains found in complement binding proteins (Figure 1–6). Truncation of the SCR motifs in P-selectin impaired the efficiency with which P-selectin could support leukocyte rolling (Patel *et al.*, 1995), suggesting that these domains are essential to project the molecular structure of P-selectin beyond the glycocalyx into the bloodstream for optimal attachment of leukocytes. Selectins also possess a transmembrane domain and a short C-terminal cytoplasmic domain.

1.3.1.1.1 L-selectin

L-selectin is constitutively expressed by all leukocytes except activated memory lymphocytes, and was the first selectin to be shown as important for the entry of leukocytes into tissue. It was originally described by Gallatin, Weissman, and Butcher in 1983 as a lymphocyte 'homing receptor' involved in the initial attachment of lymphocytes to high endothelial venules (HEV) in lymph nodes (Gallatin *et al.*, 1983). Later, L-selectin was shown to be involved in the migration of neutrophils into inflamed tissue (Jutila *et al.*, 1989). Cell activation by chemoattractants and activating factors rapidly downregulates L-selectin expression on the plasma membrane by proteolytic activity. L-selectin is cleaved at an extracellular site proximal to the cell membrane in a process known as "shedding" (Kishimoto *et al.*, 1989). L-selectin shedding is also stimulated by cross-linking L-selectin with immobilised mAb (Palecanda *et al.*, 1992).

A mAb against L-selectin inhibited rolling of leukocytes in rabbit mesentery *in vivo*, while in the same model, an anti-β2 integrin antibody had no effect on rolling but did block firm attachment of leukocytes (von Andrian *et al.*, 1991). This work, by von Andrian *et al.*, established a two-step model for leukocyte adhesion to endothelial cells under shear flow conditions *in vivo*, with the selectin mediating the rolling step and the firm attachment being subsequently mediated by β2 integrins.

The importance of L-selectin as a rolling receptor has been shown in many studies. Eosinophils have been shown to roll on L-selectin (Sriramarao *et al.*, 1994). Lymphocyte homing in L-selectin-deficient mice was significantly reduced (Steeber *et al.*, 1996) and these mice mount defective immune responses (Tedder *et al.*, 1995).

Figure 1–6 A simplified schematic of the structure of the selectin subfamily members and their main ligands

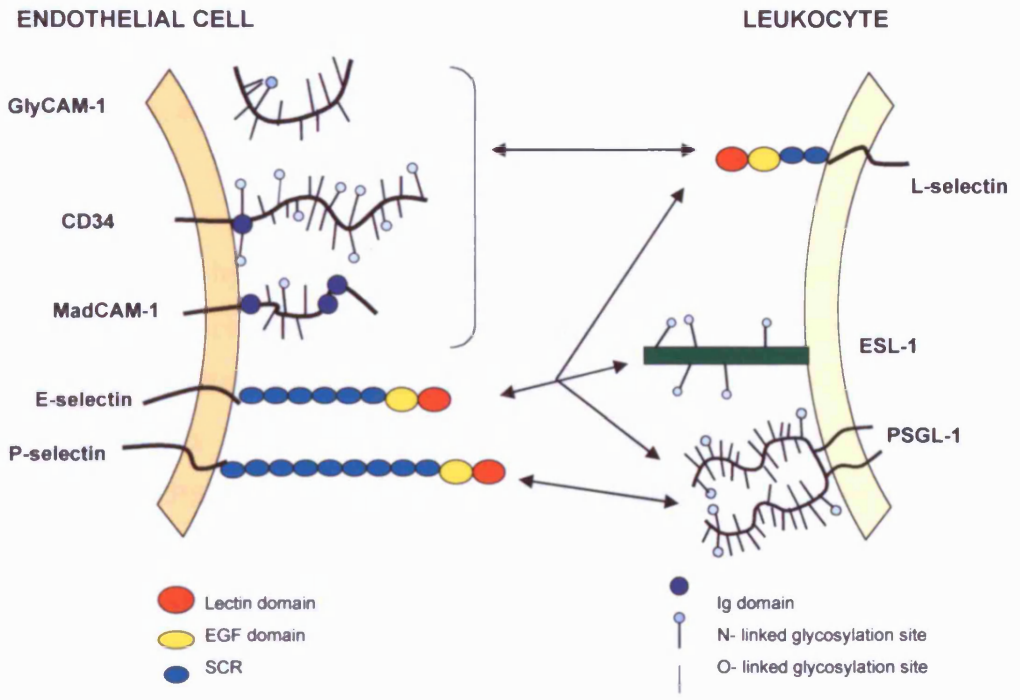


Figure 1–6 Selectins and their ligands. Abbreviations: EGF, epidermal growth factor; ESL-1, E-selectin ligand-1; GlyCAM-1, glycosylation-dependent cell adhesion molecule-1; PSGL-1, P-selectin glycoprotein ligand-1; SCR = short consensus repeats (complement-binding domains), Ig = immunoglobulin.

L-selectin is located on the tips of leukocyte microvilli (Bruehl *et al.*, 1996), and such presentation of receptors has been shown to facilitate the establishment of primary interactions between leukocytes and the endothelium under physiological shear conditions (von Andrian *et al.*, 1995). In contrast, β2 integrins, which are not able to initiate contacts under flow conditions, are excluded from microvillous processes.

1.3.1.1.2 E-selectin

E-selectin was identified by mAbs that had been raised against cytokine-activated human endothelial cells, and which blocked the binding of neutrophils. Cloning revealed its close homology to L-selectin (Bevilacqua *et al.*, 1987; Bevilacqua *et al.*, 1989). Expression of E-selectin on HUVEC is induced by cytokines such as TNF-α and IL-1β, and by lipopolysaccharide (LPS) (Bevilacqua *et al.*, 1987). Expression requires new protein synthesis, and maximal E-selectin levels are expressed at the cell surface within 3-4 hours after stimulation. Basal levels are reached again after 16-24 hours, in contrast to other cytokine-inducible adhesion molecules such as ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1). E-selectin has been shown to support rolling of neutrophils both *in vitro* (Abbassi *et al.*, 1993; Lawrence and Springer, 1993) and *in vivo* in IL-1β-stimulated rabbit mesenteric venules (Olofsson *et al.*, 1994). Bovine γ/δ (Jutila *et al.*, 1994), and human α/β and γ/δ T cell subsets (Diacovo *et al.*, 1996) also roll on E-selectin.

Eosinophils differ from neutrophils in their ability to recognise and bind vascular E- and P-selectins. It has been a consistent finding that eosinophils bind less avidly to E-selectin than neutrophils, both *in vitro* (Kitayama *et al.*, 1997b) and *in vivo* (Sriramarao *et al.*, 1996; Patel and McEver, 1997). Eosinophils express lower amounts of sLe^x compared to neutrophils (Bochner *et al.*, 1994), which may explain their weak E-selectin binding.

In contrast to P- and L-selectin deficient mice, E-selectin null mutants have no obvious abnormalities of the inflammatory response (Bullard *et al.*, 1996). A more detailed analysis of the E-selectin null mutants revealed that E-selectin was required for slow rolling (Kunkel and Ley, 1996). Treating E-selectin knockouts with anti-P-selectin reduced neutrophil emigration in a thioglycollate-induced peritonitis model of inflammation and leukocyte accumulation and oedema in a DTH skin model. In this study, wild-type mice treated with anti-P-selectin had no defects, suggesting that E-selectin and P-selectin share overlapping functions (Labow *et al.*, 1994).

1.3.1.1.3 P-selectin

P-selectin was originally found as a membrane component of human platelet storage ("alpha") granules (Hsu-Lin *et al.*, 1984). It was subsequently cloned and found to mediate binding of neutrophils to platelets and endothelial cells via its lectin domain (Johnston *et al.*, 1989; Geng *et al.*, 1990). P-selectin is also constitutively found in Weibel-Palade bodies of endothelial cells and is mobilised to the cell surface within minutes after activation by thrombogenic and inflammatory mediators (McEver *et al.*, 1989). Expression is maximal 5-10 minutes after stimulation, and P-selectin protein is rapidly cleared from the cell surface within the next hour by endocytosis. This short-lived expression makes P-selectin an ideal candidate for mediating early leukocyte-endothelial cell interactions. TNF-α also stimulates expression of P-selectin in mouse endothelial cells, with similar kinetics as that of E-selectin (Weller *et al.*, 1992). In HUVEC, P-selectin expression could not be induced by TNF-α, IL-1β, or LPS (Khew-Goodall *et al.*, 1996; Yao *et al.*, 1996). However, IL-4 and oncostatin M (a member of the IL-6-type cytokine family) were able to stimulate P-selectin transcription and protein expression in HUVEC that lasted 72 hours.

The main phenotype of P-selectin-deficient mice is the complete absence of leukocyte rolling after exteriorisation of mesenteric venules (Mayadas *et al.*, 1993). The importance of P-selectin in selective eosinophil accumulation in allergic disease has been underlined by numerous studies, outlined later in section 1.4.4.1. From these, it is clear that P-selectin is a strong candidate for the therapeutic inhibition of eosinophil influx into the airways in asthma.

1.3.1.2 Selectin ligands

Studies of the molecular basis of selectin adhesion have focused mainly on carbohydrate recognition by the lectin domain (Rosen and Bertozzi, 1994). All selectins bind to the tetrasaccharide sLe^x and its isomer sialyl Lewis A (sLe^a). L- and P-selectins, but not E-selectin, also bind to certain sulphated carbohydrates, such as heparan sulphate, that lack sialic acid and fucose (Varki, 1997). Selectins bind with higher affinity to only a few glycoproteins, most of which are mucins, i.e., glycoproteins containing multiple Ser/ Thr-linked oligosaccharides (O-glycans) and repeating peptide motifs (Kansas, 1996).

Both sialylation and fucosylation appear to be important requirements for adhesive function. Neuraminidase abolishes selectin-dependent adhesion interactions; and leukocyte adhesion deficiency (LAD) type 2, a congenital disorder of fucose metabolism, results

from absence of the sLe^x epitope on neutrophils (Etzioni *et al.*, 1992). Neutrophils from these patients do not adhere to immobilised E-selectin and fail to roll on mesenteric venules. Sufferers of LAD2 have recurrent episodes of bacterial infection, including pneumonia, without the formation of pus. This syndrome indicates a requirement for fucose in the carbohydrate ligands recognised by the selectins and illustrates the importance of selectin-mediated cell adhesion in the initial phase of leukocyte recruitment in response to infectious agents. Additionally, the α,1,3-fucosyltransferase enzyme FucT-VII is crucial for the formation of ligands for all three selectins (Maly *et al.*, 1996).

1.3.1.2.1 L-selectin ligands

To date, several ligands have been shown to support L-selectin binding. GlyCAM-1 is the most extensively characterised ligand. It is a 50 kDa secreted glycoprotein expressed mainly in the HEV of peripheral lymph nodes, consistent with its function as a specific vascular addressin for lymphocyte homing. GlyCAM-1 needs to be sulphated on oligosaccharide side chains to bind to L-selectin (Imai *et al.*, 1993). GlyCAM-1 is found in cytoplasmic granules rather than on the cell surface, arguing against a direct function for it as an adhesion molecule (Kikuta and Rosen, 1994). However, recently, GlyCAM-1 has been shown to activate β1 and β2 integrins (Hwang *et al.*, 1996; Giblin *et al.*, 1997), which is suggestive of its participation in leukocyte recruitment.

Less is known about other L-selectin ligands. The 90 kDa sialomucin CD34 is constitutively expressed on most endothelial cells and haematopoietic stem cells. The L-selectin ligand activity of CD34 depends on appropriate sulphation and glycosylation; CD34 only appears to be correctly glycosylated for L-selectin recognition in the HEV of lymph nodes (Baumheter *et al.*, 1993). However, genetic disruption of CD34 expression in mice does not result in an obvious loss of lymphocyte trafficking to lymph nodes (Cheng *et al.*, 1996).

Mucosal addressin cell adhesion molecule (MAdCAM-1) is a third L-selectin ligand, but only in the mouse. It contains immunoglobulin-like domains that mediate α4β7 integrin binding and a putative mucin-like domain that may mediate selectin binding (Berg *et al.*, 1993). Podocalyxin is a transmembrane sialomucin that is similar in structure to CD34. Podocalyxin-like protein (PCLP) is present on HEV, where it binds to both recombinant L-selectin and the HEV-specific monoclonal antibody MECA-79. Purified HEV-derived PCLP is able to support the tethering and rolling of lymphocytes under physiological flow

conditions *in vitro* (Sassetti *et al.*, 1998). P-selectin glycoprotein ligand 1 (PSGL-1), discussed in more detail below, is a 220 kDa mucin-like transmembrane protein, which is a receptor for L-, E- and P-selectin, hence the term PSGL-1 is misleading (Varki, 1997).

1.3.1.2.2 E-selectin ligands

The ligand with the highest affinity for E-selectin is E-selectin ligand 1 (ESL-1), a 150kD glycoprotein, which is a fucosylated variant of a receptor for fibroblast growth factor and is only expressed on murine neutrophils (Steegmaier *et al.*, 1995). PSGL-1 also binds to E-selectin, although in a different manner to P- and L-selectin. Core-2, sialylated and fucosylated O-glycans are required for E-selectin binding, but sulphation, which is essential for P- and L-selectin ligands, appears not to be crucial for E-selectin ligands (Li *et al.*, 1996). The binding affinity of PSGL-1 to E-selectin is at least 50-fold lower than to P-selectin, as demonstrated by competitive binding studies with neutrophils (Moore *et al.*, 1994). Memory T cells that infiltrate the skin express a unique skin-homing receptor called cutaneous lymphocyte-associated antigen (CLA), which is defined by its reactivity with a unique monoclonal antibody, HECA-452. CLA is a third ligand for E-selectin, and it was recently reported that CLA is an inducible carbohydrate modification of PSGL-1 (Fuhlbrigge *et al.*, 1997).

1.3.1.2.3 P-selectin ligands

PSGL-1 is a homodimer of two 120 kDa subunits, and was first identified as a specific ligand for P-selectin (Moore *et al.*, 1992). PSGL-1 binds all three selectins, demonstrating the highest affinity for P-selectin. It is constitutively expressed on the surface of most circulating leukocytes, and has been demonstrated to play a role in leukocyte-leukocyte, leukocyte-platelet, and leukocyte-endothelial cell interactions (McEver and Cummings, 1997).

PSGL-1 does not bind to P-selectin unless it is correctly post-translationally modified by α1, 3-fucosylation, α2, 3-sialylation and core-2 branched O-glycosylation (McEver and Cummings, 1997). Modifications must be on O-glycans, because enzymatic digestion of N-glycans or mutations that prevent N-glycan addition do not inhibit binding (Moore *et al.*, 1992). In contrast, enzymatic removal of sialic acids or O-linked glycans eliminates PSGL-1 binding to P-selectin. Human PSGL-1 is also sulphated exclusively on tyrosine residues, rather than on O-glycans (Wilkins *et al.*, 1995). Enzymatic removal of sulphate or blockade of sulphate synthesis eliminates binding of PSGL-1 to P-selectin (Sako *et al.*,

1995; Wilkins *et al.*, 1995). The majority of P-selectin binding function is found in the N-terminal 19 amino acids of PSGL-1. In contrast, E-selectin binds to the central mucin-like, O-linked, carbohydrate-rich region of the receptor.

PSGL-1 has been demonstrated to be the essential ligand mediating leukocyte adhesion to P-selectin. Studies of P-selectin binding to PSGL-1 provided the first direct evidence that leukocyte rolling under physiological shear requires the specific interaction of a selectin with a single cell surface glycoprotein. Moore and co-workers demonstrated that a blocking mAb against PSGL-1 completely inhibited P-selectin-mediated rolling of neutrophils under a range of physiologic shear stresses (Moore *et al.*, 1995). Indeed, mAb blockade of PSGL-1 function can completely inhibit neutrophil, eosinophil, basophil, and monocyte adhesion to P-selectin under static and flow conditions (Moore *et al.*, 1995; McEver and Cummings, 1997; Taylor *et al.*, 1999). Activation of neutrophils, monocytes and eosinophils was shown to downregulate surface PSGL-1, and this was associated with decreased adhesion to purified P-selectin *in vitro* (Davenpeck *et al.*, 2000). The mechanism for this remains unknown, but may possibly involve a novel sheddase.

1.3.2 Activation

The first selectin-driven interactions in the leukocyte migration paradigm serve as a pre-requisite for the second *visible* step of the cascade, that is, the arrest and firm adhesion of the rolling leukocyte via the integrin family of adhesion molecules. However, despite high levels of expression on circulating cells, integrins are generally in a functionally inactive state that needs to be converted to an active one. Thus, the true second step of the cascade is a 'triggering' step; where an activation signal enables the integrins, which do not constitutively bind ligand, to become adhesive (known as inside-out signalling). There are two classes of molecular interactions proposed to provide triggering in the cascade. Endothelial surface receptors are one possible trigger. For example, activation of the leukocyte integrins has been reported after binding to E-selectin (Lo *et al.*, 1991) and to platelet endothelial cell adhesion molecule-1 (PECAM-1) (Berman and Muller, 1995). A much more important mechanism of activation is via fast signalling mediated by soluble factors present at or near the endothelial surface. These include primarily the classical chemoattractants for myeloid cells (fMLP, C5a, LTB_4 and PAF) and the large family of chemokines, which signal via serpentine GPCRs on the leukocyte (Murphy, 1994).

Figure 1–7 The structure of the chemokine gene superfamily members

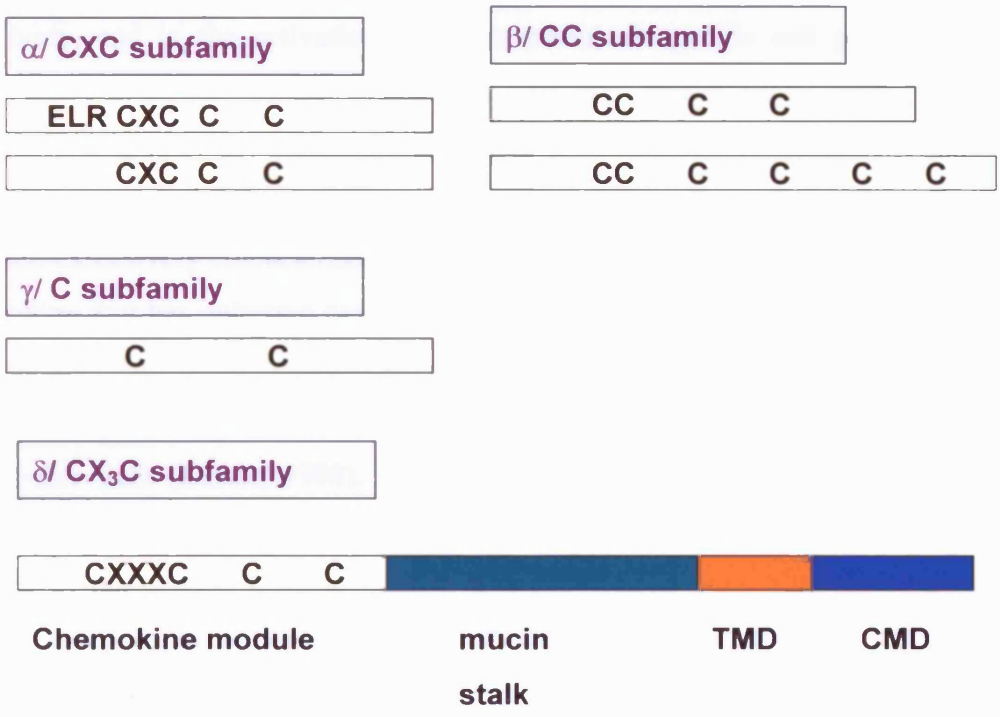


Figure 1–7 Structure of the chemokine superfamily. This simplified schematic outlines the cysteine signature motif of the primary amino acid sequence that distinguishes between members of each subfamily. Two other subdivisions can be made within the CXC and CC subfamilies, based on the presence or absence of an ELR motif immediately preceding the CXC motif, or the presence of either 4 or 6 cysteines in the CC subfamily. TMD = transmembrane domain, CMD = cytoplasmic membrane domain.

1.3.2.1 Chemokines

Chemokines, reviewed by Luster (Luster, 1998), are a growing family of small, structurally related peptides, involved in both the routine immune surveillance that occurs in the body, and in the activation and recruitment of specific cell populations during disease. They have so far been subdivided into four groups based upon their primary structure. In this, cysteine residues are arranged as C, CC, CXC, or CXXXC, where 'C' is a cysteine residue and 'X' is a different amino acid (Figure 1–7). Only two of the families have been extensively characterised, the CXC (α) and CC (β) chemokines. Lymphotactin, a chemokine that has only two cysteines (C), is a member of a third family. The fourth is represented by fractalkine, a membrane-bound glycoprotein in which the first two cysteine residues are separated by three amino acids (CXXXC) and the chemokine domain sits atop a mucin-like stalk (Luster, 1998). Because many chemokines now have more than one name, a new nomenclature for these molecules was established at the 1999 Chemokine Keystone Symposium in Colorado, USA. The chemokines were numbered following the order they were discovered and an L ('ligand') suffix was added (Zlotnik, 2000).

Chemokines mediate their biological responses by binding to target cell-surface receptors that belong to the large family of G-protein-coupled, 7TM receptors (section 1.1.6). Many chemokine-induced signalling events are inhibited by PTX, suggesting that chemokine receptors are linked to G-proteins of the G_i class. The receptors that bind CXC chemokines are designated CXCRs and those that bind to CC chemokines are CCRs. The classical function of the chemokines is to promote chemotaxis of leukocytes within tissues, but they also induce cell activation. In general, the CXC chemokines tend to act more on neutrophils, whereas the CC chemokines tend to act more on eosinophils, monocytes, basophils, and lymphocytes. Clearly some redundancy occurs, as different leukocyte subtypes may share the same chemokine receptor and different chemokines share receptor-binding capabilities (Luster, 1998), as shown in Table 1–3.

Thus, multiple pathways may be generated to achieve similar cellular responses. Most cell types express many chemokine receptors so that if one ligand or receptor is defective, an alternate set of chemokines and receptors may accomplish the same biological function.

Table 1–3 CXC, C, CX_3C and CC Chemokine/ Receptor Families

Receptor/ Ligands	Receptor/ Ligands
CXC chemokines	**CC chemokines**
CXCR1/ IL-8, GCP-2	**CCR1**/ RANTES, MCP-2/3/4, MIP-1α/β
	MIP-1δ, HCC-1/2/4, MPIF-1
CXCR2/ IL-8, GRO-α/β/γ, ENA-78	**CCR2**/ MCP-1/3/4
GCP-2	
CXCR3/ IP-10, MIG, I-TAC	**CCR3**/ Eotaxin, eotaxin-2/3, RANTES
	MCP-2/3/4, HCC-2
CXCR4/ SDF-1α/β	**CCR4**/ TARC, MDC
CXCR5/ BCA-1	**CCR5**/ MIP-1α/β, RANTES, MCP-2
C chemokines	**CCR6**/ LARC, MIP-3α, exodus-1
XCR1/ Lymphotactin, SCM-1β	**CCR7**/ 6Ckine, SLC, exodus-2
	MIP-3β, ELC, exodus-3
CXXXC chemokines	**CCR8**/ I-309
CX3CR1/ Fractalkine	**CCR9**/ TECK
Neurotactin (mouse)	**CCR10**/ CTACK

Table 1–3 Chemokine/ Receptor families. Abbreviations: BCA-1, B-cell attracting chemokine-1; 6Ckine, 6-cysteine chemokine; CTACK, cutaneous T-cell attracting chemokine; ELC (Epstein Barr virus-induced gene 1)-ligand chemokine; ENA-78, epithelial-cell-derived neutrophil-activating peptide-78; GCP-2, granulocyte chemotactic protein-2; GRO, growth regulatory oncogene; HCC, haemofiltrate CC chemokine; IP-10, interferon inducible protein-10; I-TAC, interferon inducible T cell alpha chemoattractant; LARC, liver and activation regulated chemokine; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MIG, monokine induced by γ-interferon; MIP, macrophage inflammatory protein; MPIF-1, myeloid progenitor inhibitory factor-1; SCM-1β, single cysteine motif-1β; SDF-1, stromal cell-derived factor-1; SLC, secondary lymphoid tissue chemokine; TARC, thymus and activation regulated chemokine; TCA-4, thymus-derived chemotactic agent 4; TECK, thymus-expressed chemokine.

For example, eight different CXC chemokines can bind and activate CXCR2, but if this receptor is defective, neutrophils can still be recruited through IL-8 activation of CXCR1. CC receptors bind only CC chemokines and CXC receptors bind only CXC chemokines, a restriction that may be related to different quaternary structures (Luster, 1998).

The current theory of how chemokines might change the affinity of integrins for their ligands is that they are immobilised on the surface of endothelial cells via their basic C-terminus binding to endothelial proteoglycans (Tanaka *et al.*, 1993). This permits effective chemokine presentation to the rolling leukocyte, allows the maintenance of receptor-binding activity, and prevents the chemokine from being swept away by flowing blood. The N-terminus then binds to the 7TM receptors of the leukocyte and elicits the signal mediated by G-proteins that leads to integrin activation (Dunon *et al.*, 1996). Receptor binding leads to a cascade of cellular activation, as detailed in section 1.1.6.1 and Figure 1–3. Chemokine-receptor signalling also activates small GTP-binding G-proteins of the ras and rho families, which are involved in cell motility (Laudanna *et al.*, 1996).

1.3.2.1.1 Chemokines in allergic disease

Allergic disease has been associated with the presence of several CC chemokines. RANTES was the first chemokine to be identified as a potent eosinophil chemoattractant, and neutralisation of RANTES with a receptor antagonist inhibited both lymphocyte and eosinophil recruitment (Gonzalo *et al.*, 1998). Consistent with these observations, increased RANTES mRNA expression has been demonstrated in bronchial biopsies from patients with atopic and non-atopic asthma (Ying *et al.*, 1999). MCP-3 has attracted much interest with respect to allergic disease. MCP-3 is expressed in the bronchial mucosa of mild to moderate asthmatics (Ying *et al.*, 1999), and pre-treatment of allergen-sensitised mice with an anti-MCP-3 antibody significantly inhibited allergen-induced airway inflammation and BAL eosinophilia (Stafford *et al.*, 1997).

In the past five years, three peptides with specific chemotactic activity for eosinophils, have been identified, namely, eotaxin, eotaxin-2, and eotaxin-3. Eotaxin, reviewed by Rankin *et al.* (Rankin *et al.*, 2000) was first identified in 1994 in the BAL fluid of ovalbumin-sensitised guinea pigs (Jose *et al.*, 1994). Two years later, the gene encoding human eotaxin was described and found to exhibit 58% identity with guinea pig and mouse eotaxin (Ponath *et al.*, 1996). The potency and rapid action of eotaxin in inducing selective eosinophil recruitment suggests an integral role for this protein in the early phases of the

signalling mechanism for eosinophil homing and tissue recruitment. Eotaxin expression has been localised to epithelial and endothelial cells in the asthmatic bronchial mucosa. Moreover, soluble eotaxin has been found in the serum of asthmatic patients, with concentrations correlating with the severity of disease (Rankin *et al.*, 2000).

Eotaxin increases eosinophil adhesion to cytokine-stimulated lung endothelial cell monolayers (Burke-Gaffney and Hellewell, 1996), and is chemotactic for eosinophils both *in vitro* (Garcia-Zepeda *et al.*, 1996b) and *in vivo* (Das *et al.*, 1997). Eotaxin can induce actin polymerisation and free intracellular calcium mobilisation, leading to superoxide generation and upregulation of integrins on the cell surface (Tenscher *et al.*, 1996). Thus, eotaxin not only plays an integral part in the attraction of eosinophils to inflammatory sites, but also is involved in the development of tissue damage by activating the pro-inflammatory effector functions of these cells.

Eosinophils express an eotaxin receptor, CCR3, which is a candidate for regulating the selective recruitment of this leukocyte to sites of inflammation (Daugherty *et al.*, 1996). CCR3 is a promiscuous receptor, interacting with multiple ligands, including MCP-2, -3, -4, and RANTES (Heath *et al.*, 1997). Increased CCR3 mRNA expression was found predominantly localised to eosinophils in the bronchial biopsies of asthmatics (Ying *et al.*, 1999). CCR3 is also expressed to a lesser extent on basophils, and a small subset of Th2 cells (Sallusto *et al.*, 1997). A recent report suggested that neutrophils could also express CCR3 (Bonecchi *et al.*, 1999) but this was argued against by another study, which suggested that the earlier expression was probably due to contamination by eosinophils (Hochstetter *et al.*, 2000). Hence, due to its mainly exclusive expression on eosinophils, CCR3 is an attractive therapeutic target for the treatment of eosinophilic inflammation.

Due to their cell-target specificity favouring neutrophil chemoattractants, a role for CXC chemokines in asthma is less likely, although IL-8 has been shown to be chemotactic for eosinophils *in vitro* (Schweizer *et al.*, 1994). Elevated levels of IL-8 have been demonstrated in the BAL fluid of patients with asthma compared to normal controls (Chanez *et al.*, 1996; Folkard *et al.*, 1997). Shute and co-workers detected free IL-8 in the sera and bronchial tissue of atopic asthmatics, but not normal controls. There was a positive correlation between free IL-8 and serum ECP levels found in severe disease, suggesting an association between IL-8 and eosinophil activation (Shute *et al.*, 1997). In humans, IL-8 binds to two receptors, CXCR1 and CXCR2. However, although expressed

on neutrophils, neither receptor was expressed on eosinophils in one flow cytometry study, even after priming with cytokines such as IL-4, IL-5, and TNF-α (Petering *et al.*, 1999). In contrast to humans, mice express one main chemokine receptor that binds several CXC chemokines. Ovalbumin-sensitised, aerosol challenged IL-8 receptor gene-deleted mice demonstrated a diminished capacity to recruit neutrophils to inflammatory sites (De Sanctis *et al.*, 1999). These mice also produced more antigen-specific IgE compared to control mice, suggesting an inhibitory role for the IL-8 receptor in regulating IgE production and cell migration into the airways.

1.3.3 Firm arrest

In general, two families of adhesion receptors mediate the firm arrest stage in the adhesion paradigm, the integrins, and the immunoglobulin superfamily.

1.3.3.1 Integrins

The integrins are a complex family of cell-surface glycoproteins that act as receptors for extracellular matrix proteins, or for membrane-bound counter-receptors on other cells. Each integrin is a heterodimer that contains an α and a β subunit, as represented in Figure 1–8. Each subunit has a large extracellular domain, a single membrane-spanning region, and in most cases, a short cytoplasmic domain. So far, 18 distinct α and 8 β subunits have been discovered, which can associate to form more than 20 distinct integrin heterodimers (Hynes, 1999). The α/β pairings specify the ligand-binding abilities of the integrin heterodimers. The functional state, density, and distribution of integrins on cells are regulated by lipid, cytokine, and chemokine signalling molecules and by "cross-talk" from other surface adhesion molecules. The human eosinophil integrin repertoire consists of β1 integrins; α4β1 (VLA-4) and α6β1 (VLA-6), β2 integrins; αL/β2 (CD11a/ CD18, LFA-1), αMβ2 (CD11b/CD18, Mac-1, CR3), αXβ2 (CD11c/CD18, p150,95), αDβ2 (CD11d/CD18), and the β7 integrin α4β7. Two classes of integrins immobilise rolling leukocytes on the surface of the vascular endothelium. The α4β1 and α4β7 integrins bind to endothelial VCAM-1 and MAdCAM-1, respectively, and the LFA-1 and Mac-1 integrins are ligands for the intercellular adhesion molecules (ICAMs) (Imhof and Dunon, 1995).

1.3.3.1.1 The β2 (leukocyte) integrins

The β2 heterodimers are restricted to cells of the leukocyte lineage, thus they are known as the β2 or "leukocyte" integrins. The gene for the β2 chain (95kD) is located on human chromosome 21 and encodes a cysteine-rich transmembrane protein with six N-linked extracellular glycosylation sites. The cytoplasmic tail contains sequences critical for inside-out signalling and cytoskeletal association. The extracellular portion of β2 contains an "I" ("inserted") -like, domain (see below) near the N-terminus that is highly conserved in other β subunits and is critical for ligand recognition (Lee *et al.*, 1995). The genes for human αL (177 kDa), αM (165 kDa), αX (150 kDa), and αD (100 kDa) are located in a cluster on chromosome 16 (Wong *et al.*, 1996). Each contains a distal N-terminal extracellular I domain of approximately 200 amino acids that is critical for ligand binding. The I domains of LFA-1 and Mac-1 have been crystallised, and an important part of the structure shown to be a Mg^{2+}/ Mn^{2+} binding site termed the MIDAS motif (Qu and Leahy, 1995), Figure 1–8. The I domains lie within the third repeat and are predicted to be exposed and mobile. The cytoplasmic tails of the α chains contain a GFFKR motif common to all integrin α subunits, which serves to lock the heterodimers into a low affinity conformation in the absence of activating signals, and which is involved in α/β subunit association (Hughes *et al.*, 1996).

The clinical importance of leukocyte integrin-mediated cell adhesion is emphasised by the autosomal recessive LAD type 1 syndrome, in which an affected individual has a congenital deficiency of the CD18 subunit. LAD1 varies in severity and is characterised by an absence or a greatly reduced display of all β2 integrin heterodimers on the surface of leukocytes. This results in absent or dramatically reduced accumulation of neutrophils and monocytes at extravascular sites, recurrent, life-threatening bacterial infections, and impaired tissue remodelling and wound healing (Etzioni *et al.*, 1999).

1.3.3.1.2 Inside-out and outside-in signalling

Leukocytes differ from other cells in being able to transiently regulate the activity of their integrins, which act as bi-directional signal transducers to mediate both "inside-out" and "outside-in" signalling mechanisms, reviewed for neutrophils by Williams and Solomkin (Williams and Solomkin, 1999). For normal leukocyte function, it is especially important that the process of adhesion is tightly regulated. As leukocytes circulate in the bloodstream, they must be non-adherent; otherwise, small blood vessels would rapidly

Figure 1–8 The domain structure of the leukocyte integrin heterodimers

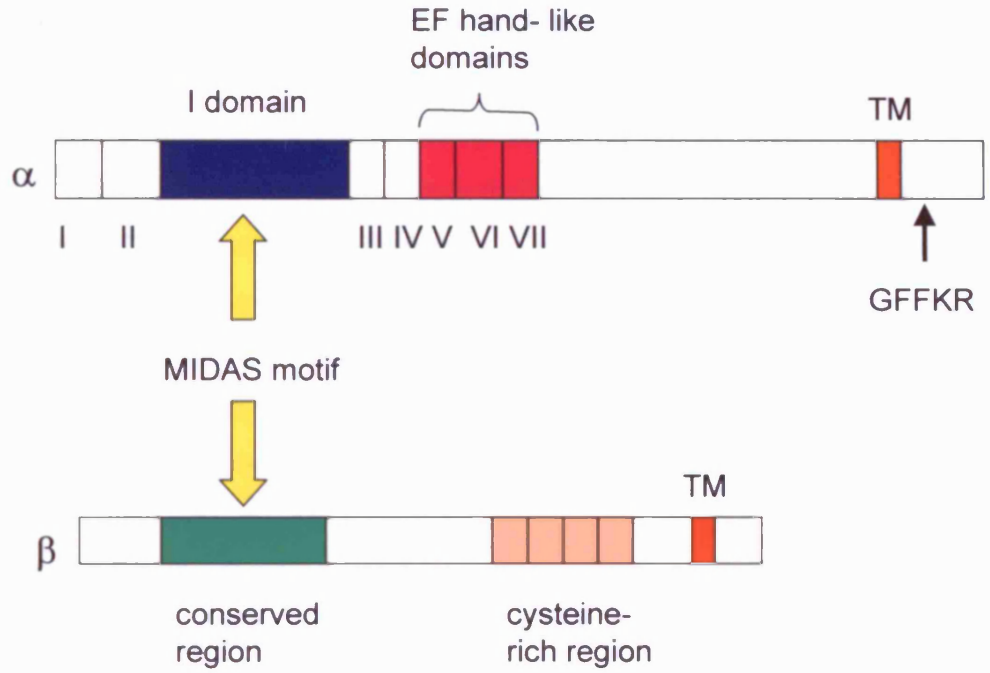


Figure 1–8 Leukocyte integrin structure. The α and β subunits both contain the MIDAS (metal ion dependent adhesion site) motif. Domains V-VII in the I domain-containing integrins have EF-hand-like cation-binding sequences with a conserved motif. The highly conserved GFFKR motif in the cytoplasmic tail of the α subunit is involved in maintaining the default low affinity conformation in the absence of activating signals. TM = transmembrane domain.

become plugged with cell aggregates. Inside-out signalling (also known as integrin "activation" or "functional upregulation") is important for recruiting leukocytes during inflammatory responses. Activation of the leukocyte by a variety of agonists, particularly endothelial-derived chemoattractants, triggers conformational changes in the β2 integrin heterodimers, which are not constitutively active on resting leukocytes. This gives the integrins heightened affinity for endothelial ligands, dramatically increasing the adhesiveness of leukocytes for endothelium. The second, outside-in signalling mechanism commences with binding of ligand to an integrin at the cell surface, which triggers intracellular signal transduction cascades. Second messengers activated by the cytoplasmic tails of integrins include G-proteins and tyrosine kinases, which have a variety of actions, such as triggering of cytoskeletal contraction and regulation of gene expression.

1.3.3.1.3 The β1 integrins

The β1 (CD29) integrin family used to be referred to as the very late antigens (VLA), as the *in vitro* expression of two of its members, VLA-1 and VLA-2 increased dramatically on lymphocytes after some weeks of growth factor stimulation (Hemler *et al.*, 1985). The member of this subfamily most relevant to allergic airway disease is VLA-4, which is the receptor for VCAM-1 and is found on monocytes, lymphocytes, basophils and eosinophils, but interestingly not on neutrophils in physiological circumstances. Hence, the VLA-4/ VCAM-1 interaction is thought to contribute to the selective eosinophil accumulation observed in allergic disease (Bochner *et al.*, 1991; Walsh *et al.*, 1991). However, under non-physiological conditions neutrophils will express α4β1, for example, after stimulation with dihydrocytochalasin B (Kubes *et al.*, 1995). Such artificially induced α4β1 enabled neutrophils to adhere to TNF-α-stimulated HUVEC under flow conditions independently of β2 integrins (Reinhardt *et al.*, 1997).

1.3.3.2 The immunoglobulin supergene family

The members of the immunoglobulin cell adhesion molecule superfamily all bear a close similarity in a 90-100 amino acid domain, which was originally observed in the constant regions of the light and heavy chains of immunoglobulins (Imhof and Dunon, 1995). This family includes a diverse range of members, such as the T cell receptors CD3, CD4, and CD8, the major histocompatibility complexes (MHC) I and II, and a number of leukocyte adhesion molecules including the ICAMs and VCAM-1.

1.3.3.2.1 The intercellular adhesion molecules (ICAMs)

The major ligand for the integrins LFA-1 and Mac-1 is ICAM-1 (CD54), which is a 90 kDa single chain glycoprotein composed of a short cytoplasmic domain, a hydrophobic transmembranous domain, and five immunoglobulin (Ig)-like extracellular domains (Rothlein *et al.*, 1986). In contrast to other integrin ligands, ICAM-1 does not contain the Arg-Gly-Asp (RGD) sequence, indicating that CD11/ CD18 integrins bind to ICAM-1 via RGD-independent sites (Staunton *et al.*, 1988). ICAM-1 is constitutively expressed on the endothelial cell surface. Expression increases progressively, reaching a plateau 24 hours after TNF-α, IL-1β, or LPS stimulation, and can remain elevated for up to 72 hours in the continued presence of these cytokines (Pober *et al.*, 1986).

ICAM-2 (CD102) is an integral membrane protein with two Ig-like domains. Expression of ICAM-2 remains unaltered by cytokine and LPS stimulation, suggesting that its role in tissue inflammation is different to that of ICAM-1. ICAM-2 seems to be the main LFA-1 ligand on resting endothelium (Staunton *et al.*, 1989). ICAM-3 is a third ligand for LFA-1, unlike ICAM-1 and ICAM-2, it is absent from vascular endothelial cells, being distinctly localised to resting leukocytes (Fawcett *et al.*, 1992). It seems unlikely that ICAM-3 plays a major role in leukocyte infiltration.

1.3.3.2.2 Vascular cell adhesion molecule-1 (VCAM-1)

VCAM-1 is a 110 kDa glycoprotein that is found, as its name suggests, on vascular endothelium (Osborn *et al.*, 1989), as well as on other cell types, including epithelium and smooth muscle. It is basally expressed on 5-20% of vessels in lymphoid tissue and is absent at other sites under resting conditions. VCAM-1 is induced by IL-1, TNF-α, LPS (Wellicome *et al.*, 1990), and IL-4 (Iademarco *et al.*, 1995), but unlike ICAM-1, not by IFN-γ. The cytokine-stimulated expression kinetics of VCAM-1 are similar to ICAM-1, with increased expression seen within 2 hours of stimulation, and upregulation lasting for 72 hours.

In vivo, selective expression of VCAM-1 is associated with the accumulation of mononuclear cells and eosinophils during chronic and allergic inflammation (Montefort *et al.*, 1992; Bentley *et al.*, 1993; Nakajima *et al.*, 1994; Jahnsen *et al.*, 1995). IL-4, a cytokine expressed by CD4+ T cells, basophils, and mast cells, is frequently detected at the same sites as VCAM-1. VCAM-1 and its ligand VLA-4 could offer a selective mechanism by which eosinophils accumulate in asthma, as discussed in section 1.4.4.1.

1.3.4 Transmigration

After binding tightly to the endothelial cell (EC), leukocytes crawl towards an EC-EC junction and quickly move between the EC into the sub-endothelial extracellular matrix (ECM) (Muller and Weigl, 1992). The ECM is a complex network of large fibrillar proteins that influence cellular functions through adhesive contacts with integrin receptors. There, leukocytes are guided to their final destination in the tissue by gradients of chemoattractants. Matrix metalloproteinases (MMPs) may be important in transmigration through the endothelial basement membrane. MMPs are a family of endopeptidases, which are capable of proteolytically degrading many components of the ECM. The proteolytic activity of MMPs is inhibited by tissue inhibitors of metalloproteinases (TIMPs) (Murphy and Docherty, 1992). The balance between levels of MMPs and TIMPs is thought to be a critical factor in regulating the breakdown of connective tissues. MMP-2 and MMP-9 cleave type IV collagen, which is an important component of the basement membrane. MMP-9 was found to be over-expressed on eosinophils accumulating in the airway walls of asthmatics (Ohno *et al.*, 1997). Inhibition of MMP-2 and MMP-9 inhibited antigen-induced recruitment of eosinophils to the airway wall and lumen and reduced antigen-induced AHR in a mouse model of allergic asthma (Kumagai *et al.*, 1999).

PECAM-1, a member of the Ig family of adhesion receptors is also thought to be important at this stage. PECAM-1 is concentrated at junctions between EC and is also expressed on eosinophils, platelets, monocytes, neutrophils, and a subset of T cells (Imhof and Dunon, 1995). Anti-PECAM-1 mAbs or soluble PECAM-1, added to either leukocytes or EC, inhibits transendothelial migration *in vitro* without interfering with leukocyte-endothelial firm adhesion (Muller *et al.*, 1993). Anti-PECAM-1 mAbs appear to block a similar mechanism *in vivo* (Bogen *et al.*, 1994).

1.3.5 Adhesion receptor expression in allergic disease

Adhesion molecule function can be regulated in several ways: by shedding (e.g. L-selectin); by increases in expression (e.g. E- and P-selectin, ICAM-1, and VCAM-1); and by changes in receptor binding affinity (e.g. integrins). A number of groups have studied expression of E-selectin, ICAM-1, and VCAM-1 in asthma and other allergic inflammatory conditions. P-selectin expression has been less widely studied, partly because of the difficulty in distinguishing between intracellular and luminal staining. In general, the results from *in vivo* allergen challenge studies match observations found in cytokine-stimulated HUVEC.

Expression of endothelial adhesion receptors is partly organ-dependent. Normal skin endothelium has a low basal expression of ICAM-1, and absent expression of E-selectin and VCAM-1, with expression of all three receptors increasing after allergen challenge (Kyan-Aung *et al.*, 1991; Leung *et al.*, 1991). In the airway, Montefort *et al.* found increased expression of ICAM-1 and E-selectin 6 hours after local allergen challenge with no increase in VCAM-1 expression (Montefort *et al.*, 1994a). Bentley *et al.* reported a trend towards increased VCAM-1 expression with a good correlation between VCAM-1 expression and eosinophil infiltration 24 hours after aerosol allergen challenge (Bentley *et al.*, 1993). In lung explants sensitised with dust mite allergen, increased ICAM-1, E-selectin and VCAM-1 was seen after challenge (Hirata *et al.*, 1998). Upregulation in this study was also mediated by a combination of IL-1β and TNF-α.

There are discrepancies among studies of the expression of these receptors in clinical asthma, probably because it is difficult to accurately quantify small increases using immunohistochemical techniques. Gosset *et al.* reported low background expression of ICAM-1, but not VCAM-1 or E-selectin in control subjects, with increases in expression of all three molecules in atopic but not non-atopic asthmatics (Gosset *et al.*, 1995). Ohkawara *et al.* agreed with these findings in corticosteroid-dependent patients with asthma (Ohkawara *et al.*, 1995). In contrast, Fukuda and co-workers detected no increase in ICAM-1 or E-selectin staining over controls, although the E-selectin Ab used cross-reacted with P-selectin (Fukuda *et al.*, 1996). However, in this study expression of VCAM-1 was upregulated. It has been suggested that a graded response of expression of these different receptors could exist depending on the severity of the disease (Hirata *et al.*, 1998).

Several adhesion molecules can be detected in soluble form circulating in the plasma, including E-selectin, ICAM-1 and VCAM-1 (Kobayashi *et al.*, 1994; Montefort *et al.*, 1994b; Koizumi *et al.*, 1995). However, so far there seems to be no clear-cut correlation between disease severity and concentration of the soluble receptor, so the significance of these studies in terms of disease pathogenesis is uncertain.

1.4 Selective eosinophil accumulation in allergic inflammation

The increase in eosinophil numbers in tissues in asthma appears to be selective, as it generally occurs without a concomitant increase in neutrophil numbers. It is estimated that there is a 50-100-fold increase in the accumulation of eosinophils over neutrophils in the airways in clinical asthma (Wardlaw, 1999). This increase occurs in sequential and

cumulative stages (Figure 1–9). Two important questions regarding the role of eosinophils in allergic inflammation are 1) the mechanisms by which eosinophils selectively accumulate in the airways and 2) the mechanisms by which eosinophils are triggered to release their toxic mediators. It is clear that inhibition of either of these steps may be an effective approach to the treatment of asthma as well as offering insights into the pathogenesis of this debilitating disease.

There are several possible mechanisms for selective eosinophil accumulation:

- a selective eosinophil chemoattractant
- prolonged eosinophil survival under the influence of cytokines such as IL-3, IL-5, and GM-CSF
- *in situ* differentiation from eosinophil precursors resident in the airways
- a selective adhesion pathway

The possibilities of each mechanism will now be briefly discussed in turn.

1.4.1 Selective eosinophil chemoattractants

Thirty years ago, ideas on eosinophil trafficking into tissues were based around the notion of a selective chemoattractant generated from mast cells. Eosinophil chemotactic factor of anaphylaxis (ECF-A), was detected in supernatants from allergen challenged guinea pig lung, and appeared to be selectively chemotactic for eosinophils (Kay *et al.*, 1971). ECF-A from human lung was isolated and characterised as two tetrapeptides, Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu (Goetzl and Austen, 1975). However, it is now believed that ECF-A has negligible activity on eosinophils, compared with later, highly potent eosinophil chemotactic factors that were identified, such as PAF (Wardlaw *et al.*, 1986). It is now thought that the effects of mast cells on eosinophil migration are largely indirect, through the generation of cytokines such as TNF-α, IL-4, and IL-13. Recently, the discovery of chemokines such as eotaxin (see section 1.3.2.1.1) has revived interest in the role of selective chemoattractants in directing migration.

1.4.2 Prolonged survival

Unless they are provided with support from growth factors, eosinophils rapidly exhibit the classic morphological changes associated with apoptosis, or programmed cell death (PCD), such as DNA laddering, nuclear and cytoplasmic condensation, and redistribution of

Figure 1–9 The multi-step process of selective eosinophil tissue accumulation in asthma

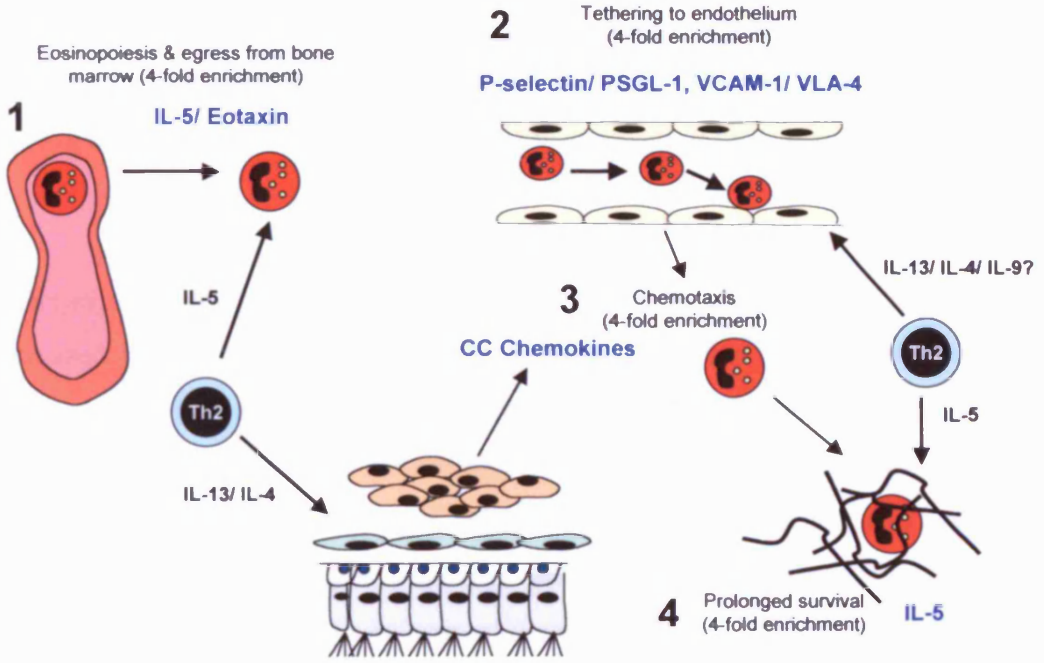


Figure 1–9 Schematic representation of the multi-step process of eosinophil recruitment into tissue. The selective accumulation of eosinophils occurs as sequential and cumulative approximately 4-fold increases, in eosinophils compared with neutrophils, at several stages in the life cycle of the cell. Each step is under separate molecular control, influenced either directly or indirectly by Th2 cytokine production. The first step involves haematopoiesis and bone marrow egress mediated by IL-5 and chemotactic signals. The second step is through cytokine upregulation of P-selectin and vascular cell adhesion molecule-1 (VCAM-1) on vascular endothelium. The third step involves selective chemotaxis under the influence of CC chemokines generated by IL-4- and IL-13-stimulated epithelial, fibroblast, and smooth muscle cells. The fourth step is prolonged survival, mediated by IL-5. PSGL-1, P-selectin glycoprotein ligand-1; VLA, very late antigen. Adapted from (Wardlaw, 1999), with kind permission.

membrane components. Apoptosis, reviewed by Simon (Simon, 2000), can be induced in response to specific ligands or stimuli that engage so-called "death receptors" of the TNF receptor family, e.g. Fas (CD95/ Apo-1). Alternatively, lack of survival factors can trigger apoptosis. *In vitro*, the hematopoietins IL-3, IL-5, and GM-CSF have been shown to prolong the survival and suppress the PCD of eosinophils (Her *et al.*, 1991; Yamaguchi *et al.*, 1991).

Direct evidence for prolonged survival was provided by a study where anti-IL-5 antibodies decreased tissue eosinophilia in cultured explants of nasal polyps (Simon *et al.*, 1997). Other pro-survival and pro-apoptotic signals exist. The apoptosis-inducing elements Bax and Bcl-x_S, and the apoptosis-inhibiting factors Bcl-2 and Bcl-x_L, are critical regulators of the apoptotic pathway (Oltvai *et al.*, 1993; Simon, 2000). Transforming growth factor-β1 (TGF-β1) production inhibits autocrine production of pro-survival cytokines *in vitro*, suggesting the possibility of a balance of these cytokines *in vivo* (Alam *et al.*, 1994).

Eosinophil apoptosis may be important in the resolution of airway inflammation in asthma. The reduction of eosinophil numbers in response to glucocorticoid treatment appeared to be governed, in part, due to increased eosinophil apoptosis (Woolley *et al.*, 1996). Triggering of eosinophil apoptosis in murine airways using an anti-Fas mAb resulted in decreased airway eosinophilia after allergen challenge (Tsuyuki *et al.*, 1995). A greater understanding of apoptosis is needed to result in the development of novel therapeutic agents that would promote the safe and rapid removal of eosinophils from tissue before they exert their toxic effects.

1.4.3 Eosinopoiesis and the role of IL-5

As discussed previously in section 1.1.1, IL-5 is crucial for eosinophil development, and there is plentiful evidence for an increase in IL-5 production in the airways in asthma. IL-5 prolongs the survival of mature eosinophils in tissue and primes them for activation and degranulation. IL-5 was primarily thought to exert its effects at a late stage of eosinopoiesis, influencing the terminal differentiation of CD34+ progenitors in the bone marrow (Ema *et al.*, 1990). Subsequently, it was demonstrated that IL-5Rα is expressed on CD34+ cells, suggesting that co-localisation may be indicative of eosinophil lineage-committed precursors (Sehmi *et al.*, 1997). Circulating CD34+ progenitors have been detected within the peripheral blood (Sehmi *et al.*, 1996); recently, it has been suggested that eosinophils may differentiate from these locally within the respiratory mucosa.

Robinson *et al.* demonstrated a significantly increased population of CD34+/ IL-5Rα mRNA+ cells in the airways of asthmatics compared with normal controls, with a correlation between numbers of these cells and asthma severity (Robinson *et al.*, 1999). Cameron *et al.* confirmed that a subset of eosinophils could differentiate from CD34+/ IL-5Rα precursors within allergic nasal mucosa using an *ex vivo* explant system, eliminating the possibility that any increases in cell number were due to eosinophil infiltration (Cameron *et al.*, 2000). Thus, there is strong evidence that, in addition to the infiltration of mature eosinophils that has been documented, a population of eosinophils could differentiate locally within allergic nasal mucosa, under the influence of IL-5. The increase in peripheral blood eosinophils in asthma is the consequence not only of increased haematopoiesis, but also of an increased rate of egress from the bone marrow. The mechanism of emigration from bone marrow sinuses is only just emerging, but is thought to be mediated by IL-5, eotaxin, α4 and β2 integrin expression, and L-selectin shedding (Collins *et al.*, 1995; Palframan *et al.*, 1998a; Palframan *et al.*, 1998b).

1.4.4 Selective adhesion pathways

The pattern of adhesion receptors expressed by eosinophils is similar to other leukocytes, although eosinophils, unlike neutrophils, express functional forms of VLA-4, VLA-6, and α4β7. Eosinophils also express CD11d/ CD18 (αdβ2), which functions as an alternative ligand for VCAM-1. The correct complimentary signals must also be present on the post-capillary endothelium in order for tissue accumulation of eosinophils to occur. To some extent, the cytokine milieu present dictates which receptors mediate adhesion. Hence, selective pressures leading to tissue accumulation may be exerted at different stages of the multi-step paradigm, as described below.

1.4.4.1 Tethering/ rolling

VLA-4 can promote both tethering and firm arrest and it has attracted the most interest as a possible receptor mediating selective eosinophil adhesion, as it is not expressed on human neutrophils under physiological conditions. The allergic cytokines IL-4 and IL-13 (section 1.4.4.1.2) upregulate the expression of VCAM-1, the ligand for VLA-4 (Schleimer *et al.*, 1992; Bochner *et al.*, 1995). Eosinophil transmigration through IL-4/ IL-13-stimulated HUVEC was dependent on VLA-4 in one study (Bochner *et al.*, 1995). Studies in animal models have demonstrated that blocking VLA-4 and VCAM-1 inhibited eosinophil migration into the lung and skin and prevented bronchial hyperresponsiveness (Bochner and Schleimer, 1994).

P-selectin is also an attractive alternative candidate for mediating eosinophil tethering. Using nasal polyps as a model of chronic eosinophilic inflammation, Symon *et al.* found that eosinophil and neutrophil adhesion to nasal polyp endothelium (NPE) was mediated by P-selectin, which was well expressed on NPE (Symon *et al.*, 1994). In this model, up to ten times more eosinophils bound to NPE than neutrophils, pointing to a selective adhesion pathway. The importance of P-selectin in eosinophil accumulation in allergic disease has been further underlined by the use of gene-deleted mice. Eosinophil accumulation was reduced in the airways of P-selectin deficient mice after allergen challenge (Broide *et al.*, 1998b). A mAb against P-selectin, but not E-selectin, reduced eosinophil influx into the pleural cavity in a mouse model of pleuritis (Henriques *et al.*, 1996). In a ragweed peritonitis model, eosinophil accumulation was reduced by 75% in P-selectin knockout mice, with an additional contribution from VCAM-1 and ICAM-1 (Broide *et al.*, 1998a).

1.4.4.1.1 Cytokines

Cytokines such as TNF-α, IL-1β, IFN-γ, and LPS act early on in the inflammatory response to induce the expression of cell adhesion receptors, by influencing immediate response genes. These cytokines have no effect on P-selectin expression in humans, although increases in expression have been seen in mouse models (Sanders *et al.*, 1992; Weller *et al.*, 1992). This observation supports the concept of a potential role of P-selectin in situations where these cytokines are not prominent. The Th2 cytokines IL-4, IL-13, and IL-9 are thought to be important in allergic disease and their potential roles in selective eosinophil accumulation are discussed below.

IL-4 and IL-13 share many biological characteristics. Both cause Ab class switching to production of IgE and IgG_4, induce cell surface expression of integrins, and induce the production of chemokines important for the recruitment of cells in allergic immune responses (Brombacher, 2000). The overlapping biological functions of IL-4 and IL-13 are due to at least one shared component of otherwise distinct receptors. The IL-4 receptor comprises an IL-4Rα chain and a common γ chain that is shared by the receptors for IL-2, IL-7, IL-9, and IL-15. The IL-4Rα chain associates with either the γ chain to form an IL-4 receptor, or with IL-13Rα1 to form a functional IL-13 receptor (Aman *et al.*, 1996; Hilton *et al.*, 1996). The IL-4Rα chain is responsible in both receptor complexes for signal transduction. Monoclonal antibodies directed against the IL-4Rα inhibit the effects of IL-13 as well as IL-4. Signal transduction via IL-4Rα includes the activation of Jak1-dependent pathways, which involves tyrosine phosphorylation of Stat6 (Curiel *et al.*,

1997). Studies in Stat6-deficient mice indicate that Stat6 activation is critically involved in both IL4- and IL-13-mediated disorders, including allergic disease (Takeda *et al.*, 1997).

IL-4 was recently shown to cause a marked increase in P-selectin mRNA and intracellular protein expression, with a more modest increase in surface expression (Yao *et al.*, 1996). Eosinophil rolling on IL-4-stimulated HUVEC was shown to be mediated by P-selectin and VCAM-1 (Patel, 1998). At the time of this study, it had not been addressed whether IL-13 could also induce P-selectin expression on HUVEC and furthermore, support eosinophil rolling.

IL-9 is a multi-functional cytokine secreted by activated T cells that has pleiotropic effects on a variety of cell types. Recently, a role for IL-9 has been suggested in allergy and asthma (Nicolaides *et al.*, 1997). IL-9 has been linked to a genetic predisposition to asthma because its encoding gene is located in the chromosomal region (5q23-q33) that includes the cytokine gene cluster implicated in bronchial inflammation associated with asthma (Doull *et al.*, 1996). The *in vivo* importance of IL-9 in the pathophysiologic mechanisms of asthma has been suggested mainly by animal models of airway inflammation. Gene-mapping studies in mice demonstrate linkage between the regulation of airway hyperresponsiveness and the IL-9 locus (Nicolaides *et al.*, 1997). Studies with IL-9 transgenic mice show that over-expression of IL-9 selectively within the lungs results in a characteristic human asthmatic phenotype, such as increased bronchial hyperresponsiveness, elevated IgE levels, and mucus hypersecretion (McLane *et al.*, 1998; Temann *et al.*, 1998). Increased expression of IL-9 mRNA has been found in human airways from atopic asthmatic subjects compared to normal subjects (Shimbara *et al.*, 2000). Recently, the expression of IL-9 and the IL-9R was evaluated in the BAL and epithelial cells of mild asthmatic airways compared with healthy airways (Bhathena *et al.*, 2000). IL-9 was not detected in the airways of either group. In contrast, IL-9R expression was found in asthmatic but not normal airway samples. Recently, neutrophils have been shown to express the IL-9R, with surface expression on asthmatic neutrophils being greater than on neutrophils from normal controls (Abdelilah *et al.*, 2001). Thus, further study of IL-9 and its receptor may provide potentially useful insights into the pathogenesis of asthma.

1.4.4.2 Activation and Firm Adhesion

The activation stage is thought to be mediated by chemoattractants on the endothelium,

signalling through PTX-sensitive GPCRs (Campbell *et al.*, 1998). This process has been modelled *in vitro* with the use of flow chambers to observe the effect of adding stimuli on leukocyte behaviour when binding to purified adhesion proteins. For example, neutrophils roll on slides coated with purified E- and P-selectin and ICAM-1, and only stop if an activating stimulus is added such as PAF or IL-8 (Zimmerman *et al.*, 1997). Many studies on eosinophil adhesion and transmigration have been carried out on HUVEC, and evidence suggests that firm arrest events are mediated via a combination of VLA-4 binding to VCAM-1 and the β2 integrins binding to ICAM-1/ ICAM-2. LFA-1 and Mac-1 are involved in CD18-mediated binding (Bochner and Schleimer, 1994; Blease *et al.*, 1998; Yamamoto *et al.*, 1998).

In summary, there are selective signals for eosinophil adhesion to endothelium at each stage of the established paradigm for leukocyte adhesion, that is, tethering, activation, and firm arrest. Additionally, the local cytokine milieu seems to be crucial in governing which adhesion receptors dominate in mediating capture and firm adhesion.

AIMS

The purpose of the studies described in this thesis was to investigate the molecular receptors and mediators involved in the multi-step paradigm of eosinophil adhesion to endothelium. The hypothesis being tested was that eosinophil-specific pathways of adhesion were involved, which were in part responsible for the relatively selective accumulation of eosinophils in diseases such as asthma. It was hoped that these studies would reveal potential targets for therapeutic intervention directed at specific inhibition of the airway eosinophilia in asthma.

Two models were chosen to investigate the adhesion paradigm. Firstly, a rotational frozen section assay (FSA) employed nasal polyp endothelium as a model of chronic airway inflammation. Secondly, human umbilical vein endothelial cells (HUVEC) were used in a parallel plate flow chamber assay, which simulated the shear conditions likely to be experienced in the post-capillary venules. HUVEC were stimulated with cytokines to mimic the milieu present in allergic inflammation. In both models, the behaviour of eosinophils on the endothelium was assessed and compared to that of neutrophils. Chemoattractants and blocking monoclonal antibodies against adhesion receptors were added to both the FSA and the flow system in order to dissect out the mediators and receptors involved.

CHAPTER TWO

Materials and Methods

2.1 Materials

2.1.1 Antibodies

All antibodies were used at optimal saturating concentrations and were monoclonal, unless otherwise stated.

2.1.1.1 Primary antibodies

Primary antibodies against adhesion receptors, cytokines, and chemokines used in the FSA and for flow cytometry are summarised in Tables 2–1, 2–2, and 2–3. Primary antibodies used in immunostaining experiments are detailed in Table 2–4. Mouse IgG1, mouse IgG2a, mouse IgG2b control antibodies were purchased from Dako Ltd., Ely, Cambs, UK.

2.1.1.2 Secondary antibodies

Rabbit anti-mouse biotinylated $F(ab')_2$ Ig, rabbit anti-mouse Ig-FITC conjugate, rabbit anti-mouse Ig unconjugated, and rabbit anti-rat Ig unconjugated were all purchased from Dako Ltd., Ely, UK.

2.1.2 Other reagents

2.1.2.1 Cytokines

Recombinant human (rh) GM-CSF, TNF-α, IL-5, IL-9, IL-13, and IL-4 were purchased from R & D Systems Europe, Abingdon, UK.

2.1.2.2 Chemokines

rh RANTES, eotaxin, and IL-8 were purchased from R & D Systems Europe, Abingdon, UK. The soluble 8 kDa chemokine portion of fractalkine was a kind gift of Tom Schall, Chemocentryx, CA, US.

2.1.2.3 Agents involved in activation experiments

Pertussis toxin (from *Bordetella pertussis*), and sodium azide were purchased from Sigma Chemical Co., Poole, UK. Platelet activating factor (PAF) was purchased from Bachem (UK) Ltd., St. Helens, UK. The metalloprotease inhibitor KD-IX-73-4 was a generous gift of Dr. T. Kishimoto, Boehringer Ingelheim Pharmaceuticals, Inc., CT, US.

Table 2–1 Selectin and Ig family molecule primary antibodies

Adhesion receptor	Clone	Blocking Ab? (Y/ N)	Origin
P-selectin	G1	Y	R. McEver, University of Oklahoma, OK, US (gift)
	G1	Y	Bender MedSystems Diagnostics (Alexis Corp Ltd.), Nottingham, UK
	RUU-SP1.18	N	K. Nieuwenhuis, University Hospital Utrecht, Utrecht, The Netherlands (gift)
L-selectin	Dreg 56	Y	T. Kishimoto, Boehringer Ingelheim Ltd., CT, US (gift)
VCAM-1	4B9	Y	R. Lobb, Biogen, Inc., Cambridge, MA, US (gift)
ICAM-1	R6.5 $(F(ab)_2)$	Y	R. Rothlein, Boehringer Ingelheim Ltd., CT, US (gift)
	15.2	Y	N. Hogg, ICRF, London, UK (gift)
ICAM-2	BT-1	Y	Serotec Ltd., Kidlington, UK

Table 2–2 Integrin primary antibodies

Integrin	Clone	Blocking Ab? (Y/ N)	Origin
CD18	7E4, AZN-L18, CLB-LFA-1, AZN-L27	unknown	VIth HLDA Workshop, Kobé, Japan, 1996
	MHM23	Y	Dako Ltd., High Wycombe, UK
	L130	Y	Becton Dickinson Ltd., Oxford, UK
CD11a	CD11a-5E4, AZN-L20, AZN-L21,	unknown	VIth HLDA Workshop, Kobé, Japan, 1996
	38	Y	Cymbus Bioscience Ltd., Southampton, UK
	SPV-L7	Y	Bradsure Biologicals Ltd., Loughborough, UK
CD11b	44	Y	Cymbus Bioscience Ltd., Southampton, UK
	2LPM19c	Y	Dako Ltd., High Wycombe, UK
CD11c	3.9	Y	Cymbus Bioscience Ltd., Southampton, UK
	KB90	Y	Dako Ltd., High Wycombe, UK
CD11d	169A, 169B, 217L, 240I	Y	D. Staunton, ICOS Corps, WA, US (gift)
CD29 (β1)	P4C10	Y	Life Technologies Ltd., Paisley, UK
CD49d (α4 / VLA-4)	HP2/1	Y	Serotec Ltd., Kidlington, UK
	HP1/2	Y	F. Sánchez-Madrid, Hospital de la Princesa, Madrid, Spain (gift)
	P4C2	Y	Chemicon International Inc., Harrow, UK
α4β7	Act-1	Y	Leukosite Inc., Cambridge, MA, US (gift)

Table 2–3 Cytokine and chemokine primary antibodies

Cytokine/ chemokine	Clone	Blocking Ab? (Y/ N)	Origin
IL-5	mAb7	Y	A. Proudfoot, Glaxo Wellcome R & D, Geneva, Switzerland (gift)
	TRFK-5	Y	A. Proudfoot, also D. Slater, Schering–Plough Ltd., Bury St. Edmonds, UK (gift)
	14611.3	Y	R & D Systems Europe, Abingdon, UK
IL-3	M1	Y	Genzyme Corporation, Cambridge, UK
GM-CSF	126.2.1.3.2	Y	Genzyme Corporation, Cambridge, UK
IL-8	B-K8	Y	Serotec Ltd., Kidlington, UK
	A5.12.14	Y	D. Washington, Genentech Inc., San Francisco, CA, US (gift)
CXCR1	9H1.5.1	Y	D. Washington (gift)
CXCR2	10H2.12.1	Y	D. Washington (gift)
Eotaxin	72D	Y	A. Proudfoot (gift)
RANTES	VL-1, VL-3, VL-4	Y	A. Proudfoot (gift)
CCR3 (eotaxin receptor)	LS63 7B11	Y	C. Mackay, Leukosite Inc., Cambridge, MA, US (gift)

2.1.2.4 Cell culture media

HUVEC were cultured in an endothelial growth medium bullet kit (Biowhittaker, Wokingham, UK), which consisted of endothelial cell basal medium (EBM) supplemented with 2% FBS, 3 mg/ ml BBE, 10 ng/ ml hEGF, 50 µg/ ml gentamicin, and 50 ng/ ml amphotericin-B. In an improvement to the culture method (see Methods section 2.2.3.1), the FBS content was increased to 12%.

Cord collection medium consisted of HBSS (Life Technologies Ltd., Paisley, UK) supplemented with 10 µg/ ml gentamicin antibiotic (Sigma Chemical Co, Poole, UK). PBS without calcium or magnesium ions (Life Technologies Ltd., Paisley, UK) was used as the HUVEC washing medium.

2.1.2.5 Miscellaneous media/ wash buffers

The wash buffer used in leukocyte purification consisted of 1x HBSS, 30mM Hepes (Life Technologies Ltd., Paisley, UK) and 5mM EDTA (Sigma Chemical Co., Poole, UK). This buffer was also used for the flow chamber assay, omitting the EDTA and supplementing with 1mM $CaCl_2$, 1mM $MgCl_2$, and 0.5% human serum albumin (HSA). Medium 199 (M199) used in the FSA and PBS were purchased from Life Technologies Ltd., Paisley, UK.

2.1.2.6 Eosinophil and neutrophil separation apparatus/ reagents

Anti-CD16 immunomagnetic beads, iron wool separation columns, and 3-way taps were bought from Miltenyi Biotech, Bisley, UK. The stands for the columns were custom-made by the Biomedical Workshop, Leicester University, Leicester, UK. High field strength 'Major' magnets were supplied by the Cromwell Tools Group, Freeman's Common, Leicester, UK. Dextran (from *Leuconostoc spp*, mw 110,000) was purchased from Fluka Chemicals Ltd., Glossop, UK. Histopaque 1083 and 1077 were purchased from Sigma Co. Ltd., Poole, UK.

2.1.2.7 Miscellaneous reagents

The streptavidin-biotin alkaline phosphatase conjugated complex used in immunostaining was purchased from Dako Ltd., Ely, UK. The New Fuchsin substrate kit used in immunostaining was purchased from Biomen Ltd., Finchampstead, UK. Human serum albumin was bought from First Link (UK) Ltd., Brierly Hill, UK. All other general chemicals (methanol, propan-1-ol, xylene, hydrochloric acid, sodium chloride, calcium and

magnesium chloride, acetone) were supplied by Fisher Scientific, Loughborough, UK or Analytical Supplies (BDH), Little Eaton, UK. Other reagents, unless otherwise stated, were supplied by Sigma Chemical Co. Ltd., Poole, UK.

2.2 Methods

2.2.1 Cell staining

2.2.1.1 Kimura stain

A stain originally developed for basophils by Kimura *et al.* was used to count white blood cells and identify eosinophils (Kimura *et al.*, 1973). The cytoplasm of eosinophils stains a light green colour while the cytoplasm of other cells remains clear. The nuclei of all the leukocytes stain purple. The stain was prepared as follows:

1) A phosphate buffer, pH 6.4, was prepared from a 1:3 mixture of 0.08M Na_2HPO_4 and 0.06M KH_2PO_4.
2) A solution of toluidine blue was made up by adding 50 ml of 0.3 M NaCl solution to 0.05 g of toluidine blue. To this mixture, 22 ml of 96% IMS was added and the volume made up to 100 ml with distilled water.
3) The stain was prepared by mixing together 11 ml of toluidine blue, 0.8 ml light green solution (0.03 g in 100 ml H_2O), 0.5 ml saturated saponin in 50% IMS and 5 ml phosphate buffer. After filtering, the mixture was ready for use. 10 µl of whole blood/ cell suspension was added to 90 µl of Kimura in an eppendorf, thoroughly mixed and transferred to a cover-slipped haemocytometer chamber for counting.

2.2.1.2 Trypan Blue stain

Trypan Blue was used for viable cell counting. The stain is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do. Staining also facilitated the visualisation of cell morphology. The stain was purchased as a pre-prepared solution (Sigma Co. Ltd., Poole, Dorset, UK). Trypan blue was mixed 1:1 (vol) with cell suspension and then transferred to a cover-slipped haemocytometer chamber for counting.

2.2.2 Isolation of peripheral blood eosinophils and neutrophils

Eosinophils

Whole human peripheral blood from normal or mildly atopic individuals (absolute count of less than or equal to 0.5×10^6 eosinophils per ml of blood) was drawn into 2×50 ml syringes, each containing 0.5 ml heparin (100U) as an anti-coagulant, using a 21G butterfly needle. A drop of blood was set aside to calculate the total white cell count and absolute eosinophil count of the donor. 10 ml 6% dextran in HBSS was added to each syringe and blood was allowed to settle out for 45 min to remove the majority of erythrocytes. The resulting plasma layers were removed via a 19G needle, then spun at 200 g for 10 min at RT. The supernatants were discarded and the remaining pellets pooled and resuspended in a small volume of wash buffer (see Methods section 2.1.2.5). This was topped up to 30 ml and then carefully layered over 2×10 ml Histopaque 1083 in universal tubes. These were spun at 395 g for 25 min at RT. The resulting monocyte layer was discarded and the universals wiped with cotton buds to remove any residue. The cell pellets were then pooled and spun down in ice cold wash buffer at 290 g, 8 min, 4 °C. The supernatant was discarded and the cell pellet gently lysed in 20 ml ice cold distilled water for 30 seconds, topped up with cold ($2 \times$) wash buffer, and spun down at 290 g, 8 min, 4 °C. The resulting pellet was then resuspended in a small volume of cold wash buffer and a sample removed for determination of total cell number, before being topped up to 50 ml and washed again as before. The supernatant was discarded. The volume of anti-CD16 immunomagnetic beads to add was calculated thus:

$$\text{Volume of beads to add } (\mu l) = \frac{\text{Total cell number}}{5 \times 10^7} (\times 50) / 2$$

The beads were added directly to the cell pellet followed by 100 µl wash buffer and the suspension was mixed gently. The cell pellet was incubated on ice for 40 min with the anti-CD16 beads to remove contaminating neutrophils, with occasional mixing. MACS separation columns were chosen appropriately depending on the total cell number (a 'B2' column for $0.5\text{-}1 \times 10^8$ cells, a 'C' column for between $1\text{-}2 \times 10^8$ cells, or a combination of these). The columns were regenerated using syringes and a 3-way tap, by flushing PBS through them to remove ethanol, followed by ice cold wash buffer, ensuring that any air trapped in the column was removed. Columns were stored at 4 °C until use.

At the end of the 40-min incubation period, the separation columns were placed in perspex holders, which were inserted between the poles of a magnet. A 3-way tap and a 23G needle were attached to the bottom of each column. Wash buffer was flushed through the tap and needle to remove any trapped air. The cell pellet was resuspended in the correct volume of cold wash buffer (2 ml per B2 column, 4 ml per C column) and loaded onto the column (s). The filtrate (CD16-ve eosinophils) was collected in a 50 ml Falcon tube on ice. Columns were then washed through with 4 times the column volume of wash buffer (16 ml for a B2 column, 32 ml for a C column), which was collected in the same tube. The collection tubes were then spun down at 290 g for 8 min at 4 °C. Meanwhile, the columns were removed from the magnet and flushed through with wash buffer to remove neutrophils. Columns were next flushed with PBS, distilled water, and finally ethanol, which was left in the columns until their next use. Columns were used repeatedly and discarded when either rusting became apparent, or if the cell yield or purity was much lower than normal.

The supernatant from the collecting tubes was discarded and the cell pellet resuspended in a small volume of assay medium for counting. Eosinophils collected using this method were routinely >95% pure and >95% viable. Contaminating cells were monocytes.

Neutrophils

Neutrophils were collected in the same way as eosinophils, except Histopaque 1077 was used in place of Histopaque 1083, and the separation process was stopped after lysis. Again, neutrophils collected by this method were routinely >95% pure and >95% viable. Contaminating cells were eosinophils.

2.2.3 Cell culture

2.2.3.1 HUVEC isolation

Method 1

Human umbilical vein endothelial cells (HUVEC) were isolated using two methods. Both were adaptations of a protocol from Jaffe *et al.* (Jaffe *et al.*, 1973). In the first, umbilical cords at least 15 cm in length, containing no clamp marks or punctures, were collected in 50-100 ml transport buffer. Cords were usually collected within 12 h of delivery, and immediately washed thoroughly under running tap water and milked of blood to remove as many clots as possible. After drying the cord was transferred to a Class II Microbiological Safety Cabinet (MSC) and irrigated on the outside thoroughly with 70% industrial

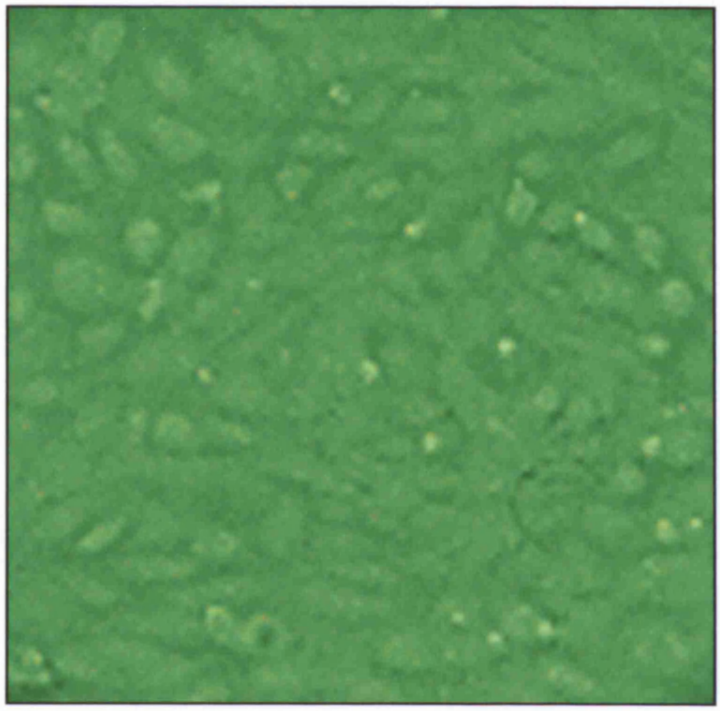
methylated spirits (IMS). A 1 cm section was cut from each end of the cord and the veins were identified and teased open. A 16G cannula was inserted into the vein at both ends and tied in place with strong, sterile suture. A 20 ml syringe was filled with washing buffer, attached to the cannula at one end, and the cord was flushed through until the buffer ran clear. The cord was wrapped in a layer of clingfilm followed by a layer of aluminium foil, and more buffer was flushed through to check that flow was still established. Next, 5-10 ml (depending on cord length) of sterile-filtered collagenase in HBSS (10 μg/ ml) was inserted from a 10 ml syringe until it reached the other end of the cord. This end was then capped off and the vein inflated slightly with more collagenase. Finally the syringe end of the vein was capped off, and the cord was incubated for 20 min at 37 °C (5% CO_2, 95% humidity).

Following incubation, the cord was rolled gently along its length to aid cell detachment, flushed through with 25 ml M199/ 2% FBS, and the cell/ enzyme mixture was collected in a 50 ml tube. Digested cells were centrifuged at 1000 rpm (200 g) for 5 min at RT. The resultant pellet was resuspended in 5 ml of supplemented EBM, transferred to a 25 cm^3 culture flask, and placed in a 37 °C incubator (5% CO_2, 95% humidity). Culture medium was changed the next day and the cells microscopically checked. By this stage, if successful, small clusters of HUVEC in active growth were evident. Only 1 out of every 3 cords provided viable primary cultures, for reasons unknown. Primary cultures were usually confluent within 3 days but occasionally took longer. Cultures that took longer than 5 days to reach confluence were discarded. Fresh medium was provided every 2-3 days. Once confluent, the HUVEC ceased division and had to be subcultured. A typical confluent HUVEC monolayer is shown in Figure 2–1.

Method 2

Method 1 was further adapted until there was a >95% success rate in culture. Briefly, cords were collected and transferred to MSCs identically to Method 1. Cords were digested similarly to Method 1, with some minor differences. Wider bore cannulae were used (12G), which ensured that no leakage occurred, the cord was distended more fully with collagenase, and the cell dislodging technique was improved. In addition, there was a higher percentage of serum in the culture medium (12%) and cells were grown in gelatin-coated tissue culture flasks. These changes resulted in a much improved HUVEC viability and growth rate.

Figure 2–1 A confluent HUVEC monolayer



2.2.3.2 Subculturing

Monolayers were washed briefly with 2 ml PBS, which was discarded. 1 ml trypsin/ EDTA (0.05%/ 0.02%), was then added, and flasks were incubated at 37 °C (5% CO_2/ 95% humidity). Cells were monitored microscopically until detached (2-4 min). Flasks were tapped sharply against a bench to aid detachment, and 1 ml HUVEC medium was added to neutralise the trypsin. Cells were removed to a 15 ml tube, centrifuged at 201 g for 5 min at RT, and the supernatant discarded. The cell pellet was resuspended in 1-3 ml EBM, whereupon cell viability was assessed with Trypan Blue (see Methods section 2.2.1.3). The cells were then resuspended in the correct volume of EBM according to cell number and the vessel (s) being used. HUVEC were used as first passage cultures in all studies undertaken.

2.2.3.2.1 Fibronectin coating method

All HUVEC culture vessels were coated with human plasma fibronectin diluted in PBS to 40 μg/ ml. The vessels were then incubated at 37 °C for 1 h, the fibronectin was removed, and the vessels were left to dry for 15 min, before being securely wrapped in aluminium foil and stored until used (within 1 month).

2.2.3.2.2 96-well plates

For static adhesion assays (refer to section 2.2.7), HUVEC were seeded into 96-well microtitre plates at a density of 10-20,000 viable cells per well in 100 µl EBM. The cells were then cultured to confluence (1-2 days).

2.2.3.2.3 Chamber slides

For some flow assays, HUVEC were seeded into plastic 2-well LabTek II chamber slides (Life Technologies, Paisley, UK). Only one well out of the two on each slide was used for culture, as this resulted in sufficient coverage of HUVEC for the flow assay. Each well had a growth area of 4 cm^2. HUVEC were seeded at a density of 100-150,000 cells per well, in 2.5 ml HUVEC medium, and cultured to confluence (1-4 days). If necessary, the culture medium was partially changed after 2 days, by removing and discarding 1 ml of spent medium and replacing it with 1 ml of fresh medium.

2.2.3.2.4 Tissue culture dishes

For the remainder of the flow assays, HUVEC were seeded into 35 mm tissue culture dishes (Corning Costar, High Wycombe, UK) that had a growth area of 8 cm^2. Seeding density was raised to 200-250,000 per dish, in 2.5 ml HUVEC medium. Cells were cultured to confluence (1-4 days), changing culture medium after 2 days if necessary.

2.2.3.3 Cytokine stimulation of HUVEC

HUVEC monolayers were grown until approximately 90% confluent before stimulation with the appropriate cytokines. In 96-well plates, wells had 50 µl of medium aspirated, which was replaced with 50 µl of cytokine-containing medium to result in the correct final concentration of cytokine. Control wells had 50 µl of untreated medium added. In chamber slides and tissue culture dishes, 1 ml of medium was removed and replaced with 1 ml cytokine-containing medium to result in the correct final concentration of cytokine. HUVEC were stimulated with IL-4 (20 ng/ ml) and IL-13 (5 ng/ ml) for 48 h, and with TNF-α (50 ng/ ml) for 6 h. These concentrations were found to be optimum in dose response experiments carried out by Dr. G. Woltmann.

2.2.4 Flow cytometry

A basic, single-colour protocol was used, which focused on measuring the mean and median fluorescence intensity produced by fluorescent-labelled antibodies and ligands binding to specific cell-associated molecules.

2.2.4.1 Indirect labelling of leukocytes for FACS analysis

FACS buffer consisted of PBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1% BSA. Cells were kept at 4 °C throughout the experiment, unless otherwise stated. Freshly isolated eosinophils or neutrophils were washed and resuspended in ice-cold FACS buffer at 2-5 × 10^6/ ml. 100 µl cell suspension was added to each assay tube. The appropriate amount of primary antibody was added, the cells were vortexed briefly, and tubes were incubated for 15 min on ice. Next, cells were washed by adding 1 ml FACS buffer to the tubes, and spinning them for 7 min at 200 g, 4 °C. The supernatant was tipped off and the resulting cell pellet was resuspended in 100 µl buffer, before addition of a FITC-labelled second antibody (1:10 dilution) for 15 min, 4 °C. Cells were washed as before and resuspended in 300-400 µl FACS buffer. Cells were analysed using a Becton-Dickinson FACScan® (Becton Dickinson, Cowley, Oxfordshire, UK) and Lysis II software. Propidium iodide (PI) at a final concentration of 1 µg/ ml was added to the tubes in order to gate on live cells. PI is a fluorescent stain for nucleic acids, hence only binds to dead, lysed cells.

2.2.4.2 Indirect labelling of HUVEC for FACS analysis

Confluent HUVEC monolayers were trypsinised (section 2.2.3.2), washed, and resuspended in ice-cold FACS buffer at 2-5 × 10^6/ ml. 100 µl cell suspension was added to each assay tube and the method continued as detailed for leukocytes (section 2.2.4.1).

2.2.5 Immunohistochemistry

Immunostaining was used to investigate the profile of adhesion receptors expressed by nasal polyps, nasal biopsies, and nasal turbinates.

2.2.5.1 Subjects and study design

2.2.5.1.1 Nasal polyp study

Nasal polyps were obtained from patients undergoing polypectomy at the ENT department of the Leicester Royal Infirmary. The patients' medical histories were not known in detail,

but patients were not receiving topical corticosteroid treatment. Seven subjects were evaluated, although not for all the adhesion receptors studied (n = 4-7). Human tonsils were obtained from patients undergoing tonsillectomy as control tissue for some antibodies (n = 3).

2.2.5.1.2 Nasal biopsy and inferior turbinate study

Subjects with symptomatic perennial allergic rhinitis were recruited from the staff and patients of Glenfield Hospital and the ENT outpatients department of the Leicester Royal Infirmary. All subjects with rhinitis had symptoms consistent with perennial rhinitis and at least one positive allergic skin prick test to common aeroallergens, including house dust mite, cat, dog, and grass pollen. None of the subjects were receiving steroid treatment. Nasal tissue from patients with allergic rhinitis was obtained by nasal biopsy (n = 7) or resection of inferior turbinates (n = 4). Normal controls included nasal biopsies from healthy volunteers (n = 6) and inferior turbinate material (n = 3) resected from patients with a deviated septum or mechanical nasal obstruction.

Turbinate tissue was trimmed into pieces approximately 1 cm^3, and mounted onto cork discs, snap-frozen, and stored in the vapour phase of liquid nitrogen until use. Nasal biopsies were approximately 2 mm^2 and were embedded in lamb's kidney for support. They were then placed on cork, snap-frozen in liquid nitrogen, and stored as above until use.

2.2.5.1.3 Adhesion receptor staining

In both studies, tissue was sectioned and immunostained for the following adhesion receptors: the pan-endothelial marker PECAM-1, the immunoglobulin family members ICAM-1 and VCAM-1, and the selectin family members P- and E-selectin. The presence of eosinophils was assessed using a marker for the eosinophil granule protein, MBP. Stained and mounted sections were blinded before being graded according to a visual 5-point scoring scale. The primary mAbs used to detect these markers and the scoring scale are shown in Table 2–4.

2.2.5.2 Silane coating of slides

Microscope slides were coated with 3-aminopropyltriethoxysilane in acetone, which enabled tissue to adhere optimally to slides. Silane coating was carried out in a fume hood. Slides were racked and coated by dipping in a 2% solution of silane in acetone, followed

by two brief dips in 100% acetone, and finally two brief dips in distilled water. The racks were placed on tissues to remove excess liquid from the slides, which were left to air dry.

2.2.5.3 Tissue sectioning

Frozen tissue was sectioned using a Bright FS cryostat (Bright Instrument Co Ltd., Huntingdon, UK). The Stamper–Woodruff frozen section assay required tissue to be slightly thicker than immunostaining experiments (8 μm and 6 μm respectively). This was necessary to allow cells layered on top of the tissue to interact with the endothelium. Sections were cut at temperatures of –20 °C and –30 °C, with biopsies requiring the cooler cutting temperature compared to nasal polyps.

2.2.5.4 Immunostaining procedure

At all times during the following procedure, care was taken to ensure that the sections did not dry out, and all incubations were carried out in a humidified chamber. TBS wash buffer was made up from 0.05M Tris and 0.15M NaCl, pH 7.6. 6 μm frozen tissue sections were cut and allowed to air dry. A hydrophobic marker pen was used to draw a circle around the sections. Slides were then fixed, either in acetone for 10 min, RT, or 4% paraformaldehyde (for P-selectin) in PBS for 5 min, RT. Following fixation, non-specific binding was blocked by incubation in 20% rabbit serum in TBS for 10 min, RT. Excess serum was tipped off and 100 μl of appropriately diluted primary antibody in TBS was added to the sections (Table 2–4). After incubation for 60 min at RT with gentle rotation (or overnight at 4 °C, stationary), excess unbound antibody was removed with a 5 min wash in TBS. Slides were then incubated with 100 μl of appropriately diluted biotinylated secondary antibody (1:400 in TBS, 30 min, RT, gentle rotation). Immediately after the secondary antibody was applied, the avidin-biotin complex (ABC) solution was prepared, by mixing 1 μl of streptavidin with 1 μl biotinylated alkaline phosphatase and 1ml TBS. Excess unbound secondary antibody was washed off (5 min in TBS) and 100 μl of the ABC solution was added to each section for 30 min at RT with gentle rotation. Finally, excess ABC solution was washed off as above and slides were replaced in the humid chamber ready for staining with substrate solution.

Table 2–4 Primary mAbs used for immunohistochemical staining of adhesion receptors and eosinophils in upper airway tissue (nasal polyps, nasal biopsies and nasal turbinates)

Clone (all monoclonal)	Specificity (human)	Isotype (murine)	Working concentration or dilution	Source
MoIgG	(rabbit-anti-mouse)	IgG1	1:20	Dako Ltd. (Ely, UK)
EN4	PECAM-1	IgG1	Supernatant, 1:20	TCS Biologicals (Botolph Claydon, UK)
1.18	P-selectin	IgG1	Purified Ig, 1 μg/ ml	Gift from Dr. H. K. Nieuwenhuis (Utrecht University Hospital, Utrecht, Netherlands (gift)
BBIG-E4	E-selectin	IgG1	Purified Ig, 1 μg/ ml	R&D Systems Europe (Abingdon, UK)
BBIG-V1	VCAM-1	IgG1	Purified Ig, 1 μg/ ml	R&D Systems Europe (Abingdon, UK)
BBIG-I1	ICAM-1	IgG1	Purified Ig, 1 μg/ ml	R&D Systems Europe (Abingdon, UK)
BT-1	ICAM-2	IgG1	Purified Ig, 10 μg/ ml	Serotec Ltd. (Oxford, UK)
BMK13	MBP	IgG1	Purified Ig, 10 μg/ ml	TCS Biologicals (Botolph Claydon, UK)

Key for Grading Immunostaining in Tissue

I = Intensity of substrate staining according to a visual scale of 0 to 5 where 0 = no staining, 1 = very weak, 2 = weak, 3 = moderate, 4 = strong, 5 = very strong.
E = Extent of stain through tissue according to a visual scale of 0 to 5 where 0 = 0%, 1 = 0-10%, 2 = 10-30%, 3 = 30-50%, 4 = 50-75%, 5 = >75%

2.2.5.5 New Fuchsin substrate

Fresh substrate was always prepared immediately prior to use. 50 µl of New Fuchsin chromogen solution was combined with 50 µl of New Fuchsin activator solution, and mixed by repeated gentle pipetting for at least 30 seconds. Thorough mixing was essential for successful colour development. 5 ml of Tris buffer (0.1M Tris/ HCl, pH 8.2) was added to the chromogen-activator mixture and mixed well. Finally, 400 µl of substrate solution was added to the mixture. At this stage, levamisole was added if endogenous alkaline phosphatase activity was suspected. 100 µl of substrate was added to the tissue sections and incubated until the colour developed (5-25 min). Slides were washed and counter-stained as detailed in section 2.2.5.4, dried overnight, and mounted in XAM, a xylene-based organic mounting medium.

2.2.6 Stamper–Woodruff frozen section assay

2.2.6.1 Nasal polyp collection and freezing

After excision, polyps were placed into sterile 50 ml pots and transferred immediately to the laboratory. The time taken from polyp excision to freezing was usually 30 min-1 h. On arrival at the laboratory, the polyp was examined for any gross abnormalities (which, if found, excluded the polyp from being used), and cut into pieces about 0.5 cm^3. Where possible, pieces were chosen which could visibly be seen to contain blood vessels. The polyp was mounted on cork and lowered into liquid nitrogen until the nitrogen just covered the cork, but left the polyp above the surface. After a few seconds, polyps were completely submerged for a few more seconds, then left in nitrogen until ready to be placed into cryotubes labelled with the date, patient reference, and tube number. It was usual to obtain several pieces of tissue from one patient (n = 2-10). Polyps were stored in the vapour phase of liquid nitrogen until ready for use. Each polyp had some sections removed from it, which were stained with May Grünwald-Giemsa. This stain allowed assessment of the tissue for blood vessels. Only polyps that contained more than 100 blood vessels were used for frozen section assays.

2.2.6.2 Nasal biopsies

Nasal biopsies were taken from consenting volunteers already undergoing broncho-alveolar lavage. These were mounted, with the epithelium upright, in OCT on a piece of cork. The cork was then placed on a beaker of frozen isopentane suspended in liquid nitrogen. This method enabled the tissue to freeze slowly, and avoided bubbles forming in the OCT, which disturbed the tissue.

2.2.6.3 Frozen section assay

Eosinophils or neutrophils were resuspended at 5×10^6/ ml in M199, adding EGTA where necessary. Cells were stored on ice while 8 μm frozen sections of tissue were cut and placed on silane-coated microscope slides. These were allowed to air-dry before the assay for at least 30 minutes. A hydrophobic marker pen was used to draw a circle around the section. For adhesion blockade experiments, there were two possible options depending on whether the marker being studied was cell- or endothelial-expressed. For a cellular marker, cells were incubated with the appropriately diluted mAb for 15 min at RT, prior to the FSA. For an endothelial marker, 100 μl of appropriately diluted mAb was used to cover tissue sections and left on for 30 min with gentle rotation, before excess Ab was tipped off against a tissue and the FSA continued. Slides were placed in an incubation tray and 100 μl of cell suspension was carefully pipetted over the tissue sections. The incubation trays were then placed onto a Belly Dancer shaker machine (Scotlab, Lanarkshire, UK), and rotated at speed 5 (70 rpm), 30 min, RT. Following rotation, excess medium and cells were drained from the sections by tipping the edge of the slide onto a tissue. Slides were then fixed in 2% gluteraldehyde in PBS for 30 min without rotation, and washed in PBS for 10 min with rotation at setting 3 (56 rpm). Following washing, slides were placed in an incubation tray for staining with May Grünwald-Giemsa.

2.2.6.4 May Grünwald-Giemsa stain

Four drops of filtered neat May Grünwald-Giemsa stain were added to each tissue section using a pipette and left for 1 min. Next, four drops of dH_2O were added on top of the May Grünwald-Giemsa and left for 4 min. Slides were then washed in dH_2O for 5 min, with the rotation speed at setting 3. Slides were removed, had excess liquid wiped away from around the sections, and were replaced in the incubation tray. Three drops of Giemsa stain (1:20 dilution in dH_2O) were added to the sections for 10 min. Slides were transferred to racks once more and washed in dH_2O as before. Sections were air-dried overnight before being mounted in XAM for assessment.

2.2.7 Quantitative adhesion assay for granulocytes based on ^{51}Cr labelling

A static adhesion assay for measurement of eosinophil adherence to endothelial cells was performed as previously described (Lamas *et al.*, 1988). The assay is useful in defining adhesion-stimulatory or inhibitory substances that might act on either the leukocyte or the endothelial cells. Using specific antibodies or other blocking reagents, the precise receptor-ligand pairs responsible for the leukocyte adhesion can be characterised.

2.2.7.1 ^{51}Cr labelling

Purified eosinophils were washed into M199/ 2% FBS and resuspended in a 15 ml tube at a concentration of 5×10^6/ ml for labelling with $Na^{51}CrO_4$ (sodium chromate). 3 mCi of $Na^{51}CrO_4$ was added per 5×10^6 cells and cells were incubated at 37 °C for 1 h, with occasional gentle shaking. After incubation, cells were washed twice with M199/ 2% FBS in order to remove unincorporated radioactivity (243 g, 8 min, RT), and resuspended in M199/ 2% FBS at a concentration of 1.5×10^6/ ml for the assay. 100 µl of cell suspension was aliquoted into each of three tubes that were kept to one side as controls, in order to calculate percentage adhesion later.

2.2.7.2 Treatment of eosinophils and endothelial cells

Adhesion assays were performed on HUVEC cultured to confluence in 96-well plates. Plates were washed with assay medium by removing the culture medium already in the wells, adding 50 µl of assay medium, shaking the plate gently to wash, and finally removing and discarding this medium. This was done just prior to commencing the assay and care was taken not to let the endothelial cells dry out.

HUVEC monolayers were stimulated prior to the assay, where appropriate, with cytokines as follows: TNF-α, 50 ng/ ml, 6 h; IL-4, 20 ng/ ml, 48 h; IL-13, 5 ng/ ml, 48 h. In some experiments, HUVEC were subsequently treated with histamine at a physiologically relevant concentration (10^{-5} M, 15 min). Blocking antibodies were used to determine the adhesion molecules involved in eosinophil adhesion to endothelium. Antibodies against cell-expressed receptors were incubated with eosinophils for 15 min at RT, and 100 µl of appropriate antibody-containing cell suspension was added to the endothelial cells. Antibodies against endothelial receptors were added to the plate in 20 µl of medium for 30 min at RT, and 100 µl of radiolabelled granulocyte suspension was added on top, without removing the antibody from the plate. Each condition was performed in triplicate. Upon

completion of the antibody incubations, plates were incubated for 30 min at 37 °C, to allow eosinophils to adhere to the endothelial cells. The three control tubes containing untreated cells were also incubated.

2.2.7.3 Removal of non-adherent cells

After incubation, plates were washed by topping up the wells with M199/ 2% FBS, so that a meniscus of fluid could be seen above each well. Plates were then covered with adhesive Mylar sealing tape, inverted and centrifuged at 18 g for 5 min, RT. After spinning, the sealing tape was removed with the plates still inverted, and non-adherent cells were gently flicked out. 50 µl of trypsin-EDTA (0.05%/ 0.02%) was added to each well to remove endothelial cells and adherent eosinophils. Plates were wrapped in aluminium foil and frozen, along with the control tubes for later assessment (usually within 24 h).

2.2.7.4 Assessment of percentage adhesion

Once a plate was ready to be assessed, it was left to thaw and the contents of the wells were transferred to labelled plastic test tubes. Each well was washed out with 50 µl of assay medium, which was also added to the appropriate tube. The number of eosinophils in each well was assessed by counting the γ-emission from each tube in a gamma counter (Auto-Gamma counting system, Packard, Meriden, UK). The percentage of adherent eosinophils was calculated by averaging the triplicates for that condition, and comparing this value with the average value of the three control tubes. Percentage adhesion was calculated as the percentage difference between the control and the test condition.

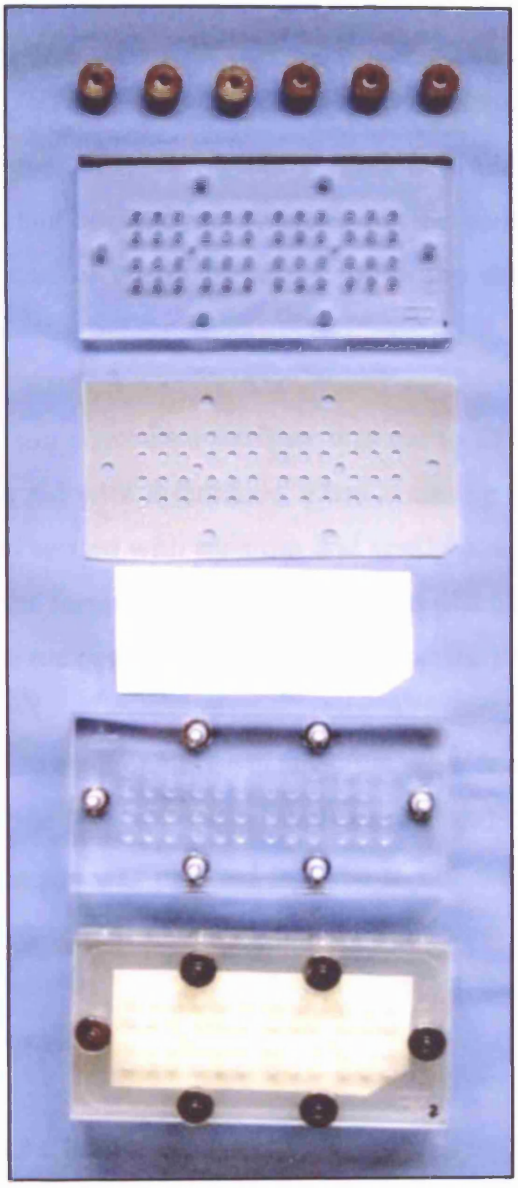
2.2.8 Microchemotaxis assay

A modified Boyden chamber microchemotaxis assay (Falk *et al.*, 1980) was used to assess the migration of eosinophils and neutrophils towards a chemoattractant.

2.2.8.1 Chemotaxis equipment

A 48-well microchemotaxis assay chamber (Figure 2–2) was purchased from Neuroprobe Inc., Cabin John, MD, US). 8 µm nitrocellulose membrane filters were supplied by Sartorius Instruments Ltd., Belmont, UK.

Figure 2–2 A microchemotaxis assay chamber



Screws

Top portion of chamber

Rubber gasket

8μm nitrocellulose filter

Bottom portion of chamber

Assembled chamber

2.2.8.2 Chemotaxis method

The chemotaxis chamber and all its components were rinsed in dH_2O and dried thoroughly before and after each use. Care was taken at all times during the assay to ensure that no grease or foreign particles got into the chamber/ on the chemotaxis membrane. 25 µl chemoattractant or diluent control was added to the lower wells of the chamber. It was important not to over- or under-fill the wells. Wells that were filled correctly had a small meniscus of chemoattractant above the rim, which allowed a seal to form between the membrane and the plate.

An 8 µm nitrocellulose filter was cut to fit exactly over the wells, and one corner was cut off to aid with orientation when counting cells after staining of the filter. The filter was lightly wetted with medium and gently placed over the lower wells, taking care that no air bubbles formed. A rubber gasket was placed over the top of the chemotaxis filter, followed by the top portion of the chamber, and the chamber was screwed firmly together.

Purified eosinophils or neutrophils were resuspended in M199/ 2% FBS at a concentration of 4-5 $\times$ 10^6/ ml, and treated appropriately (see individual results sections). 50 µl of cell suspension was pipetted into the top of the chamber wells, and immediately the chamber was placed in a humid box to minimise evaporation. The box was then placed in an incubator at 37 °C for 90 min to allow the cells to migrate. All tests were performed in triplicate.

2.2.8.3 Chemotaxis filter staining

After incubation, the chamber was carefully disassembled, taking care not to damage the nitrocellulose filter. The filter was gently lifted off the lower wells using fine forceps, and placed into a staining trough containing IMS for 30 min to fix the cells. Subsequently, staining of the filter was carried out in a fume hood, as follows:

1. Haematoxylin, 5 min
2. Tap water, 5 min, until colour of filter turned dark purple
3. IMS, 2 min
4. Propan-1-ol, 2 min
5. Propan-1-ol/ xylene, 2 min
6. Xylene, 40 min

After staining, the filter was cut into four sections vertically (3 $\times$ 4 wells each), and quickly placed upside down onto four numbered microscope slides, the numbers aiding decoding

of the tests. The filter was then mounted in xylene-based mountant and coverslipped. It was important not to let the filter dry out during staining. After mounting, the filter was stored at 4 °C until analysis. Usually it could be left for 48 h before showing signs of drying out, if this occurred, the coverslip was gently removed with xylene, and the filter was re-mounted.

2.2.8.4 Chemotaxis filter analysis

A permanent marker pen was used to mark out the filter well areas on the slide. Slides were analysed using the × 40 objective of a microscope. Within each well, the cells that had migrated to the bottom of the filter were counted. Since the filter was mounted upside down, these cells were the ones to first come into view as the focus was moved down. It was important to keep the plane of focus constant within a well area. Each well area allowed 7-10 fields to be assessed and tests were performed in triplicate wells, giving an average value of number of cells migrated/ field of between 20-30 fields.

2.2.9 Laminar Flow assay

A flow chamber assay was used to investigate eosinophil and neutrophil adhesion to endothelial cells at physiological flow rates. This assay allows visualisation of cell adhesion under well-defined wall shear stresses that are similar to those existing *in vivo* in postcapillary venules. It was subsequently possible to quantify the different events of cell adhesion by selective image acquisition and image processing.

2.2.9.1 Materials

35 mm sterile tissue culture dishes were purchased from Corning Costar, High Wycombe, UK. Dual plastic 2 cm^2 Lab Tek II chamber slides were bought from Life Technologies Ltd., Paisley, UK. "Silastic" medical grade tubing was purchased from Sani-Tech (UK) Ltd., Havant, UK. Additional tubing was supplied by Glycotech, Rockville, MD, US. 1 ml, 10 ml, and 50 ml syringes were supplied by Southern Syringe Co. Ltd., London, UK. Small 3-way taps were purchased from Miltenyi Biotech, Bisley, UK. High vacuum grease was bought from Merck Ltd., Poole, UK. A Perthése 0.5 mm silicon gasket was purchased from Osteotec Ltd., Christchurch, UK. Additional silicon gaskets were supplied by Glycotech. An Edwards 'Vacuubrand' model RZ2 vacuum pump was purchased from Fisher Scientific, Loughborough, UK.

Two parallel plate flow chambers were used. The first was a rectangular 3D polycarbonate

chamber (Figure 2–3), kindly donated by M. Lawrence, University of Virginia, Charlottesville, Virginia, US), which was used in conjunction with the LabTek II slides and OsteoTec gaskets. This chamber became worn over time, hence the remainder of the experiments used a round 3D parallel plate flow chamber (Glycotech, Rockville, MD, US), shown in Figure 2–4 in conjunction with the 35 mm culture dishes. This chamber has been described in detail by Brown *et al.*, 1999.

2.2.9.2 Method

To simulate the fluid shear stresses present in the microvasculature, a parallel plate geometry was used for the flow chamber (Lawrence *et al.*, 1987). The chamber consisted either of the chamber slide or tissue culture dish (depending on the chamber used) seeded with a confluent monolayer of endothelial cells, that was attached to a polycarbonate chamber base. These two flat surfaces were held apart by a lightly greased silicon gasket. The chamber was attached, using connectors and silastic tubing, to a vacuum pump, cell/ buffer reservoir and an automated syringe pump (model 22, Harvard Apparatus Inc., Edenbridge, UK), which was used to control flow rates. Experiments were conducted at shear rates of either 1 ml/ min or 1.5 ml/ min. Figure 2–5 is a photograph of the flow assay set-up.

After purification, leukocytes were washed twice in flow assay buffer (HBSS/ Hepes/ 0.5% HSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$), to remove EDTA from the separation buffer, and resuspended at a concentration of 5×10^6/ ml in 50 ml Falcon tubes. Where appropriate, cells were stimulated with 100 ng/ ml PTX for 2 h, 37 °C, with occasional gentle mixing. For IL-5 dose response experiments, cells were treated with 0.05, 0.5, or 5 ng/ ml of IL-5 at 37 °C for 30 mins. In experiments examining the combined effects of IL-5 plus PTX, IL-5 was used at 0.5 ng/ ml. Medium controls were incubated alongside treated cells. When incubations were complete, leukocytes were gently resuspended in additional flow assay buffer at a final concentration of 1.0×10^6 cells/ ml, for perfusion in the flow chamber. HUVEC were grown to confluence in dishes/ slides and stimulated appropriately (see section 2.2.3).

Figure 2–3 Parallel plate flow chamber (supplied by M. Lawrence)

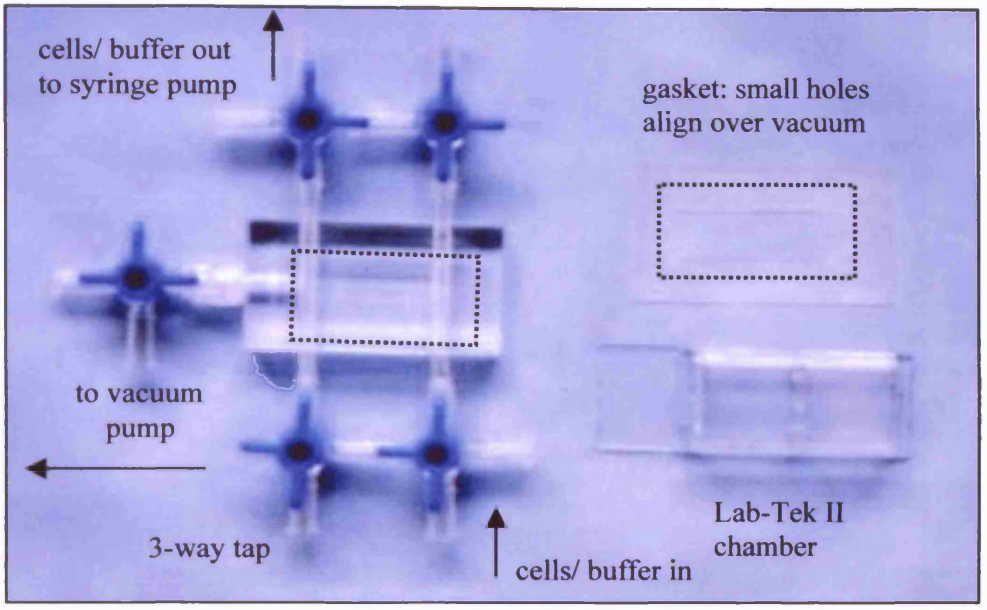


Figure 2–4 Parallel plate flow chamber (Glycotech Corps, Rockville, MD, US)

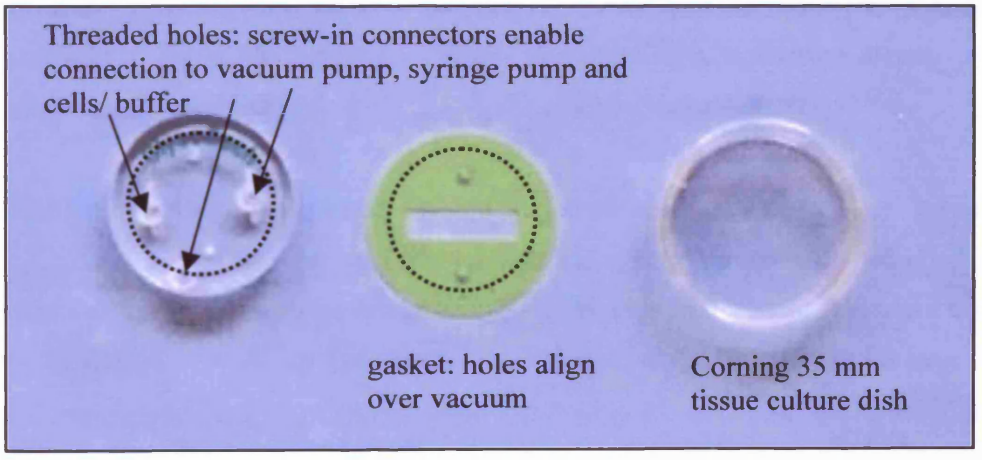
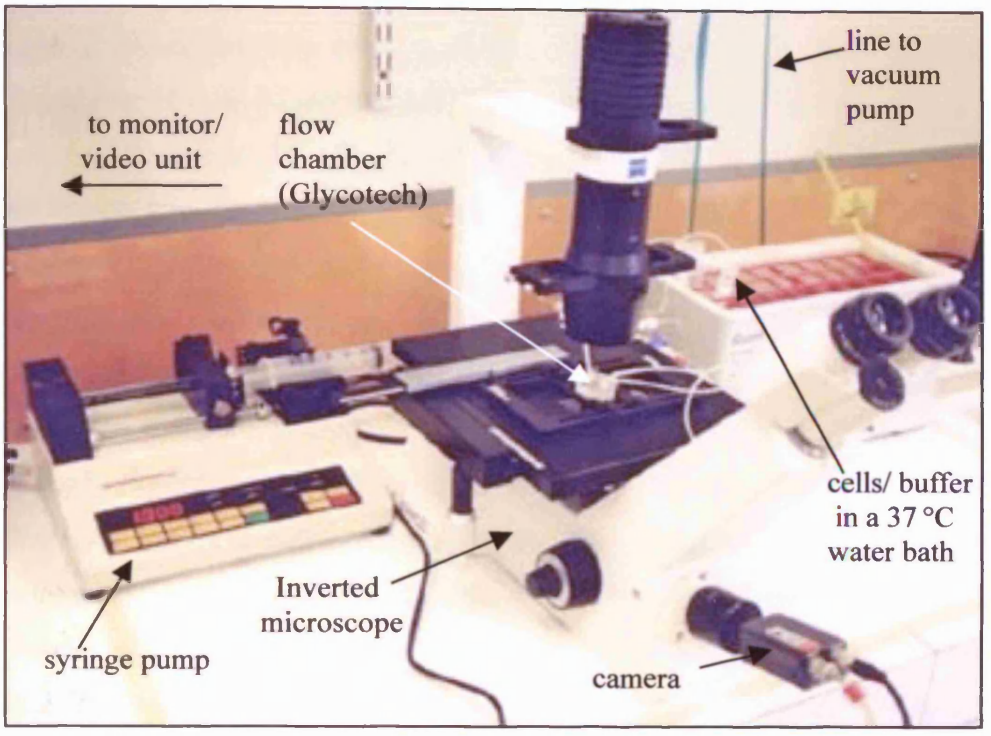


Figure 2–5 Laboratory set-up of parallel plate flow chamber assay



For blocking experiments, either leukocytes or HUVEC were pre-incubated for 15-30 min with function blocking mAbs. Most tests using eosinophils (except where stated) were carried out in singlicate, due to constraints with the availability of cells.

The assembled chamber was placed on the stage of a Zeiss Axiovert 25 inverted microscope (Zeiss, Jena, Germany) and the vacuum and syringe pumps switched on. Flow assay buffer was drawn through the chamber and all taps and tubing before each test using a syringe, to ensure that all air bubbles were excluded. Cells were perfused over the endothelial monolayer using the syringe pump attached to the outlet side of the chamber, and leukocyte-endothelial interactions were monitored with a × 40 objective. In experiments with the Lawrence chamber, the shear stress used was 1.5 dynes/ cm^2; with the Glycotech chamber, it was 1 dyne/ cm^2. For some experiments, an activating stimulus dissolved in flow buffer (RANTES, eotaxin, IL-5, or PAF) was introduced into the system from a Falcon tube reservoir in the water bath. Tubing was washed out thoroughly after an activation stimulus between tests by drawing flow buffer through the system.

2.2.9.3 Evaluation of tethering and accumulation, and enumeration of arrested, rolling and detached cells

Images of leukocytes interacting with the endothelium during flow were captured on a CCD camera (Hitachi Model KP-M1E/ K) and videotaped using a time-lapse videocassette recorder (JVC, model SR-L900E), video monitor (Hitachi, model VM1202K), and video timer (For.A Co. Ltd., Model VTG-33). Leukocytes were perfused for 2 min to obtain sufficient cell-endothelial interactions. Total cell accumulation was quantified by counting neutrophils and eosinophils in 15-20 video frames after a 2-min interaction for each condition. Rolling velocity was calculated by securing a sheet of acetate over the video monitor, and using a fine-tipped marker to track the movement of individual cells over at least 1 min of footage. A calibrating graticule was then used to equate the distance moved on the video screen to that on the acetate by the cell in μm/ sec. At least 2 different fields were observed, and at least 10 cells from each field were tracked (minimum 20 cells). The percentage of rolling compared to adherent cells was also determined over an average of at least 3 fields for a minimum period of 30 seconds. In some experiments, an activating stimulus was perfused through the chamber after the initial 2 min interaction for 4 min, before being washed out with flow assay buffer. Leukocyte behaviour was observed before, during, and after the addition of the stimulus.

2.2.10 Statistics

Statistical differences between experimental groups were evaluated using the unpaired Student's t-test. Where data were not normally distributed, or when sample numbers were low, the Mann-Whitney U test was used as a non-parametric method for the comparison of two independent random samples. Statistical calculations were carried out using Arcus Quickstat (Camcode, Cambridge, UK). P values ≤ 0.05 were considered significant.

CHAPTER THREE

Characterisation of the integrins involved in eosinophil and neutrophil adhesion to nasal polyp endothelium using the FSA

3.1 Introduction

As discussed, nasal polyps can be used as a research model that offers a unique opportunity for the study of leukocyte adhesion to inflamed upper airway endothelium. In recent years, the Stamper–Woodruff frozen section assay (FSA), which was originally used to model lymphocyte homing to HEV (Stamper, Jr. and Woodruff, 1976), has been adapted by Symon *et al.* to study eosinophil and neutrophil binding to nasal polyp endothelium (NPE) (Symon *et al.*, 1994). Using this model, both cell types were found to bind specifically to blood vessels, and adhesion was almost completely inhibited with mAbs against P-selectin and PSGL-1. Eosinophils also bound more avidly than neutrophils to NPE and to P-selectin under shear flow conditions. A possible explanation for this was thought to be due in part to eosinophils expressing a structurally and functionally distinct isoform of PSGL-1 to neutrophils (Symon *et al.*, 1996).

Thus, the selectin stage of the multi-step paradigm of adhesion described in the introduction has been successfully investigated for eosinophils and neutrophils using the FSA. No data have been published on the contribution of the integrin and activation signals involved for these cells using a nasal polyp model, with one exception. In the study by Symon *et al.* (Symon *et al.*, 1994), of the integrins looked at, only a mAb directed against the α chain of Mac-1 caused significant inhibition of eosinophil adhesion, and this was very modest in degree. Hence, the purpose of this chapter was to determine whether eosinophil and neutrophil binding in the FSA was integrin-dependent, consistent with the established multi-step paradigm of leukocyte adhesion. The activation step will be examined in chapter 4.

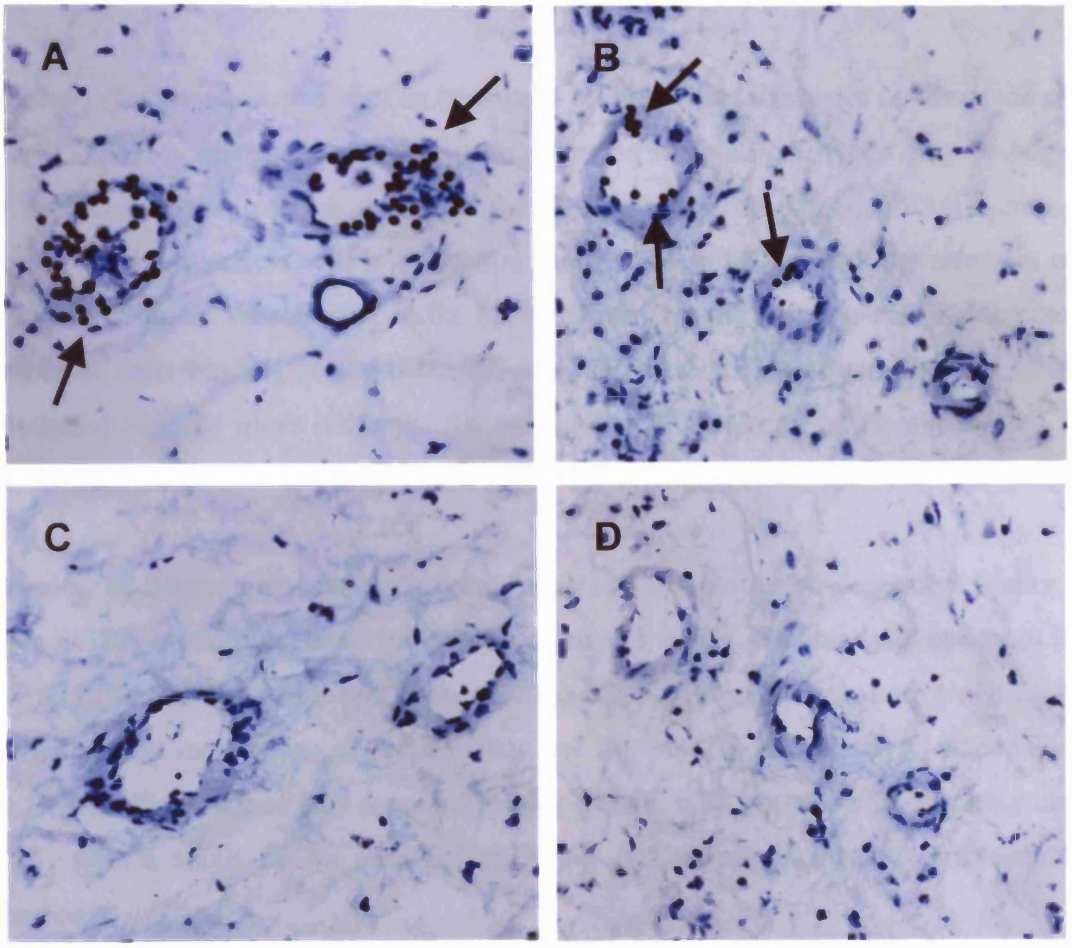
As one of the laboratories involved in the 6th Human Leukocyte Differentiation Antigen (HLDA) workshop, we obtained a blind panel of monoclonal antibodies against adhesion structures, including eight raised against the β2 integrins (Table 3–1).

3.2 Results

3.2.1 Eosinophil and neutrophil adhesion to NPE is cation-, rotation speed-, temperature-, and concentration-dependent

The FSA was already well established in the laboratory and was initially practised under supervision. As previously shown, eosinophils and neutrophils bound selectively to blood vessels with little background binding to stromal structures (Figure 3–1, a) and b)). Two

Figure 3–1 Eosinophils and neutrophils bind specifically to NPE. The FSA was carried out as shown in the Methods, section 2.2.6. Representative example of the pattern of eosinophil and neutrophil binding with (C and D) and without (A and B) EGTA. A and C: eosinophils; B and D: neutrophils. Arrows denote cells bound.



adherent cells were taken as the minimum to record a positive blood vessel, although most positive vessels bound considerably more cells. As previously stated, eosinophils bound in greater numbers than neutrophils to NPE, both in the percentage of positive blood vessels, and in the number of cells binding per vessel.

Adhesion was cation-dependent, as treatment of either the leukocytes or the tissue sections with EGTA almost completely abrogated adhesion for both cell types (Figure 3–1 c) and d)). Decreasing the rotation speed of the Belly Dancer used in the FSA increased the number of blood vessels within the tissue that bound cells, and also the numbers of cells binding per blood vessel (Appendix 1). However, as the rotation speed decreased, the number of cells binding in the tissue stroma increased, making quantification of specific endothelial binding more difficult. An optimum rotation speed of 70 rpm (speed 5) was therefore adopted.

Adhesion of eosinophils and neutrophils was cell concentration-dependent, being barely above EGTA-treated levels at cell densities below 1×10^6 cells/ ml, with optimum binding at 5×10^6 cells/ ml (data not shown). Above 7×10^6 cells/ ml, background adhesion throughout the section was observed (data not shown). As previously reported (Symon *et al.*, 1994), adhesion was also temperature dependent, with optimal binding being observed at RT, with a small fall in adhesion at 37 °C and an approximately 50% reduction in adhesion at 4 °C.

After a leukocyte has been captured by receptors on the endothelium, it becomes loosely tethered to the endothelial surface and begins to roll. It may then detach, or alternatively, arrest and flatten, enabling it to transmigrate. To arrest, leukocytes need to become activated through outside-in signalling. This occurs via integrins binding to endothelial-expressed counter-receptors that are members of the immunoglobulin family and include ICAM-1, ICAM-2, and VCAM-1.

The effect of inhibiting the β2 integrins was studied. A detailed examination of the role of CD18 in eosinophil and neutrophil binding was undertaken using an extensive panel of mAbs, available either commercially, or through the 6th HLDA workshop. Tables 3–1 and 3–2 summarise the mAbs used. The reactivity of the HLDA mAbs was first tested using flow cytometry. Experiments were carried out on cells from three different volunteers. Adhesion blockade studies were only carried out with mAbs that showed good reactivity

with eosinophils in the flow cytometry assays (mean of 3 experiments > 35% binding, Appendix 2). Eight HLDA mAbs directed against CD18, CD11a, and CD11b were investigated in the FSA. mAbs against CD11c were not provided by the HLDA workshop. Fifteen mAbs were investigated in total.

3.2.2 Eosinophil and neutrophil adhesion to NPE is CD18-dependent

Figure 3–2 shows inhibition of CD18 using a mAb clone (MHM23) that was representative of six antibodies directed against CD18. Consistent inhibition of both eosinophil (range 35.9 to 55.6%) and neutrophil (range 57.6 to 78.5%) adhesion was observed.

3.2.3 Eosinophils and neutrophils demonstrate a difference in usage of CD11α chains for adhesion to NPE

The β chain of the leukocyte integrins must dimerise with an individual CD11α chain in order to be functional, therefore the effect of inhibiting each chain was examined (CD11, a, b, and c), illustrated in Figure 3–3. Again, consistent effects were seen for each cell type with each α chain, and one representative clone is shown. The results are shown as percentage inhibition of cell adhesion for clarity. In the case of eosinophils, inhibition was seen with antibodies against all three integrins. Eosinophils and neutrophils used CD11b to adhere equally. Eosinophils favoured CD11a usage, and there was a small contribution for eosinophils from CD11c. In contrast, anti-CD11a antibodies did not inhibit neutrophil adhesion; instead, neutrophils were consistently inhibited by mAbs against CD11c. A single experiment was undertaken using mAbs against CD11d, which was recently discovered as a fourth member of the β2 integrin family. There was little inhibitory effect of clones 169A, 169B, 217L or 240I in this experiment (Figure 3–4).

Table 3–1 Summary of β2 integrin Abs used from 6th HLDA workshop (Kobé, Japan)

Code	Specificity	Clone	Origin	Isotype	% +ve FACS (eos, $n = 3$)	Eos % inhibition ± SEM (n), *P* value	Neut % inhibition ± SEM (n), *P* value
A014	CD18	7E4	mouse	IgG1	85.33	47.29 ± 9.07 (7) $p < 0.001$	-21.86 ± 30.4 (4) n/s
A086	CD18	AZN-L18	mouse	IgG1	90.02	35.97 ± 9.92 (7) $p < 0.0007$	1.48 ± 7.8 (4) n/s
A089	CD18	AZN-L27	mouse	IgG1	93.87	52.46 ± 7.13 (7) $p < 0.0006$	59.07 ± 15.4 (4) $p < 0.05$
A078	CD11a	CD11a-5E4	mouse	IgG1	40.89	60.66 ± 9.00 (6) $p < 0.007$	Not done
A087	CD11a	AZN-L20	mouse	IgG1	85.17	28.1 ± 8.00 (7) $p < 0.0001$	Not done
A088	CD11a	AZN-L21	mouse	IgG2b	91.54	25.41 ± 11.5 (7) $p < 0.0006$	1.49 ± 7.85 (4) $p < 0.01$
A001	CD11b	LT-11	mouse	IgG1	82.79	16.73 ± 17.33 (3) $p < 0.04$	57.94 ± 8.48 (4) $p < 0.016$
A015	CD11b	Bear-1	mouse	IgG1	86.62	2.26 ± 9.58 (3) n/s	-19.74 ± 19.43 (4) n/s

Key: (n) = number of experiments

n/s = not significant

Table 3–2 Summary of commercially available β2 integrin Abs used in function blocking studies

Source	Specificity	Clone	Origin	Isotype	Epitope targeted	Eos % inhibition ± SEM (n), *P* value	Neut % inhibition ± SEM (n), *P* value
Dako	CD18	MHM23	mouse	IgG1	I domain	55.56 ± 5.86 (7) $p < 0.0001$	67.67 ± 7.04 (9) $p < 0.002$
Becton Dickinson	CD18	L130	mouse	IgG1	I domain	46.33 ± 4.68 (4) $p < 0.001$	78.48 ± 8.4 (4) n/s
Monosan	CD11a	SPV-L7	mouse	IgG1	Unknown	27.12 ± 12.86 (4) $p < 0.01$	-87.1 ± 26.38 (4) n/s
Becton Dickinson	CD11a	G25.2	mouse	IgG2a	EF hand repeats	22.45 ± 4.16 (4) $p < 0.0003$	-23.43 ± 30.41 (5) n/s
Cymbus Bioscience	CD11a	38	mouse	IgG2a	Unknown	36.87 ± 3.63 (3) $p < 0.003$	28.95 ± 6.49 (3) n/s
Becton Dickinson	CD11b	D12	mouse	IgG2a	EF hand repeats	15.11 ± 5.44 (4) $p < 0.0006$	23.78 ± 13.6 (5) $p < 0.005$
Dako	CD11b	2LPM19c	mouse	IgG1	I domain	27.98 ± 6.83 (11) $p < 0.0001$	25.16 ± 7.17 (5) $p < 0.0005$
Cymbus Bioscience	CD11b	44	mouse	IgG1	I domain	31.02 ± 10.11 (4) $p < 0.006$	54.03 ± 9.41 (7) $p < 0.003$
Dako	CD11c	KB90	mouse	IgG1	C-terminal domain	39.64 ± 15.89 (4) $p < 0.03$	56.64 ± 9.59 (4) $p < 0.02$
Bradsure Biologicals	CD11c	SHCL-3	mouse	IgG2b	N-terminus (not I domain)	28.51 ± 12.56 (3) $p < 0.03$	30.41 ± 24.88 (3) $p < 0.01$
Cymbus Bioscience	CD11c	3.9	mouse	IgG2b	I domain	21.82 ± 9.13 (3) $p < 0.01$	42.38 ± 6.34 (3) $p < 0.01$

Key: See Table 3–1, page 88

Figure 3–2 Eosinophil and neutrophil adhesion to NPE is CD18-dependent. Eosinophils and neutrophils from different donors were purified (Methods, section 2.2.2) and incubated for 15 min at RT with anti-CD18 mAb clone MHM23, control antibody, or EGTA-treated assay medium, before use in the FSA (Methods, section 2.2.6.2). Filled columns, eosinophils, $n = 7$; hatched columns, neutrophils, $n = 9$. Values shown indicate means ± SEM, ** $P < 0.005$, *** $P < 0.0001$.

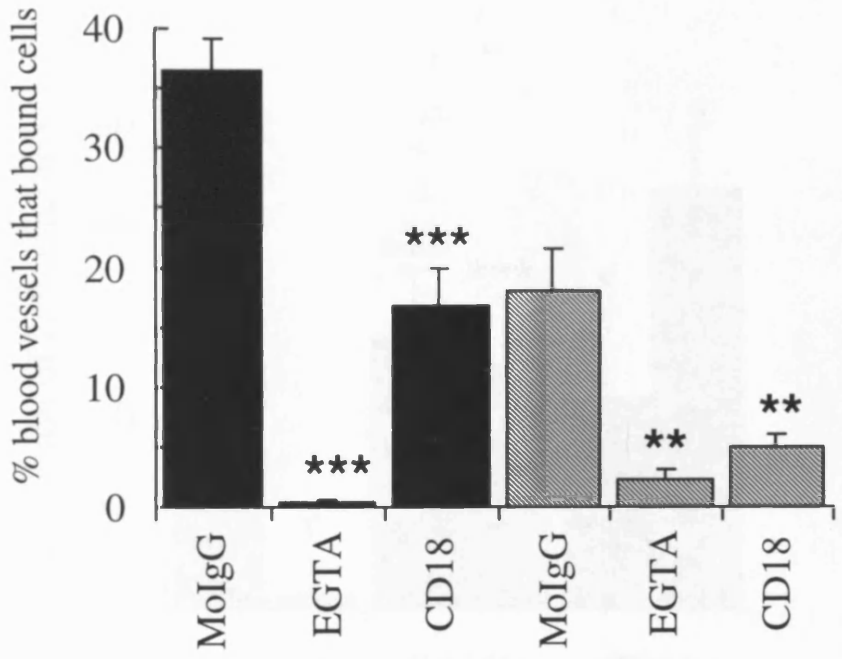


Figure 3–3 Contribution of individual CD11α-chain integrins to eosinophil and neutrophil adhesion to NPE in the FSA. Eosinophils and neutrophils from different donors were purified (Methods, section 2.2.2) and incubated for 15 min at RT with anti-CD11a (AZN-L21), anti-CD11b (2LPM19c), or anti-CD11c (3.9), before use in the FSA (Methods, section 2.2.6.2). Filled columns, eosinophils, n = 3-11; hatched columns, neutrophils, n = 3-5. Values represent the mean percentage inhibition of cell adhesion compared to isotype-matched control antibodies ± SEM, * P <0.01, *** P < 0.0005.

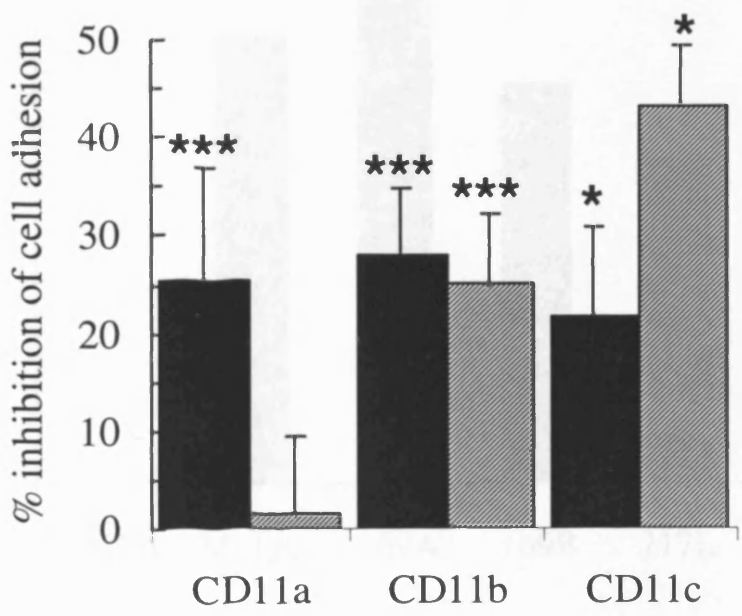


Figure 3–4 Effect of a panel of CD11d antibodies on eosinophil adhesion to nasal polyp endothelium. Eosinophils from different donors were purified (Methods, section 2.2.2), and incubated for 15 min at RT with MoIgG control, EGTA-treated assay medium, or anti-CD11d mAb clones 169A, 169B, 217L, or 240I, before use in the FSA (Methods, section 2.2.6.2). $n = 1$.

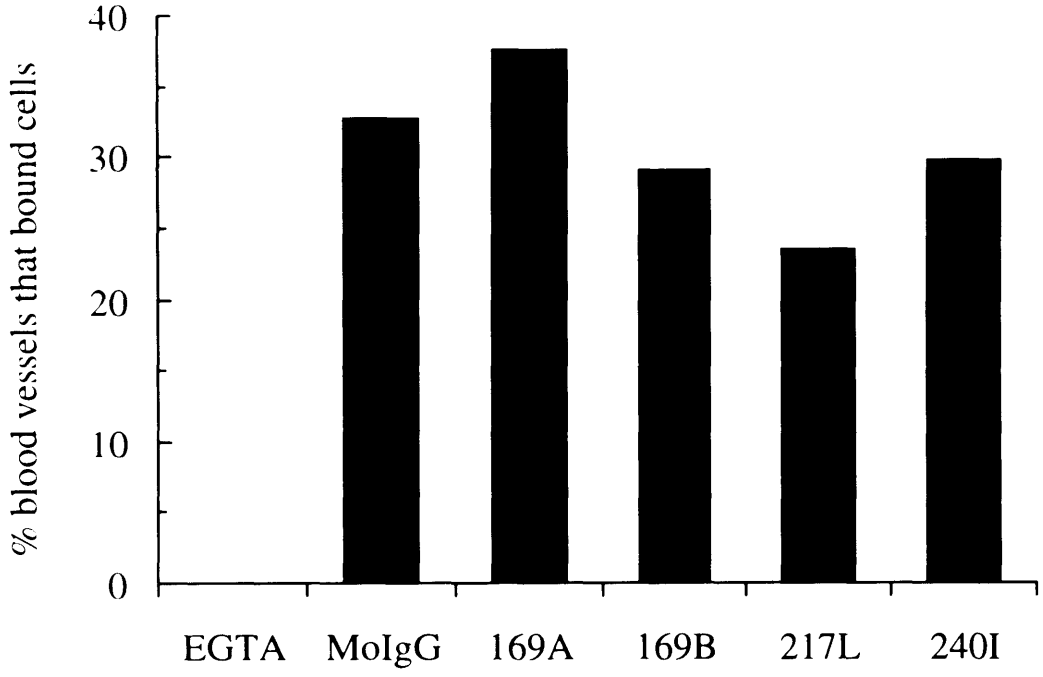
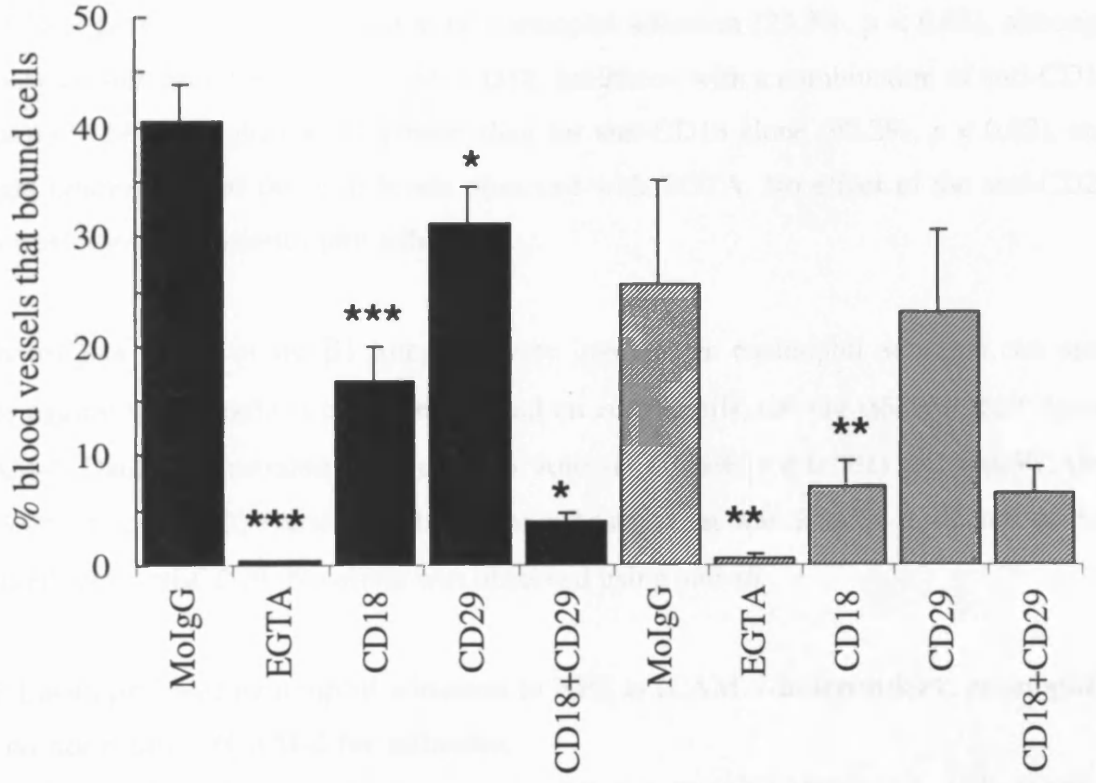


Figure 3–5 Eosinophil, but not neutrophil, adhesion to NPE is mediated by β1 integrins. Eosinophils and neutrophils from different donors were purified and incubated for 15 min at RT with anti-CD18 (β2, clone MHM23), anti-CD29 (β1, clone P4C10), control antibody, or EGTA-treated assay medium, before use in the FSA (Methods, section 2.2.6.2). Filled columns, eosinophils, n = 3-10; hatched columns, neutrophils, n = 3. Data shown indicate mean values ± SEM, * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.



The percentage inhibition of cell adhesion by anti-CD18 was significantly greater for neutrophils (67.7%) than for eosinophils (55.6%) ($p < 0.05$ for mAb MHM23). This raised the possibility of other integrins being involved in eosinophil adhesion. Therefore, the effects of a known blocking mAb directed against CD29 (β1), clone P4C10, were examined. Data are shown in Figure 3–5.

3.2.4 Eosinophil adhesion to NPE requires a contribution by the β1 integrins

Anti-CD29 gave consistent inhibition of eosinophil adhesion (23.3%, $p < 0.05$), although this was considerably less than for anti-CD18. Inhibition with a combination of anti-CD18 and anti-CD29 was significantly greater than for anti-CD18 alone (90.2%, $p < 0.03$), and brought binding almost down to levels observed with EGTA. No effect of the anti-CD29 mAb was observed on neutrophil adhesion.

To investigate which of the β1 integrins were involved in eosinophil adhesion, we used mAbs against the integrin α chains expressed on eosinophils, α4 and α6, and their ligand VCAM-1. Data are illustrated in Figure 3–6. Anti-α4 (19.6%, $p < 0.001$) and anti-VCAM-1 (13.0%, $p < 0.0003$) caused inhibition of adhesion that was similar in degree to that observed with anti-CD29. No effect was observed using anti-α6.

3.2.5 Eosinophil and neutrophil adhesion to NPE is ICAM-1-independent; eosinophils also do not require ICAM-2 for adhesion

The principle endothelial counter-receptor for CD18 is ICAM-1 (Diamond *et al.*, 1990), a second ligand is ICAM-2 (Staunton *et al.*, 1989). The effect of function blocking mAbs against these receptors was examined in the FSA, shown in Figures 3–7 and 3–8. No inhibition of eosinophil or neutrophil adhesion was seen with the well-known blocking mAb against ICAM-1, clone R6.5 (Figure 3–7, work undertaken in collaboration with Dr. F. Symon, unpublished data). However, blocking mAb clone 15.2 moderately inhibited eosinophil adhesion to NPE in two experiments (mean % inhibition 14.4% ± 1.95, not shown). Eosinophils did not use ICAM-2, as designated by the blocking mAb BT-1 (Figure 3–8). Studies were not carried out on neutrophils with anti-ICAM-2.

Figure 3–6 Eosinophil adhesion to NPE uses CD49d and VCAM-1 but not CD49f. Eosinophils from different donors were purified (Methods, section 2.2.2) and incubated for 15 min at RT with anti-CD49d (α4, clone HP2/1), anti-CD18 (β2, clone MHM23), anti-CD29 (β1, clone P4C10), CD49f (α6, clone 4F10), control antibody, or EGTA-treated assay medium, before use in the FSA (Methods, section 2.2.6.2). VCAM-1 (clone 4B9) or control antibody was incubated on the endothelium for 30 min at RT. n = 3-8. Values represent the mean percentage inhibition of cell adhesion compared to isotype-matched control antibodies ± SEM, * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0005$.

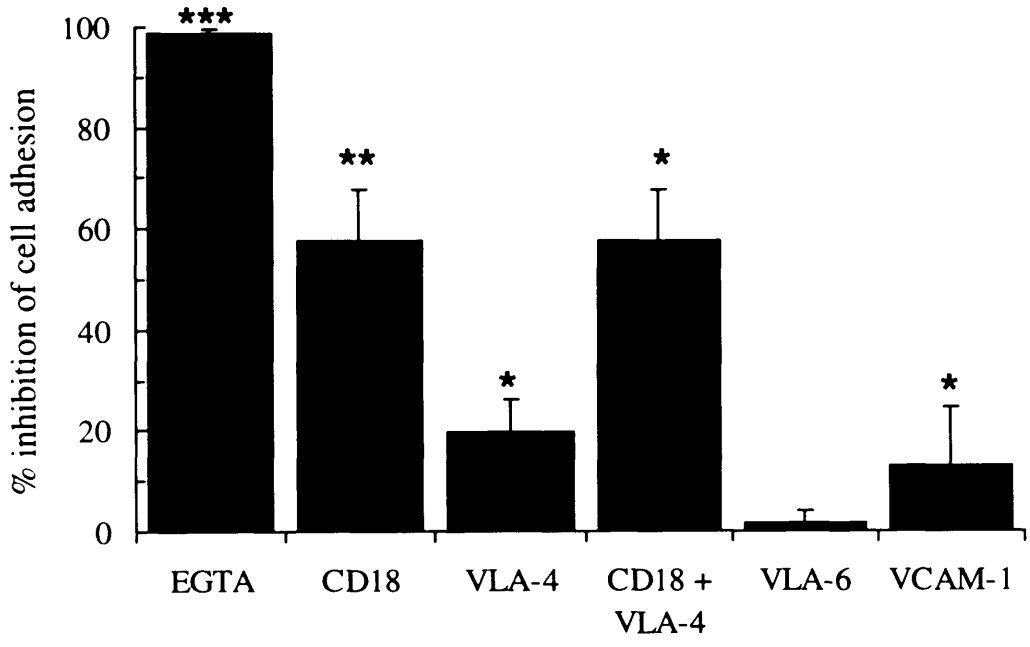


Figure 3–7 Eosinophil and neutrophil adhesion to NPE is not mediated by ICAM-1. Eosinophils and neutrophils from different donors were purified (Methods, section 2.2.2) and layered over nasal polyp sections that had been incubated with anti-ICAM-1 (clone R6.5), control antibody, or EGTA-treated assay medium for 30 min, RT, before continuing with the FSA as described in the Methods, section 2.2.6. Filled columns, eosinophils, n = 3; hatched columns, neutrophils, n = 5. Data shown represent the mean values ± SEM, * $P < 0.05$.

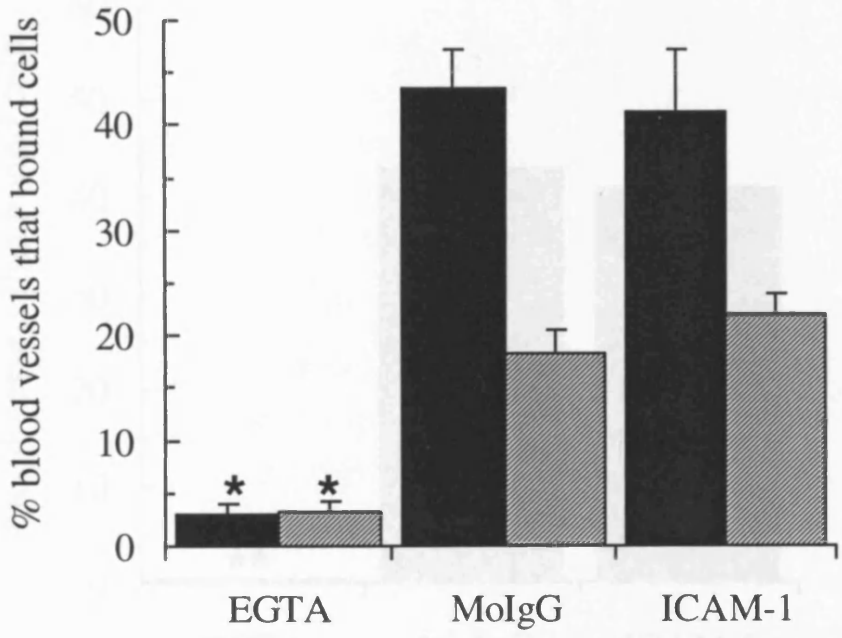
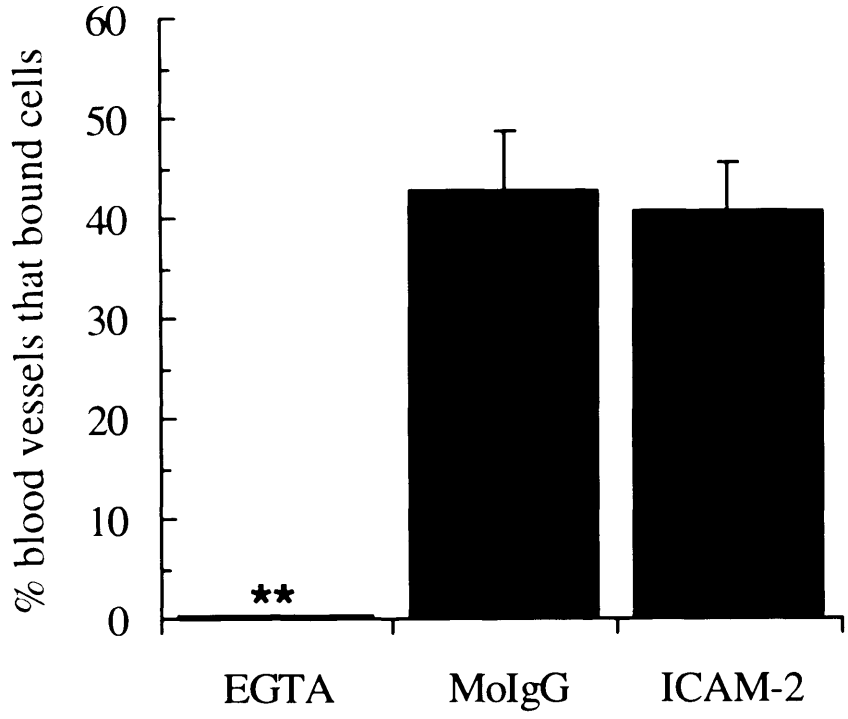


Figure 3–8 ICAM-2 does not mediate eosinophil adhesion to NPE. Eosinophils from different donors were purified (Methods, section 2.2.2) and layered over frozen sections of nasal polyp that had been incubated with anti-ICAM-2 (clone BT-1), control antibody, or EGTA-treated assay medium for 30 min, RT, before continuing with the FSA as described in the Methods, section 2.2.6. n = 4. Data shown indicate mean values ± SEM, ** $P <$ 0.005.



3.2.6 Expression of adhesion receptors on mucosal endothelium in the upper airways

So far, it has been demonstrated that the nasal polyp can model the integrin-mediated interactions between leukocytes and the endothelium in the multi-step paradigm of adhesion and migration from peripheral blood to sites of tissue inflammation. The next stage was to confirm that the nasal polyp was a valid model to study these steps, by demonstrating that this tissue contains the necessary ligands required for such interactions. Nasal polyp has previously been assessed for adhesion receptor presence (Symon *et al.*, 1994); however, it was useful to verify this with the tissue that was used for the FSAs in the current study. In addition, it was necessary to corroborate that the lack of inhibition with known endothelial ligand blockers for which the cellular component was effectively inhibited (e.g. ICAM-1 on endothelium and CD18 on cells), was not due to an absence of the ligand in the tissue.

Nasal polyps are by definition a model of disease for which there is no control tissue. Adhesion receptor expression in nasal biopsies and nasal turbinates from normal subjects was studied; the data were compared with those from patients with allergic rhinitis. This was undertaken to confirm that adhesion receptor expression in nasal polyp was comparable to expression in the nose in a diseased state for which there were controls, hence strengthening the use of the nasal polyp in the FSA. Any differences in the pattern of expression of adhesion receptors between normal and allergic subjects were noted. P-selectin expression in particular was looked at, as expression studies on this candidate for selective eosinophil accumulation in allergic airway disease have been very limited. In particular, no previous studies have investigated the tissue expression of P-selectin in subjects with allergic rhinitis.

3.2.6.1 Nasal Polyp study

Polyps were sectioned and immunostained for adhesion receptors, as detailed in Methods, section 2.2.5. Stained and mounted sections were blinded before being graded according to a visual 5-point scoring scale, as shown in Methods, Table 2–4. Adhesion receptor expression in nasal polyps is summarised in Figure 3–9 and Table 3–3. The mean values of intensity and extent of staining are shown.

Table 3–3 Expression of adhesion receptors in nasal polyp tissue (n = 4-7), supplementary to Figure 3–9. Data shown are mean intensity and extent of substrate staining (± SEM) using a semi-quantitative 5-point scale (see Table 2–4 in Methods).

	EN4	P-selectin	MBP	ICAM-1	ICAM-2	VCAM-1	E-selectin
Intensity	5 (0)	4.6 (0.2)	4.9 (0.1)	1.8 (0.4)	3.5 (0.3)	1.8 (0.5)	1.3 (0.3)
Extent	3.9 (0.3)	3 (0.3)	3.7 (0.6)	1.6 (0.2)	1.8 (0.3)	1.8 (0.5)	1.5 (0.5)

3.2.6.1.1 Nasal polyp endothelium expresses ICAM-1, ICAM-2, VCAM-1, and E-selectin sparsely

EN4 was used as a positive control for blood vessels, as this mAb is raised against the pan-endothelial receptor, PECAM-1. EN4 stained well in both intensity and extent, as shown in Figure 3–9 and Table 3–3. Figures 3–10 b) and c) demonstrate EN4 staining, which is representative of the upper range of the scale used to grade staining in all polyps, the intensity (I) and extent (E) scores are 5 and 3.9, respectively. The negative marker control, MoIgG, was not expressed (intensity = 0, extent = 0) in all immunostaining experiments, as shown in Figure 3–10 a).

ICAM-1 was expressed weakly to moderately on blood vessels, epithelium, mucus glands, and individual stromal cells in nasal polyps. Data are summarised in Figure 3–9 and Table 3–3. Figure 3–11 a) shows weak expression of ICAM-1 on a blood vessel, with stronger expression on neighbouring cells. ICAM-2 stained blood vessel endothelium specifically and quite strongly, but the extent of the staining was moderate (Figure 3–9 and Table 3–3).

Both VCAM-1 and E-selectin were expressed weakly or not at all on polyps (Figure 3–9, Table 3–3), and expression was specific to blood vessel endothelium. An example of VCAM-1 staining is shown in Figure 3–11 b), which visually demonstrates the lower range of the grading scale for intensity, I = 1. E-selectin staining is not shown, but expression was similar. Human tonsil was a good positive control for the ICAM-1, VCAM-1, and E-selectin antibodies, which all stained strongly in this tissue (data not shown).

3.2.6.1.2 P-selectin is well expressed on NPE

P-selectin was well expressed in the majority of nasal polyps, with a mean intensity and extent score of 4.6 and 3, respectively (Figure 3–9, Table 3–3). The trend was for more than half of those vessels that stained with EN4 to also stain positively for P-selectin. An example of P-selectin expression is shown in Figure 3–11 c), which illustrates the range of staining seen for this adhesion receptor, although most vessels stained at the highest intensity.

3.2.6.1.3 Nasal polyps contain large numbers of eosinophils

An antibody against eosinophil major basic protein was used to locate eosinophils in the tissue. Data are shown in Figure 3–9 and Table 3–3. The majority of polyps showed eosinophils to be present in large numbers with MBP, staining over half the tissue, mean value of E = 3.7, shown in Figure 3–12 a). The staining was very strong, and eosinophils were often seen to be degranulating, as illustrated in Figure 3–12 b).

.

Figure 3–9 Expression of a) selectins and eosinophil MBP, and b) immunoglobulin family adhesion receptors in nasal polyp tissue (n = 4-7). 6 μm sections of nasal polyp were immunostained (Methods, section 2.2.5). The intensity (I) and extent (E) of the final colour reaction were graded using a scale from 0-5, where 0 = no expression and 5 = very strong expression (see Table 2–4 for key). The mean value is shown for each parameter. EN4 is shown as a positive control for blood vessels.

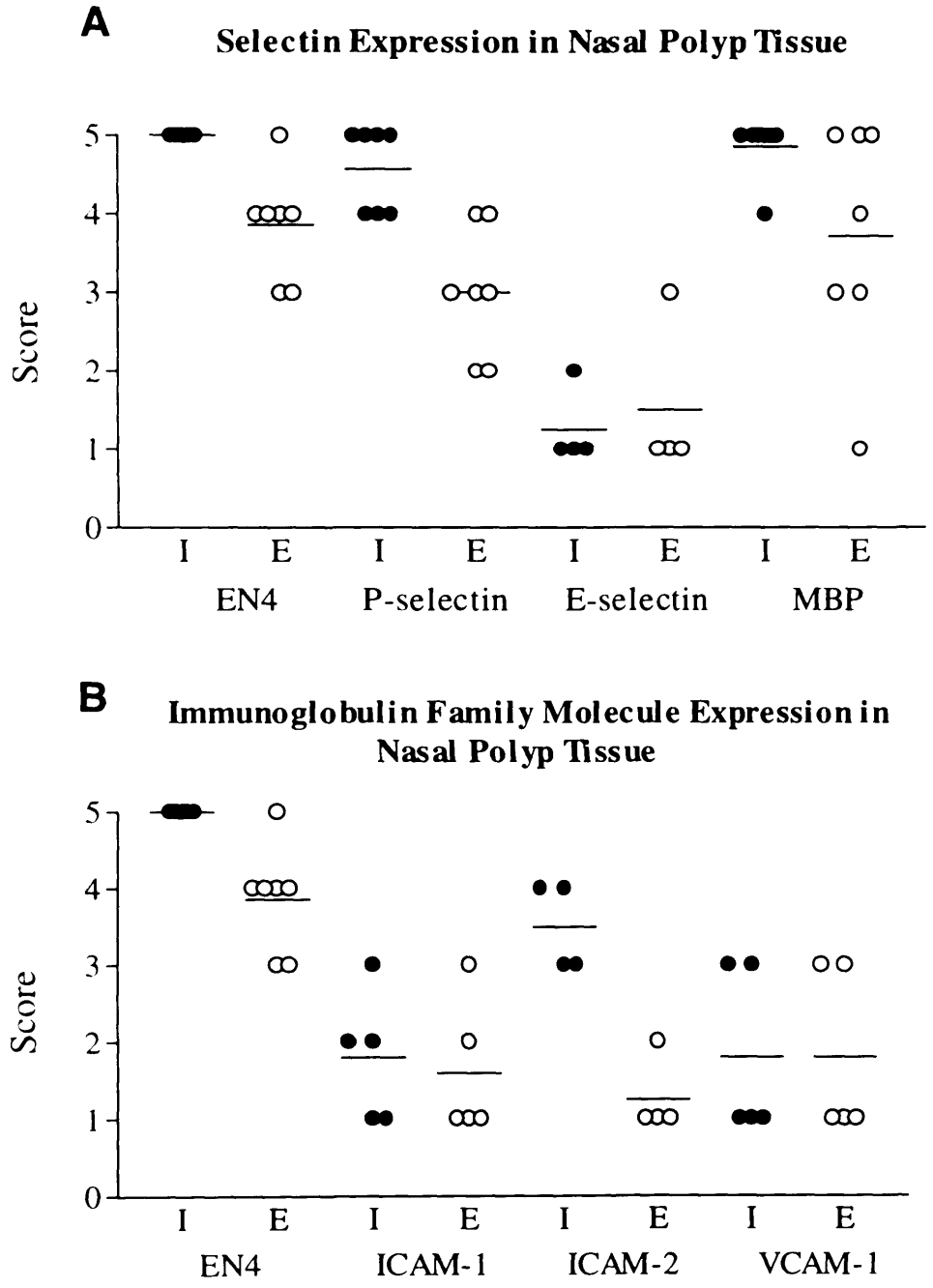


Figure 3–10 Staining of nasal polyp with a pan-endothelial marker. 6 μm cryosections of acetone-fixed human nasal polyp tissue were immunostained for PECAM-1 (Methods, section 2.2.5). B) PECAM-1, magnification × 100 shows an example of the maximum immunostaining score for blood vessels, intensity (I) = 5, extent (E) = 5. C) PECAM-1 at a higher magnification, × 400, I = 5. Control staining, A) shows an isotype-matched MoIgG which was negative (I = 0, E = 0) in all experiments, magnification × 100.

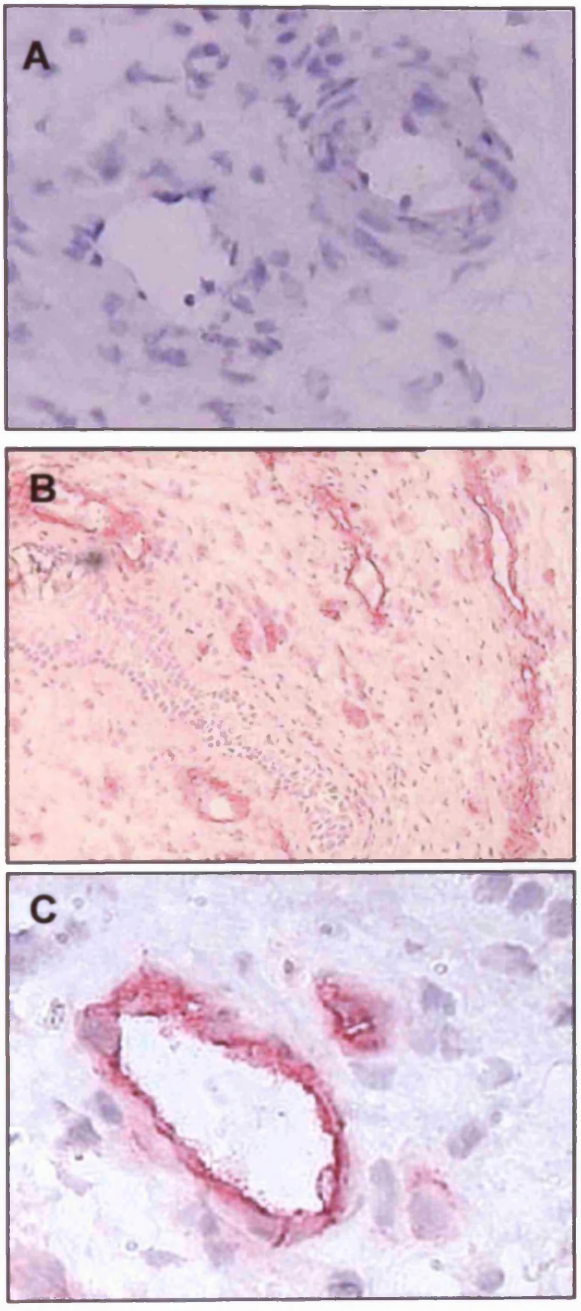


Figure 3–11 Staining of nasal polyp for ICAM-1, VCAM-1, and P-selectin. 6 μm cryosections of human nasal polyp tissue were immunostained for A) ICAM-1, shows an example of a variable score, with a blood vessel staining weakly (small arrow, I = 2) and stromal cells staining more strongly (large arrows, I = 3-4), magnification × 200, acetone-fixed. B) VCAM-1, stained blood vessels very weakly, I = 1, magnification × 200, acetone-fixed. C) P-selectin, stained blood vessels very specifically. Generally, intensity was maximum (right hand side, I = 5), although occasionally blood vessels stained weakly (left hand side, I = 2), magnification × 200, paraformaldehyde-fixed.

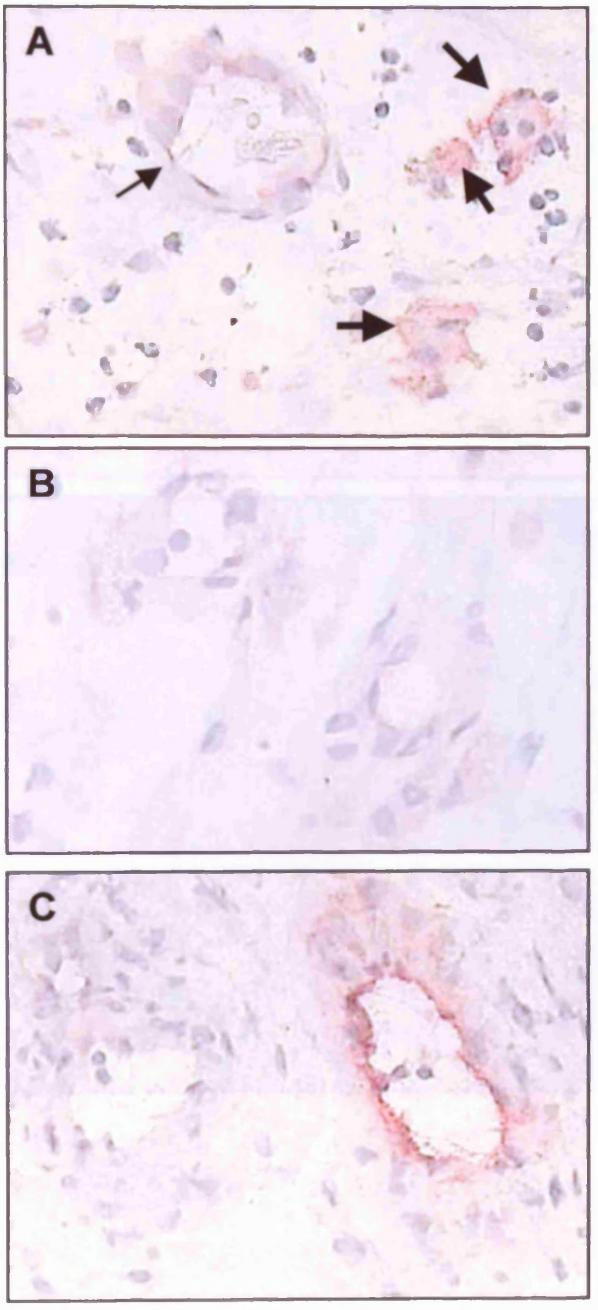
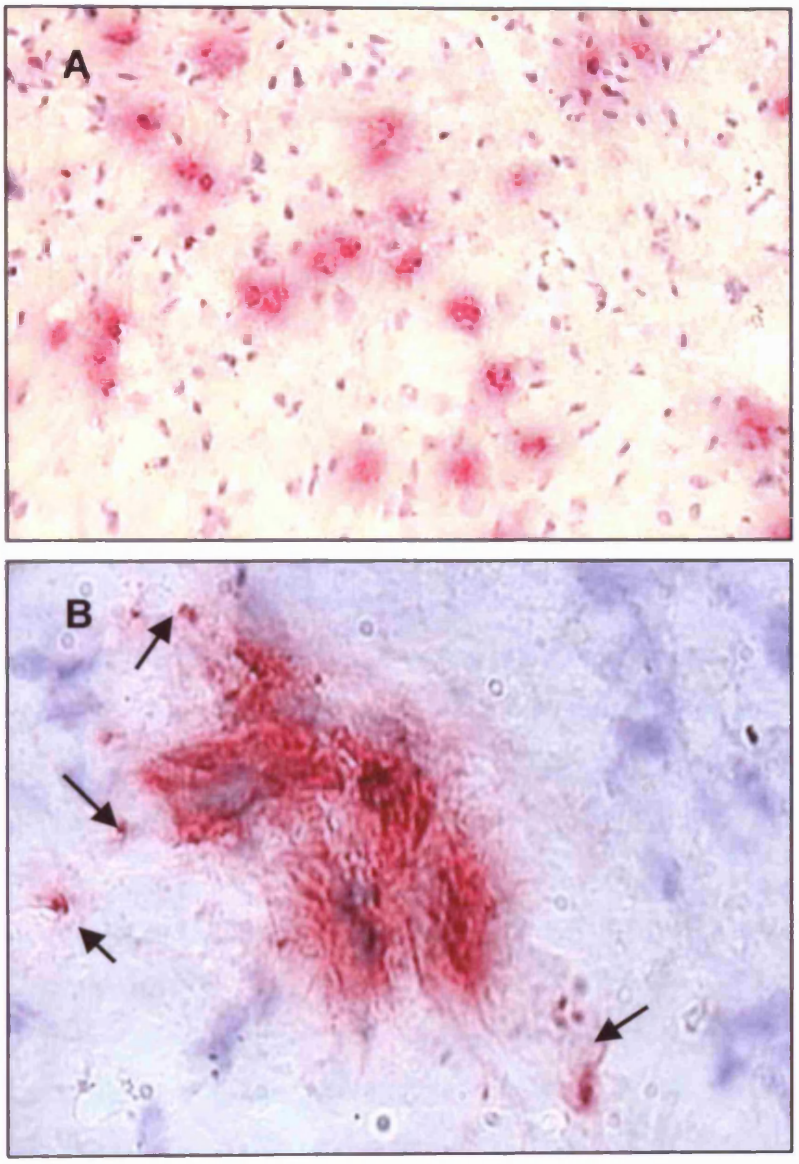


Figure 3–12 Staining of nasal polyp for the presence of eosinophils. 6 μm cryosections of acetone-fixed human nasal polyp tissue were immunostained for: A) the eosinophil granule protein, major basic protein (MBP), magnification × 100. Numerous vivid red eosinophils are scattered throughout the polyp section, intensity (I) = 5, extent (E) = 4. B) MBP staining at a higher magnification (× 400) shows an individual eosinophil that has degranulated, releasing its contents into the surrounding tissue (arrows), I = 5.



3.2.6.2 Nasal biopsy/ nasal turbinate study

Biopsies and turbinates were sectioned and immunostained for adhesion receptors as detailed in Methods, section 2.2.5. Stained and mounted sections were blinded, then graded according to a visual 5-point scoring scale, as shown in Methods, Table 2–4. Adhesion receptor expression in this study is summarised in Figure 3–14 and Table 3–4 as the mean value of intensity and extent of staining, depending on the receptor being examined. It was decided that showing intensity only was valid for EN4, P-selectin, E-selectin, ICAM-1, and VCAM-1, as unlike nasal polyps, the biopsy samples were too small for extent to be a valid measurement for these receptors. Extent is shown for MBP only. Due to small sample numbers, the data for the allergic and control subjects were pooled for biopsies and turbinates. Staining patterns of nasal turbinates are illustrated in Figure 3–13. Nasal biopsy staining patterns were similar.

3.2.6.2.1 VCAM-1, E-selectin, and ICAM-1 are poorly expressed in both normal and allergic nasal mucosa

There was very weak VCAM-1 and E-selectin expression in both normal and allergic subjects, and ICAM-1 expression was modest (Figure 3–14 and Table 3–4). There was no significant difference in the expression of any of these receptors between the normal and allergic groups. Expression of VCAM-1 and E-selectin was confined to blood vessels. ICAM-1 stained blood vessel endothelium, mucus glands, epithelium, and some individual stromal cells.

3.2.6.2.2 P-selectin expression and eosinophil infiltration are upregulated in the nasal mucosa of allergic subjects

P-selectin was increased in subjects with allergic rhinitis compared to controls, although this did not reach significance for pooled data (Figure 3–14 and Table 3–4). However, a significant difference ($p < 0.05$) was seen in P-selectin expression in nasal turbinates between the normal (mean intensity 1 ± 0.57) and rhinitic (mean intensity 3 ± 0.0) groups (Appendix 3 a), although the sample numbers were small ($n = 3$ and 4, respectively). There was no difference in P-selectin expression between the normal and allergic nasal biopsy groups alone (Appendix 3 b). P-selectin expression was exclusively seen in vessel areas. There was a significant eosinophilic tissue infiltration in the disease group compared to normal controls, as assessed by extent of anti-MBP specific staining (Figure 3–14, Table 3–4, $p < 0.02$).

Figure 3–13 Representative immunostaining of 6 μm inferior turbinate sections using monoclonal antibodies against P-selectin (paraformaldehyde-fixed), VCAM-1, EN4, and MBP (all acetone-fixed). Staining from allergic subjects is shown. Magnification × 100.

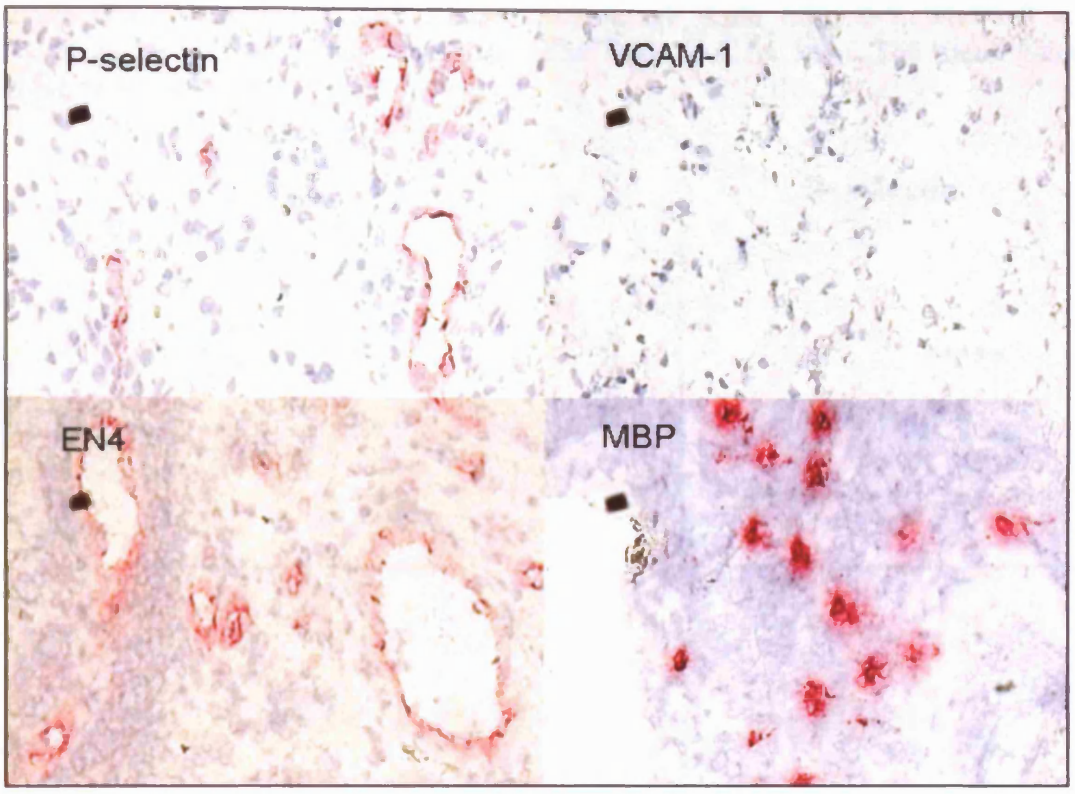
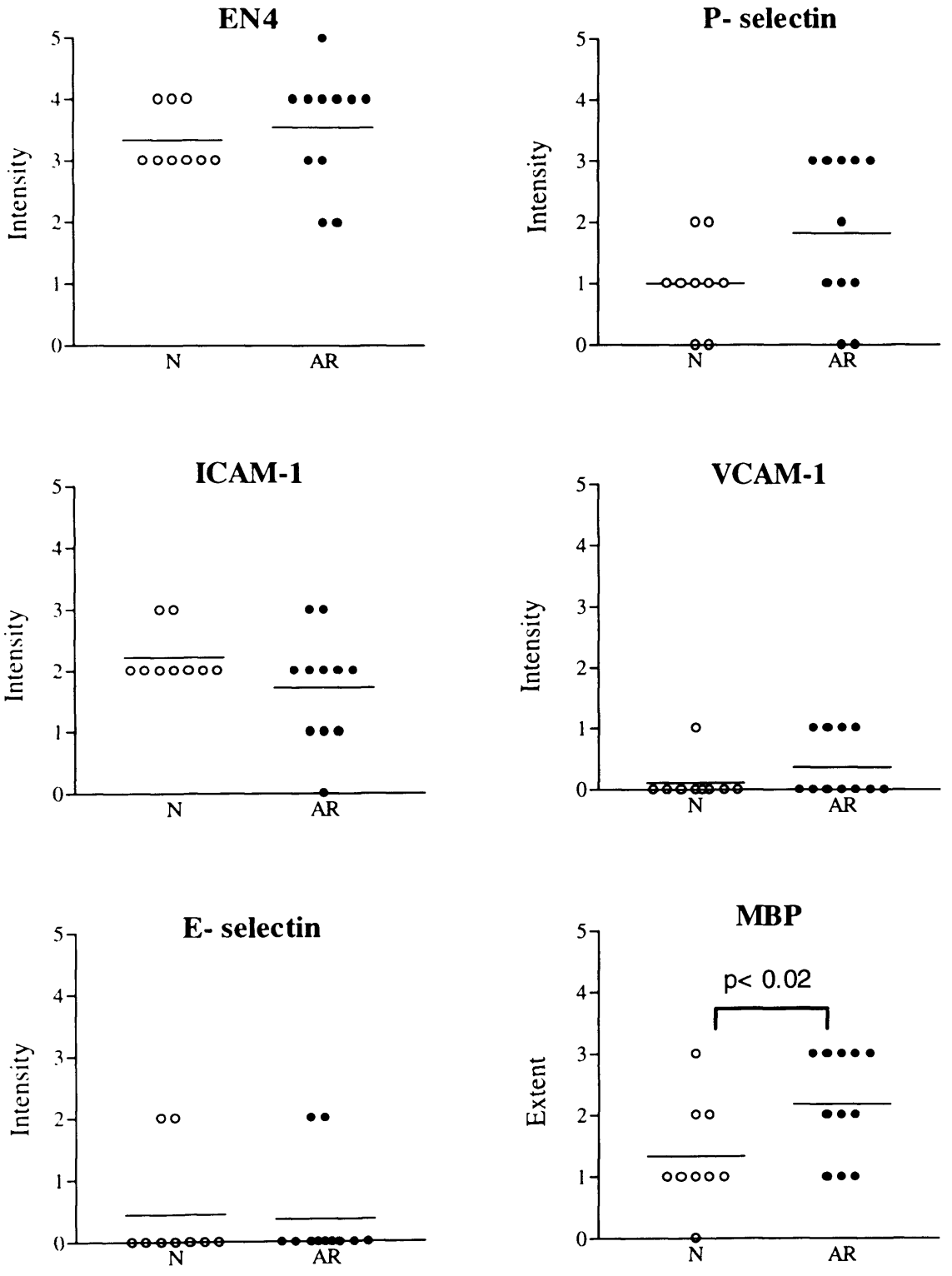


Table 3–4 Expression of adhesion receptors in nasal mucosa, supplementary to Figure 3–14. Data shown are mean extent (for MBP) and intensity (all other receptors) of substrate staining (± SEM), using a semi-quantitative 5-point scoring scale (see Table 2–4 in Methods). The control group comprised of 6 nasal biopsies and 3 inferior turbinate resections, and the allergic rhinitis group comprised of 7 nasal biopsies and 4 inferior turbinate resections.

	EN4	P-selectin	MBP	ICAM-1	VCAM-1	E-selectin
Normal	3.3 (0.2)	1.0 (0.3)	1.3 (0.3)	2.2 (0.1)	0.1 (0.1)	0.4 (0.3)
Allergic Rhinitis	3.5 (0.3)	1.8 (0.4)	2.2 (0.3)	1.7 (0.3)	0.4 (0.2)	0.4 (0.2)

Figure 3–14 Expression of EN4, P-selectin, ICAM-1, E-selectin, and MBP in normal and allergic nasal tissue. Allergic rhinitis (AR) group = 7 nasal biopsies and 4 inferior turbinates. Control (N) group = 6 nasal biopsies and 3 inferior turbinates. Sections were immunostained as shown in the Methods, section 2.2.5. The intensity/ extent of the final colour reaction was graded using a semi-quantitative scale from 0-5, where 0 = no expression and 5 = very strong expression (see Table 2–4 for key). The mean value is shown for each parameter.



3.3 Summary

Using the nasal polyp frozen section assay model, both eosinophils and neutrophils required CD18 for firm adhesion to NPE, and shared a requirement for CD11b. Eosinophil, but not neutrophil adhesion, was inhibited by CD11a, with the exception of one mAb clone (38). Both eosinophils and neutrophils used CD11c to bind, although neutrophil adhesion was consistently inhibited to a greater extent. Eosinophils, but not neutrophils, demonstrated a modest but significant requirement for β1, which was presumably working through VLA-4 and VCAM-1, since mAbs against these receptors also caused inhibition, but to a lesser extent. The endothelial counter-receptor (s) for CD18 on eosinophils remains unknown, as no effect on adhesion was seen when ICAM-1 and ICAM-2 were blocked.

A semi-quantitative examination of the expression of adhesion receptors in three models of upper airway inflammation was undertaken. This was to validate the use of the nasal polyp as a model in the FSA, and to add to the paucity of data that exists on adhesion molecule expression in chronic human allergic disease. Nasal biopsy and nasal turbinate specimens contained adhesion receptors in similar proportions to nasal polyp tissue. There were many blood vessels present in both tissue types, as demonstrated by staining with a pan-endothelial marker. Similarly to nasal polyp, P-selectin staining was strong and extensive in both biopsies and turbinates, while VCAM-1 and E-selectin expression was rather weak and patchy, or absent. Immunoreactivity of ICAM-1 was weak to moderate in all three tissues. Large numbers of MBP-positive eosinophils were present in all polyps, and in biopsies and turbinates, with a significant increase in eosinophil number in the allergic rhinitic group compared to the control group, when the data were pooled.

3.4 Discussion

The Stamper–Woodruff frozen section assay was recently adapted to study eosinophil and neutrophil adhesion to nasal polyp endothelium (Symon *et al.*, 1994). mAbs against P-selectin and PSGL-1 almost completely inhibited binding of eosinophils using this model. The purpose of Chapters 3 and 4 was to determine whether eosinophil and neutrophil binding in this assay was integrin- and activation-dependent, consistent with the multi-step model of adhesion described above.

Eosinophil and neutrophil adhesion to NPE was crucially dependent on the β2 integrins using this assay. Previous studies using cytokine-stimulated HUVEC have also

demonstrated that eosinophil and neutrophil adhesion and transmigration are β2 integrin-dependent (Kimani *et al.*, 1988; Smith *et al.*, 1989; Tonnesen *et al.*, 1989; Ebisawa *et al.*, 1992; Moser *et al.*, 1992). In these studies, LFA-1 and Mac-1 appeared to be involved for both cell types. This is in contrast to the observation that neutrophils did not use LFA-1 in the nasal polyp model. This may be related to the state of activation of either the LFA-1 epitope targeted by the mAb, or the counter-structure being recognised on the endothelium.

All four α subunits of the β2 integrins contain a 200 amino acid inserted or 'I' domain that is critical for ligand binding (Diamond *et al.*, 1993). The I domains of LFA-1 and Mac-1 also contain a cation binding site, referred to as the MIDAS motif (Lee *et al.*, 1995; Qu and Leahy, 1995). Ligand binding to all integrins requires divalent cations, suggesting that the I domain may influence activation as well as ligand binding. It was considered that the epitopes targeted by the antibodies used in this study might have been important in determining adhesion of eosinophils and neutrophils to NPE. Tables 3–1 and 3–2 show the CD18 mAbs studied; information on the epitopes targeted was discovered for some of the commercially available mAbs but none of the workshop mAbs. The majority of antibodies used were targeted against the I domains. There did not appear to be a correlation between the epitope targeted and the degree of inhibition of adhesion.

The most recently identified and fourth member of the β2 integrin family, CD11d/ CD18, is expressed on eosinophils, and has been shown to be an alternative ligand for VCAM-1 in both static and flow adhesion assays (Grayson *et al.*, 1998; Van der Vieren *et al.*, 1999). A panel of anti-CD11d mAbs had little inhibitory effect on eosinophil adhesion to NPE in the current study, although only one preliminary experiment was performed and further experiments would be required to confirm the lack of an effect. CD11a and CD11b bind ICAM-1, however, CD11d does not appear to; instead, it selectively binds the non-endothelially expressed receptor ICAM-3, as well as VCAM-1. The full relevance of CD11d in allergic disease remains to be determined.

The counter-receptor on NPE recognised by the leukocyte integrins was not identified. ICAM-1 is expressed on NPE, as shown here and by others (Symon *et al.*, 1994; Demoly *et al.*, 1998; Tingsgaard *et al.*, 1998). ICAM-1 on HUVEC has been reported to contribute to eosinophil (Ebisawa *et al.*, 1992) and neutrophil (Luscinskas *et al.*, 1991; Issekutz *et al.*, 1999) transmigration. Lack of inhibition of eosinophil adhesion to NPE was previously reported (Symon *et al.*, 1994) with an anti-ICAM-1 mAb that inhibits both the Mac-1 and

LFA-1 binding sites (Diamond *et al.*, 1991). In the present study, a modest inhibitory effect was seen with clone 15.2, but this was only in two experiments. Previously unpublished work by Symon using clone R6.5 was also incorporated into this study, again, this clone had no effect on eosinophil or neutrophil adhesion. This was despite this mAb being able to inhibit leukocyte adhesion to endothelium in other systems (Lawrence *et al.*, 1990; Issekutz *et al.*, 1999).

Blocking ICAM-2 also had no effect on eosinophil adhesion, despite this receptor being well expressed on NPE. ICAM-2 inhibition had no effect on neutrophil adhesion to NPE in one experiment (not shown). No other studies have investigated the effect of ICAM-2 inhibition on eosinophil or neutrophil adhesion to NPE. However, ICAM-2 was the major counter-receptor involved for the β2 integrin component of T-lymphocyte adhesion to NPE (Symon *et al.*, 1999). ICAM-2 was a major contributor to LFA-1-mediated neutrophil transmigration through HUVEC in another study (Issekutz *et al.*, 1999). Surprisingly, eosinophil accumulation in the lung interstitium was increased in ICAM-2-deficient mice after allergen challenge (Gerwin *et al.*, 1999). The mechanism of this observation is unclear.

From these observations, it appears that the principle counter-ligand for the β2 integrins expressed by inflamed airway endothelium as modelled by the nasal polyp remains to be defined. Other adhesion receptors are present on NPE, including lymphocyte vascular adhesion proteins 1 and 2 (L-VAP-1 and -2). These adhesion receptors are expressed in the HEV of peripheral lymph nodes. VAP-1 has been shown to mediate lymphocyte binding to HEV in lymphatic tissues in the FSA, in a sialic acid-dependent manner (Salmi *et al.*, 1997). VAP-2 has also been implicated in T cell adhesion to vascular endothelium (Airas *et al.*, 1995). However, although they are well expressed on NPE, no role has been found for these receptors in mediating eosinophil or neutrophil adhesion to nasal polyp (Dr. F. Symon, personal communication).

Inhibition using the anti-CD18 mAbs averaged 57% for eosinophils and 68% for neutrophils. It was postulated that this difference was due to the ability of eosinophils to additionally use β1 integrins, particularly VLA-4. There is a considerable body of evidence from animal models suggesting that VLA-4/ VCAM-1 interactions may be important in controlling eosinophil accumulation in allergic inflammation (Bochner *et al.*, 1991; Weg *et al.*, 1993; Nakajima *et al.*, 1994). In the present study, an anti-CD29 antibody consistently

inhibited eosinophil adhesion. No effect on neutrophil adhesion was seen, which is consistent with the lack of expression of β1 on neutrophils under physiological conditions. A combination of anti-CD18 and anti-CD29 mAbs had a significantly greater inhibitory effect than anti-CD18 alone. This synergy suggests some redundancy in the adhesion process, where loss of binding via β2 may be compensated by binding via β1 and vice-versa.

Ridger and co-workers recently investigated the role of CD29/ CD49 integrin heterodimers in neutrophil migration in a mouse model of pulmonary inflammation (Ridger *et al.*, 2001). They demonstrated that neutrophil migration to LPS and a murine CXC chemokine was inhibited by anti-CD29. In addition, the effect of CD29 was not increased when blocked in combination with CD18, suggesting that in this model CD29 and CD18 do not mediate the same adhesion pathway. The differences to the current study may be due in part to the differences in migration occurring in lung capillaries compared to postcapillary venules, and the dissimilarities between mouse and human models.

In the FSA, similar degrees of inhibition were observed with mAbs against VLA-4 and VCAM-1 as those observed with anti-CD29, although results were less consistent than those with anti-CD29. Two other antibodies against VLA-4 (clones P4C2 and HP1/2, data not shown) also demonstrated variable inhibition. Nevertheless, the data suggests that the β1 component of eosinophil adhesion was through VLA-4/ VCAM-1. Previously, anti-VCAM-1 failed to inhibit eosinophil adhesion to NPE, although a different antibody was used in those experiments (Symon *et al.*, 1994). It is likely that the VLA-4 receptor on nasal polyp is VCAM-1, as this receptor was expressed, albeit weakly, on nasal polyp endothelium. The variable inhibition seen with VLA-4/ VCAM-1 may be due to the relatively minor contribution these interactions make to eosinophil binding in this assay.

The α4 subunit also associates with the β7 subunit on eosinophils. Integrin α4β7 is constitutively expressed on eosinophils and binds VCAM-1, and additionally, fibronectin and MAdCAM-1 (Walsh *et al.*, 1996). Little or no inhibition of eosinophil adhesion to NPE was seen with two antibodies directed against α4β7 (data not shown, clones Act-1 and HP1/2, which also targets α4β1). Eosinophils from mildly allergic donors were found to consistently express the integrin α6 subunit (Georas *et al.*, 1993). No inhibition of eosinophil adhesion was seen in the FSA using a blocking mAb against α6. No other

studies have shown an inhibitory effect of α6β1 on eosinophil adhesion to endothelium, however, α6β1 has recently been shown to mediate neutrophil tethering and arrest on laminin under physiological shear flow (Kitayama *et al.*, 2000).

The *in vivo* and *in vitro* data in recent literature support the view that α4β1 integrin-dependent leukocyte rolling and adhesion occurs via VCAM-1 (Alon *et al.*, 1995; Berlin *et al.*, 1995; Abe *et al.*, 1996; Sanz *et al.*, 1997). No other ligand for α4β1 has been shown to mediate leukocyte rolling under shear flow conditions. This has been challenged by Johnston *et al.*, who recently studied the recruitment of leukocytes under flow conditions from whole blood to endothelium in a rat model of chronic vasculitis (Johnston *et al.*, 2000). In this model, recruitment was α4β1- and not α4β7-dependent, but the ligand for α4β1 was not VCAM-1, as dictated by three separate approaches to inhibit this receptor.

Novel mechanisms of eosinophil adhesion to endothelium in the FSA were considered. Fractalkine is synthesised as a type I transmembrane protein on activated endothelial cells. Its unique CX_3C chemokine domain is attached to a mucin stalk, a transmembrane domain, and an intracellular domain of unknown function. A soluble form of fractalkine can be generated by proteolytic cleavage at the base of the mucin stalk (Bazan *et al.*, 1997). It has been suggested that the fractalkine receptor, CX_3CR1, acts primarily as an adhesion molecule, rather than as a signalling molecule. Fractalkine on endothelium interacting with CX_3CR1 on leukocytes mediated the initial capture, firm adhesion, and activation of circulating lymphocytes (Fong *et al.*, 1998). It was hypothesised that fractalkine might mediate adhesion of eosinophils to NPE in the FSA. Experiments were undertaken in which the soluble 8 kDa chemokine portion of fractalkine was incubated with eosinophils before carrying out the FSA as normal. Some inhibition of adhesion to NPE was seen, although this did not reach significance (mean % adhesion untreated cells 34.7 ± 3.6, mean % adhesion fractalkine-treated cells 27.3 ± 8.5, n = 3, data not shown). This 8 kDa fragment had no chemotactic activity for eosinophils in one experiment (data not shown). At the time, commercially available monoclonal antibodies against fractalkine were not available to take these studies further. So far, no studies have investigated a role for fractalkine in eosinophil or neutrophil adhesion to endothelium, although studies with monocytes are more promising (Chapman *et al.*, 2000).

Limited information is available about the expression of adhesion molecules in chronic

allergic human disease. There is evidence that several adhesion molecules are important in the pathogenesis of lung diseases such as bronchial asthma (Montefort *et al.*, 1993). Most experimental data exists predominantly from animal models that implicate the selectins in the process of leukocyte rolling in inflammatory conditions. Human tissue sections are a desirable source material for determining the level of expression of these receptors in clinical disease. Where large amounts of tissue are unavailable, biopsy studies are an option. Hence, to add to the current paucity of data available, adhesion receptor expression was examined in three different examples of nasal tissue.

In the present study, the expression of E-selectin and VCAM-1 on NPE was found to be very weak or absent. Additionally, levels of these adhesion molecules were not raised in the allergic biopsies and turbinates compared to the control tissue, being very weak in both groups. Previously, E-selectin was well expressed on NPE (29% of tissue), whereas VCAM-1 expression was absent or very weak (5% of tissue) (Symon *et al.*, 1994). The difference in staining with E-selectin in the current study could be due to the different antibody used. However, in the study by Symon *et al.*, the E-selectin mAb was found to cross-react with P-selectin. Symon *et al.* additionally found negligible inhibition of eosinophil adhesion to NPE with blocking mAbs against both E-selectin and VCAM-1, which agrees with the weak staining in the present study.

Other studies have found a more significant role for VCAM-1. Jahnsen *et al.* examined eosinophil infiltration and expression of VCAM-1 on nasal polyps and nasal turbinates from the same patients (Jahnsen *et al.*, 1995). In this study, both the number of eosinophils and the proportion of vessels positive for VCAM-1 were significantly increased in nasal polyps compared with the nasal turbinates. There was also a strong correlation between the numbers of VCAM-1 positive vessels and eosinophil, but not neutrophil numbers, suggesting a VCAM-1-mediated extravasation of eosinophils. This group further extended these *in situ* observations by culturing nasal polyp endothelial cells and demonstrating expression of VCAM-1 induced by the allergy-associated cytokines IL-4 and IL-13 (Jahnsen *et al.*, 1997). In the same study, VCAM-1 was functional, as shown by antibody-mediated inhibition of leukocyte adhesion to polyp-derived microvascular endothelial cells in a static assay.

ICAM-1 staining of NPE in the current study was moderate, as shown previously (Symon *et al.*, 1994). ICAM-2 was well expressed, although as stated, blocking mAbs against both

of these receptors had no effect on adhesion of eosinophils in the FSA, suggesting the presence of an uncharacterised ligand for CD18. P-selectin was well expressed on NPE, as demonstrated previously (Symon *et al.*, 1994). A limitation of studying P-selectin expression in tissue sections is the inability to distinguish reliably between P-selectin stored intracellularly with P-selectin expressed on the endothelial surface. This is because sectioning the tissue will inevitably split open the plasma membrane of at least some endothelial cells. Paraformaldehyde was chosen as a fixative for all tissue sections stained with P-selectin over acetone or other permeabilising fixatives to minimise this factor.

No previous studies have investigated the tissue expression of P-selectin in patients with allergic rhinitis. A novel finding of this project was the increased expression of P-selectin in nasal biopsies and inferior turbinate material from patients with symptomatic perennial allergic rhinitis compared to normal controls. There was a significant increase in P-selectin expression and eosinophil numbers in allergic compared to control nasal turbinates, but not in allergic compared to control nasal biopsies. However, the sample numbers were small, and the significance of the individual turbinate study was lost when these data were pooled.

Recently, adhesion molecule expression and eosinophil infiltration in snap-frozen nasal polyp biopsies was compared before and a month after treatment with a topical intranasal corticosteroid, fluticasone, or a placebo control. Both P-selectin expression and activated eosinophil infiltration (as assessed by EG2 staining) were reduced significantly following the treatment course with fluticasone (Hamilos *et al.*, 1999). This was accompanied by a significant reduction of IL-4 and IL-13 mRNA positive cells within the tissue. In contrast, fluticasone did not significantly reduce expression of endothelial VCAM-1. These observations point to a greater role for P-selectin than VCAM-1. However, work by Tingsgaard *et al.* compared adhesion molecule expression in nasal polyps and inferior turbinates obtained from patients with nasal polyposis before and after topical budesonide treatment (Tingsgaard *et al.*, 1999). They found higher expression of P-selectin in the turbinate tissue but higher VCAM-1 expression in the polyp tissue, in contrast to our study. Expression of both molecules, and eosinophil infiltration, was reduced by the glucocorticoid treatment.

In a limited bronchoscopic biopsy study (personal observation, data not shown) using paraformaldehyde-fixed tissue, expression of P-selectin in six asthmatic subjects was

compared to expression in six normal controls. Expression levels were again determined semi-quantitatively and were found to be higher in the asthmatic group compared to normal controls but these differences did not reach significance. Vrugt *et al.* also found no significant differences between the levels of P-selectin expression in a bronchoscopic study comparing 8 normal controls with 15 subjects with severe corticosteroid-dependent asthma and 15 subjects with mild asthma (Vrugt *et al.*, 2000). Another recent study found no differences in the percentage of P-selectin positive blood vessels between normal controls and two groups of subjects with nocturnal asthma (ten Hacken *et al.*, 1998). However, the percentage of VCAM-1 positive vessels in biopsies of the asthmatic patients was higher than in biopsies of the healthy controls.

Feuerhake *et al.* suggested that a permanent low-grade endothelial activation state might exist in normal airway mucosa, which could be due to the normal antigen exposure via the air (Feuerhake *et al.*, 1998). No expression of P-selectin or other adhesion molecules was seen in alveolar capillary blood vessels in their study on normal lung. However, P-selectin was well expressed in all other segments of the bronchial and pulmonary circulation, and expression was higher overall than for ICAM-1, E-selectin, and VCAM-1. Our group has found P-selectin to be well expressed in the normal lung (W. Monteiro, personal communication). Subtle levels of luminal endothelial P-selectin expression may be sufficient or even required to capture circulating eosinophils rather than neutrophils. This is supported by the observation that soluble P-selectin levels were not increased in the plasma of asthma patients compared to normal controls, nor found in sputum supernatants (Dr. G. Woltmann, personal communication).

One limitation of the current study, and one that is frequent when evaluating immunostaining, is that it was semi-quantitative. Previous studies on endothelial adhesion receptors have enumerated the number of positive blood vessels stained by an endothelial marker, and the number of positive staining vessels with the test adhesion receptor (Symon *et al.*, 1994; Feuerhake *et al.*, 1998; Tingsgaard *et al.*, 1998). This enables a percentage calculation of the whole section stained. This could have been done with the nasal polyp but not the nasal biopsies or turbinates as the sections contained too few blood vessels to result in an accurate quantification. Another limitation is that expression of adhesion receptors may rely to some extent on which mAb clone is used, or the method of fixation or staining. This may account for the discrepancies in results seen in some of the studies.

In summary, so far I have demonstrated a difference in integrin usage between eosinophils and neutrophils for binding to endothelium in a model of chronic allergic inflammation. These differences are important when considering the therapeutic blockade of eosinophils in allergic diseases such as asthma. The endothelial counter-receptor (s) for CD18 in this model remain to be defined. Overall, the finding of significantly increased P-selectin expression and eosinophil infiltration, and weak or absent expression of VCAM-1 and E-selectin in allergically inflamed nasal mucosa supports an active role of P-selectin in these eosinophilic diseases. VCAM-1 would not appear to play as large a role, although other studies disagree (Lee *et al.*, 1994; Jahnsen *et al.*, 1995; Tingsgaard *et al.*, 1999). Additionally, the lack of inhibition of leukocyte adhesion to NPE with ICAM-1 is not due to an absence of this receptor on nasal polyp endothelium.

CHAPTER FOUR

Use of the FSA to evaluate the activation mechanisms involved in eosinophil and neutrophil adhesion to nasal polyp endothelium

4.1 Introduction

The involvement of integrins and immunoglobulin family members in the multi-step paradigm of leukocyte adhesion to endothelium has been investigated in Chapter 3. These receptors mediate arrest of the leukocyte after the initial tethering step. However, an activation signal is required in order for the integrins to become functional. The activation signal is thought to be supplied by chemoattractants, expressed on the endothelial surface, bound either to specific receptors or the proteoglycan-rich endothelial glycocalyx (Tanaka *et al.*, 1993).

Most work on the activation step of the migration process has been undertaken with neutrophils, in which the β2 integrins are the predominant integrins mediating endothelial adhesion. The signal to activate the β2 integrins on neutrophils is thought to be supplied by low molecular weight chemoattractants such as fMLP and PAF, and by the chemokine IL-8 (Diamond and Springer, 1994; Murphy, 1994). Little work has been undertaken on the activation step in other leukocytes, especially eosinophils. The aim of this section was to determine whether eosinophil and neutrophil adhesion to endothelium was activation-dependent consistent with the multi-step adhesion paradigm, using the *ex vivo* FSA, which has so far been shown to model the selectin- and integrin-mediated steps.

4.2 Results

On both eosinophils and neutrophils, the CD18 integrins need to be activated through outside-in signalling to be able to bind to their counter-structures. The requirement of an activation step for eosinophil adhesion to NPE was suggested by the previously reported observation that adhesion was temperature-dependent (Symon *et al.*, 1994). Optimum adhesion was observed at RT, with a small fall in adhesion at 37 °C, and an approximately 50% reduction in adhesion at 4 °C. It was first investigated whether activation of the cells prior to adding them to the tissue had any effect on adhesion. To avoid any effects of *in vivo* priming, normal donors were used for all activation experiments.

4.2.1 GM-CSF and RANTES are not involved in promoting eosinophil adhesion to NPE

Several humoral mediators and cytokines are capable of activating the effector functions of eosinophils. GM-CSF and RANTES are two mediators thought to be important in asthma. There is increased expression of GM-CSF in the epithelium of bronchial biopsy specimens from asthmatics (Sousa *et al.*, 1993), and increased circulating concentrations have been

detected in patients with acute severe asthma (Brown *et al.*, 1991). Similarly, RANTES has been shown to be an activating factor for human eosinophils (Alam *et. al.*, 1993; Ying *et al.*, 1999) and RANTES expression is increased in bronchial biopsies of asthmatics compared to normals (Ying *et al.*, 1999). It was hypothesised that GM-CSF and RANTES were candidates for priming eosinophils for increased adhesive function in the nasal polyp FSA model.

Incubation with GM-CSF (10^{-9} M) had no effect on either eosinophil or neutrophil adhesion to NPE (Figure 4–1). Pre-activating eosinophils with RANTES (10^{-6} M) also had no effect on eosinophil adhesion (data not shown, % binding medium control 38.03 ± 4.33 versus % binding RANTES 34.78 ± 6.38, $n = 2$).

4.2.1.1 L-selectin shedding and Mac-1 upregulation as markers of eosinophil and neutrophil activation

As discussed in section 1.3.1, L-selectin initiates the interaction of leukocytes with activated endothelium, and L-selectin shedding is one important aspect of the normal physiologic regulation of leukocyte adhesive function. Lymphocyte surface expression of L-selectin is characteristically downregulated in response to cell activation (Jutila *et al.*, 1989; Kishimoto *et al.*, 1989). In contrast, Mac-1 surface expression increases upon cell activation. Eosinophils express both L-selectin and Mac-1. Factors that activate eosinophils to cause selective down- or upregulation of adhesion molecule expression may be likely to modulate their adhesion to endothelium.

L-selectin shedding involvement was investigated by examining the effect of GM-CSF and azide on the modulation of L-selectin expression on purified human eosinophils and neutrophils using flow cytometry. Mac-1 involvement was also studied. Data are shown in Figures 4–2, 4–3, and 4–4.

Figure 4–1 Eosinophil and neutrophil adhesion to NPE is activation-dependent. Eosinophils and neutrophils from different donors were purified (Methods, section 2.2.2) and incubated with one of the following agents, before use in the FSA (Methods, section 2.2.6.2): GM-CSF, 10^{-9} M, 30 min, 37 °C; azide, 0.65% final concentration, 30 min, 37 °C. Filled columns, eosinophils, n = 3-4; hatched columns, neutrophils, n = 4-8. Values represent the means ± SEM, * $P < 0.02$, ** $P < 0.001$, *** $P < 0.0001$.

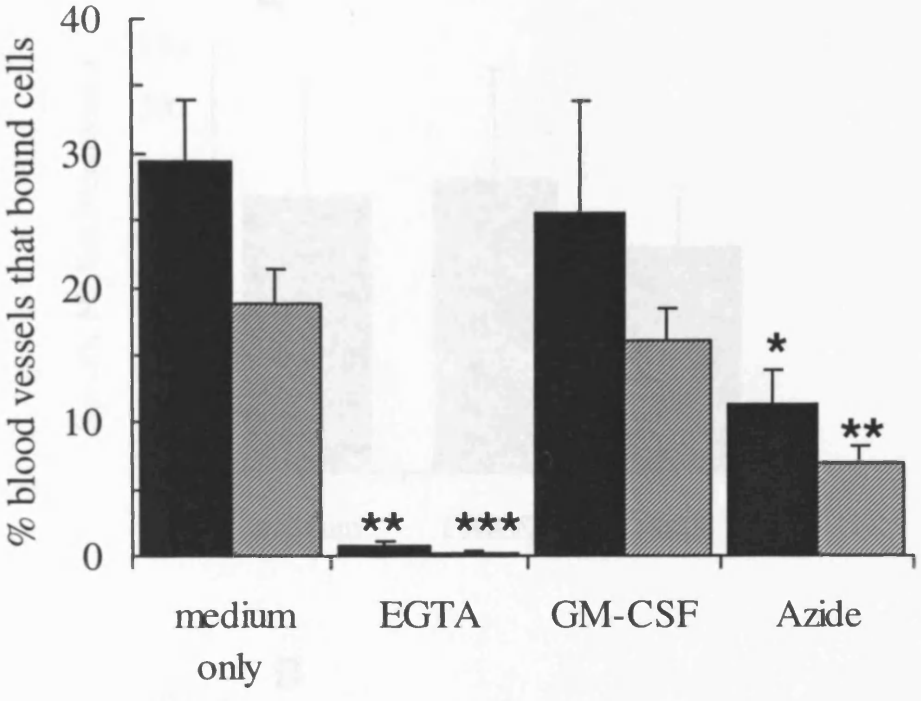


Figure 4–2 Effect of activation on surface expression of L-selectin on A) eosinophils, and B) neutrophils, after stimulation with GM-CSF (10^{-9} M, 30 min, 37 °C) and azide (0.65%, 30 min, 37 °C). Eosinophils and neutrophils were purified (Methods, section 2.2.2) and incubated with activators as above, before being assessed for expression of L-selectin using flow cytometry (Methods, section 2.2.4). Data are expressed as specific median fluorescence (test value minus isotype-matched control value) ± SEM. Eosinophils, n = 3, neutrophils, n = 5-6, * $P < 0.05$.

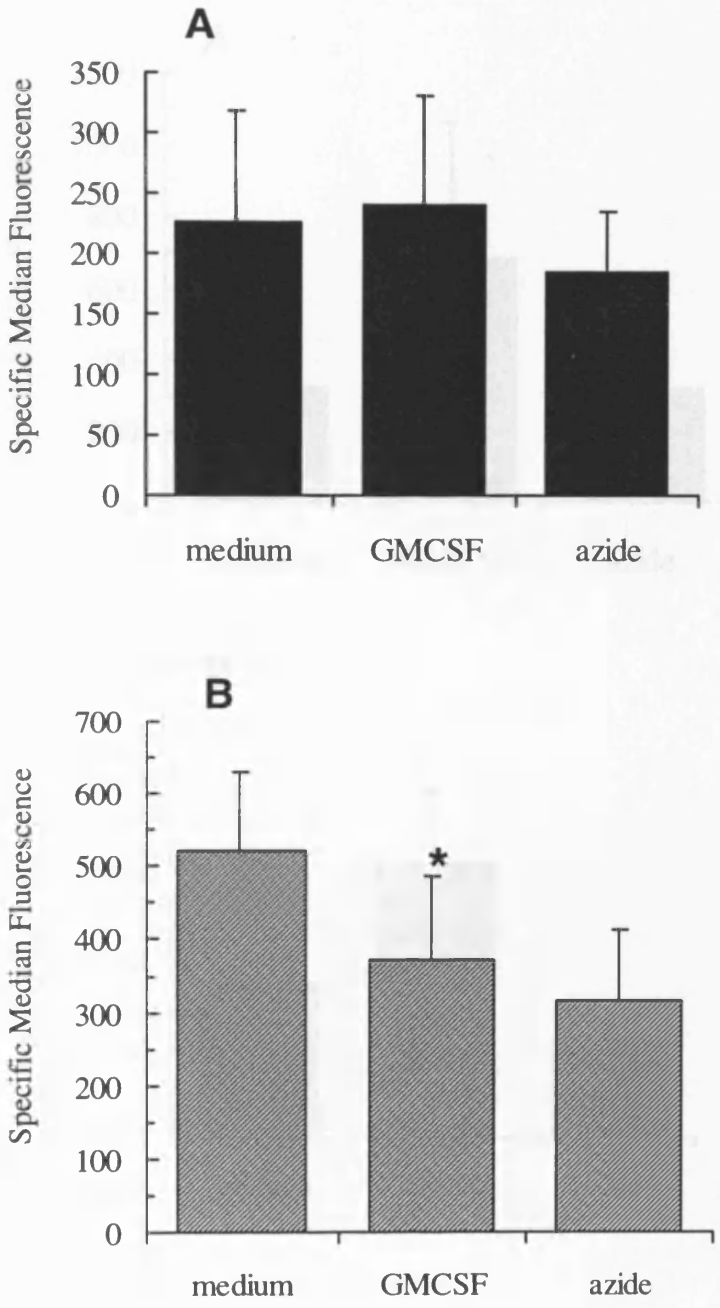


Figure 4–3 Effect of activation on surface expression of Mac-1 on A) eosinophils and B) neutrophils after stimulation with GM-CSF (10^{-9} M, 30 min, 37 °C) and azide (0.65%, 30 min, 37 °C). Eosinophils and neutrophils were purified (Methods, section 2.2.2) and incubated with activators as above, before being assessed for expression of Mac-1 using flow cytometry (Methods, section 2.2.4). Data are expressed as specific median fluorescence (test value minus isotype-matched control value). Eosinophils, n = 3, neutrophils, n = 5-6. Data shown are ± SEM.

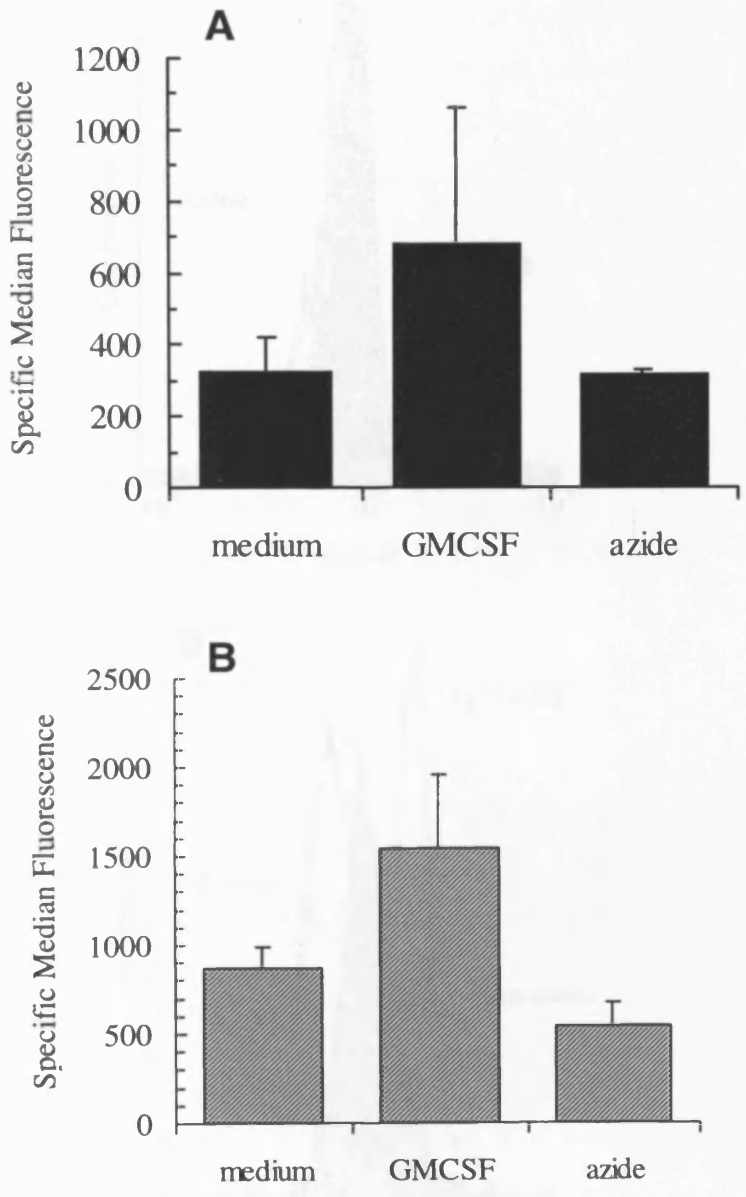
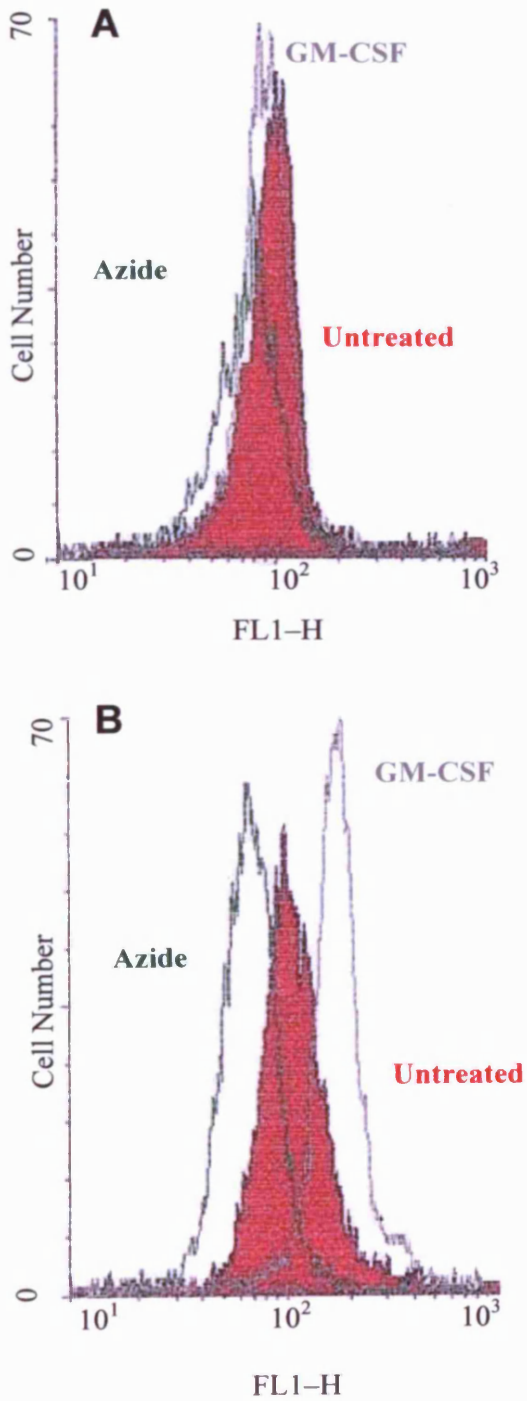


Figure 4–4 Representative FACS histograms for Figures 4–2 and 4–3, demonstrating the effect of GM-CSF (10^{-9} M, 30 min, 37 °C) and sodium azide (0.65%, 30 min, 37 °C) on A) L-selectin, and B) Mac-1 expression on eosinophils and neutrophils. Eosinophils and neutrophils were purified (Methods, section 2.2.2) and incubated with activators as above, before being assessed for expression of L-selectin and Mac-1 using flow cytometry (Methods, section 2.2.4). FACS histogram shown is for neutrophils, and is representative of experiments for both cell types.



Overall, neither GM-CSF nor azide had a significant effect on eosinophil L-selectin shedding (Figure 4–2 a). Activation of neutrophils with these agents caused slightly more shedding than from eosinophils, and the result with GM-CSF was significant (Figure 4–2 b). Mac-1 appeared to be upregulated on both eosinophils and neutrophils by GM-CSF, while azide had no effect (Figure 4–3 a) and b)). A representative example of the flow cytometry traces for these experiments is shown in Figure 4–4. An attempt was made to carry out the flow cytometry experiments in conjunction with the FSA, to see if there was a correlation with the effects of the activating stimulus on marker expression with that on adhesion to nasal polyp. However, no consistent data was generated due to insufficient numbers of cells being available to perform both tests on the same day. In the few experiments that were done, there was no correlation between marker expression and adhesion (data not shown).

4.2 1.2 Effect of protease inhibition on eosinophil and neutrophil adhesion to NPE

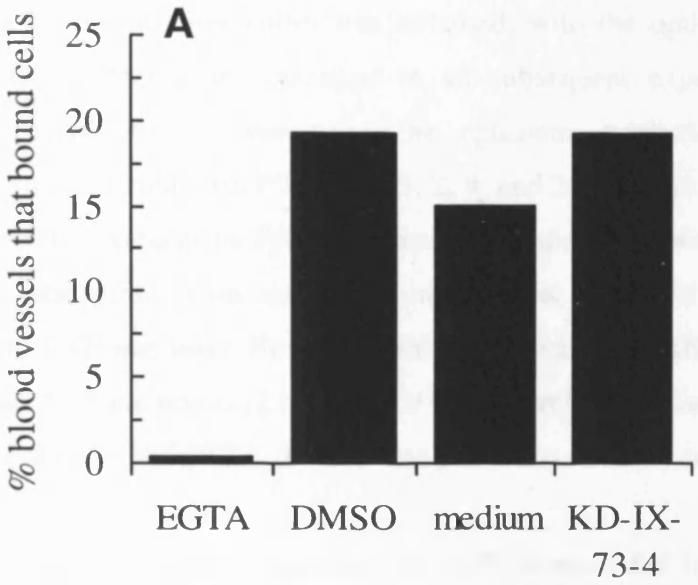
KD-IX-73-4 is a hydroxamic acid-based metalloprotease inhibitor peptide that has been shown to inhibit L-selectin proteolysis on lymphocytes (Feehan *et al.*, 1996). We obtained some of this peptide to use in the FSA to further investigate a role for L-selectin shedding in regulating eosinophil and neutrophil adhesion to nasal polyp. Preliminary experiments (eosinophils, n = 1, neutrophils, n = 2) demonstrated little effect of KD-IX-73-4 on adhesion of both cell types to NPE (Figure 4–5).

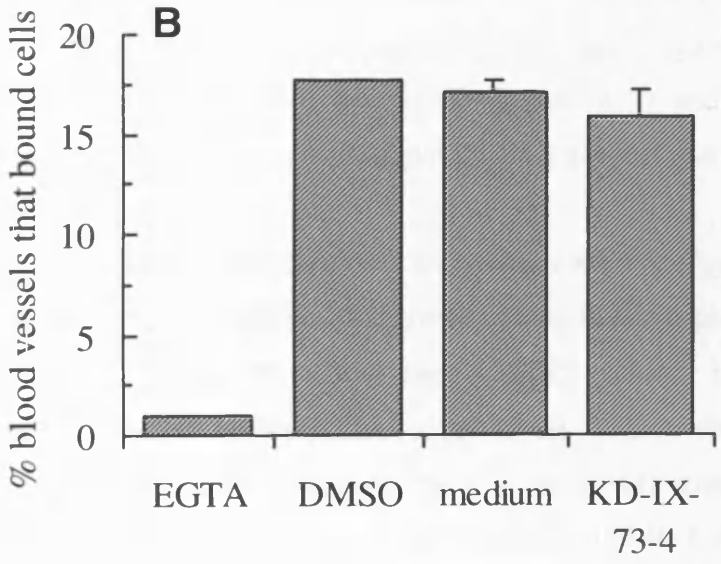
4.2.2 Leukocytes require energy to adhere to NPE as shown by potent inhibition of adhesion with azide

Since mediator priming had little effect on leukocyte adhesion in the FSA, another possibility was that the cells were becoming activated as they made contact with the endothelium. To test this, cells were treated with sodium azide, a non-specific inhibitor of cell metabolism. Azide treatment (0.65% final concentration) inhibited adhesion of both cell types by approximately 60%, as shown in Figure 4–1 (eosinophils 61.6% inhibition ± 2.6, neutrophils 62.9% inhibition ± 1.2). This observation offered support for the idea that cell activation occurring during the assay was required for effective adhesion.

The majority of chemokines and chemoattractants signal via GPCRs, many of which are PTX-sensitive (Luster, 1998). The inhibitory action of azide led to the investigation of the possibility that cell activation was occurring through PTX-sensitive serpentine receptors.

Figure 4–5 Effect of the protease inhibitor KD-IX-73-4 on A) eosinophil and B) neutrophil adhesion to NPE. Eosinophils and neutrophils were purified (Methods, section 2.2.2) and incubated with EGTA-treated assay medium, KD-IX-73-4 (25 µg/ ml), DMSO or medium control for 30 min, 37 °C, before use in the FSA (Methods, section 2.2.6.2). Eosinophils, $n = 1$, neutrophils, $n = 2$. Data shown are ± SEM.





4.2.3 Eosinophil chemotaxis to PAF is inhibited by PTX

Initially, chemotaxis assays were performed to discover the optimum conditions for the use of PTX in the FSA. PAF elicited directional locomotion of eosinophils, in a time- and dose-dependent fashion, at concentrations from 10^{-5} to 10^{-9} M, as previously described (Wardlaw *et al.*, 1986). Figure 4–6 shows the dose response of eosinophil chemotaxis to PAF. A bell-shaped concentration curve was obtained, with the optimum concentration being 10^{-6} M. This concentration was used in all subsequent experiments with PAF (chemotaxis and activation). To determine the optimum incubation time for PTX, eosinophils were pre-incubated with PTX for 0.5, 2, 4, and 24 h before chemotaxis to PAF (Figure 4–7). Control migration to PAF decreased over the 24 h period. PTX inhibited migration at each time point, with maximum inhibition at 4 h (at the 24 h time point, control migration to PAF was low). However, since there was little difference in inhibition between the 2 h and 4 h time points (2 h: 49 cells/ hpf control, 7.2 cells/ hpf PTX, 4 h: 45.7 cells/ hpf control, 3.2 cells/ hpf PTX), the 2 h time point was used for convenience.

4.2.4 Neutrophil, but not eosinophil, adhesion to NPE is mediated by a PTX-sensitive GPCR

A striking difference was observed in the FSA using PTX, with complete inhibition of neutrophil adhesion to NPE but no effect on eosinophil adhesion (Figure 4–8). This was despite PTX specifically inhibiting both eosinophil (Figure 4–7) and neutrophil (not shown) chemotaxis to PAF, which demonstrated that the PTX reagent was functional.

The agents involved in eosinophil and neutrophil activation were investigated further with the PAF antagonist WEB 2086 (10^{-5} M) and function blocking IL-8 receptor antibodies (25 μg/ ml) against CXCR1 (clone 9111.5.1) and CXCR2 (clone 10H2.12.1). The effectiveness of these reagents was determined prior to the FSA in chemotaxis assays (Figure 4–9). As expected, WEB 2086 almost completely inhibited neutrophil migration to PAF but had no effect on migration towards IL-8. Similarly, anti-IL-8 receptors did not block neutrophil migration to PAF. Individually, anti-CXCR1 and -R2 both inhibited neutrophil adhesion to IL-8 by approximately 50%, the effect increased to 70% when both antibodies were used together.

Figure 4–6 Dose response of eosinophil chemotaxis to platelet activating factor (PAF). Eosinophils were purified and used in a standard chemotaxis assay towards PAF (Methods, section 2.2.8), at a range of concentrations from 10^{-5} to 10^{-9} M. The optimum dilution, 10^{-6} M, was used in all subsequent assays. The figure represents one experiment undertaken in triplicate, and is representative of one other experiment.

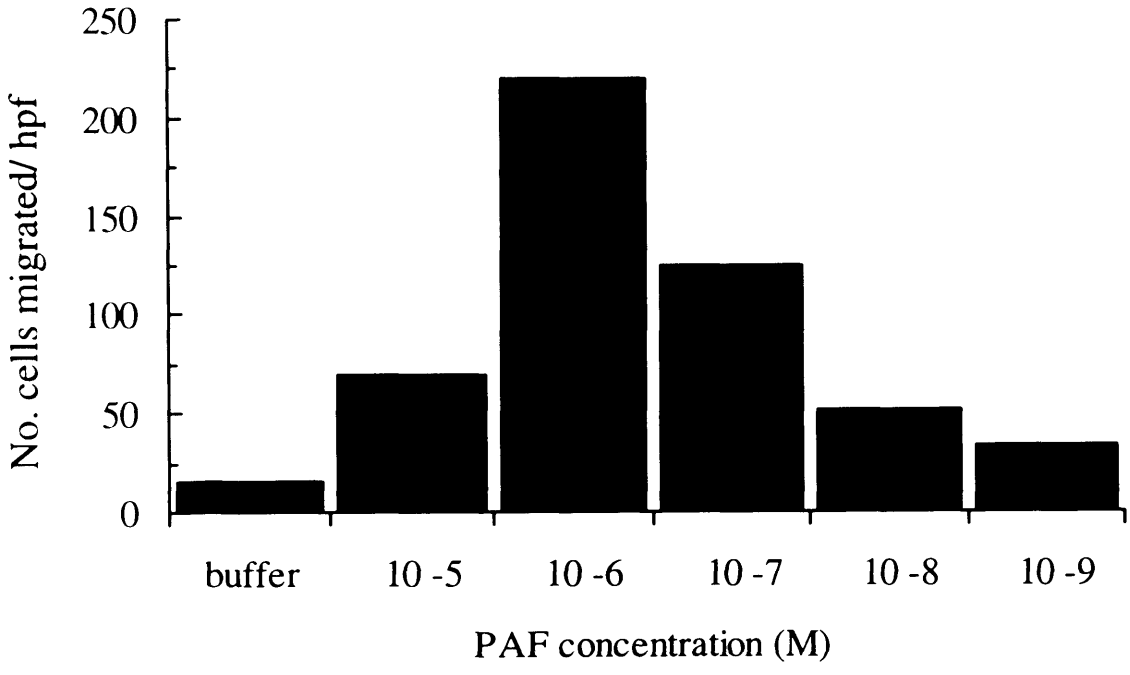


Figure 4–7 Time course of PTX inhibition of eosinophil migration to PAF. Eosinophils were purified and pre-incubated with 100 ng/ ml PTX for 0.5, 2, 4 and 24 h before being used in a standard chemotaxis assay towards 10^{-6} M PAF (Methods, section 2.2.8). The figure represents one experiment undertaken in triplicate, and is representative of one other experiment.

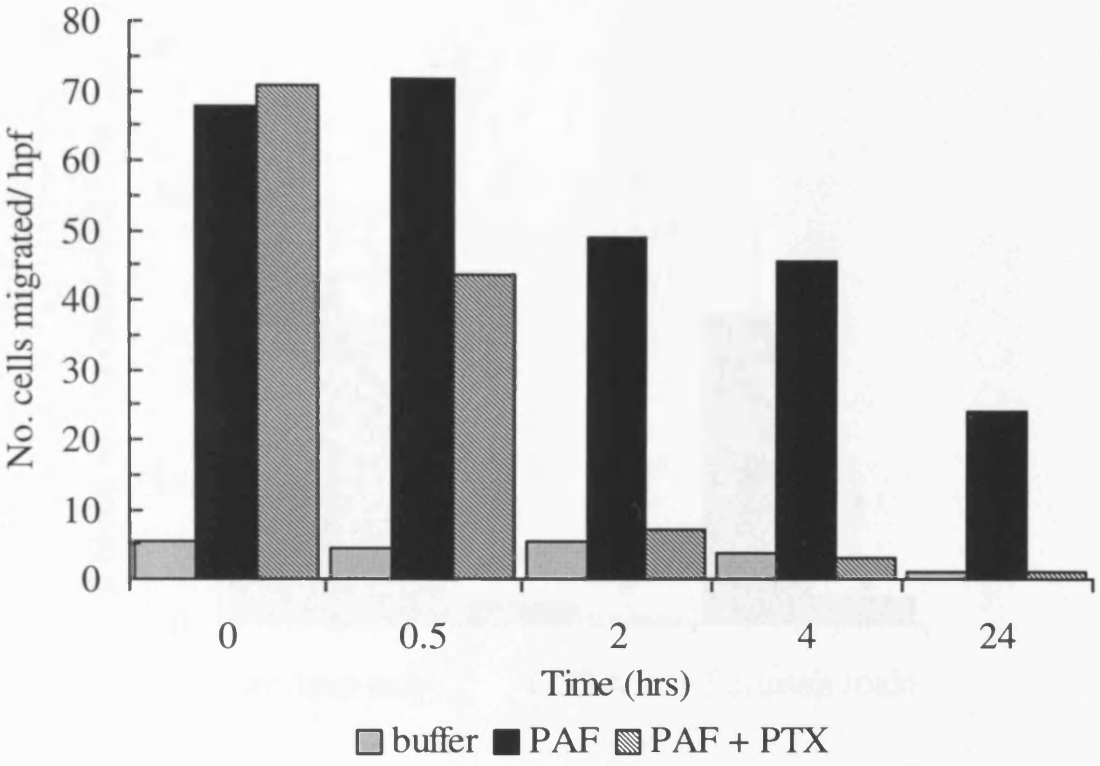


Figure 4–8 Neutrophils, but not eosinophils, signal via a PTX-sensitive, seven transmembrane receptor to bind to NPE. Eosinophils and neutrophils from different donors were isolated (Methods, section 2.2.2), and incubated with PTX (100 ng/ ml) for 2 h at 37 °C, before use in the FSA (Methods, section 2.2.6.2). Filled columns, eosinophils, $n = 4$; hatched columns, neutrophils, $n = 3$. Values represent the means ± SEM, * $P < 0.05$.

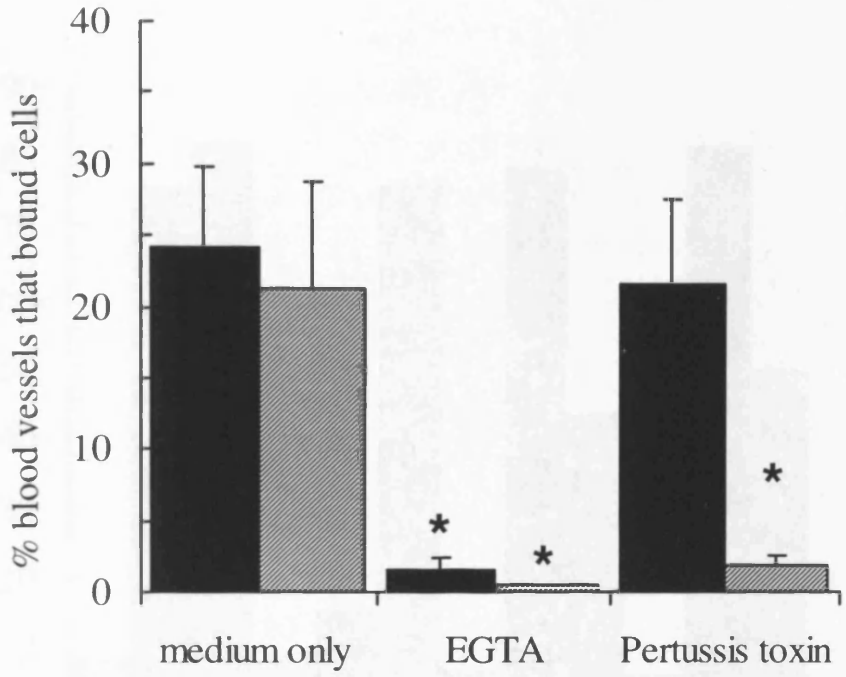
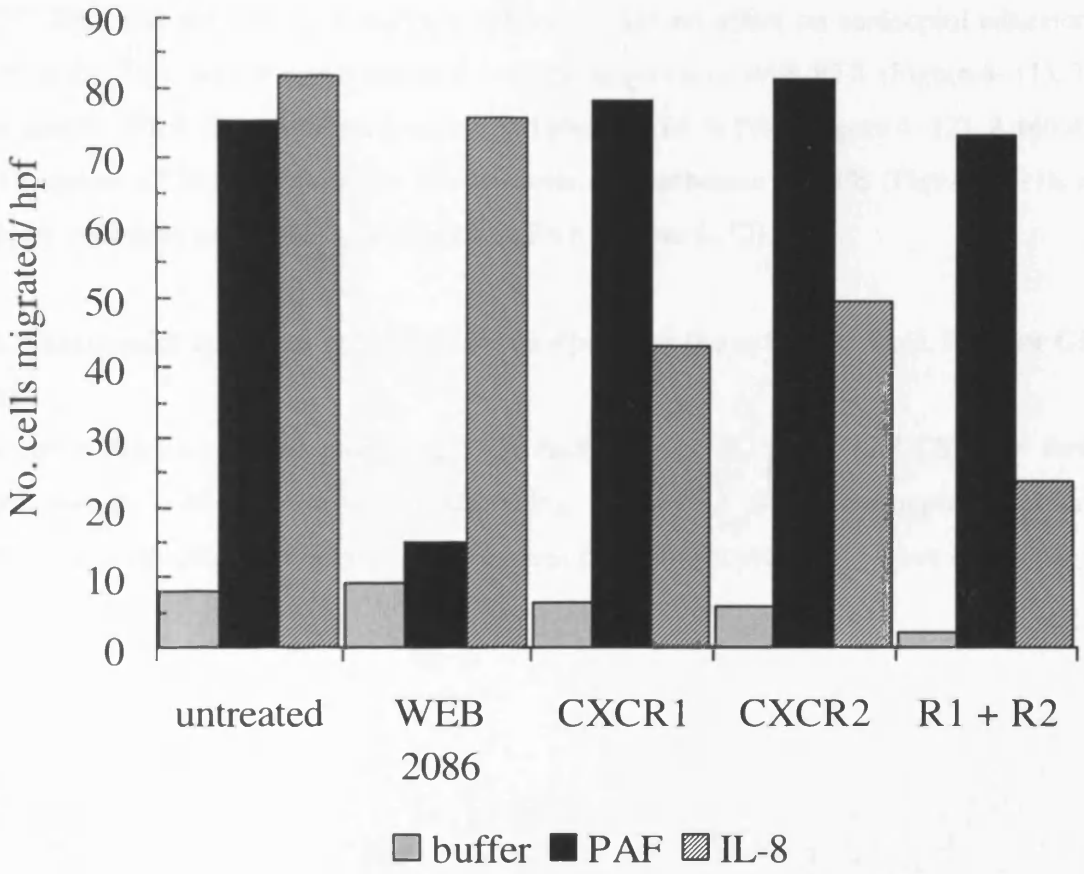


Figure 4–9 Neutrophil migration to PAF is inhibited by WEB 2086 but not CXCR1 and -R2, whereas neutrophil migration to IL-8 is inhibited by CXCR1 and -R2 but not WEB2086. Neutrophils from different donors were isolated and used in a chemotaxis assay towards PAF at 10^{-6} M, or IL-8 at 100 ng/ ml (Methods, section 2.2.8). Cells were incubated with WEB 2086 (PAF antagonist, 10^{-5} M) or anti-IL-8 receptors (25 µg/ ml) for 15 min at RT prior to chemotaxis. The figure represents one experiment undertaken in triplicate and is representative of one other experiment.



4.2.5 Neutrophil adhesion to NPE is mediated by PAF and IL-8

In the FSA, neutrophil adhesion was inhibited by WEB 2086 and anti-CXCR1 and -R2 (Figure 4–10). An anti-IL-8 mAb (clone A5.12.14) also inhibited neutrophil adhesion by a mean of 46% (MoIgG control, 19.61% ± 1.22 versus anti-IL-8 mAb, 10.6 ± 1.16%; $n = 3$, $p < 0.05$, data not shown).

4.2.6 Eosinophil activation for adhesion to NPE is not via PAF, IL-8, or CCR3

WEB 2086 and the anti-IL-8 receptor antibodies had no effect on eosinophil adhesion to NPE in the FSA, which was consistent with the observation with PTX (Figure 4–11). This was despite WEB 2086 inhibiting eosinophil chemotaxis to PAF (Figure 4–12). A blocking mAb against CCR3 also failed to inhibit eosinophil adhesion to NPE (Figure 4–11), also despite inhibiting eosinophil chemotaxis to PAF (Figure 4–12).

4.2.7 Eosinophil adhesion to NPE is not mediated by the cytokines IL-3, IL-5, or GM-CSF

The pro-inflammatory eosinophil growth factors IL-3, IL-5 and GM-CSF are further candidates for mediating eosinophil signalling. In the FSA model, eosinophil adhesion to NPE was unaffected by blocking mAbs against these three cytokines (Figure 4–13).

Figure 4–10 Neutrophil adhesion to NPE is mediated by PAF and IL-8. Neutrophils from different donors were isolated (Methods, section 2.2.2) and treated with CXCR1 (clone 9111.5.1), CXCR2 (clone 10H2.12.1), the PAF antagonist WEB2086 (10^{-5} M), control antibody, or EGTA-treated assay medium for 15 min at RT, before use in the FSA (Methods, section 2.2.6.2). n = 5-6. Values represent the means ± SEM, * $P < 0.02$, ** $P < 0.005$, *** $P < 0.0009$.

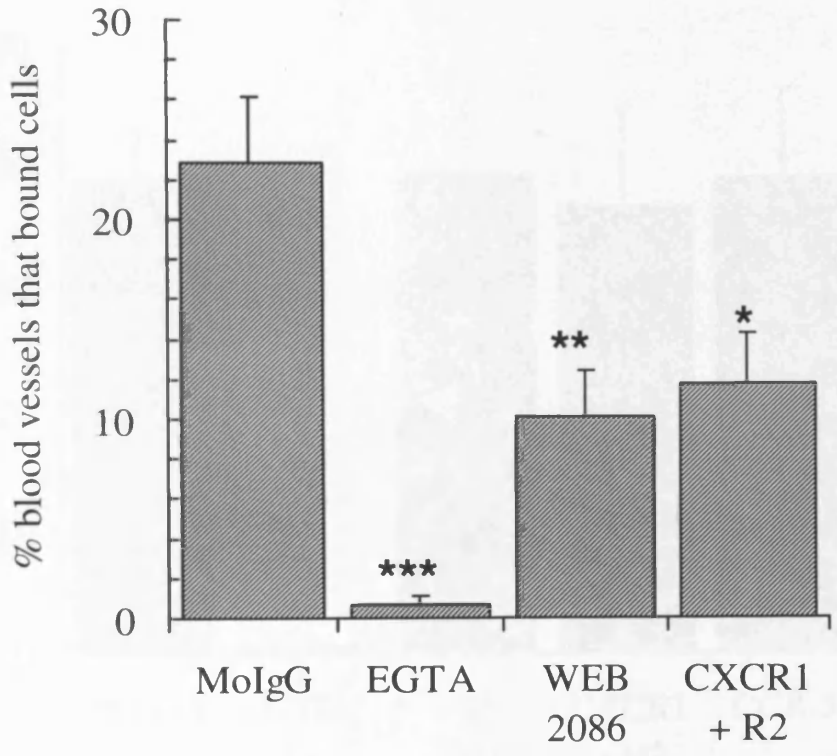


Figure 4–11 Eosinophil adhesion to NPE is not mediated by PAF, IL-8, or eotaxin. Eosinophils from different donors were purified (Methods section 2.2.2) and treated with CXCR1 (clone 9111.5.1), CXCR2 (clone 10H2.12.1), CCR3 (anti-eotaxin receptor clone 7B11), the PAF antagonist WEB 2086 (10^{-5} M), control antibody, or EGTA-treated assay medium for 15 min at RT before use in the FSA (Methods, section 2.2.8). n = 3-5. Values shown indicate means ± SEM, *** $P < 0.0001$.

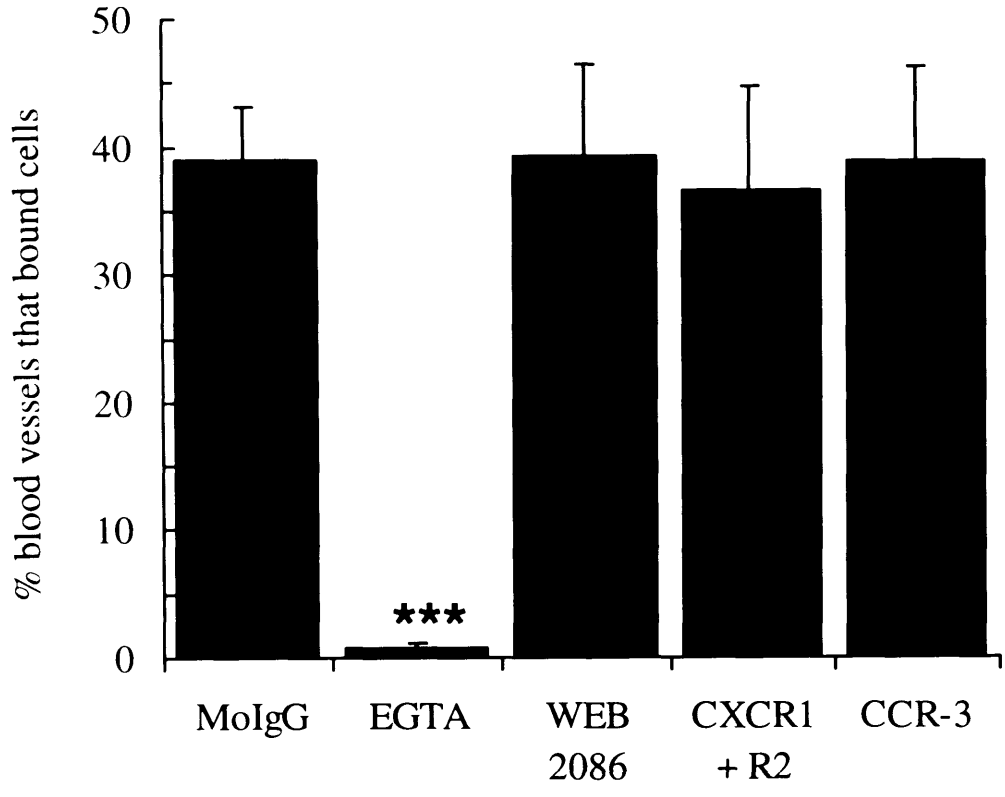


Figure 4–12 Inhibition of eosinophil chemotaxis to PAF and eotaxin by selective receptor antagonists. Eosinophils from different donors were isolated and used in a standard chemotaxis assay towards control buffer, PAF (10^{-6} M), or eotaxin (100 ng/ ml) (Methods, section 2.2.8). Eosinophils were incubated with mAb CCR3 (25 µg/ ml), or WEB 2086 (10^{-5} M) for 15 min at RT, prior to chemotaxis. The figure represents one experiment undertaken in triplicate, and is representative of one other experiment.

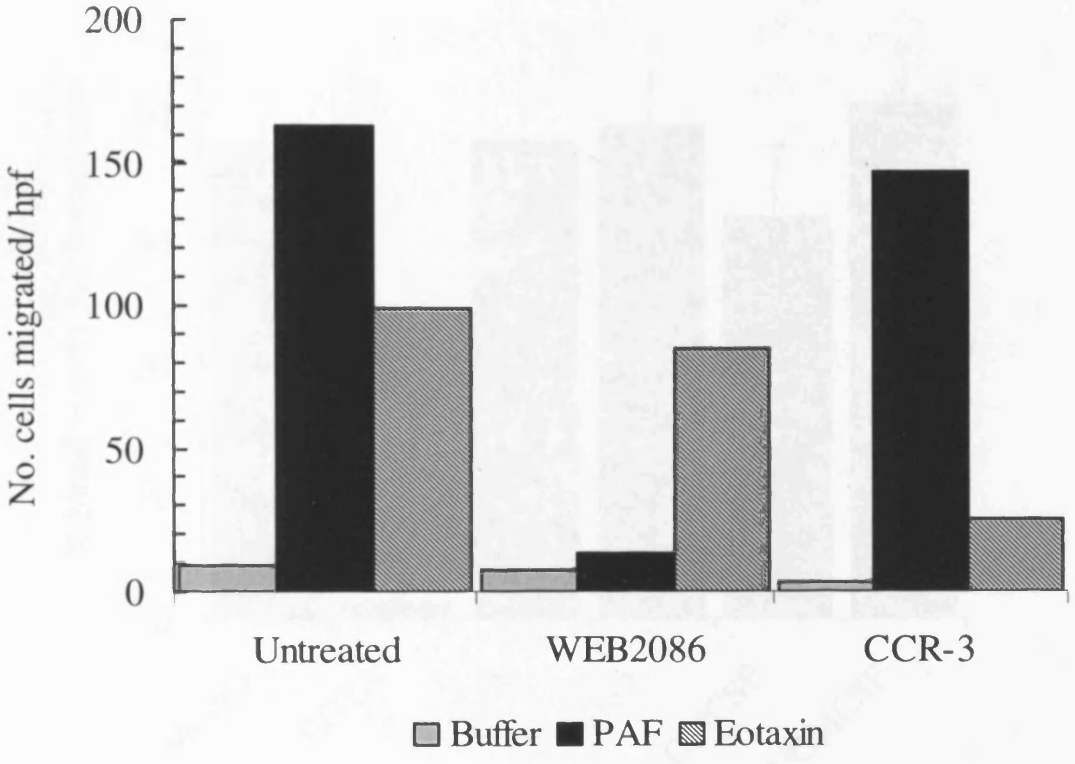
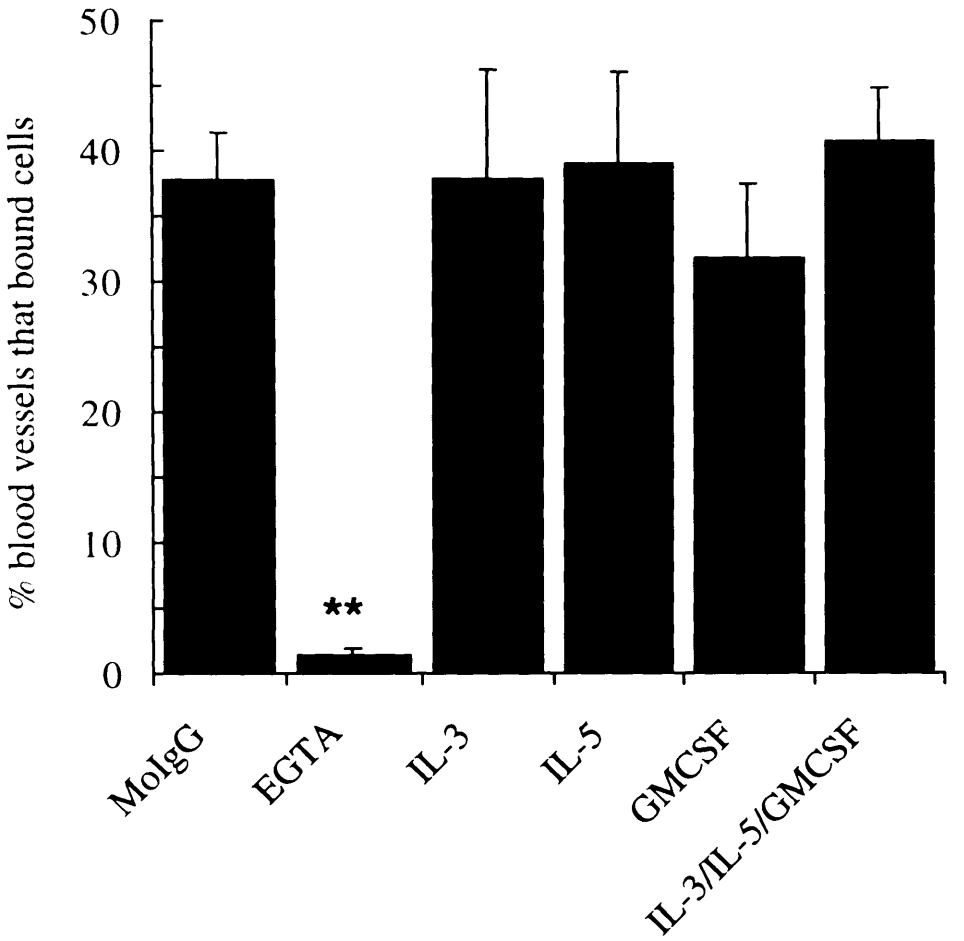


Figure 4–13 Eosinophil adhesion to NPE is not mediated by IL-3, IL-5, or GM-CSF. Eosinophils from different donors were purified (Methods, section 2.2.2) and incubated for 15 min at RT with anti-IL-3, anti-GM-CSF, anti-IL-5 clone mAb7, control antibody, or EGTA-treated assay medium before use in the FSA (Methods, section 2.2.6.2). $n = 3$. Values shown indicate means ± SEM, ** $P < 0.005$.



4.3 Summary

Using the nasal polyp frozen section assay model, stimulating eosinophils with GM-CSF or RANTES before addition of the cells to NPE had no effect on adhesion. Binding of both eosinophils and neutrophils to NPE was inhibited by up to 50% by incubation with azide. In addition, neutrophil adhesion was completely inhibited by PTX, and inhibited by approximately 50% by the PAF antagonist WEB2086 and antibodies directed against CXCR1 and CXCR2. In contrast, eosinophil adhesion was unaffected by PTX, WEB2086, anti-CCR3, or anti-IL-8 receptor mAbs. Similarly, mAbs against the eosinophil growth factors IL-3, IL-5, and GM-CSF had no effect on adhesion.

4.4 Discussion

Although the involvement of selectins and integrins in leukocyte transmigration appears secure, there is still some uncertainty about the activation signal. It is well known that the leukocyte integrins need to be activated through outside-in signalling to be able to bind to their endothelial counter-structures. Most work on the activation step has been undertaken with neutrophils and lymphocytes, few studies have investigated the signals required for eosinophil adhesion to endothelium.

The importance of cell activation in the FSA was explored. This aspect had been touched upon briefly by Symon *et al.*, whose data suggested a requirement for an eosinophil activation step (Symon *et al.*, 1994). Eosinophil adhesion to NPE was temperature-dependent, with optimal adhesion occurring at RT. A fall in adhesion was seen at 37 °C, this decreased to approximately 50% at 4 °C. These observations were extended further. Eosinophils of allergic subjects have been shown to be in a more active state in comparison to eosinophils from nonatopic subjects (Bass *et al.*, 1980). To avoid any effects of *in vivo* priming, normal donors were used for activation experiments with both eosinophils and neutrophils.

Firstly, the effects of cell stimulation on adhesion were studied. GM-CSF has previously been reported as upregulating CD18-dependent adhesion (Moser *et al.*, 1992). In the current study, there was no mean effect of GM-CSF on either eosinophil or neutrophil binding to NPE over four experiments for both cell types. The chemokine RANTES has previously been shown to induce eosinophil migration across HUVEC monolayers that is β2 integrin-dependent (Ebisawa *et al.*, 1994). Pre-activation of eosinophils with RANTES had no effect on adhesion in the FSA.

An alternative approach to look at an activation requirement was to inhibit cell metabolism, hence energy production, which is essential for activation. The metabolic inhibitor sodium azide is a general inhibitor of cell metabolism that prevents ATP production by inhibiting cytochrome oxidase c and ATP synthase. Azide significantly inhibited adhesion of both eosinophils and neutrophils to NPE, offering support that cell activation occurring during the assay was required for effective adhesion. Another study used azide to show that leukocyte activation was necessary for adhesion; in this case, for neutrophils to be able to bind to activated platelets under flow (Yeo *et al.*, 1994). Once it was established that activation was involved in the FSA, the individual mechanisms involved for both cell types were explored.

Firstly, the effects of PTX were studied; this inhibits the function of the majority of known chemoattractant GPCRs. PTX has been shown to inhibit chemokine-induced chemotaxis (Arai *et al.*, 1997) and cell adhesion (Campbell *et al.*, 1996). A striking difference was observed between eosinophils and neutrophils in the FSA, with complete inhibition of neutrophil adhesion, but no effect on eosinophil adhesion. The agents involved in neutrophil activation were further defined using the PAF antagonist WEB2086 and anti-IL-8 receptor mAbs. Neutrophil adhesion was inhibited by WEB2086 and anti-CXCR1 and -R2. This is consistent with previous studies in which both PAF and IL-8 were involved in neutrophil activation during adhesion to endothelium (Lorant *et al.*, 1993; Gaboury *et al.*, 1994; Rainger *et al.*, 1995). Rainger and co-workers dissected the separate kinetics of activation of neutrophils via IL-8 and PAF, by blocking PAF and IL-8 receptors in an *in vitro* flow model using HUVEC (Rainger *et al.*, 1997). IL-8 and PAF induced arrest of neutrophils rolling on P-selectin to promote stationary adhesion via β2 integrins. In the FSA, variability was observed in the amount of inhibition with the PAF antagonist and the anti-IL-8 receptor mAbs, suggesting that for a given polyp and blood donor the cells were predominantly being activated by either PAF or IL-8.

The inhibitory effect of the anti-IL-8 mAb confirmed the involvement of this cytokine, but does not exclude a role for other CXC chemokines. In addition, other chemoattractants such as LTB_4 or C5a may be involved in neutrophil activation, because PTX was more effective at blocking adhesion than the combined effect of WEB2086 and the CXCR1 and -R2 antibodies (data not shown, % inhibition combined = 58.9 ± 14.5, $P < 0.05$, $n = 4$). Both C5a and LTB_4 have been shown to enhance neutrophil adhesion to endothelium via β2 integrins (Tonnesen *et al.*, 1989; Jagels *et al.*, 2000).

The polyp tissue was the likely source of PAF and IL-8, although it is not certain that PAF and IL-8 were being presented to the neutrophil expressed on the surface of the endothelium. It is possible that other cell types in the polyp, such as T-cells, fibroblasts, endothelial cells, mast cells, epithelial cells, and resident eosinophils, were releasing chemotaxins into the assay medium during the experiment. IL-8 has been shown to be generated in substantial quantities by nasal epithelium from patients with rhinitis (Calderon *et al.*, 1997). There was a significant correlation between myeloperoxidase (MPO) levels and neutrophil numbers and between MPO and IL-8 levels in the lavage of patients with chronic sinusitis in another study, suggesting that IL-8 might activate neutrophils (Demoly *et al.*, 1997). Allen *et al.* also demonstrated a substantial presence of IL-8 in nasal polyp tissue (Allen *et al.*, 1997b). In the present study, a diffuse staining pattern was seen when the nasal polyp tissue was stained with the anti-IL-8 mAb (data not shown). Thus, local production of IL-8 could be an important factor in the sustained recruitment of neutrophils in nasal polyposis, as well as in their activation.

Eosinophil adhesion was unaffected by PTX, WEB2086, the anti-IL-8 receptor antibodies, and an antibody against CCR3, which is the major chemokine receptor so far identified on peripheral blood eosinophils. This was despite PTX inhibiting chemotaxis to PAF and eotaxin, and WEB2086 and the anti-CCR3 mAb inhibiting chemotaxis to PAF and eotaxin respectively, which demonstrated that the reagents used were functional. A potent PAF antagonist also had no significant effect on eosinophil tethering, arrest, or accumulation on TNF-α- and IFN-γ-activated HUVEC when used alone or with an anti-CCR3 mAb in a study by Kitayama *et al.*, (Kitayama *et al.*, 1998). In the same study, PTX only moderately reduced eosinophil arrest and accumulation. Nonetheless, the involvement of CD18 integrins, which require activation to be functional, together with the inhibitory effect of azide and reduced binding at 4 °C, suggest that an activation step was occurring in the current experiments. PTX does not uncouple chemokine receptors from all classes of G-proteins, so a role for chemokine signalling in mediating adhesion cannot be ruled out. For example, studies have shown that CCR2 and CXCR1 couple to multiple G-proteins that are not PTX-sensitive (Arai and Charo, 1996; Kuang *et al.*, 1996). Eosinophils have not been shown to express CCR2, and inconsistencies exist in the literature with respect to the sensitivity of eosinophils to IL-8 (Ebisawa *et al.*, 1994; Erger and Casale, 1995); a recent study concluded that neither CXCR1 nor CXCR2 are expressed on eosinophils (Petering *et al.*, 1999). However, there may be an as yet undiscovered chemokine or receptor involved.

It cannot be ruled out that the method used to isolate the eosinophils may have had an effect on eosinophil function when studying activation in the FSA. It has been shown that eosinophils isolated using immunomagnetic beads are more activated than those isolated via Percoll gradients, as measured by increased LTC_4 production, O_2^- generation, and cell surface marker expression (Sedgwick *et al.*, 1996). In a study by Blom *et al.*, CD16 bead-isolated cells showed heightened chemotactic responses to C5a and PAF (Blom *et al.*, 1995). In contrast, Casale *et al.* suggested that immunomagnetically isolated eosinophils responded more poorly to PAF and LTB_4 than those isolated using Percoll (Casale *et al.*, 1999). CD16-isolated eosinophils show no chemotactic response to IL-8, in contrast to Percoll-purified cells (Rozell *et al.*, 1996). The data are conflicting, but suggest that comparisons between eosinophil functions from different studies should take into account the use of different isolation protocols when determining the relevance to *in vivo* settings.

PAF is an effective, though non-specific, chemoattractant for eosinophils, and has been shown to enhance CD18-dependent adhesion to HUVEC (Wardlaw *et al.*, 1986; Kimani *et al.*, 1988). Several studies have evaluated the role of IL-8 in eosinophil activation and migration, resulting in conflicting data. Some *in vitro* studies failed to show a chemotactic effect of IL-8 on eosinophils (Schroder *et al.*, 1987), whereas others succeeded. However, the successful studies used cytokine-primed eosinophils (Schweizer *et al.*, 1994), or cells from allergic asthmatic (Erger and Casale, 1995) or eosinophilic (Sehmi *et al.*, 1993) subjects. As discussed above, the eosinophil separation protocol has been shown to affect the outcome of the chemotactic response to IL-8, and it is uncertain whether eosinophils express CXCR1 and -R2. Therefore, it is perhaps unsurprising that no effect was seen in the FSA with the IL-8 receptor antibodies. However, recently, IL-8 was shown to cause a transient arrest of rolling, unprimed eosinophils on TNF-α-activated HUVEC under flow conditions, which was α4- and β2 integrin-dependent (Ulfman *et al.*, 2001); further studies may reveal a more prominent role for IL-8 in eosinophil activation.

There is an increasing amount of literature demonstrating that several CC chemokines are effective, and often highly specific, eosinophil chemoattractants that are expressed in eosinophilic inflammation, including nasal polyps (Ponath *et al.*, 1996; Stellato *et al.*, 1997). These chemokines signal primarily via CCR3. RANTES, C5a, and MCP-3 were able to enhance adhesion of eosinophils to purified VCAM-1 via VLA-4 and purified ICAM-1 via Mac-1 (Weber *et al.*, 1996). In contrast, eotaxin (but not RANTES) was able to enhance adhesion to endothelial cells via a VLA-4-dependent pathway (Burke-Gaffney

and Hellewell, 1996). It is therefore unclear why eosinophil activation was not mediated by nasal polyp-derived chemoattractants including chemokines. The role of CC chemokines in allergic disease may be predominantly to direct eosinophil chemotaxis to the mucosal surface, rather than to mediate the activation step in endothelial adhesion. It is possible that chemokines that can signal through the CCR1 receptor are playing a part in eosinophil activation in the FSA. However, this is unlikely, because CCR1 almost certainly signals through a PTX-sensitive receptor (Nardelli *et al.*, 1999; Sabroe *et al.*, 1999).

Kitayama and colleagues recently demonstrated a modest reduction in eosinophil adhesion to HUVEC under shear flow with a mAb against CCR3 (Kitayama *et al.*, 1998). This is in contrast to what was found in the present study using the same CCR3 mAb. However, the effect Kitayama *et al.* observed required a combination of TNF-α and IFN-γ, whereas IL-4, a cytokine more relevant to allergic inflammation, did not result in CC chemokine production by HUVEC. The recent identification of the CX_3C subfamily of chemokines, which can enhance adhesion through a PTX-insensitive mechanism (Bazan *et al.*, 1997), means that it remains possible that a chemokine is triggering the activation step. However, there did not appear to be a role for fractalkine in mediating eosinophil adhesion to NPE in preliminary experiments (also refer to section 3.4).

Eosinophil growth factors, in particular IL-3, IL-5, and GM-CSF, are highly effective at enhancing CD18-dependent eosinophil adhesion (Walsh *et al.*, 1990; Moser *et al.*, 1992). Antibodies directed against these cytokines did not inhibit eosinophil adhesion in the FSA, for unknown reasons. It is possible that IL-3, IL-5, and GM-CSF, or their receptors, were not expressed by the nasal polyp endothelium we used. However, all three cytokines have been shown to be present in NPE, and associated with infiltrating cells, primarily eosinophils (Allen *et al.*, 1997a).

Adhesion receptors not only have to be activated in order to bind their ligands, but must also become disengaged in order for cells to extravasate and move through tissues. Such disengagement can occur through deactivation or proteolytic shedding. L-selectin shedding is one of the major mechanisms of leukocyte deadhesion, allowing leukocytes to move from a rolling to a firm arrest stage. Although other cell markers also show a downregulation upon activation (e.g. CD14, CD16, CD44), the shedding of L-selectin is distinct because of its rapidity and resistance to protease inhibitors (Kishimoto *et al.*, 1989; Bazil and Strominger, 1994; Preece *et al.*, 1996).

We examined the effect of activation on the differential expression of cellular adhesion molecules on the surface of purified human eosinophils and neutrophils. GM-CSF caused a modest but significant L-selectin shedding on neutrophils, but not eosinophils. Azide gave variable results with both eosinophils and neutrophils, causing shedding in some experiments, but not others; overall, the effect was not significant. More recently, azide has been shown to induce a rapid L-selectin shedding from neutrophils, indicating that the maintenance of L-selectin on the neutrophil surface requires energy consumption (Gomez-Gaviro *et al.*, 2000). In the present study, GM-CSF upregulated the surface expression of Mac-1 on both eosinophils and neutrophils, but this was not significant. Azide had little effect on Mac-1 expression on either cell type. On eosinophils, both Mac-1 surface expression and affinity for adhesion increase after stimulation, following mobilisation of intracellular ligand to the surface, and through a conformational change in β2 integrin (Lundahl *et al.*, 1993; Neeley *et al.*, 1993; Weber *et al.*, 1996). Significant increases in Mac-1 and decreases in L-selectin expression on both eosinophils and neutrophils were shown with stimulation with PAF and fMLP (Neeley *et al.*, 1993). In contrast, IL-5 selectively affected the surface expression of Mac-1 and L-selectin in eosinophils but not neutrophils (Neeley *et al.*, 1993).

An attempt was made to link the FSA with the flow cytometry experiments; however, this was difficult due to insufficient cell yield. In the three experiments performed (two on eosinophils, one on neutrophils), there was no effect of L-selectin shedding or Mac-1 upregulation caused by activation with GM-CSF or azide on adhesion to NPE. An unidentified metalloproteinase is believed to be responsible for L-selectin shedding, the activity of which can be effectively inhibited by the well characterised hydroxamic acid based metalloprotease inhibitor KD-IX-73-4 (Feehan *et al.*, 1996). KD-IX-73-4 was used to further investigate a role for L-selectin shedding in regulating eosinophil and neutrophil adhesion to NPE. In preliminary experiments, KD-IX-73-4 demonstrated little effect on adhesion of both cell types to NPE. Previously, Symon *et al.* found no effect on adhesion of eosinophils to NPE using a function blocking mAb against L-selectin (Symon *et al.*, 1994). Allport *et al.* did not show any regulatory function for L-selectin shedding using KD-IX-73-4 in terms of neutrophil attachment, rolling, or migration, on and through TNF-α-treated HUVEC (Allport *et al.*, 1997). In contrast, in a more recent study, KD-IX-73-4 inhibited L-selectin shedding on neutrophils, which decreased leukocyte rolling velocity and increased rolling flux in the mouse cremaster muscle (Hafezi-Moghadam and Ley, 1999). The role of L-selectin in eosinophil rolling is believed to be of less importance than

P- and E-selectin. L-selectin was shown to mediate eosinophil adhesion to HUVEC, but this was at 4 °C (Knol *et al.*, 1994). Combining the FSA and flow cytometry data suggests that L-selectin shedding is not involved in eosinophil or neutrophil adhesion to NPE.

In summary, there was a striking difference between eosinophils and neutrophils in the activation step required for firm arrest on allergically inflamed endothelium, using a rotational assay. Binding of both cell types was energy-dependent as demonstrated by inhibition of adhesion with azide. Neutrophils adhered via PAF, IL-8, and possibly other mediators that signal via PTX-sensitive GPCRs. Eosinophils did not appear to use PAF, eotaxin, IL-8, or the eosinophil growth factors, IL-3, IL-5, and GM-CSF for signalling, nor did they signal via PTX-sensitive GPCRs. Additionally, L-selectin did not appear to be important for eosinophil adhesion to NPE.

CHAPTER FIVE

Characterisation of the receptors involved in eosinophil and neutrophil adhesion to cytokine-stimulated HUVEC under shear flow conditions

5.1 Introduction

So far, the role that individual adhesion receptors and activating agents have to play in the selective accumulation of eosinophils in allergic inflammation has been analysed using a model of chronic inflammation centred on a rotational assay. However, *in vivo*, selectin-dependent attachment occurs under fluid shear to result in eosinophils rolling along the vascular endothelium. In the case of lymphocytes, α4 integrins have also been shown to be important in the rolling stage of migration as well as the firm attachment step (Alon *et al.*, 1994; Alon *et al.*, 1995; Berlin *et al.*, 1995). To fully describe the adhesive sequence, it is necessary to identify the adhesion receptors and activating stimuli, and to effectively block their individual activities under conditions of flow. A more dynamic model of leukocyte-endothelial interactions was employed to attempt to gain further insight into the adhesive events that would be likely to occur *in vivo*.

The aim of the next two chapters was to examine in detail the molecular mechanisms of eosinophil and neutrophil adhesion to endothelium using a well-described *in vitro* parallel plate flow chamber assay (Lawrence *et al.*, 1987; Brown *et al.*, 1999). This system allows direct microscopic examination of leukocyte-endothelial interactions, under a range of defined laminar flow conditions that mimic blood flow in post-capillary venules. It was used to investigate the involvement of selectins, integrins (Chapter 5) and activation signals (Chapter 6) in the adhesion of eosinophils and neutrophils to HUVEC stimulated with different cytokines relevant to the inflammatory events occurring in allergic disease.

Studies have not previously been undertaken to examine whether the allergic cytokine IL-13 can, like IL-4, induce P-selectin expression on HUVEC and support eosinophil rolling. The first aim of this chapter was to address this question. IL-9 is also associated with allergic disease, and the effects of IL-9 on endothelial cells have not been reported. The second aim of this chapter was to investigate the pattern of expression of adhesion receptors on IL-9-stimulated HUVEC. Additionally, we explored whether IL-9-treated HUVEC could support eosinophil adhesion under shear flow conditions.

5.2 Results

5.2.1 IL-13 and IL-9 selectively induce P-selectin expression on HUVEC

The work with IL-4- and IL-13-stimulated HUVEC under flow was carried out in collaboration with Dr. G. Woltmann (Woltmann *et al.*, 2000). Expression data with IL-4 and IL-13 is provided courtesy of Dr. Woltmann and is essential for background information to supplement the flow experiments performed by myself. Expression of P-selectin on unstimulated HUVEC was either absent or barely detectable using flow cytometry. It was confirmed, as previously shown (Yao *et al.*, 1996), that IL-4 induced a consistent, if modest increase in surface P-selectin expression (Figure 5–1 a) and b), SMF medium, 1.54 ± 0.66, SMF IL-4, 6.39 ± 1.51, n = 7). IL-13 induced P-selectin expression to a similar degree (Figures 5–1 a) and b), SMF medium 1.54 ± 0.66, SMF IL-13, 5.97 ± 1.11, n = 7). IL-13 had no effect on E-selectin expression, which was absent on unstimulated cells, or ICAM-1, which was constitutively highly expressed on unstimulated cells (Figure 5–1 b). VCAM-1 was induced on HUVEC by both IL-4 and IL-13, at a level similar to P-selectin (Figure 5–1 b). In addition, the increase in P-selectin expression was dose-dependent, was readily apparent by 24 hours, and was maintained for up to at least 48 hours in culture (Appendices 4 and 5).

Interestingly, IL-9 also induced an increase in surface P-selectin expression, which was also modest but consistent (Figures 5–2 a) and 5–3 a) and b), SMF medium, 3.97 ± 1.87, SMF IL-9, 6.98 ± 2.61, n = 7). However, this expression did not reach significance due to two outlying data points. IL-9 had no effect on expression of VCAM-1, E-selectin, or ICAM-1 (data not shown). The IL-9 receptor was consistently expressed on a small population of unstimulated HUVEC, as shown in Figure 5–2 b) and Figure 5–3 c).

5.2.2 Eosinophils use P-selectin, VLA-4 and VCAM-1 to adhere to HUVEC in a static assay

Before evaluating the contribution of adhesion receptors under flow conditions, a static adhesion assay for the measurement of eosinophil adhesion to TNF-α-, IL-4-, and IL-13-stimulated HUVEC was performed. This was to determine whether the upregulation of P-selectin on confluent HUVEC after stimulation with IL-4 and IL-13, was functionally

Figure 5–1 IL-4 and IL-13 induce surface P-selectin and VCAM-1 expression in HUVEC. A) Representative FACS histogram demonstrating P-selectin expression in medium- and cytokine-treated HUVEC (shaded histogram, P-selectin, open histogram, isotype-matched control). B) IL-4 (20 ng/ ml) and IL-13 (5 ng/ ml) induced a significant increase in expression of both P-selectin and VCAM-1 after 48 h in culture. $n = 7$. Data are expressed as specific median fluorescence ± SEM, $P < 0.05$.

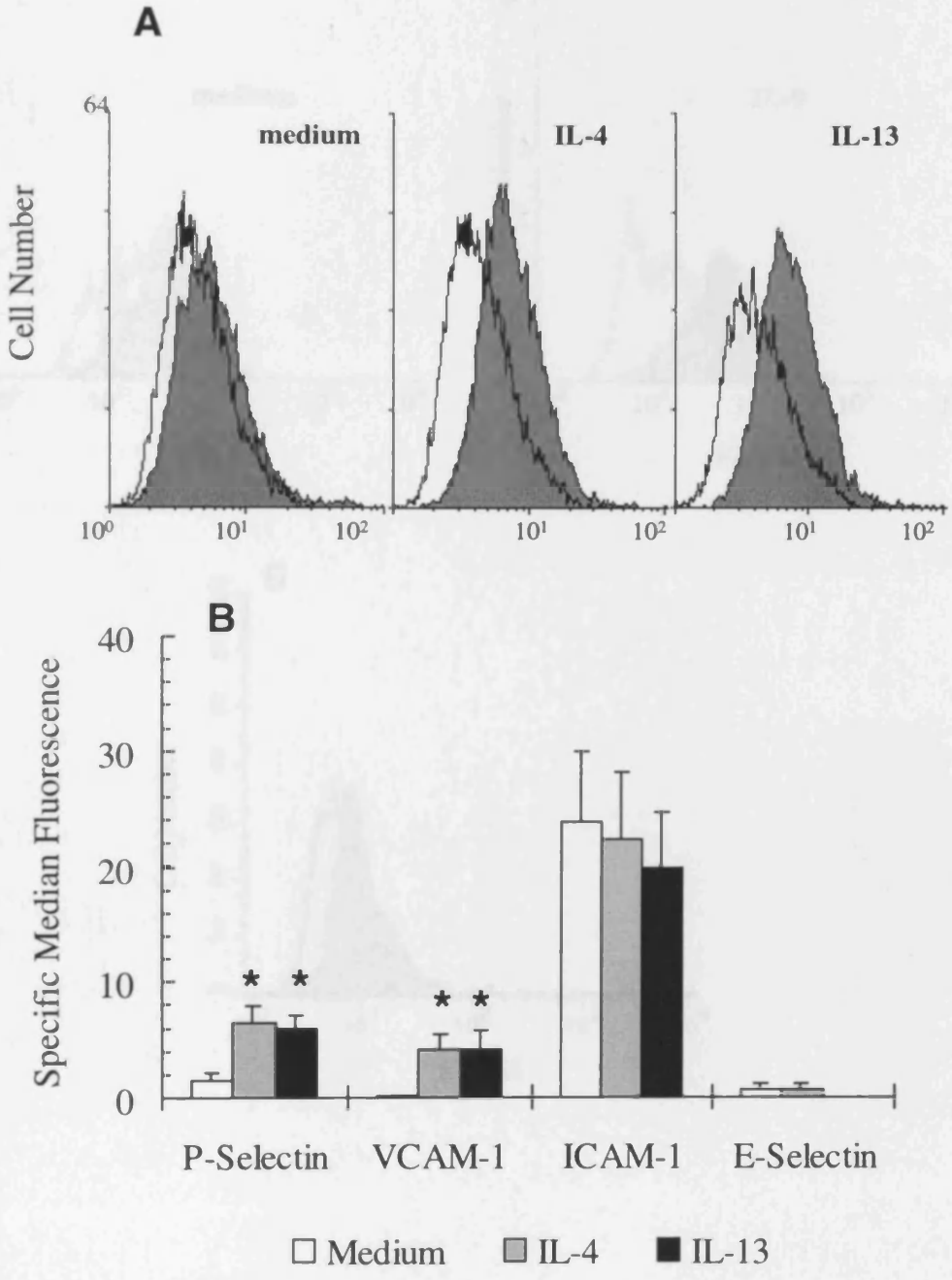


Figure 5–2 IL-9 induces surface P-selectin expression on HUVEC. A) Representative FACS histogram from an experiment demonstrating P-selectin expression on medium- and IL-9-treated (50 ng/ ml, 24 h) HUVEC (shaded histogram, P-selectin, open histogram, isotype-matched control). B) The IL-9 receptor is present on unstimulated HUVEC (shaded histogram, IL-9 R, open histogram, isotype-matched control, 1 histogram representative of 3 experiments).

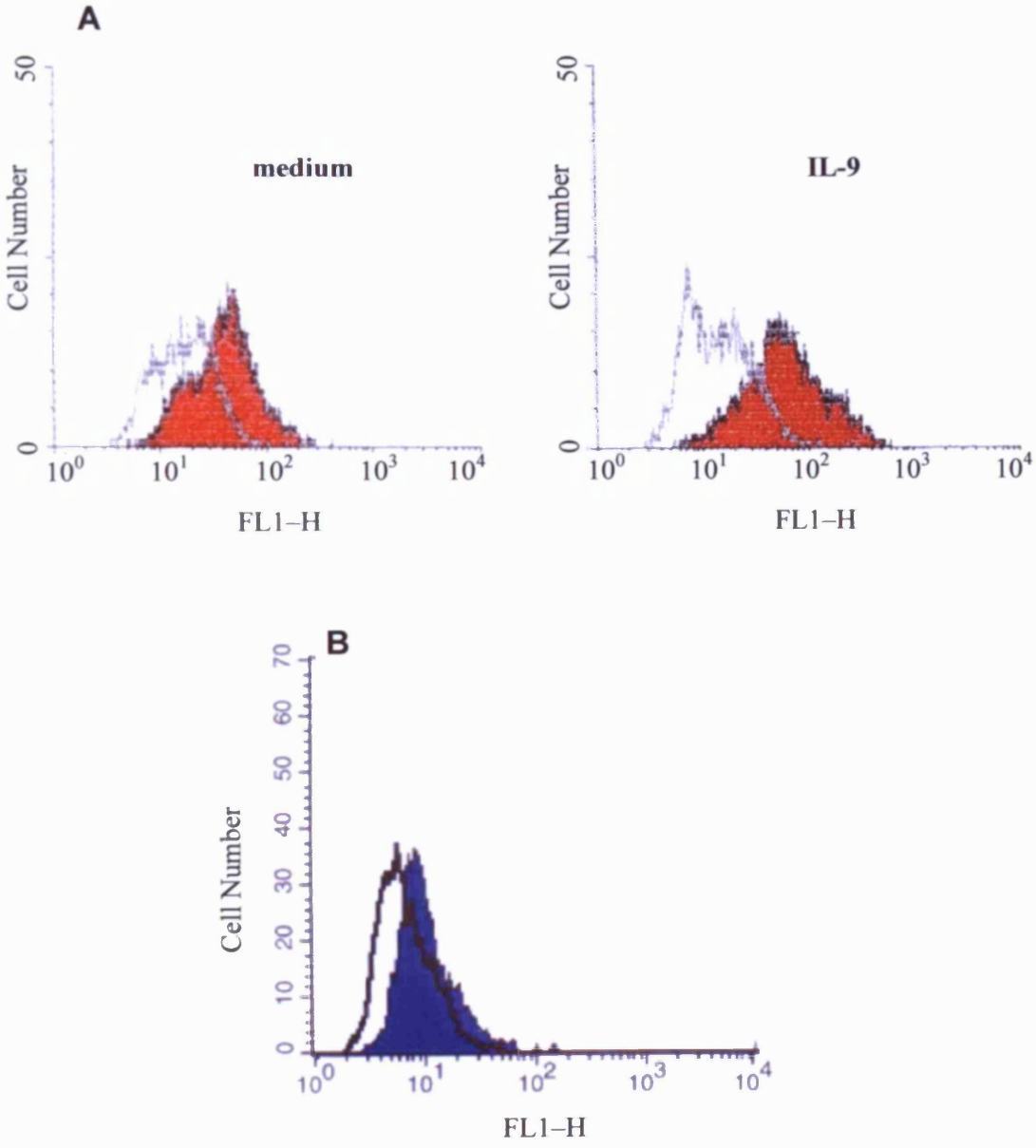
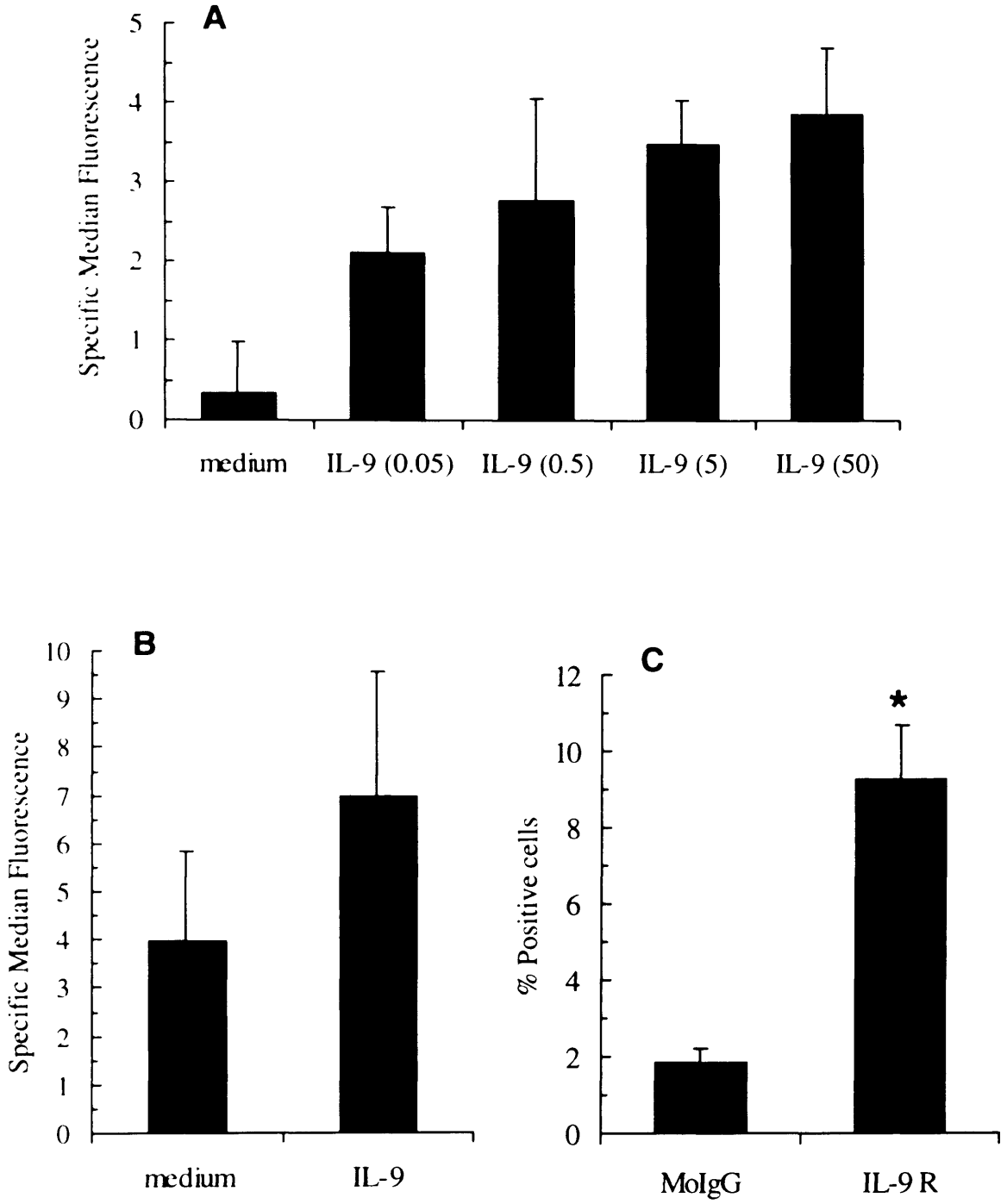


Figure 5–3 IL-9 induces an increase in surface P-selectin expression in HUVEC. A) Dose response with IL-9 after 24 h in culture (n = 4). B) Untreated and IL-9-treated HUVEC, 50 ng/ ml IL-9, 24 h (n = 7). C) The IL-9 receptor is expressed on unstimulated HUVEC, n = 3, * $P < 0.05$. All data are expressed ± SEM.



significant for eosinophil adhesion. As histamine causes rapid upregulation of granule-stored P-selectin to the cell surface, the adhesion assay was also performed with histamine stimulation (10 min) of the endothelial cell monolayer. HUVEC were stimulated with TNF-α, IL-4, or IL-13, with or without histamine at physiologically relevant concentrations, in order to maximise P-selectin expression. Eosinophils were incubated with either control Ab or blocking P-selectin mAb clone G1, and the adhesion assay was carried out as detailed in the Methods, section 2.2.7. The results are shown in Figure 5–4.

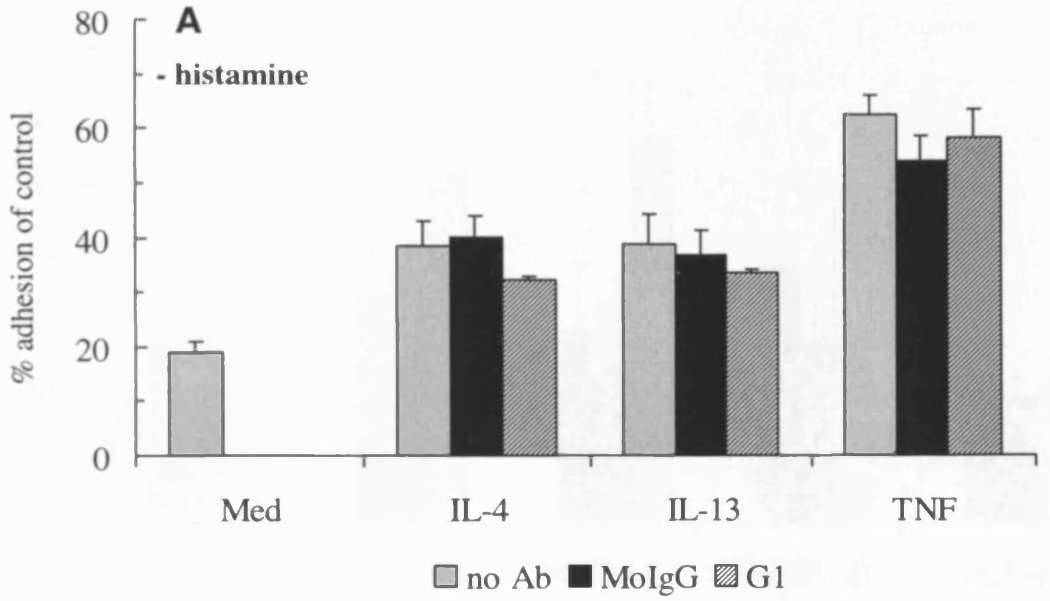
Histamine increased eosinophil adhesion to IL-4- and IL-13-stimulated cells, although this did not reach significance. Histamine had no effect on eosinophil adhesion to TNF-α-stimulated HUVEC. Blocking P-selectin had little effect on eosinophil adhesion to HUVEC stimulated with each individual cytokine. However, eosinophil adhesion to IL-4 or IL-13-stimulated HUVEC that had been pre-incubated with histamine and blocked with P-selectin, was consistently reduced over four experiments (% inhibition IL-4, 39.7% ± 5.08, % inhibition IL-13, 40% ± 5.6). This effect was significant for both cytokines.

The functional significances of VCAM-1 and VLA-4 were also assessed using blocking mAbs against these receptors. There was no effect of blocking VCAM-1 on eosinophil adhesion to HUVEC stimulated with TNF-α, IL-4, or IL-13 (Figure 5–5). In contrast, anti-VLA-4 reduced adhesion to HUVEC stimulated with each of these cytokines. This was significant for IL-4- (% inhibition 44.9 ± 0.475, $p < 0.001$) and TNF-α- (% inhibition 57.4 ± 2.01, $p < 0.01$), but not IL-13- (% inhibition 32.5 ± 2.9) stimulated HUVEC.

5.2.3 Eosinophils but not neutrophils adhere to IL-4- and IL-13-stimulated endothelium under flow

Eosinophil behaviour was subsequently studied under shear flow conditions. The flow assay was already established in the lab with the first flow chamber used (M. Lawrence, see Methods section 2.2.9.2 and Symon *et al.*, 1996. However, in that system purified proteins and not endothelial cells were the substrate. Nevertheless, a flow rate in between the ones used previously (1.5 dynes/ cm^2) was adopted for the current assays with cytokine-stimulated HUVEC. In preliminary experiments at this shear stress (not shown), both eosinophils and neutrophils attached to the HUVEC in sufficient numbers to analyse, hence it was decided not to change the shear stress. All experiments with IL-4-and IL-13-stimulated HUVEC were performed at 1.5 dynes/ cm^2 at room temperature.

Figure 5–4 Histamine enhances eosinophil adhesion to IL-4-, and IL-13-, but not TNF-α-stimulated HUVEC, and the enhanced adhesion is mediated by P-selectin. Eosinophils were purified and incubated with $Na^{51}CrO_4$, before assessing adhesion to HUVEC in a static assay (Methods, section 2.2.7). Blocking P-selectin mAb clone G1 or MoIgG (both at 25 μg/ ml) was added to the HUVEC for 30 min before the start of the adhesion incubation. HUVEC were incubated with 10^{-5} M histamine for 10 min before the adhesion incubation where appropriate. A) without histamine, B) with histamine, $n = 4 \pm$ SEM, * $P < 0.05$.



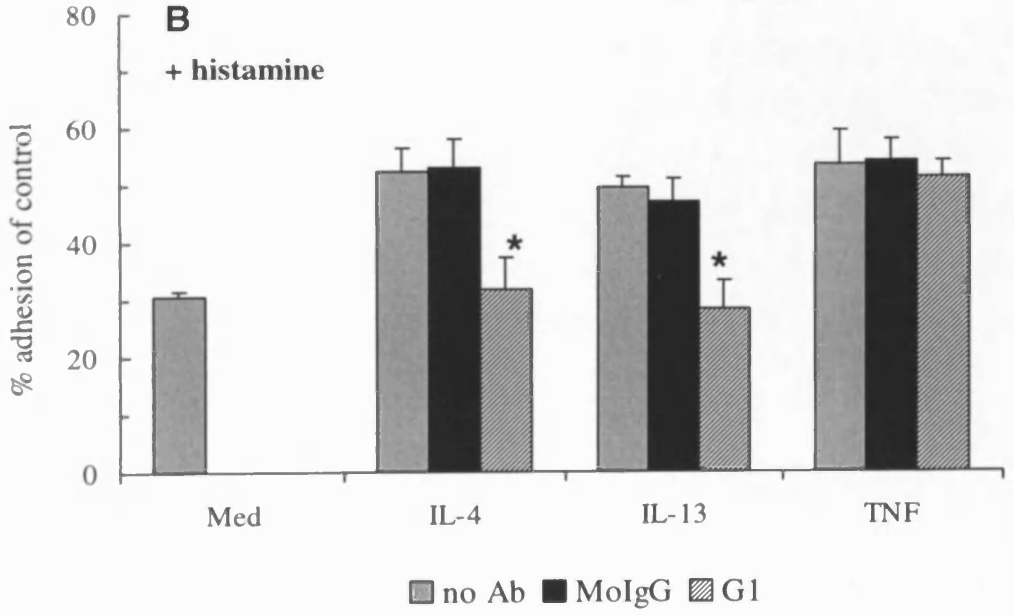
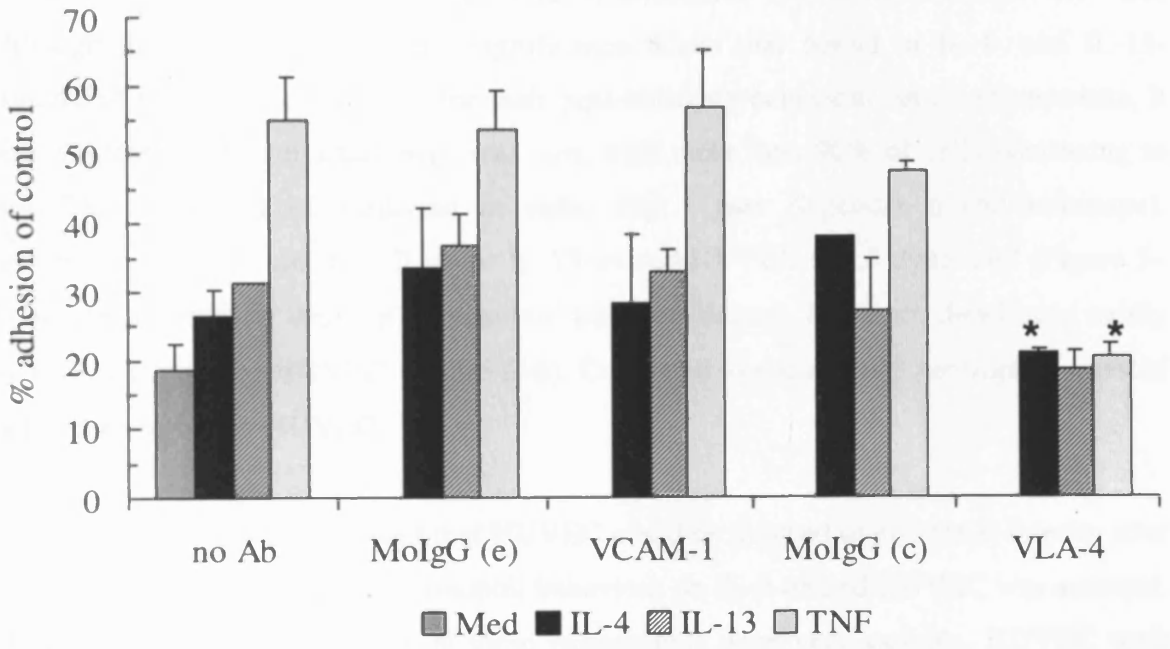


Figure 5–5 Eosinophil adhesion to cytokine-stimulated HUVEC is mediated by VLA-4. Eosinophils were purified and incubated with $Na^{51}CrO_4$ before assessing adhesion to HUVEC in a static assay (Methods, section 2.2.7). Before the start of the adhesion incubation, the HUVEC/ eosinophils were treated as follows: MoIgG or blocking VCAM-1 clone 4B9 (25 µg/ ml) was added to the HUVEC for 30 min; eosinophils were incubated for 30 min with MoIgG or blocking VLA-4 clone HP2/1 (25 µg/ ml). (e) = endothelium, (c) = cells, $n = 3 \pm$ SEM, * $P < 0.05$.



Initially, a comparison was made between eosinophil and neutrophil binding capacities on HUVEC stimulated with IL-4, IL-13, and TNF-α. At a shear stress of 1.5 dyne/ cm^2, eosinophils and neutrophils adhered to unstimulated HUVEC in low numbers (6.6 ± 2.93 and 10.4 ± 4.59 cells per field respectively, Figure 5–6). There was a significant increase in the total number of eosinophils that accumulated after 2 minutes on IL-4- and IL-13-treated HUVEC, compared to medium-treated cells (Figure 5–6). Numbers of accumulated eosinophils were almost equivalent to those accumulating on TNF-α-treated endothelium. In general, more eosinophils bound to IL-13-stimulated over IL-4-stimulated HUVEC, although this trend did not reach significance. Cells that bound to IL-4- and IL-13-stimulated HUVEC were studied for their post-tethering behaviour. In all experiments, it was observed that firm attachment was rare, with more than 90% of cells continuing to roll. This behaviour is illustrated in video clip 1 (see Appendix 6 and videotape). Neutrophils did not adhere to IL-4- or IL-13-treated HUVEC at 1.5 dyne/ cm^2 (Figure 5–6), or at a lower shear stress of 1 dyne/ cm^2 (data not shown). However, they bound avidly to TNF-α-stimulated HUVEC (Figure 5–6). Compared to eosinophils, neutrophils arrested rather than rolled on HUVEC.

The flow cytometry data showed that HUVEC could be induced to express P-selectin after stimulation with IL-9. Hence, eosinophil behaviour on IL-9-treated HUVEC was assessed. However, the data generated from these experiments were very variable. HUVEC were incubated with 50 ng/ ml IL-9 (the optimum concentrations from the FACS data) for 24 h and 48 h. There were no repeatable patterns of behaviour in two experiments at 24 h and three at 48 h, with duplicate or triplicate conditions. Eosinophils always rolled rather than arrested, but the movement was extremely fast and jerky (illustrated in video clip 4, see Appendix 6 and videotape), hence velocity could not be measured accurately using an acetate. Cells skipped continuously rather than making any firm attachments. Binding levels were also very inconsistent between triplicates.

5.2.4 Eosinophils adhere to IL-13-stimulated endothelium predominantly via P-selectin/ PSGL-1 interactions

To determine which receptors mediated eosinophil binding to IL-4- and IL-13-stimulated HUVEC, eosinophils were pre-treated with blocking mAbs against PSGL-1 and VLA-4, or the endothelial cells with a blocking mAb against P-selectin and VCAM-1 (Figures 5–7 and 5–8). Binding with control MoIgG was in the region of 32 cells per high power field.

Figure 5–6 Eosinophils, but not neutrophils, adhere to IL-4- and IL-13-stimulated HUVEC under flow. Eosinophils and neutrophils were purified and their binding to confluent cytokine-stimulated HUVEC monolayers studied (Methods, section 2.2.9). Leukocytes were allowed to bind to the endothelial cells at 1.5 dynes/ cm^2 for 2 min, and the average number of cells bound per high power field (hpf) from 10 random fields was counted. Shaded bars, eosinophils, hatched bars, neutrophils. n = 4-10 ± SEM, * $P < 0.05$, ** $P < 0.005$.

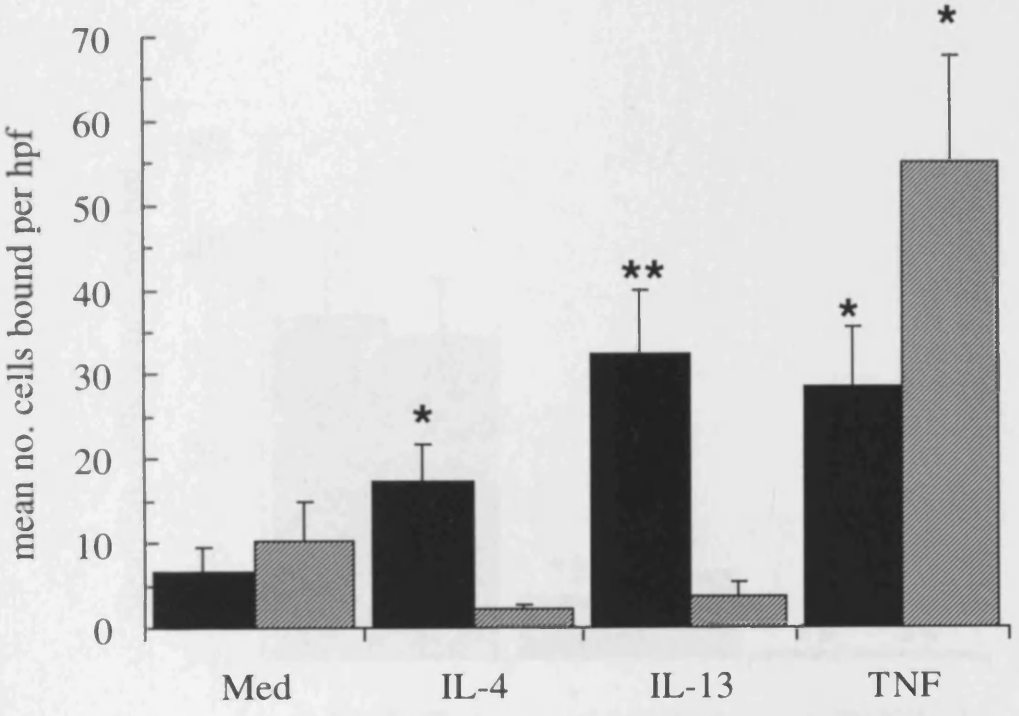


Figure 5–7 Eosinophil adhesion to IL-4- and IL-13-stimulated HUVEC under flow is PSGL-1- and VLA-4-dependent. Eosinophils were purified and incubated with either MoIgG, anti-VLA-4 clone HP1/2 (40 μg/ ml) or anti-PSGL-1 clone PL-1 (25 μg/ ml) for 15 min at RT, before their binding to confluent cytokine-stimulated HUVEC monolayers was studied (Methods, section 2.2.9). Eosinophils were allowed to bind to the endothelial cells at 1.5 dynes/ cm^2 for 2 min, and the average number of cells bound per high power field (hpf) from 10 random fields was counted. Shaded bars, IL-4, hatched bars, IL-13. n = 3 ± SEM, * $P < 0.02$, ** $P < 0.006$.

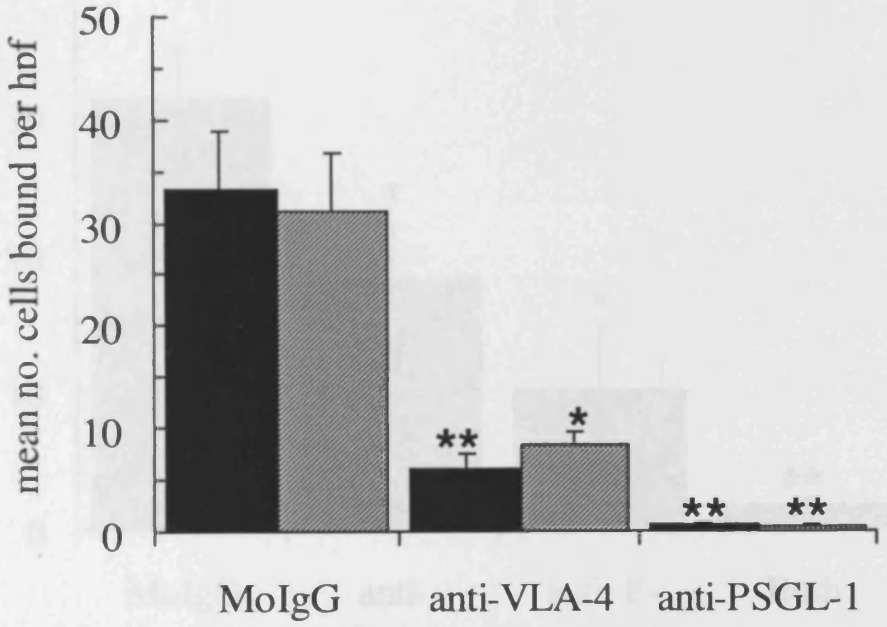
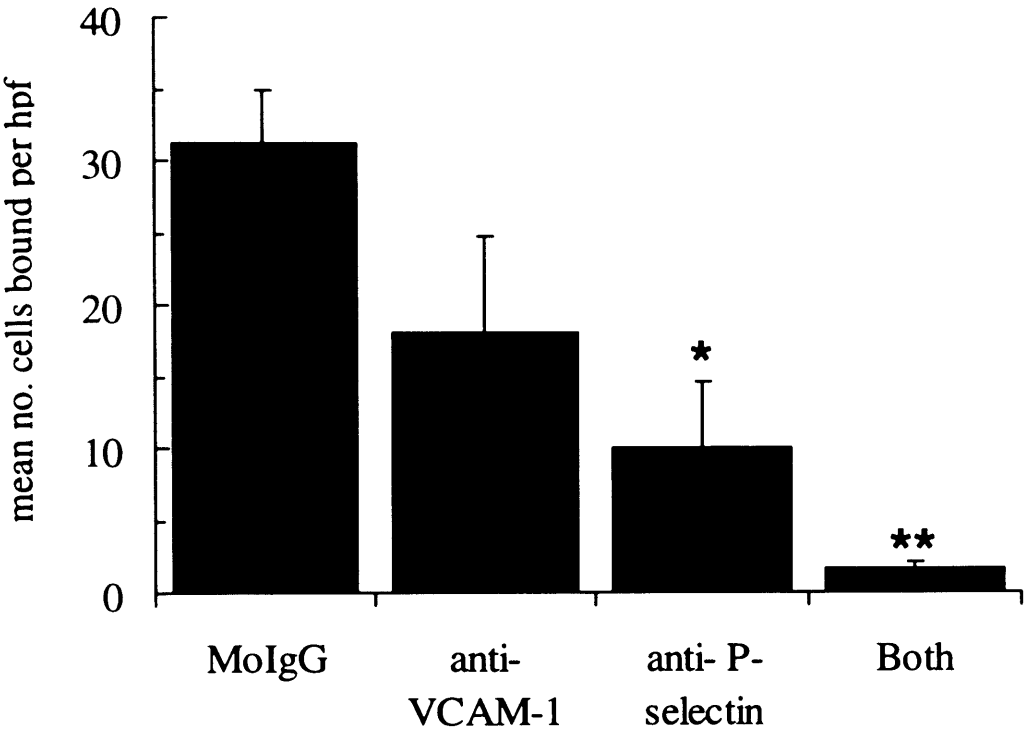


Figure 5–8 Eosinophil adhesion to IL-13-stimulated HUVEC under flow is P-selectin- and VCAM-1-dependent. Eosinophils were purified and incubated with either MoIgG, anti-VLA-4 clone 4B9 (25 μg/ ml), or anti-P-selectin clone G1 (25 μg/ ml) for 15 min at RT, before their binding to confluent IL-13-stimulated HUVEC monolayers was studied (Methods, section 2.2.9). Eosinophils were allowed to bind to the endothelial cells at 1.5 dynes/ cm^2 for 2 min, and the average number of cells bound per high power field (hpf) from 10 random fields was counted. $n = 3 \pm$ SEM, * $P < 0.02$, ** $P < 0.006$.



An antibody against PSGL-1 completely ablated binding, whereas anti-VLA-4 inhibited binding by approximately 70% on both IL-13- and IL-4-treated HUVEC. An antibody against P-selectin also reduced binding significantly (68% inhibition) on IL-13-stimulated HUVEC. In contrast, VCAM-1 blockade was not significant, although a moderate inhibitory effect was seen. Blockade of both P-selectin and VCAM-1 reduced binding to almost zero, and was more effective than blockade of either receptor alone.

5.3 Summary

The Th2-associated allergic cytokines IL-13 and IL-9 selectively induced surface P-selectin expression on HUVEC in a dose-dependent manner. It was also confirmed that IL-4 could induce P-selectin, as previously shown. Eosinophil, but not neutrophil rolling was supported on IL-13- and IL-4-stimulated HUVEC. The IL-13-stimulated rolling, which has not been previously shown, was mainly mediated by a combination of P-selectin/ PSGL-1 and, to a lesser extent, VCAM-1/ VLA-4 interactions.

5.4 Discussion

Eosinophils, in marked contrast to neutrophils, constitutively express the α4β1 integrin, VLA-4 (Dobrina *et al.*, 1991). Consequently, over the past decade, much of the work focussing on a selective eosinophil transmigration pathway has concentrated on a VLA-4/ VCAM-1 receptor pairing. This study has confirmed that VCAM-1 is selectively induced by the Th2 cytokines IL-4 and IL-13, which have previously been detected at both the mRNA and protein level in allergic airway disorders (Schleimer *et al.*, 1992; Bochner *et al.*, 1995). However, the VCAM-1 expression induced on endothelium by IL-4 and IL-13 in the current study was modest. In support of this, other studies have shown that IL-4- and IL-13-induced VCAM-1 expression is generally weak to moderate, unless other inflammatory cytokines such as IL-1β or TNF-α are present (Iademarco *et al.*, 1995; Blease *et al.*, 1998). In addition, the expression of VCAM-1 in the upper and the lower airway in asthma, rhinitis, and nasal polyposis is generally weak and variable (Bentley *et al.*, 1993; Beck *et al.*, 1996; Fukuda *et al.*, 1996).

Nevertheless, although selectins are thought to primarily mediate rolling interactions, it has also been suggested that VLA-4 can play a role in this process. *In vitro*, the VLA-4/ VCAM-1 pathway has been shown to mediate leukocyte tethering, rolling, and adhesion under shear (Alon *et al.*, 1995; Berlin *et al.*, 1995). Several studies have demonstrated that leukocyte subclasses interact differently with VCAM-1. α4 integrins on T cells participate in both tethering and rolling (Alon *et al.*, 1995; Berlin *et al.*, 1995), whereas α4 on monocytes does not participate in tethering, but instead acts to support firm adhesion (Luscinskas *et al.*, 1996). Other *in vitro* studies have proposed initial selectin-dependent leukocyte tethering and rolling, followed by subsequent VLA-4/ VCAM-1-dependent adhesion (Luscinskas *et al.*, 1994; Luscinskas *et al.*, 1996). *In vivo*, selectins have been shown to be required for initial tethering, whereas VLA-4/ VCAM-1 played a role in subsequent rolling and adhesion (Kanwar *et al.*, 1997). Eosinophils were shown to

transmigrate through IL-4- and IL-13-stimulated endothelium in a VLA-4-dependent manner (Bochner *et al.*, 1995). Also, several studies have demonstrated that anti-VLA-4 and anti-VCAM-1 mAbs are effective at blocking eosinophil migration to tissue using various animal models of eosinophilic migration (Hakugawa *et al.*, 1997; Hohki *et al.*, 1997; Sagara *et al.*, 1997).

Counter to the arguments in favour of VCAM-1/ VLA-4 interactions as the major selective mechanism for eosinophil migration, the present study found only a minor role for VCAM-1 and VLA-4 in eosinophil adhesion to NPE in the FSA. Weak expression of VCAM-1 was found on nasal polyp, nasal turbinate, and nasal biopsy mucosa (Chapter 3). Eosinophils have been shown to use VLA-4 to tether to TNF-α-activated HUVEC (Ulfman *et al.*, 1999), and the generation of TNF-α is associated with systemic aspects of inflammation, which are unlikely to feature prominently in asthma. Additionally, whilst eosinophils used VLA-4 and L-selectin to roll on IL-1β-activated rabbit mesentery, blocking these molecules still resulted in 50% of rolling interactions persisting (Sriramarao *et al.*, 1994). Nevertheless, the current study, using flow assay experiments, confirmed a minor, but significant, role for the VLA-4/ VCAM-1 pathway, working in conjunction with P-selectin. In the static adhesion assay, anti-VLA-4 significantly reduced adhesion to IL-4- but not IL-13-activated HUVEC. However, there was no significant effect of blocking VCAM-1 on eosinophil adhesion under these circumstances. Hence, it seems that the importance of the VLA-4/ VCAM-1 pathway in allergic disease remains in question, and appears to be dependent upon the model chosen to investigate adhesion.

As discussed in section 1.4.4.1, P-selectin is an attractive alternative candidate for mediating eosinophil tethering. Cytokines such as TNF-α, IL-1β, and LPS induce the expression of VCAM-1, E-selectin, and ICAM-1 on endothelium, by upregulating early rapid response genes. These cytokines, which are not thought to be as important in asthma as Th2-produced cytokines, have no effect on P-selectin expression in humans, although they do increase expression in mice (Sanders *et al.*, 1992; Weller *et al.*, 1992). P-selectin was initially thought to be only transiently expressed on endothelial cells upon stimulation with histamine or thrombin. However, evidence for chronic P-selectin expression in a number of inflammatory diseases, including nasal polyposis, was supported by the observation that the Th2 cytokine IL-4 could induce the prolonged expression of P-selectin on HUVEC (Yao *et al.*, 1996), an observation confirmed in the current study. In a study perfusing whole blood over IL-4-stimulated HUVEC under shear flow, recruitment was

selective for eosinophils (Patel, 1999). Despite this, many asthmatics and patients with nasal polyposis are not atopic and express low levels of IL-4, but increased amounts of the related cytokine IL-13 (Humbert *et al.*, 1997a).

IL-4 and IL-13 both signal via a shared receptor component, IL-4Rα, resulting in both cytokines mediating similar biological functions (Curiel *et al.*, 1997). This study has shown that IL-13, like IL-4, can selectively induce P-selectin expression on human endothelial cells. This is important, because it reinforces the evidence that P-selectin is an adhesion receptor associated with human Th2-type inflammatory responses. In a study by Hamilos and co-workers using nasal polyps, P-selectin but not VCAM-1 expression was inhibited by glucorticoid treatment (Hamilos *et al.*, 1999). The reduced expression of P-selectin here correlated with a reduction in the expression of IL-13 mRNA. Moreover, blocking the effects of IL-13 in a murine model of asthma was recently shown independently by two groups to reverse features of allergic asthma (Kroegel *et al.*, 1996; Wills-Karp *et al.*, 1998).

The Th2 cytokine IL-9 is located close to a cytokine gene cluster on chromosome 5, which includes IL-3, IL-4, IL-5, GM-CSF, and IL-13 (Postma *et al.*, 1995). Genetic markers for asthma and bronchial hyperresponsiveness have been mapped to this region in humans. As discussed in section 1.4.4.1.2, studies in animal models have implicated IL-9 as being important in the pathophysiologic mechanisms of asthma (Nicolaides *et al.*, 1997; McLane *et al.*, 1998; Temann *et al.*, 1998; Levitt *et al.*, 1999). The effects of IL-9 on HUVEC have not been previously reported. It was postulated that one mechanism of action for IL-9 could be the induction of P-selectin on vascular endothelium, resulting in selective eosinophil recruitment. IL-9 induced surface P-selectin expression on HUVEC in a dose-dependent manner after 24 h in culture, with the optimum concentration for expression being 50 ng/ ml. However, these data narrowly missed reaching significance after four experiments due to outlying data points. The IL-9 receptor was also significantly expressed on unstimulated HUVEC. Interestingly, IL-9 stimulation of HUVEC had no effect on the surface expression of ICAM-1, E-selectin, or VCAM-1.

The levels of P-selectin expression induced by IL-4 and IL-13 in the present study, although significant, were modest. This made it important to determine the functional relevance of this observation. Initially, a static adhesion assay was used to examine P-selectin function in eosinophil adhesion to IL-4 and IL-13-stimulated HUVEC. In these

experiments, histamine was used to stimulate HUVEC to induce maximal P-selectin expression. Indeed, blocking P-selectin significantly reduced eosinophil adhesion to IL-4- and IL-13- but not TNF-α-stimulated HUVEC only in the presence of histamine, suggesting that extremely low levels of P-selectin may not be sufficient to support adhesion.

The P-selectin promoter in humans does not have a traditional NFκB transcription site (Pan and McEver, 1995). This is in contrast to other human endothelial adhesion receptors, and the P-selectin promoter in mice, and explains why cytokines such as TNF-α have no effect on P-selectin expression. The P-selectin promoter was recently shown by Khew-Goodall and co-workers to contain two functional Stat6 binding sites (Khew-Goodall *et al.*, 1999). IL-4 induced maximum, prolonged, activation of one of the Stat6 binding sites and elevated levels of Stat6 were detectable as long as 48 hours after IL-4 stimulation. This sustained activation would theoretically result in an increased duration of P-selectin mRNA synthesis. Both prolonged Stat6 activation and prolonged P-selectin expression was dependent on the continuous presence of IL-4 (Khew-Goodall *et al.*, 1999). Increased mRNA for P-selectin has been demonstrated in HUVEC after IL-13 stimulation (Dr. G. Woltmann, personal communication). The mechanism of action for this is likely to be similar to that of IL-4, i.e. by inducing *de novo* transcription through activation of the Stat6 pathway. The VCAM-1 promoter does not contain any Stat6 binding sites (Khew-Goodall *et al.*, 1999), and hence possibly explains the inability of IL-9 to upregulate VCAM-1 expression. Interestingly, IL-4 inhibits TNF-α induction of E-selectin expression through a Jak/ Stat6 pathway via competition with NFκB binding (Bennett *et al.*, 1997). It was suggested that the reasons for these different immunomodulatory effects of IL-4 may serve to determine the leukocyte subtypes that extravasate at different stages of the inflammatory response (Khew-Goodall *et al.*, 1999).

The reason why eosinophils were found to preferentially bind IL-13-stimulated HUVEC over neutrophils was unclear. Neutrophils have previously been shown to adhere to histamine-stimulated HUVEC (Jones *et al.*, 1993), and the levels of P-selectin expression seen in this study were similar to expression seen in the current study after histamine stimulation. One idea is that stable binding of eosinophils could require interactions with both P-selectin and VLA-4, a route not available to neutrophils as they lack VLA-4. Another explanation is that eosinophils might preferentially bind P-selectin at the low level of luminal expression induced by IL-13. Alternatively, both factors may play a role. The

inhibitory effect of anti-VLA-4/ VCAM-1 and anti-PSGL-1/ P-selectin in this study demonstrated that the former may at least be part of the explanation.

Previous observations demonstrated that eosinophils bind in greater numbers than neutrophils to purified P-selectin under flow conditions (Symon *et al.*, 1996; Kitayama *et al.*, 1997b; Edwards *et al.*, 2000). The difference in binding in one study was more obvious at the lower dilutions of P-selectin used (Symon *et al.*, 1996). This has been give further credance by some recent elegant work by Edwards *et al.*, in which eosinophils preferentially bound P-selectin at lower site densities compared to neutrophils (Edwards *et al.*, 2000). They used a cone-plate viscometer integrated with a flow cytometer to detect eosinophil and neutrophil adhesion to P-selectin-expressing CHO transfectants in free cell suspensions. It was found that eosinophils could selectively bind to cells expressing very low cell membrane densities of P-selectin (~20 sites/ μm^2). Thus, the selectivity of eosinophil adhesion to P-selectin could reflect the greater efficiency with which eosinophils form initial attachments to P-selectin under flow. Additionally in this study, eosinophils showed increased rolling with increasing P-selectin site density, whereas neutrophil rolling showed a plateau. This suggested that eosinophils use high levels of P-selectin for rolling more efficiently than neutrophils.

A major finding of this study was that eosinophils, but not neutrophils, were able to bind IL-4- and IL-13-stimulated HUVEC under flow conditions, through PSGL-1 binding to P-selectin. Using monoclonal blocking antibodies, it was evident that VCAM-1 and P-selectin worked synergistically to support eosinophil adhesion. Patel *et al.* recently showed the immediate arrest of eosinophils on HUVEC treated with IL-4 for 24 hours (Patel, 1998). We found that eosinophils rolled, but did not undergo firm arrest. The use of 22°C in our flow chamber rather than 37°C might have resulted in slower kinetics of activation, however additional experiments were performed at 37 °C (data not shown), with the same outcome. Hence, the reasons for this difference are unclear, but may be related to the earlier time point in Patel's study.

As IL-9 also induced P-selectin expression on HUVEC, it was decided to determine whether IL-9-stimulated HUVEC could support eosinophil adhesion under flow conditions. The optimum concentration of IL-9 from the expression studies (50 ng/ ml) was chosen to stimulate HUVEC for 24 h and 48 h, and eosinophils were perfused over the HUVEC at 1 dyne/ cm^2 in the flow assay. However, the data from these experiments were

variable and no repeatable pattern of behaviour was seen. In the experiments performed, the numbers of eosinophils tethering to IL-9-stimulated HUVEC were lower than seen with IL-4- and IL-13-stimulated endothelium, and were inconsistent between tests. Eosinophils did appear to roll rather than arrest, however, the cells moved extremely quickly out of the field of view, and adhesion parameters (velocity, % rolling vs. arrest) could not be quantitated accurately with the equipment available.

It was unclear why stimulation of HUVEC with IL-9 in the flow assay gave inconclusive results. IL-9 on its own may be insufficient to induce a level of P-selectin expression that supports eosinophil rolling, unlike IL-13. Alternatively, IL-13, but not IL-9, induced the expression of VCAM-1 on HUVEC. With IL-13 stimulation, there was a minor VCAM-1/ VLA-4 component, which appeared to work in conjunction with P-selectin/ PSGL-1. IL-9 may not support this component, which may be required for eosinophil adhesion. Further work is needed on defining the parameters of the assay for IL-9. For instance, although the optimum concentration of IL-9 to upregulate P-selectin expression on HUVEC was 50 ng/ ml using flow cytometry, this may not be optimal in the flow assay. Similarly, the incubation time needs to be accurately determined. Time- and concentration-dose response experiments need to be carried out under flow conditions.

Eosinophils bind less avidly to E-selectin than neutrophils (Bochner *et al.*, 1994; Sriramarao *et al.*, 1996; Kitayama *et al.*, 1997b), which may be explained by their lower expression of sLe^x compared to neutrophils (Bochner *et al.*, 1994). Eosinophils would perhaps be less likely to use E-selectin to roll on endothelium in a milieu where allergy-associated cytokines such as IL-4 and IL-13, which promote P-selectin expression, are present. IL-4, IL-13, and IL-9 had no effect on E-selectin expression on HUVEC in the present study. The function of PSGL-1 is heavily dependent on its state of glycosylation. Differences in the pattern of glycosylation between eosinophil and neutrophil PSGL-1 could also be responsible for the differences in binding efficiency to P-selectin (Symon *et al.*, 1996; Somers *et al.*, 2000).

In conclusion, it has been shown that IL-13 as well as IL-4 can induce prolonged expression of P-selectin at levels of expression that support eosinophil, but not neutrophil adhesion. This offers strong support for an important role for PSGL-1/ P-selectin in directing the preferential adhesion of eosinophils to vascular endothelium, in diseases that are characterised by increased expression of Th2 cytokines, such as asthma. IL-9 can also

induce P-selectin expression, although the functional relevance of this is unclear.

CHAPTER SIX

Characterisation of the activation steps involved in eosinophil adhesion to TNF-α-stimulated HUVEC under shear flow conditions

6.1 Introduction

It was demonstrated that eosinophils and neutrophils undergo different patterns of activation when binding to nasal polyp endothelium in the FSA (Chapter 4). A requirement for cell activation was shown for both cell types, as both eosinophil and neutrophil binding was β2 integrin-dependent, and was inhibited by azide. However, although it was possible to dissect out the events mediating neutrophil adhesion to NPE, the quest for the activation mechanisms involved in eosinophil adhesion proved unsuccessful. Adhesion in the FSA was not inhibited by PTX or anti-CCR3, suggesting that alternative endothelial-associated pathways might exist for activation of the eosinophil β2 integrins. The purpose of this section was to explore the eosinophil activation step under flow conditions.

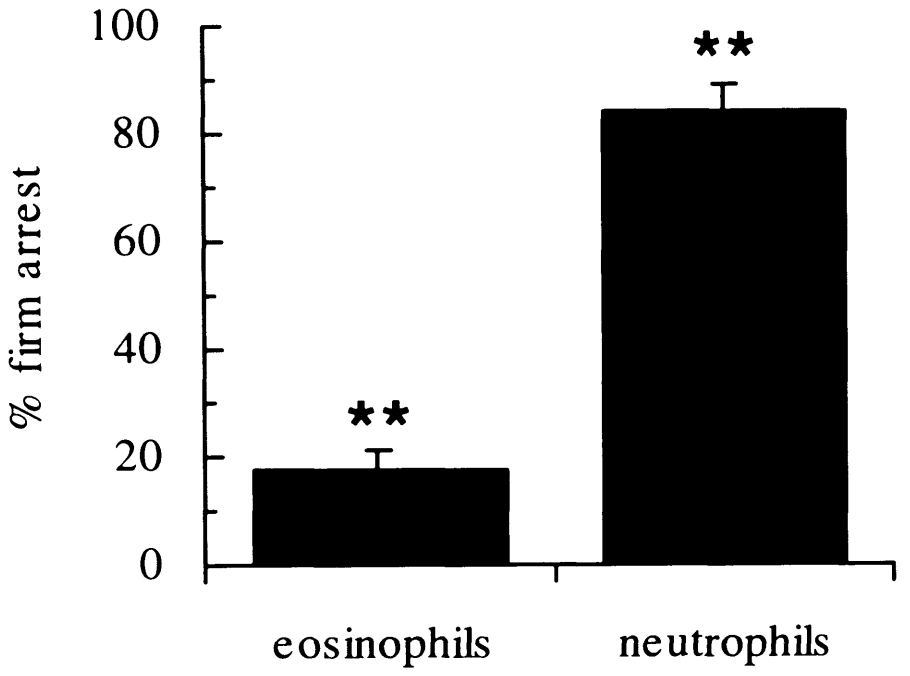
The flow chamber in the experiments in Chapter 5 became excessively worn, hence was replaced. The new chamber (Glycotech) was used at a lower shear stress of 1 dyne/ cm^2, as leukocytes did not bind in sufficient numbers to analyse at 1.5 dyne/ cm^2. In addition, it was felt that the method would be further improved if cells were kept at a physiological temperature. In two preliminary experiments studying eosinophils on TNF-α-stimulated HUVEC, binding was increased at 37 °C compared to room temperature (not shown). Hence, all experiments in Chapter 6 were performed at 1 dyne/ cm^2 at 37 °C.

6.2 Results

6.2.1 Eosinophils roll, but neutrophils arrest on TNF-α-stimulated HUVEC

A comparison was made between the behaviour of eosinophils and neutrophils on TNF-α-stimulated HUVEC (Figure 6–1). In each experiment, the majority of neutrophils were found to arrest upon touching the endothelium (% arrested 84.2 ± 5.4, n = 6, Figure 6–1 and video clip 3, Appendix 6 and videotape). Neutrophils often appeared to have a migratory phenotype, extending pseudopodia and moving across the HUVEC surface. This phenomenon can be seen in video clip 3 (Appendix 6 and videotape). In contrast, eosinophils were generally seen to roll (% arrested 20 ± 6, n = 6, Figure 6–1 and video clip 2, Appendix 6 and videotape).

Figure 6–1 Eosinophils, but not neutrophils, roll on TNF-α-stimulated HUVEC under flow conditions. Eosinophils and neutrophils were purified and HUVEC monolayers stimulated with 50 ng/ ml TNF-α for 6 h as described in the Methods. Leukocytes were allowed to interact with HUVEC under flow conditions at 1 dyne/ cm^2. After a 2-min interaction, the numbers of eosinophils and neutrophils rolling and arrested were quantified on 5-10 fields and expressed as percentage rolling vs. firmly adherent. $n = 6 \pm$ SEM, ** $P < 0.005$.



There was occasionally some variability seen, with a higher proportion of eosinophils arresting than usual. Likewise, in some experiments, more neutrophils rolled than was the norm. Overall, the data suggested that one or more signals generated by the stimulated HUVEC were enough to cause the arrest of neutrophils, but not eosinophils.

6.2.2 Exogenous chemoattractants cause eosinophils to arrest and change shape

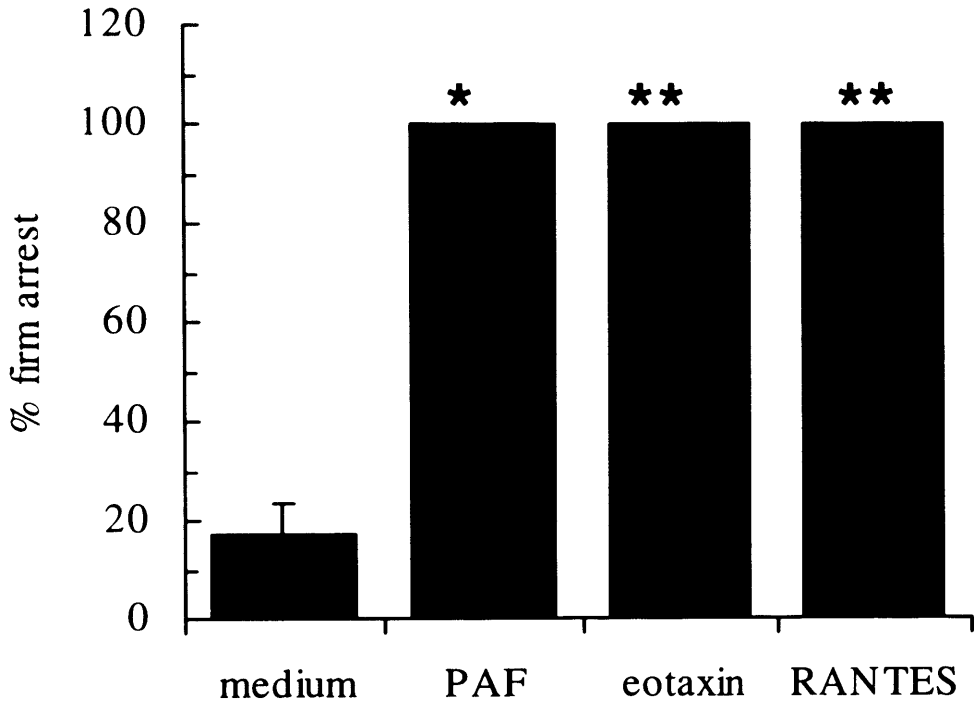
To demonstrate whether eosinophils could arrest on TNF-α-stimulated HUVEC in a similar fashion to neutrophils, the effect of an exogenous activating signal on eosinophil behaviour was studied. As shown in Figure 6–2, the lipid mediator PAF (10^{-5} M), and the chemokines RANTES and eotaxin (both 100 ng/ ml) converted the rolling phenotype seen with medium only to a completely arrested phenotype. This is demonstrated visually in video clip 2 (Appendix 6 and videotape), which shows eosinophils arresting upon addition of PAF to the system (time of arrest on video = 00:47:42). With all mediators added exogenously there was a 'dead volume' delay of between 60 and 120 seconds, which was the time taken for the substance to reach the flow chamber from its source of a tube in the water bath. The dead volume varied according to the length of the tubing, which was changed often to prevent medium building up inside and disturbing the flow. In effect, as soon as the chemoattractant entered the flow chamber, the eosinophils arrested.

The effect seen with PAF was identical for eotaxin and RANTES. Shape change was also seen upon addition of all three chemoattractants, with a considerable proportion of eosinophils taking on a migratory phenotype, extending pseudopodia, and becoming flattened (as seen with neutrophils in section 6.2.1). This effect was not quantitated, but was partially reversible for eotaxin and RANTES. After re-addition of medium without chemoattractant to the system, RANTES- and eotaxin-stimulated eosinophils began to roll again after 2-3 min. However, the majority of cells that had been activated with PAF did not tend to regain shape, remaining arrested 5 min after PAF had been washed out (not shown).

6.2.3 PTX-sensitive GPCR signalling does not appear to be involved in eosinophil adhesion to TNF-α-stimulated endothelium under shear flow conditions

The possibility that eosinophil activation was occurring via signalling through PTX-sensitive serpentine receptors was investigated. Eosinophils were incubated with PTX for 2 hours, before being allowed to interact with TNF-α-stimulated HUVEC in the flow assay.

Figure 6–2 Eosinophils arrest on TNF-α-stimulated HUVEC under flow in the presence of exogenous chemoattractants. Eosinophils were purified and HUVEC monolayers stimulated with 50 ng/ ml TNF-α for 6 h (Methods, section 2.2.9.2). Eosinophils were allowed to interact with HUVEC under flow conditions at 1 dyne/ cm^2. After a 2-min interaction, exogenous PAF (10^{-6} M), eotaxin (100 ng/ ml), or RANTES (100 ng/ ml) was added to the flow system. The numbers of eosinophils rolling and arrested were counted on 5-10 fields and are illustrated as percentage of cells that firmly arrested. Basal arrest levels are shown by medium only. n = 2-6 ± SEM, * $P < 0.05$, ** $P < 0.005$.



PTX incubation had no overall effect on the mean total numbers of eosinophils binding to the HUVEC per field viewed, as illustrated in Figure 6–3 a). Similarly, PTX incubation did not significantly affect eosinophil velocity (Figure 6–3 b)), or the percentage of cells that rolled compared to those that firmly arrested (Figure 6–3 c)). Taken together, the data suggested that signalling through PTX-sensitive GPCRs was not involved in eosinophil adhesion to TNF-α-stimulated HUVEC in this system.

6.2.4 Eosinophil adhesion to TNF-α-stimulated HUVEC requires IL-5 priming

The data so far suggested that eosinophils were not receiving a signal generated from TNF-α-stimulated endothelium to arrest, or that, unlike for neutrophils, the signal alone was insufficient, and eosinophils required additional priming. Eosinophils could arrest, as shown by addition of exogenous PAF, RANTES, and eotaxin.

The cytokine IL-5 regulates the development and function of eosinophils and it has been demonstrated that IL-5 plays a significant role in promoting the preferential accumulation of eosinophils during allergic inflammation. IL-5 can also lead to eosinophil priming, which can result in an enhanced adhesion and transmigratory response, as well as modulation of receptor expression. Eosinophils were incubated with IL-5 at a range of concentrations to study the priming effect of IL-5 on eosinophil behaviour in the flow system.

As illustrated in Figure 6–4, it was found that at low concentrations (0.05 ng/ ml), IL-5 had no effect on the percentage of eosinophils that were rolling (mean % arrest medium only 17.05 ± 0.65, mean % arrest IL-5 18.85 ± 2.15). At 0.5 ng/ ml, IL-5 caused over 40% of eosinophils to arrest (mean % medium only 15.98 ± 3.49, mean % IL-5 43.9 ± 9.43), however, there was little shape change indicative of transmigration. At the highest dose of 5 ng/ ml, IL-5 caused arrest of the entire cell population, which was accompanied by shape change (mean % medium only 24.5 ± 10.23, mean % IL-5 99.33 ± 0.42), and the arrest lasted for at least 5 minutes.

It was also investigated whether IL-5 could prime eosinophils when added to the flow chamber without pre-incubation, as shown for PAF, RANTES, and eotaxin. The outcome of these experiments was variable.

Figure 6–3 Eosinophils do not appear to signal via PTX-sensitive GPCRs to bind to TNF-α-stimulated HUVEC under flow. HUVEC monolayers were stimulated with 50 ng/ ml TNF-α for 6 h (Methods, section 2.2.9.2). Eosinophils were purified and incubated with 100 ng/ ml PTX for 2 h, before being allowed to interact with HUVEC under flow at 1 dyne/ cm^2. After a 2-min interaction, the following parameters were quantified on 10-15 fields: A) the total number of eosinophils binding, B) eosinophil rolling velocity, and C) eosinophil behaviour once attached. $n = 10 \pm$ SEM.

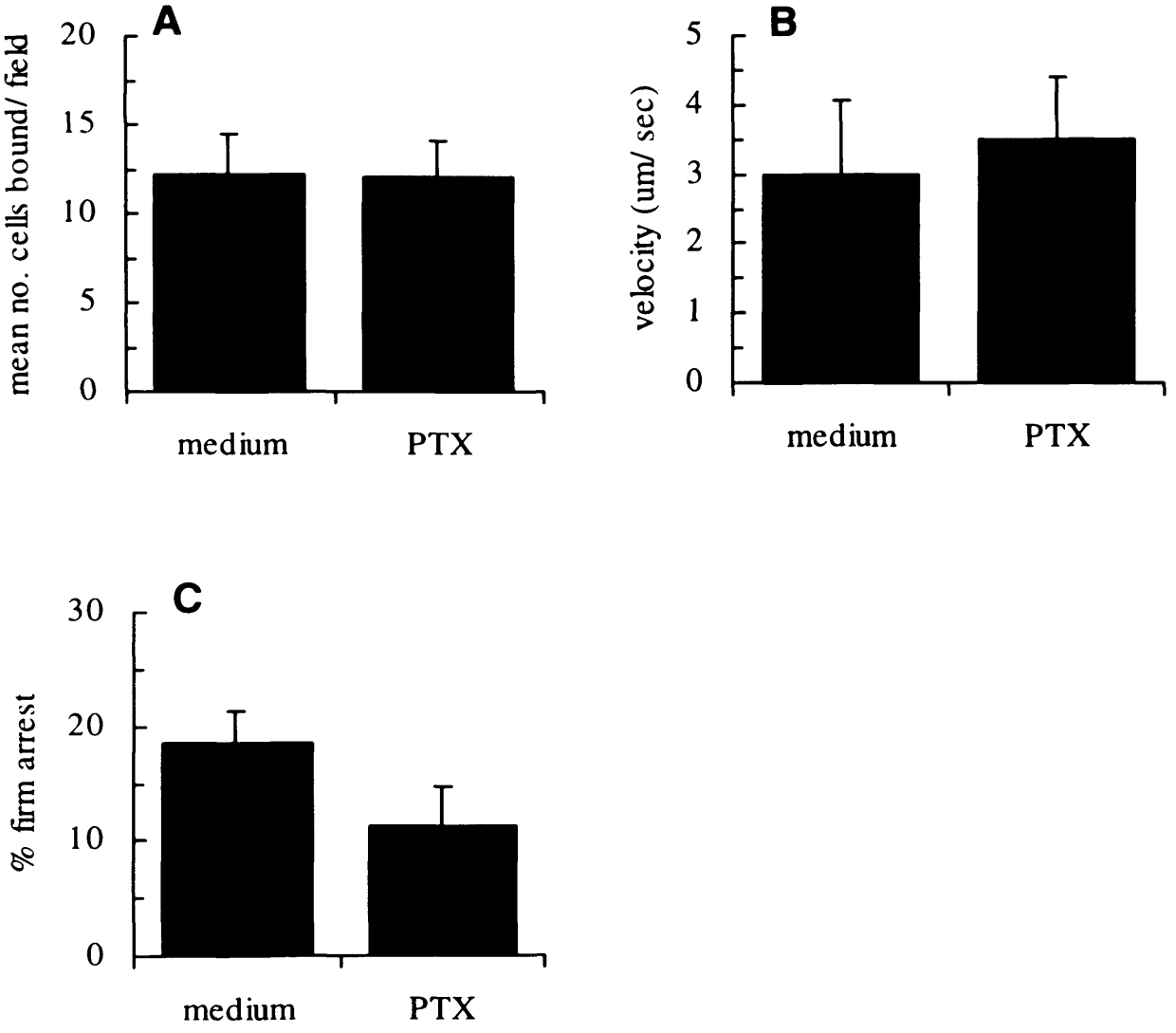
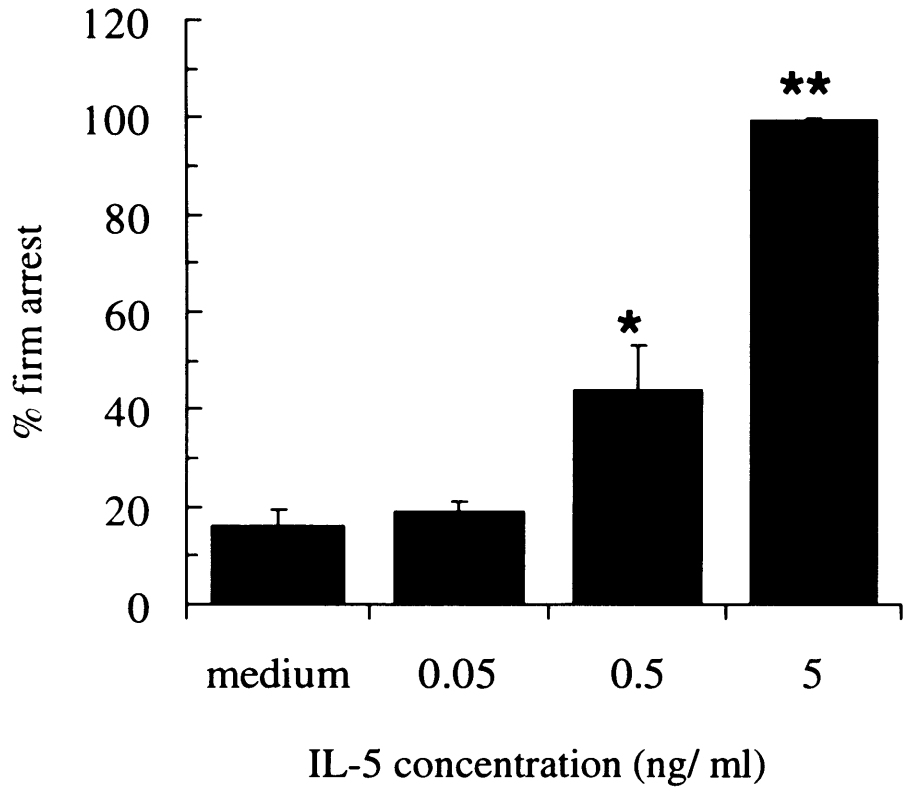


Figure 6–4 Eosinophil adhesion to TNF-α-stimulated HUVEC requires IL-5 priming for firm arrest. Eosinophils were purified and monolayers of HUVEC were treated with 50 ng/ ml TNF-α for 6 h (Methods, section 2.2.9.2). Unstimulated eosinophils, and eosinophils pre-treated with IL-5 at varying concentrations for 30 min at 37 °C, were allowed to interact with HUVEC under shear flow at 1 dyne/ cm^2. After a 2-min interaction, the numbers of eosinophils rolling and arrested were counted on 5-10 fields and are illustrated as the percentage of cell arrest. Basal arrest levels are shown by a medium only control. n = 3-9 ± SEM, * $P < 0.05$, ** $P < 0.005$.



IL-5 at 5 ng/ ml caused 75% arrest compared to 13% arrest with medium in one experiment, in another experiment, 95% of cells arrested compared to 3% with medium only, but cells then detached again within a minute. Hence, the effect of IL-5 added exogenously was unclear, but appeared to be more transient than when incubated directly with the cells.

6.2.5 The mechanism of IL-5 priming appears to be direct rather than indirect via chemoattractant signalling

So far, it has been shown that IL-5 can prime eosinophils to arrest on HUVEC, however, the exact nature of the signal being generated was unknown. To determine if the signal was being generated through GPCRs, eosinophils were incubated with IL-5 and PTX simultaneously. 0.5 ng/ ml IL-5 was used to partially prime the cells, and the effect on numbers of cells binding, velocity, and rolling vs. arrest behaviour was noted. Like PTX alone, PTX + IL-5 had little effect on the total numbers of eosinophils bound, and although cell velocity was moderately increased, this observation did not reach significance (Figure 6–5).

Eosinophils converted from a mostly arrested phenotype with IL-5, to a mostly rolling phenotype when incubated with both IL-5 and PTX at 0.5 ng/ ml in the first experiment undertaken (not shown, IL-5 80% arrested, IL-5 + PTX 2.8% arrested). However, increasing the number of experiments to seven resulted in more variable behaviour patterns. Two more experiments followed the trend of the first one, however, the overall effect of IL-5 priming and PTX incubation did not reach significance (Figure 6–6). Thus it seems that IL-5 was acting directly to cause arrest, via β2 integrin activation, rather than indirectly, via chemoattractant signalling.

Figure 6–5 PTX has no effect on the total number of IL-5 primed eosinophils bound to TNF-α-activated HUVEC, or their rolling velocity. Eosinophils were purified and monolayers of HUVEC were treated with 50 ng/ ml TNF-α for 6 h (Methods, section 2.2.9.2). Unstimulated eosinophils, and eosinophils pre-treated with either IL-5 (0.5 ng/ ml, 30 min), PTX (100 ng/ ml, 2 h) or both together (all at 37 °C) were allowed to interact with HUVEC under shear flow at 1 dyne/ cm^2. After a 2-min interaction, total numbers of eosinophils bound, and eosinophil rolling velocity were quantified on 10-15 fields, n = 7-10 ± SEM.

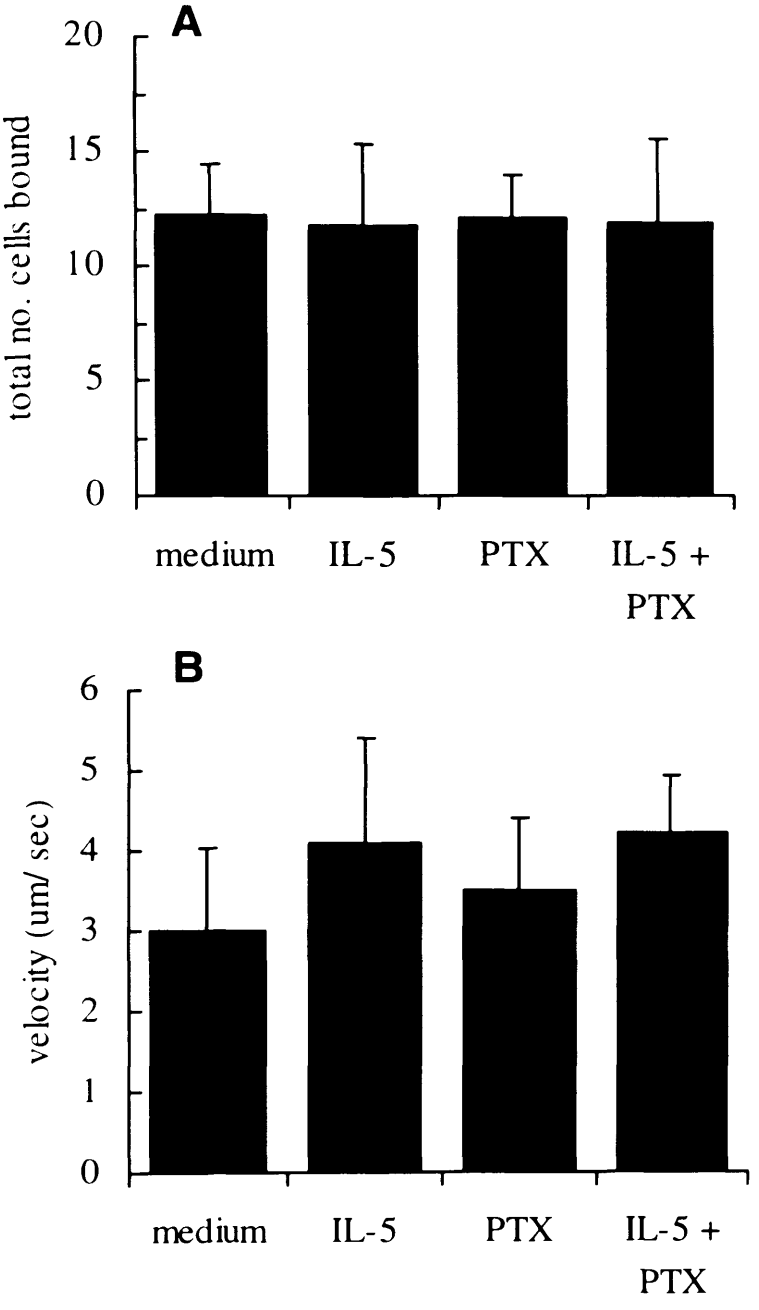
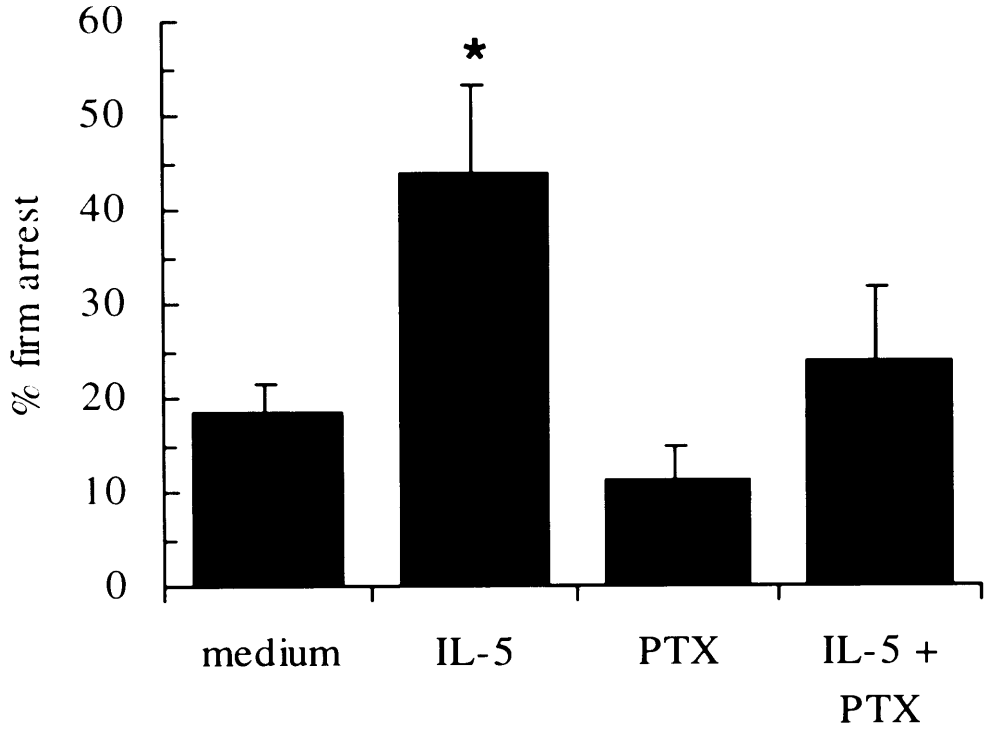


Figure 6–6 IL-5 priming does not mediate eosinophil firm arrest on TNF-α-stimulated HUVEC via PTX-sensitive GPCRs. Eosinophils were purified and monolayers of HUVEC were treated with 50 ng/ ml TNF-α for 6 h (Methods, section 2.2.9.2). Unstimulated eosinophils, and eosinophils pre-treated with either IL-5 (0.5 ng/ ml, 30 min), PTX (100 ng/ ml, 2 h) or both together (all at 37 °C) were allowed to interact with HUVEC under shear flow at 1 dyne/ cm^2. After a 2-min interaction, total numbers of eosinophils bound, and eosinophil rolling velocity was quantified on 10-15 fields, n = 7-10 ± SEM.



6.3 Summary

Eosinophils rolled, but neutrophils spontaneously arrested on TNF-α-stimulated HUVEC in a parallel plate flow chamber assay. However, eosinophils did arrest immediately upon addition of the chemoattractants PAF, RANTES, and eotaxin. Priming eosinophils prior to the assay with IL-5 also caused arrest on HUVEC. Incubation with IL-5 in the presence of PTX had no effect on eosinophil behaviour in the flow assay, suggesting that IL-5 was acting directly via β2 integrin upregulation, rather than indirectly via chemoattractant signalling, to cause arrest. Thus, it appears that an additional step priming in the adhesion cascade may be required for eosinophils to respond to an activating stimulus and arrest on inflamed endothelium.

6.4 Discussion

The observation that eosinophils rolled, but neutrophils arrested on TNF-α-activated HUVEC suggested that neutrophils were receiving sufficient activating signals from the HUVEC to increase integrin adhesiveness and cause arrest, but eosinophils were not. This contrasts with other work where neutrophils have been shown to roll on TNF-α-stimulated HUVEC under flow. In a study by Bahra *et al.*, nearly all neutrophils rolled (~ 90%) at low concentrations of TNF-α (2-5 U/ ml), but more cells arrested as the concentration of TNF-α increased, with approximately only 20% still rolling at 1000 U/ ml (Bahra *et al.*, 1998). In Bahra's study, arrest was inhibited with anti-CD18, suggesting that HUVEC were able to present activating agent (s) to neutrophils at high levels of TNF-α stimulation. Anti-CD18 was not used to confirm this in the present system.

The concentration of TNF-α has been shown to determine whether leukocytes roll on the endothelium, or proceed with transmigration, with low concentrations favouring the former, and higher concentrations the latter (Luu NT *et al.*, 2000). Neutrophils may not have rolled in our system due to the difference in experimental procedure. Bahra and co-workers isolated their neutrophils using a different method, and used a different flow system, growing HUVEC in microslides rather than tissue culture dishes. In addition, it is uncertain what the 50 ng/ ml of TNF-α used in the current study translates to in U/ ml, so it is possible that rolling was not seen because the TNF-α concentration was not low enough. Work by Kitayama *et al.* agrees with the current study, with neutrophils arresting on TNF-α-activated HUVEC (Kitayama *et al.*, 1997b). Thus, in response to high levels of TNF-α, endothelial cells may generate many activators of neutrophils e.g., PAF, IL-8, and

unknown factors, which cause them to arrest, but are not sufficient to cause eosinophil arrest.

In a study by Ulfman *et al.*, eosinophils rolled on TNF-α-stimulated HUVEC (7h, 100 U/ ml), which is in agreement with the current data (Ulfman *et al.*, 1999). In contrast, Kitayama *et al.* demonstrated the immediate arrest of both eosinophils and neutrophils on HUVEC activated with 100 U/ ml TNF-α for 6h (Kitayama *et al.*, 1997b). The difference in behaviour between these particular studies may be due to the difference in passage of the HUVEC (p2-3 in the former study, and primary cultures or p1 in the latter). Additionally, the shear stresses and cell concentrations used by the two groups were different. However, the conditions in the current study were identical to those used by Kitayama *et al*, so it is unclear why the outcomes were different. Further confounding factors may be subtle differences in the models used, for example, differences in the experimental set-up, or differences in the basal activation states of the eosinophil donors used, or in the expression of adhesion receptors on HUVEC, depending on when the umbilical cords were collected post-partum.

Eosinophils immediately arrested on TNF-α-activated endothelium upon addition of the exogenous chemoattractants PAF, eotaxin, and RANTES to the flow chamber. These chemoattractants have previously been shown to upregulate eosinophil adhesion to either purified proteins or endothelium (Burke-Gaffney and Hellewell, 1996; Wardlaw, 1999). Several groups have investigated the production of eosinophil-active chemoattractants by endothelium. Eotaxin, RANTES, and MCP-4 mRNAs were generated from HUVEC stimulated with IFN-γ or TNF-α plus IFN-γ (Marfaing-Koka *et al.*, 1995; Garcia-Zepeda *et al.*, 1996a; Garcia-Zepeda *et al.*, 1996b). TNF-α stimulation alone had no effect in these studies. Similarly, Kitayama *et al.* required both TNF-α and IFN-γ to stimulate endothelium when they demonstrated inhibition of eosinophil adhesion to HUVEC with a mAb against CCR3 (Kitayama *et al.*, 1998). However, endothelial cells are capable of producing chemoattractants such as PAF and IL-8 after incubation with TNF-α alone (Smart and Casale, 1994). Thus, in the present situation, either HUVEC were not generating the necessary signals for eosinophils to arrest as was the case for neutrophils, or additional stimulation was necessary in order for the cells to respond to existing signals.

A novel observation of this study was the immediate arrest and spreading of eosinophils upon addition of exogenous activators. PAF induced an irreversible arrest and shape

change of eosinophils on TNF-α-activated HUVEC, while arrest induced by eotaxin and RANTES, although long-term, was partially reversible when flow buffer without the activating agent re-entered the system. Exogenous IL-8 was shown to induce a transient arrest of eosinophils to TNF-α-activated HUVEC in a very recent study by Ulfman and co-workers (Ulfman *et al.*, 2001), but no shape change was seen. In fact, few studies have commented on the shape change of eosinophils adherent to activated endothelium, although neutrophil shape change during transmigration has been noted by many (Rainger *et al.*, 1997; Bahra *et al.*, 1998). Eotaxin and RANTES have been shown to induce a rapid, measurable, shape change of eosinophils in suspension (Sabroe *et al.*, 1999). Exogenous eotaxin caused the immediate arrest of eosinophils on purified VCAM-1 (Kitayama *et al.*, 1997b). In agreement with this study, Ulfman *et al.* demonstrated the flattening and long-term arrest of eosinophils when eotaxin was added to eosinophils rolling on TNF-α-activated HUVEC (Ulfman *et al.*, 2001). However, in that study eosinophils did not regain shape and begin rolling again, whereas they did in the current study.

The integrins involved in the chemoattractant-/ chemokine-induced arrest of eosinophils to TNF-α-activated HUVEC were not investigated. RANTES has been shown to regulate the avidity of β1 and β2 integrins expressed on the same eosinophil (Weber *et al.*, 1996). PAF and eotaxin, but not RANTES induced β2- and VLA-4-mediated eosinophil adhesion to HUVEC in static assays (Burke-Gaffney and Hellewell, 1996; Wardlaw, 1999). Studies of eosinophil adhesion to HUVEC generally show that activation and firm arrest are mediated by a combination of VLA-4/ VCAM-1 and CD18/ ICAM-1, the importance of either pathway depending on the cytokines used to stimulate the endothelium (Bochner and Schleimer, 1994; Blease *et al.*, 1998; Yamamoto *et al.*, 1998; Wardlaw, 1999).

It has been shown that a receptor-operated event on eosinophils is essential for chemoattractant-induced eosinophil recruitment *in vivo* (Teixeira *et al.*, 1997). The possibility that eosinophil activation via chemoattractants or chemokines was occurring through PTX-sensitive GPCRs was investigated. Eosinophils were incubated with PTX before being added to the flow assay. PTX incubation had little effect on the number of cells bound per field, cell velocity, or the percentage that firmly arrested. In another study, PTX decreased eosinophil arrest and accumulation on TNF-α and IFN-γ-activated HUVEC; however, while the reduction was significant it was very modest (17% for arrest, 23% for accumulation) (Kitayama *et al.*, 1998). In agreement with the current work, Teixeira *et al.* concluded that PAF activated eosinophils independently of G_i, although they

used guinea pig and not human eosinophils (Teixeira *et al.*, 1997). In another study, C5a, fMLP, LTB_4, IL-8, PAF, and RANTES elicited eosinophil chemotaxis that was only partially inhibited by PTX (O'Flaherty *et al.*, 2000). Chemoattractants commonly exhibit a wide range of PTX sensitivities, and PTX may inhibit some, but not all GPCRs expressed on eosinophils (Elsner *et al.*, 1998; O'Flaherty *et al.*, 2000).

It was next investigated whether eosinophils required an additional stimulus to be able to respond to an activating agent and firmly arrest on HUVEC. Priming enables cells to remain quiescent until provoked by the second stimulus, and it is known that resting eosinophils require priming to become fully capable of mediator release. IL-5 was chosen as a priming candidate partly because the IL-5R is absent on neutrophils and is rarely, if ever, expressed on other leukocytes (Neeley *et al.*, 1993). Also, the essential role of IL-5 in enhancing eosinophil activation and accumulation during allergic inflammation has been well documented (Hamelmann and Gelfand, 1999; Gleich, 2000). In asthma, IL-5 levels are often elevated in the lung and blood (Alexander *et al.*, 1994). Although IL-5 is only weakly chemotactic on mature eosinophils, it seems to have an important role as a cofactor, by its ability to initiate or enhance the response to other eosinophil chemotactic factors.

Incubation with IL-5 elicited the firm arrest of eosinophils to TNF-α-stimulated HUVEC under flow in a concentration-dependent fashion, which is a novel observation. There was no effect of IL-5 at 0.05 ng/ ml, ~45% of eosinophils arrested at 0.5 ng/ ml, and arrest of the entire population was seen at the highest dose of 5 ng/ ml. Eosinophils showed a dramatic shape change that was indicative of transmigration only at the highest concentration. The arrest with 5 ng/ ml IL-5 lasted for at least five minutes. However, since the average cell number binding to the HUVEC was approximately three times lower at 5 ng/ ml than at 0.5 ng/ ml, the latter dose was used to partially prime eosinophils in further experiments with PTX. When added without pre-incubation, exogenous IL-5 at 5 ng/ ml appeared to cause a partial, transient arrest of the population, as opposed to the total, long-term arrest seen with PAF, RANTES, and eotaxin, although only two experiments were performed. This observation is similar to the recent effect Ulfman *et al.* saw with IL-8, which induced a transient arrest (0.5-2 min) of eosinophils on TNF-α-activated HUVEC that ceased when the stimulus was washed out with flow buffer. This led them to suggest that for resting eosinophils an additional stimulus aside from IL-8 was

needed to induce long-term adhesion; cytokines such as IL-5, IL-4, and eotaxin were put forth as candidates.

It was postulated that the IL-5 could be causing eosinophil arrest in one of two ways. The first mechanism would be direct and would occur via activation of β2 integrins on the eosinophil. This is not a novel mechanism as IL-5 has previously been shown to enhance eosinophil adhesion to albumin-coated plates, plasma-coated glass, and HUVEC, via upregulation of β2 integrins (Walsh *et al.*, 1990; Kato *et al.*, 1998). Secondly, IL-5 could have primed the eosinophils to respond to a chemoattractant stimulus generated by the HUVEC, subsequently resulting in arrest. The second hypothesis was tested by examining the behaviour of IL-5-primed eosinophils incubated with PTX. Although there was some conversion from an arrested to a rolling phenotype, overall, PTX had no significant effect on eosinophil behaviour, suggesting that a chemoattractant signalling via GPCRs was not involved in mediating arrest.

Hence, the exact nature of the signal being generated was not characterised and requires further study. It especially needs to be determined whether IL-5 priming is having a direct or indirect effect on eosinophils. One other study has examined the effect of an agent on eosinophil arrest on cytokine-stimulated endothelium under flow conditions in the duration of the studies undertaken for this thesis. Ulfman *et al.* showed that a GPCR partially mediated the effects of IL-8 on eosinophil arrest to TNF-α-activated HUVEC under flow (Ulfman *et al.*, 2001). It remains possible that a chemokine is triggering arrest in the present study via a PTX-insensitive mechanism, as with the CX_3C subfamily of chemokines (Bazan *et al.*, 1997). PTX only moderately reduced eosinophil arrest and accumulation on TNF-α- and IFN-γ-treated HUVEC in a study by Kitayama *et al.* (Kitayama *et al.*, 1998). As discussed in Chapter 4, some chemokine receptors couple to G-proteins that are not PTX-sensitive (Arai and Charo, 1996), so it is possible that an unidentified chemokine was involved.

Therefore, eosinophils may require a combination of agents signalling through as yet unknown receptors to become activated and arrest. IL-5 may not be the only cytokine responsible for the *in vivo* priming of eosinophils. IL-5 was sufficient to prime eosinophils to respond to IL-8 or RANTES *in vitro* in one study (Schweizer *et al.*, 1994) but not another (Lampinen *et al.*, 1999). There is often a synergism between a leukocyte-activating cytokine and a chemoattractant. Both IL-5 and chemotactic factors exist simultaneously in

the microenvironment in inflammatory conditions (Humbert *et al.*, 1997b). Recent reports have suggested that there is cooperation between IL-5 and eotaxin during eosinophil accumulation in animal models (Collins *et al.*, 1995). Shahabuddin *et al.* demonstrated that pre-incubation of eosinophils with IL-5 significantly enhanced RANTES- and eotaxin-induced transendothelial migration across resting HUVEC (Shahabuddin *et al.*, 2000). Intracellular mechanisms exist to integrate the activities of these two different receptor classes. For example, following IL-5 priming of human peripheral blood eosinophils, chemotactic agents such as fMLP, IL-8, and RANTES promote transient activation of the extracellularly regulated kinases (ERKs) ERK1 and ERK2 (Bates *et al.*, 2000). The intracellular signalling pathways operating in the current study need to be investigated further.

In summary, a major difference has been shown between the behaviour of eosinophils and neutrophils during transmigration using TNF-α-stimulated HUVEC under flow conditions as a model of inflamed endothelium. Eosinophils appear to require a priming stage before they can respond to an activating stimulus and arrest on endothelium via integrins.

CHAPTER SEVEN

Summary, General Discussion, and Future Work

7.1 Summary and General Discussion

During the past 5 years, many aspects of the multi-step model of leukocyte binding to endothelium originally proposed by Butcher (Butcher, 1991) have been confirmed. However, the understanding of the processes involved in eosinophil adhesion to endothelium is still fragmentary and was the subject of the studies in this thesis. The hypothesis being tested was that eosinophil-specific pathways of adhesion exist, which may in part be responsible for the relatively selective eosinophilia seen in allergic inflammation. Eosinophils have few major differences in the profile of adhesion receptors expressed on their surface compared to other leukocytes. Despite this relative lack of selectivity in expression, it was questioned that there were functional differences in adhesion receptor usage, and in the signalling mechanisms used by eosinophils to adhere to endothelium compared to other leukocytes.

The aims of this thesis have been met in that two different adhesion assays have been employed to dissect the mechanisms involved in eosinophil adhesion to endothelium. The major contribution for selective migration of eosinophils appeared to be at the capture step. Here, P-selectin, and to a lesser extent VCAM-1, operated with their respective ligands to tether eosinophils, but not neutrophils, to Th2 cytokine-stimulated endothelium. Important differences in the activation step have been emphasised between the two cell types that could result in further levels of specificity. Finally, another stage may be added to the multi-step paradigm of eosinophil transmigration, in that eosinophils may require priming to be able to respond to an endothelial-generated stimulus and arrest.

In drawing physiological conclusions from this study, it is necessary to compare and contrast the two models, and to consider how closely the conditions used mimic the actual events occurring *in vivo*. Nasal polyps were an attractive candidate to use for the FSA, as they were abundantly available and eosinophilic, with their pathogenesis being similar to that of rhinitis and asthma. Leukocyte adhesion receptors did not need to be artificially induced, as with HUVEC. However, a disadvantage is that polyps are heterogeneous pieces of tissue, containing many different cell types, which are capable of releasing undefined mediators that could have contributed to the activation studies. It is possible that leukocyte-derived agents could have accumulated and influenced leukocyte responses in the FSA, but have been washed away in the presence of flow. On the other hand, HUVEC are a macrovascular, non-pulmonary cell type. The pattern and quantity of adhesion receptor expression on HUVEC may not reflect the expression of adhesion receptors *in*

vivo in allergic inflammation. However, in early passages, HUVEC can be manipulated to express the relevant adhesion molecules and signals for leukocyte adhesion and their responses seem to show parallel responses to intravital observations.

In all the flow studies, HUVEC were used at p1, as passaging of cells could have resulted in the loss of P-selectin and inappropriate alteration of the expression of other adhesion molecules. Other investigators have used HUVEC at higher or lower passages; this must be taken into account when comparing data between groups. In addition, this and other *in vitro* studies have used purified suspensions of one type of leukocyte, i.e. eosinophils, neutrophils, or monocytes. It has been argued that this approach does not assess the influence of the physical properties of the blood on adhesion; also, the purification procedure may activate the cells and alter their adhesive behaviour, making interpretation of results difficult (Patel, 1999). Recently studies have been performed where whole blood has been perfused over purified selectins, VCAM-1 (Reinhardt and Kubes, 1998), and activated HUVEC (Patel, 1999), which goes some way to avoiding this problem.

Both the FSA and the flow assay have emphasised that differences exist between the mechanisms used by eosinophils and neutrophils to adhere to endothelium. This different behaviour may reflect specialisation for different functions in disease. It appears from this and other studies that signalling pathways from chemoattractant receptors are integrated differently in neutrophils and eosinophils (Kitayama *et al.*, 1997a). The results for neutrophils agreed in both the FSA and the flow assay, and confirmed the current data in the literature. However, a discrepancy was seen when comparing eosinophil adhesion in the two models. Adhesion to NPE in the FSA was PTX-insensitive, supporting this, adhesion was not mediated via known chemoattractants signalling via PTX-sensitive GPCRs. However, in the flow assay, eosinophils arrested on cytokine-activated HUVEC when these chemoattractants were added exogenously, yet this mechanism was also PTX-insensitive. This reflects the possibility that unknown variables may exist in different models. Indeed, it remains possible that currently undiscovered molecules might exist *in vivo*, which are responsible for selective eosinophil migration. With hindsight, TNF-α may not be sufficient to create an environment where eosinophil arrest is favoured, instead, more allergic cytokines, such as IL-4 and IL-13 may be required.

In conclusion, much effort has gone into elucidating the molecular basis for selective eosinophil recruitment in asthma and related diseases in recent years. An important feature

of the adhesion paradigm outlined at the start is that it should be possible to inhibit eosinophil recruitment at each stage. In this study, P-selectin/ PSGL-1 interactions were pivotal to eosinophil arrest on Th2-cytokine-stimulated endothelium, therefore are potential targets for the therapeutic inhibition of eosinophil migration. The results of further developments with humanised anti-IL-5 mAbs, and VLA-4 and CCR-3 antagonists are also awaited with interest. Nonetheless, the inhibition of only one mediator may be insufficient to inhibit eosinophil accumulation. For instance, complete abrogation of eosinophil migration has not yet been observed by inhibiting any single chemokine (Yang *et al.*, 1998). There are integrated roles for the many different molecules present during allergic inflammation relative to eosinophil transmigration; there appears to be both synergism and redundancy between leukocyte-activating cytokines and chemoattractants, and in adhesion receptor usage. The best therapeutic strategy in preventing eosinophilia may be to suppress the production of the Th2-associated cytokines that seem to integrate and control the multiple molecular events directing recruitment. There is much motivation for continuing research in the area of eosinophil adhesion to endothelium, as the potential clinical benefits of a clear understanding of the interaction between these two cell types are enormous.

7.2 Future Work

Future studies must concentrate on the identification of the signalling events that mediate the firm arrest of eosinophils to endothelium *in vivo*. The following approaches could be taken to characterise this important step:

- Further functional studies need to be undertaken with inhibitors against other chemoattractants and their receptors, which could be incorporated into the FSA and flow assay. Potential candidates are MIP-1α, MCP-3, and MCP-4, levels of which are increased in asthma (Tillie-Leblond *et al.*, 2000), and which signal through eosinophil-expressed receptors other than CCR3 (CCR1, -2, and -5). CCR2 can couple to G-proteins that are not PTX-sensitive (Arai and Charo, 1996), it remains to be seen whether as yet undiscovered receptors exist that could mediate eosinophil adhesion.

- It will be necessary to further dissect components of the GPCR-linked signalling pathways of eosinophils and neutrophils. As well as activation of G-proteins, for certain chemokines, receptor binding activates the ERK/ MAPK pathway in multiple cell types (Coffer *et al.*, 1998). The addition of antagonists of eosinophil activation to the FSA and the flow assay (e.g. genistein, wortmannin, herbimycin A, others?) would provide an insight into the specific kinases mediating adhesion.

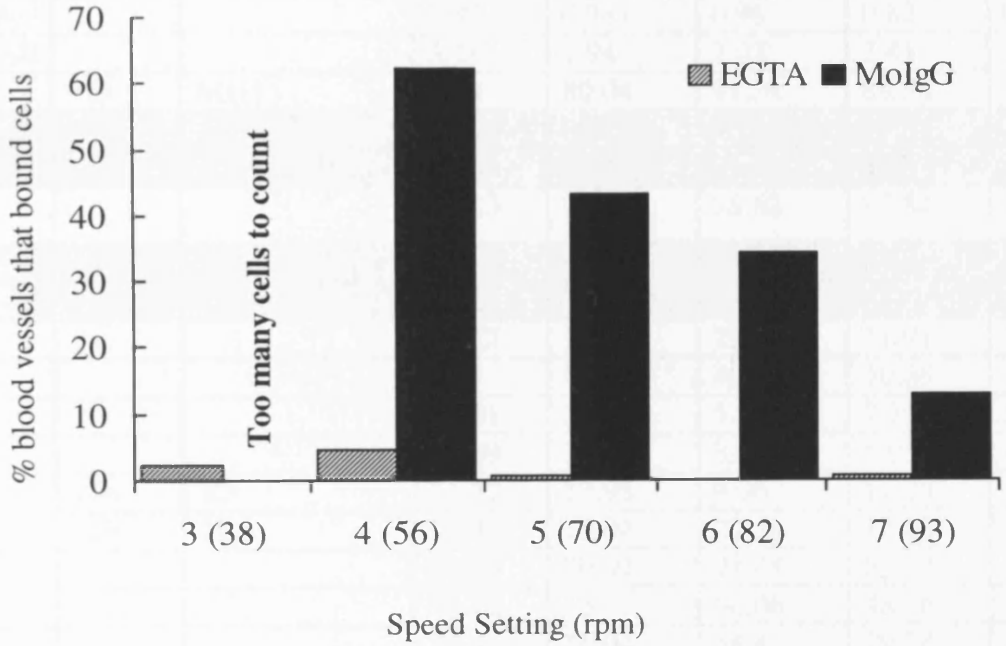
- Data in this thesis suggests that eosinophils may require priming by IL-5 in order to become fully activated for firm adhesion. It needs to be determined whether other signals could be involved in priming. For example, recently IL-8 was shown to cause a transient arrest of eosinophils on TNF-α-stimulated HUVEC (Ulfman *et al.*, 2001). It also needs to be determined whether IL-5 or unknown factors are acting directly to prime eosinophils, or indirectly, via an already existing signal generated by the HUVEC, or via the generation of intermediate active metabolites.

- It would be relevant to perform the above suggestions for future studies on low passages of human pulmonary endothelial cells, in a Th2-type cytokine environment, to mimic the conditions that predominate in asthma as closely as possible.

APPENDICES

1

Eosinophil adhesion to nasal polyp endothelium is rotation speed-dependent. Eosinophils from different donors were purified (Methods, section 2.2.2), treated with EGTA where necessary, and layered over nasal polyp sections for 30 min, RT, at different rotation speeds. The FSA was then continued (Methods, section 2.2.6.3). At speed 3, there were too many cells to count binding to blood vessels accurately. At speed 4, there was a lot of adhesion to background structures as well as to blood vessels. Therefore, speed 5 was chosen as the optimum rotation speed, based on this experiment (n = 1) and previous studies by Symon *et al.*, 1994.



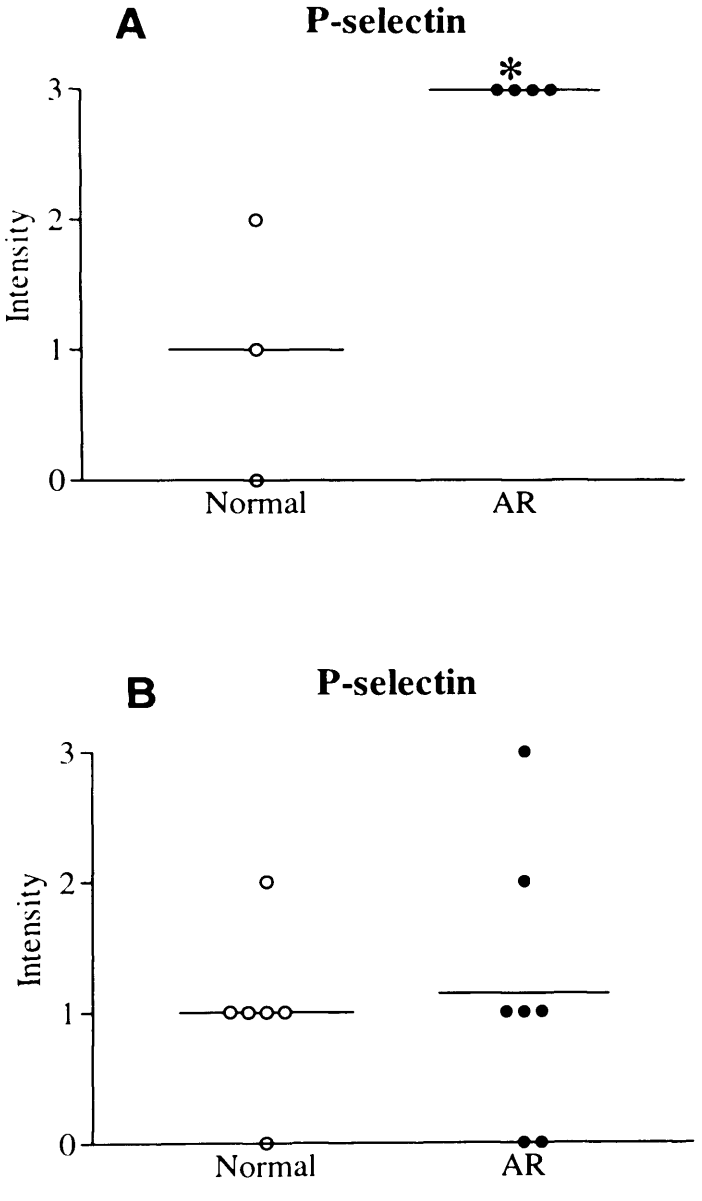
2

HLDA Workshop flow cytometry experiments showing percentage positive binding of Abs to eosinophils. Three different experiments were carried out on three different donors. All experiments were carried out blind, according to Methods, section 2.2.4.1. The tests were decoded after all experiments had been analysed. Highlighted rows show CD11/ 18 Abs used in subsequent FSAs.

			Mean of percentage positive cells ± standard deviation				
Test	**CD11/ CD18**	**Clone**	**Expt. 1**	**Expt. 2**	**Expt. 3**	**Mean**	**SD**
MoIgG			5.1	5.2	9.1	6.47	2.28
Eos only			0.89	0.58	0.98	0.82	0.21
No 1st Ab			3.28	1.94	2.02	2.41	0.75
Ref 2	11a	MI111	94.4	80.04	91.24	88.56	7.55
Ref 4	11b	44	93.22	92.28	88.24	91.25	2.65
Ref 10	18	CLB-LFA-1	93.53	83.46	91.22	89.40	5.28
A013			99.13	95.78	98.62	97.84	1.81
A014	18	7E4	53.58	79.92	92.48	85.33	6.46
A015	11b	Bear1	81.29	81.44	97.12	86.62	9.10
A016			32.27	9.5	23.96	21.91	11.52
A021			22.7	4.46	4.82	10.66	10.43
A023			13.31	2.54	5.72	7.19	5.53
A024			10.59	3.42	3.84	5.95	4.02
A028	11b	X5	11.72	12.98	9.06	11.25	2.00
A036	11b	2H3	17.93	13.04	20.26	17.08	3.68
A042			90.99	70.92	93.44	85.12	12.36
A043			77.22	13	56.06	48.76	32.73
A044			33.41	16.04	38.4	29.28	11.74
A045			15.75	7.58	20.84	14.72	6.69
A046			58.29	17.78	71.76	49.28	28.10
A047			68.43	5.26	8.9	27.53	35.47
A052			6.04	4.62		5.33	1.00
A054	11b	MEM170	38.91	24.68	33.2	32.26	7.16
A078	11a	CD11a-5E4	43.04	18.9	45.72	35.89	14.77
A079	11a	CD11a-6B7	5.63	7.9		6.77	1.61
A086	18	AZN-L18	89.18	84.98	95.9	90.02	5.51
A087	11a	AZN-L20	84.42	87.98	83.12	85.17	2.52
A088	11a	AZN-L21	89.52	91.36	93.74	91.54	2.12
A089	18	AZN-L27	91.95	93.74	95.92	93.87	1.99
A093			4.79	2.5		3.65	1.62

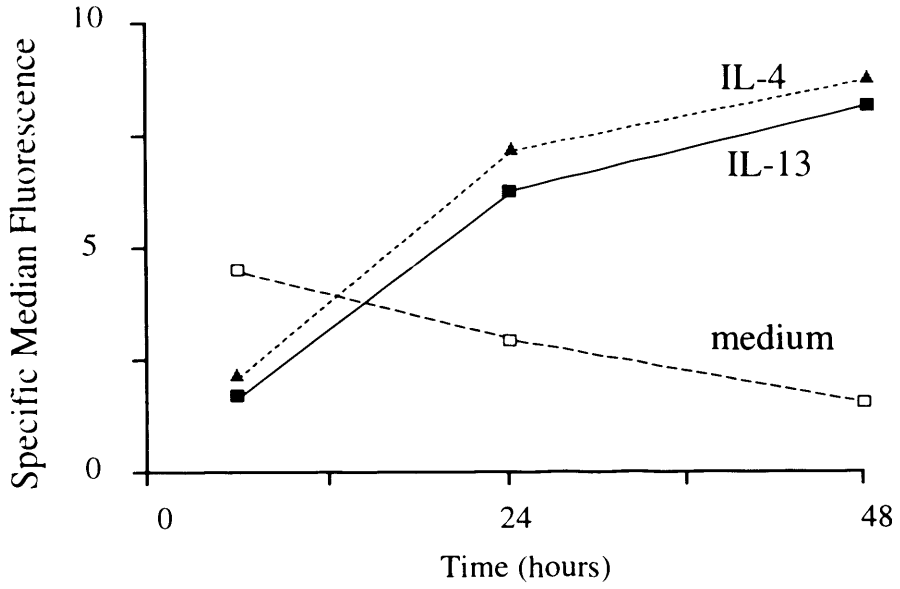
3

Expression of P-selectin in A) normal (N), allergic (AR) nasal turbinates, and B) normal (N) and allergic (AR) nasal biopsies. Sections were immunostained as shown in the Methods, section 2.2.5. The intensity/ extent of the final colour reaction was graded using a semi-quantitative scale from 0-5, where 0 = no expression and 5 = very strong expression (see Table 2–4 for key). The mean value is shown for each parameter. P-selectin expression was exclusively seen in vessel areas. A significant difference was seen in P-selectin expression between normal and allergic subjects in the turbinate (n = 3-4) but not biopsy (n = 6-7) group, * $P < 0.05$.



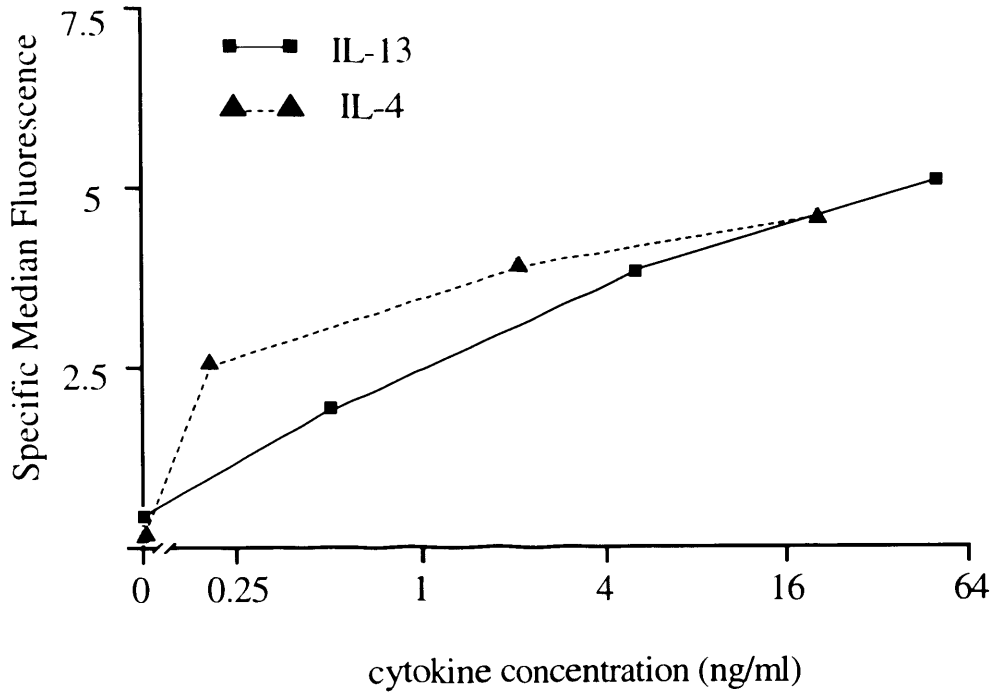
4

Time course of P-selectin expression on IL-4- and IL-13-stimulated HUVEC. HUVEC were grown to confluence and stimulated with IL-4 (20 ng/ ml) or IL-13 (5 ng/ ml) (Methods, section 2.2.3) for 6, 24, and 48 h, before being assessed for expression of P-selectin using flow cytometry (Methods, section 2.2.4). Data are expressed as specific median fluorescence with the MoIgG control values subtracted. Expression in cytokine-stimulated cells was near maximal at 24 h and maintained for at least 48 h, (data shown from one experiment representative of two). Figure courtesy of Dr. G. Woltmann.



5

Dose response of surface P-selectin expression on IL-4- and IL-13-stimulated HUVEC. HUVEC were grown to confluence and stimulated with increasing concentrations of IL-4 or IL-13 (Methods, section 2.2.3) for 48 h, before being assessed for expression of P-selectin using flow cytometry (Methods, section 2.2.4). Data shown are from one experiment representative of two. Figure courtesy of Dr. G. Woltmann.



6

Appendix to video clips. Examples of eosinophil and neutrophil behaviour on HUVEC stimulated with different cytokines.

Clip No.	Position on video	Date/ Time on video (start-end)	Cells/ substrate
1	1	12-02/ 00:02:02-00:03:15	Eosinophils/ IL-13-stimulated HUVEC
2	2	00-00/ 00:47:31-00:49:01	Eosinophils/ TNF-α-stimulated HUVEC (arrest with addition of PAF)
3	3	05-04/ 00:25:32-00:29:30	Neutrophils/ TNF-α-stimulated HUVEC
4	5	09-09/ 00:42:18-00:43:12	Eosinophils/ IL-9-stimulated HUVEC

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