HUMAN LUNG MAST CELL AND EOSINOPHIL ADHESION TO BRONCHIAL EPITHELIUM

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

By

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January 2001

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Statement of Originality

The accompanying thesis submitted for the degree of Doctor of Philosophy entitled 'Human Lung Mast Cell and Eosinophil Adhesion to Bronchial Epithelium', is based on work conducted by the author in the Department of Medicine at the University of Leicester during the period October 1995 to October 1999. All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. None of this work has been submitted for another degree in this or any other university.

Devika Sanmugalingam January 2001

Human lung mast cell and eosinophil adhesion to bronchial epithelium

Devika Sanmugalingam, University of Leicester, January 2001

Abstract

Allergic asthma is characterised by bronchial mucosal inflammation and is reflected by mucosal mast cell activation and selective eosinophil recruitment, survival and degranulation. The resultant epithelial damage and desquamation is thought to contribute to bronchial hyperresponsiveness. Cell-cell interactions are central to immune system function and control cell localisation, activation and mediator release. Dysregulated cell adhesion may contribute to the histopathological damage observed in asthma.

Asthma is initiated at the mucosal surface upon aeroallergen exposure and mast cell activation. Release of eosinophil cationic proteins is thought to be responsible for epithelial damage. In this study, human peripheral blood eosinophil and human lung mast cell (HLMC) adhesion to bronchial epithelium were investigated. A high proportion of HLMC adhered to epithelial cell monolayers compared to eosinophils. In both cases, adhesion was cation-independent, and was not inhibited by function-blocking ICAM-1 mAb, despite increased basal epithelial ICAM-1 expression upon cytokine activation.

HLMC adhesion was not modulated by function blocking mAb to cell adhesion molecule families, preincubation with carbohydrates, HLMC activation (SCF or TGF-β), or epithelial activation (cytokines). A significant reduction in adhesion was observed upon pretreatment with anti-IgE, pronase, β-galactosidase or endo-α-N-acetylgalactosaminidase (HLMC) or 4% paraformaldehyde (epithelium). No evidence for galectin involvement in adhesion was observed.

Eosinophil adhesion to alveolar epithelium was not modulated by eosinophil activation with PAF. Adhesion to bronchial epithelium was enhanced upon activation of both eosinophils (Mn^{2+}) and epithelium (cytomix). A proportion of the enhanced adhesion was β_2 (CD18) integrin-mediated.

In conclusion, the adhesion mechanisms of mast cells and eosinophils to bronchial epithelium were different, possibly relating to their divergent *in vivo* functions. The differences in proportions of adherent mast cells and eosinophils to bronchial epithelium during asthma may result in the widely documented lack of mast cells compared to eosinophils in sputum and BAL *in vivo*.

Acknowledgments

I would especially like to thank Professor A.J. Wardlaw, Dr R. Norman, Professor D. Barnett, Dr C. Hewitt, Dr R. James and Dr A. Walls for their support and advice during the writing-up and examination of this thesis.

In addition, I would like to thank Dr K. Hallam, Dr P. Bradding and the departments of respiratory medicine (Glenfield Hospital) and Biochemistry (AstraZeneca). I would also like to thank the staff and patients (who donated blood and lung tissue), theatre staff, surgeons and pathology department at Glenfield Hospital for their cooperation in obtaining the samples required for this project.

I would also especially like to thank my family for their support.

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Abbreviations

A549	alveolar epithelial cell line
αIgE	anti-IgE
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
BALT	bronchus-associated lymphoid tissue
BEAS-2B	SV-40 transformed bronchial epithelial cell line
BHR	bronchial hyperresponsiveness
BMMC	bone-marrow-derived cultured mast cells
BPE	bovine pituitary extract
BrdU	bromodeoxy uridine
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CEA	carcinoembryonic antigen
c-*kit*	stem cell factor receptor (CD117)
cpm	counts per minute
^{51}Cr	chromium-51 radioisotope
CRD	carbohydrate recognition domain
CsA	cyclosporine A
CTMC	connective tissue-type mast cells
cytomix	mixture of cyokines (50ng/ml each IFN-γ, IL-1β, TNF-α)
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DTH	delayed type hypersensitivity
EAR	early asthmatic response
ECACC	European collection of animal cell cultures
ECP	eosinophil cationic protein
ECM	extracellular matrix
EDN	eosinophil-derived neurotoxin
EDTA	ethylene diamine tetra acetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbant assay
EPO	eosinophil peroxidase
FACS®	registered trademark of Becton Dickinson and company for fluorescence-activated cell sorter
FAK	focal adhesion kinase
FcεRI	high affinity IgE receptor
FcεRII	low affinity IgE receptor
FCS	foetal calf serum

bFGF	basic fibroblast growth factor
FITC	fluorescein isothiocyanate
Fn	fibronectin
GAG	glycosaminoglycan
G-CSF	granulocyte colony-stimulating factor
GDP	guanosine diphosphate
GM-CSF	granulocyte/macrophage colony-stimulating factor
GTP	guanosine triphosphate
HABP	hyaluronan-binding protein
HBSS	Hank's balanced salt solution
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HLA	human histocompatibility leukocyte antigens
HLMC	human lung mast cell
HMC-1	human mast cell line
HMT	histamine methyl transferase
HSPG	Heparan sulphate proteoglycan
ICAM-1,-2,-3	intercellular adhesion molecule-1,-2,-3
IFN-γ	interferon-γ
Ig	immunoglobulin
IgSF	immunoglobulin superfamily
IL	interleukin
IMEM	Iscove's modified DMEM
IP_3	inositol triphosphate
ITAM	immunoreceptor tyrosine activation motifs
LAR	late asthmatic response
LT	leukotriene
M199	medium 199
mAb	monoclonal antibody
MACS	magnetic cell separation system
MAdCAM-1	mucosal vascular addressin
MAFA	mast cell function-associated antigen
MBP	major basic protein
MCP	monocyte chemotactic protein
MC_T	mast cells containing tryptase only
MC_{TC}	mast cells containing tryptase and chymase
MEM	minimum essential medium
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein-1α
MMC	mucosal-type mast cells
MoIgG	mouse IgG
MPC-1	magnetic particle concentrator
mRNA	messenger RNA

NEAA	non-essential amino acids
NF-AT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa B
NHBE	normal human bronchial epithelial cells
OD	optical density
OPD	o-phenylenediamine dihydrochloride
PAF	platelet activating factor
PAR	protease-activated receptor
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PECAM-1	platelet/endothelial cell adhesion molecule
PG	prostaglandin
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PMA	phorbol myristate acetate
PP2B	protein phosphatase 2B
PSGL-1	p-selectin glycoprotein ligand
PTK	protein tyrosine kinase
RANTES	regulated upon activation, normal T expressed and presumably secreted
RBL-2H3	Rat basophilic leukaemia cell line
RGD	arginine-glycine-asparagine
RPE	R-phycoerythrin
RPMI	Roswell Park Memorial Institute (culture medium)
RT	room temperature
RTK	receptor tyrosine kinase
SAGM	small airway growth medium
SAM	[^{3}H]methyl-S-adenosyl methionine
SCF	stem cell factor
TBS	Tris-buffered saline
TGF-β	transforming growth factor-β
T_H	T helper lymphocyte
TNF-α	tumour necrosis factor-α
TNS	trypsin-neutralising solution
U	unit (for enzyme activity)
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VLA	very late antigen

Chapter 1: Introduction

1.1 Asthma

Human bronchial asthma is characterised by widespread and variable, reversible airflow limitation, airway hyper-responsiveness to non-specific stimuli (bronchoconstriction) and inflammation (Bochner et al, 1994). Asthma is initiated at the bronchial mucosal surface and involves the systemic immune system, resulting in a T_H2 phenotype (IL-4 and IL-5 cytokine production), increased serum IgE level, increased mast cell activation, infiltration of eosinophils into the airway mucosa and lumen, eosinophil degranulation and epithelial damage (Djukanovic et al, 1990). Asthma may be manifested in three stages (a rapid spasmogenic phase in response to inhaled allergen, late sustained phase and a subacute, chronic inflammatory phase) (Busse et al, 1993). The immediate response to local allergen challenge has been associated with pulmonary mast cell activation. The late phase of asthma has been associated with eosinophil infiltration and prolonged survival.

1.1.1 Epidemiology

Increasing asthma prevalence has been reported in the U.K. and world-wide (Burney et al, 1990). The rising prevalence of asthma was first noted in Birmingham schoolchildren (Morrison-Smith et al, 1971) and was reflected nationally throughout England (Burney et al, 1990). In addition, an international rise in asthma mortality has also been observed, (although wide variations between countries examined were found) (Jackson et al, 1988). Between 1959 and 1966 the rise in mortality was especially apparent in the 10 to 14 year old age group, where a sevenfold increase in mortality was observed (Speizer et al, 1968; Inman and Adelstein, 1969). Mortality from asthma has risen despite advances in knowledge, development of novel therapies and increased pharmacological management of symptoms ('the asthma paradigm'). Recently, the increase in asthma mortality has been attributed to errors in asthma management (Jackson et al, 1988).

1.1.2 Clinical presentation

Characterisation of asthma as bronchial constriction and reversible airway obstruction led to increased use of β-adrenergic agonists to alleviate the clinical symptoms of wheeze, dyspnoea, chest tightness and cough. This was linked to a rise in mortality in several countries in the early 1960s (Inman and Adelstein, 1969).

Bronchial mucosal inflammation has recently been associated with the development of asthma. Inflammation is reflected by the presence of mast cell and T lymphocyte activation, marked eosinophilia, increased vascular permeability and oedema, epithelial desquamation and sub-epithelial basement membrane thickening, mucous gland hyperplasia and mucus hypersecretion, and bronchial smooth muscle hypertrophy and dysregulated contractility (Djukanovic, 1990). The degree of airway inflammation may correlate with disease severity and may be involved in the early pathogenesis of asthma (Bousquet et al, 1991; Laitinen et al, 1993).

1.1.3 Aetiology

The aetiology of asthma is not fully understood. It is currently thought to be a multifactorial disease, and symptoms result from mucosal inflammation resulting from the complex interaction of genetic predisposition and environmental triggers.

Genetic susceptibility

Asthma and airway hyper-responsiveness is often associated with atopy. Atopy is a genetic predisposition to synthesise inappropriate levels of immunoglobulin E (IgE) directed against innocuous external antigens. First-degree relatives of asthmatics have a higher prevalence of asthma and atopy compared to relatives of non-asthmatic patients (Laitinen et al, 1997). Asthma is thought to be an autosomal dominant inherited trait (Cookson and Hopkin, 1988), but has not been linked to a single gene, and possibly involves polygenic inheritance and/or genetic heterogeneity.

Several different genes contribute to the heritable component of asthma. Linkage to asthma and atopy have been studied for a number of candidate genes. Chromosome 5 possesses the T_H2 cytokine cluster (IL-3, IL-4, IL-5, IL-9 and IL-13 production

important for IgE isotype switching, eosinophil survival and mast cell proliferation), chromosome 6 confers T_H2 response predisposition (MHC class II region) and chromosome 11q13 possesses the FcεRI β chain locus (Cookson et al, 1989).

Environmental factors

The recent rate at which the mortality of asthma has increased, and the lack of complete concordance between identical twins has suggested that other factors, apart from underlying genetic factors also contribute to the asthma syndrome. Factors such as socioeconomic status, rural environment, diet and sibship size (hygiene hypothesis) have been implicated in increased atopic allergy (Strachan et al, 1999). In addition novel and established environmental pollutants may exacerbate asthma (such as NO_2 or SO_2 from increased traffic pollution). Other factors may be maternally-related (Jones et al, 1998).

1.1.4 Airway pathology in asthma

The pathophysiological changes occurring in asthma have mainly been studied by examination of sputum, tissue (autopsy and biopsy) and bronchoalveolar lavage (BAL) fluid. Observations include airway occlusion with mucus, infiltration of mucosa and lumen with inflammatory cells, thickening of the subepithelial basement membrane, airway smooth muscle hypertrophy and epithelial desquamation (Djukanovic et al, 1990).

Sputum

The presence of bronchial epithelial cells in asthmatic sputum was one of the first pathological differences noticed during acute asthmatic attacks and sheets of exfoliated epithelium were termed 'Creola bodies' (Naylor, 1962). Increased eosinophil number, major basic protein (MBP), Charcot-Leyden crystals and Curschmann's spirals were also observed in asthmatic sputum.

Autopsy

Autopsies of fatal asthma show hyperinflation of the lungs, hypertrophy and hyperplasia of bronchial smooth muscle, congestion due to excessive mucus secretion and plugging of bronchi and oedema (Dunnill, 1960). Plasma proteins from microvascular leakage and oedema, transmigrated activated eosinophils and desquamated epithelial cells were found in mucus plugs. Immunohistochemical staining of autopsy tissue revealed denuded epithelium, goblet cell hyperplasia, eosinophil infiltration of the tissue and collagen deposition in the epithelial sub-basement membrane region (Dunnill et al, 1969).

Biopsy

Tissue from living mild or asymptomatic asthmatics was studied using fiberoptic bronchoscopy. Results confirmed that pathologic changes such as focal epithelial damage and fragility were not artefacts due to sampling. These studies also showed inflammatory changes were apparent early in the course of the disease and in asymptomatics (Cutz et al, 1978; Laitinen et al, 1985; Laitinen et al, 1993). Similar structural and cellular results to autopsy studies of inflamed airways were found (submucosal gland hyperplasia, bronchial smooth muscle hypertrophy, increased oedema, eosinophil infiltration of the bronchial mucosa, epithelial sub-basement membrane collagen deposition and epithelial desquamation) (Roche et al, 1989).

Epithelial damage and increased numbers and activation of inflammatory cells (mast cells and eosinophils) have been described in living stable and mild asthmatics using bronchoscopy (Laitinen et al, 1985; Beasley et al, 1989; Bousquet et al, 1990). Severity of symptoms paralleled the degree of mucosal inflammation (classified by degree of epithelial shedding and number and activation status of infiltrated mast cells and eosinophils) (Beasley et al, 1989). In addition increased epithelial intercellular spaces and preferential cleavage above basal cells indicated increased ciliated epithelial cell fragility (Ohashi et al, 1992). Shed ciliated columnar epithelial cells occurred in adherent groups and were viable ("Creola bodies"), suggesting selective cleavage at adherence junctions between epithelial cells (desmosomes) (Naylor, 1962). Increased epithelial fragility, damage, shedding and turnover of

ciliated columnar cells was selective to asthmatics, and was thought to be a result of eosinophil activation and degranulation (Frigas et al, 1986). MBP was often found at sites of epithelial fragility and damage in status asthmaticus, and similar histopathologic damage was observed when human and guinea pig tracheal epithelium was treated with MBP (Filley et al, 1982; Motojima et al, 1989).

Lamina propria mast cells exhibited increased degranulation in mild and severe asthmatic but not control subjects, supporting the suggestion that mast cell degranulation and mediator release may occur continuously within the bronchial mucosa of atopic asthmatic subjects (Beasley et al, 1989). Bronchial biopsy studies have also identified increased T cell activation in asthma (Azzawi et al, 1990). In addition, the asthmatic bronchial mucosa produces increased concentrations of proinflammatory and chemotactic cytokines (IL-5, GM-CSF, MCP-1 and RANTES) (Hamid et al, 1991; Salvi et al, 1999; Sousa et al, 1993; Sousa et al, 1994; Wang et al, 1996a).

Bronchoalveolar lavage (BAL)

BAL studies have confirmed the increased activation of inflammatory cells in asthma. There was an increased proportion of activated $CD4^+$ IL-$2R^+$ HLA-DR^+ T lymphocytes in asthmatic BAL (Metzger 1987) and elevated eosinophil number and activation (measured by increased basic protein release). However, although there was a comparative absence of mast cells in asthmatic BAL (Wardlaw et al, 1988; Gibson et al, 1993), BAL mast cells from atopic asthmatic subjects exhibited increased spontaneous release of histamine (Flint et al, 1985). Products from activated mast cells (histamine, tryptase), eosinophils (cationic proteins, PAF, TGF-β), T cells (IL-4, IL-5, IL-13) and epithelium (RANTES, GM-CSF; TGF-β) are elevated in BAL fluid (Wenzel 1988; Broide 1991; Redington et al, 1997).

1.2 Cell adhesion molecules

Inflammation in asthma is characterised by eosinophil infiltration and degranulation. In addition, it is also accompanied by mucosal T cell and mast cell activation. Localisation and function of cells is dependent on surface adhesion molecule interactions. Cell-cell interactions may be homotypic or heterotypic, and interactions also occur with extracellular matrix molecules. Cell adhesion is crucial to the normal development of tissue architecture and also has a pathological role in inflammation and tumour metastasis (Gumbiner, 1996). Immune system cells transmigrate along a chemotactic gradient by regulating the adhesiveness of their surface adhesion molecules between adherent and non-adherent states (Springer et al, 1994). Aggregation of cell adhesion molecules leads to signal transduction (via protein kinases and phosphatases) and results in changes in cell activity (including activation, degranulation, chemotaxis) and control of cell function (cell growth, differentiation and gene expression) (Hynes, 1992).

1.2.1 Cell adhesion molecule families

Cell adhesion molecules are divided into families based on similarities in their structural characteristics (Figure 1.1) (Hynes, 1992)). Adhesion molecule families involve protein-protein (integrin, immunoglobulin superfamily (IgSF), cadherin) and protein-carbohydrate (selectin, proteoglycan) mechanisms. Examples of adhesion molecules involved in immune cell interactions are shown (Figure 1.2) (Springer, 1990). Compared to leukocyte interaction with epithelium, the ligand-receptor pairs involved in leukocyte adhesion and transendothelial migration have been well characterised. Selectin/carbohydrate binding initiates loose tethering and rolling on the endothelium, activated leukocyte-endothelial adhesion is mediated by integrin/IgSF member interaction and leukocyte transendothelial extravasation involves homophilic leukocyte and endothelial CD31 interaction (Carlos and Harlan, 1994; Hogg and Berlin, 1995) (Figure 1.3). Migration through the extracellular matrix and subsequent cell function involves β_1 and β_2 integrins respectively (Wardlaw, 2000).

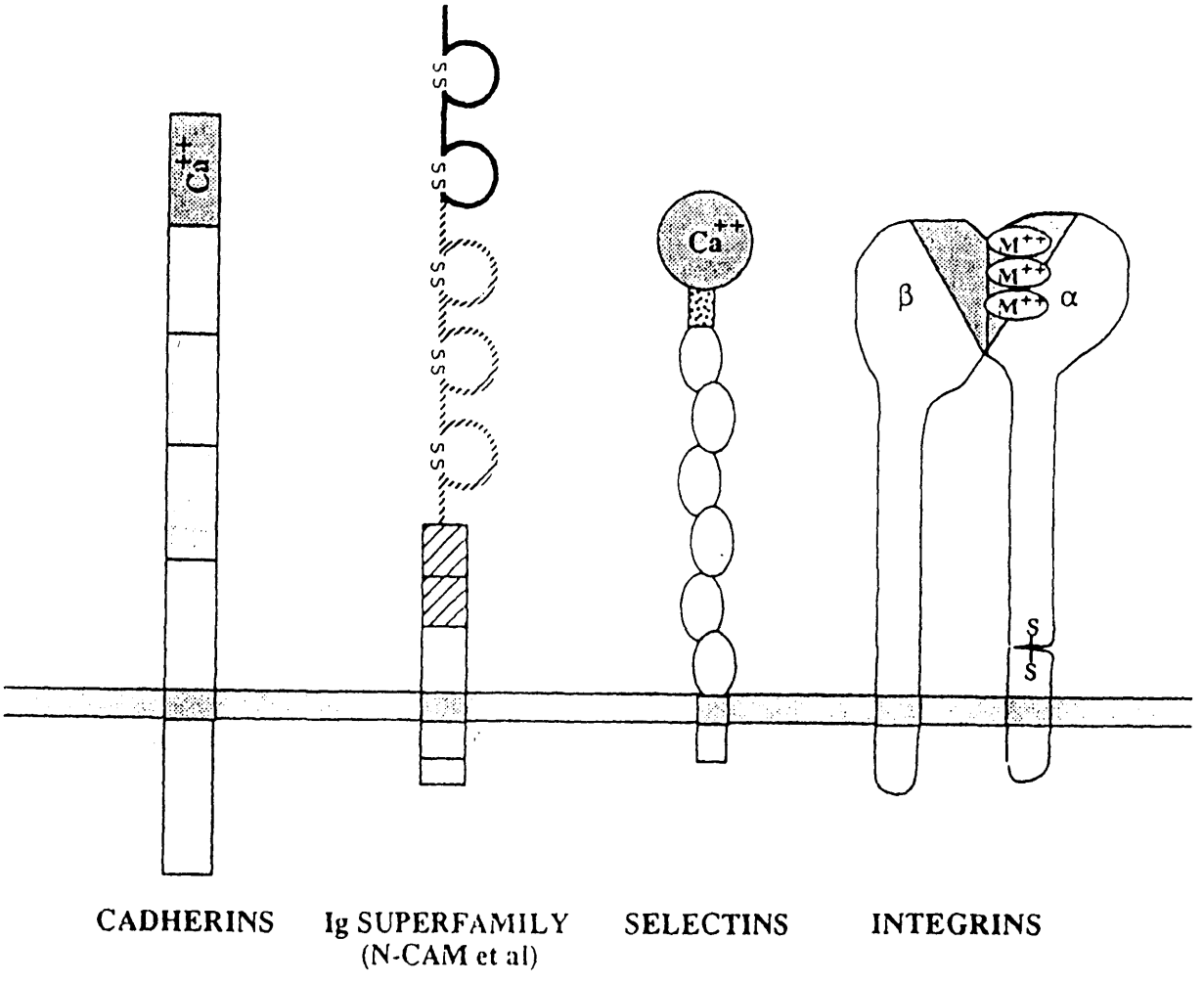


Figure 1.1 **Major families of cell adhesion molecules**
General structural characteristics of the cadherin, immunoglobulin (Ig) superfamily, selectin and integrin families are shown. Calcium-or magnesium ion binding sites for cadherin, selectin and integrin cell adhesion molecules are shown (Hynes, 1992).

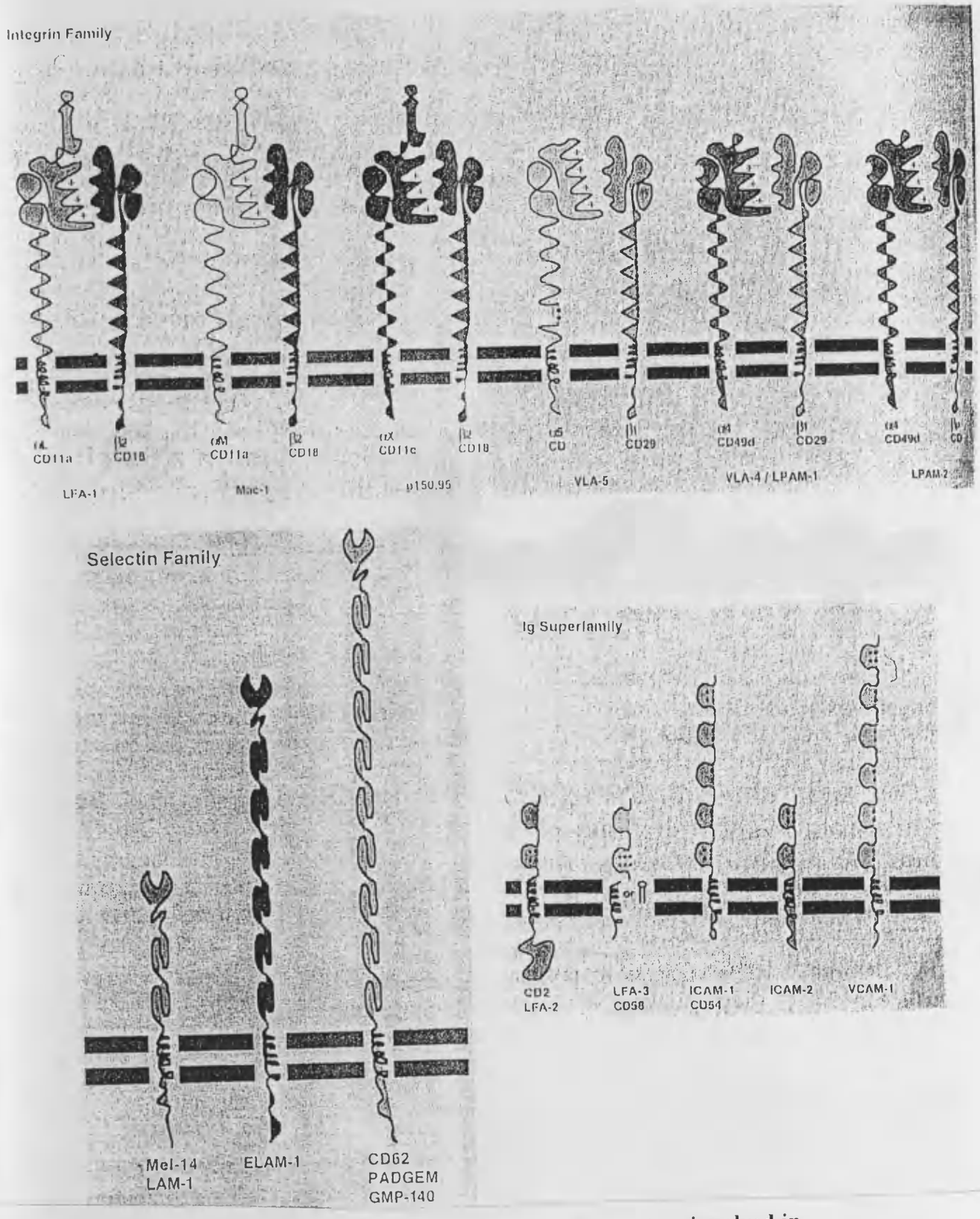


Figure 1.2 **Representative cell surface adhesion receptors involved in Immune cell interactions** (integrin, selectin and IgSF families are shown to scale) (Springer T.A., 1990)

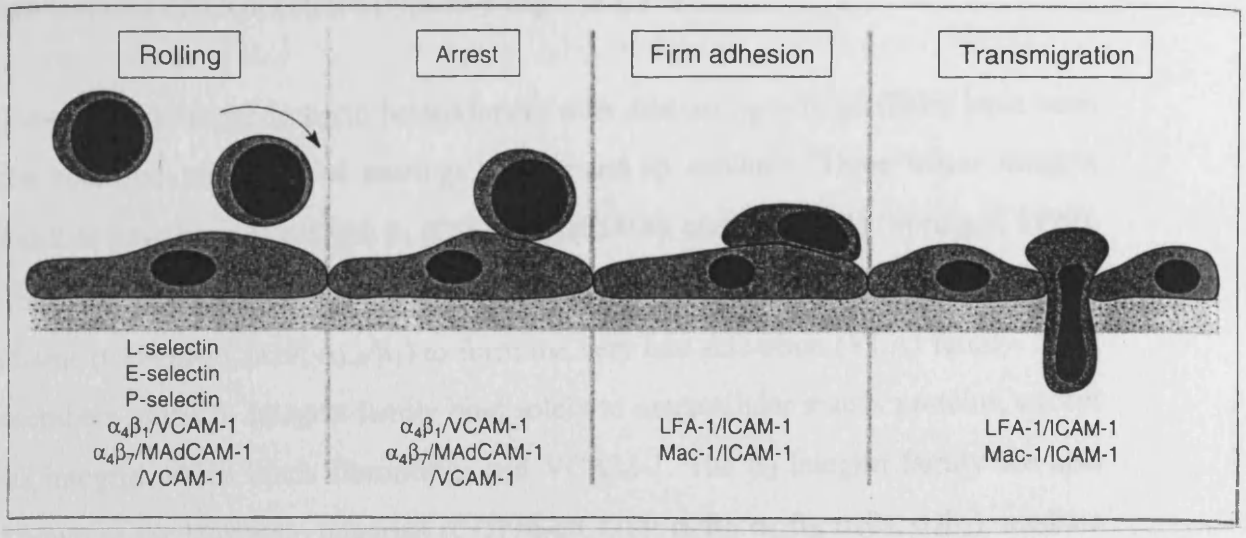


Figure 1.3 **Diagram of sequential adhesion molecule interactions during the leukocyte-endothelial adhesion cascade** (rolling, arrest, firm adhesion and transmigration involve different cell adhesion molecule pairings, as well as activation stimuli **(Hogg and Berlin, 1995).**

1.2.2 Protein-Protein interactions

(i) Integrin

The integrin family of type I transmembrane glycoprotein heterodimers (non-covalently linked α and β subunits) have a globular extracellular domain containing the divalent cation-dependent ligand binding site (Hynes, 1992). Selectivity of integrins for a given ligand is controlled by expression and activation state. Ligand affinity is regulated by activation-dependent conformational change and requires extracellular divalent cations (typically Mg^{2+} or Ca^{2+}).

Currently 20 known integrin heterodimers with distinct ligand specificity have been characterised, made up of pairings of 17α and 8β subunits. Three major integrin families have been classified: β_1 (CD29), β_2 (CD18), and β_3 (CD61) (Springer, 1990). The β_1 integrin family consists of the single β chain (CD29) combining with 9 α-chains (CD49a-f/CD29; α_{1-9}/β_1) to form the very late activation (VLA) family. Most members of the β_1 integrin family bind solely to extracellular matrix proteins, except α_4 integrin which binds fibronectin and VCAM-1. The β_2 integrin family are also known as the leukocyte integrins (CD11a-d/CD18; $\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_x\beta_2$, $\alpha_d\beta_2$), mediate cell-cell adhesion and counterreceptors include ICAM-1,-2 or –3, iC3b and fibrinogen. The β_3 family are known as the cytoadhesins (CD41/CD61, CD51/CD61; gpIIb/IIIa, $\alpha_v\beta_3$). In general, integrin ligands include IgSF members, extracellular matrix proteins, bacterial and viral proteins, coagulation and fibrinolytic factors and complement proteins. Integrin conformational change upon activation may be achieved through inside-out and/or outside-in signalling (e.g. chemoattractant) although Mn^{2+} is able to directly activate integrins.

Several integrins are localised at focal contacts - where actin bundles terminate and are surrounded by a ring of vinculin and talin (Critchley, 2000). Other cytoplasmic proteins like α-actinin have the ability to interact with the cytoplasmic domain of integrins. Ligand binding may result in focal adhesion assembly, and tyrosine phosphorylation of both cytoskeletal components and signalling molecules. FAK is a key component in the assembly of focal contacts, which influence cytoskeletal

organisation and signal transduction (Burridge and Chrzanowska-Wodnicka, 1996). Signalling provides a mechanism for the adhesion and subsequent de-adhesion required during integrin-mediated cell migration. Most integrin cytoplasmic domains are short except the β_4 chain of $\alpha_4\beta_6$, specifically concentrated at epithelial hemidesmosomes (Garrod, 1986).

(ii) Immunoglobulin gene superfamily (IgSF)

Members of this large and diverse group of single chain transmembrane glycoproteins are characterised by a variable number of immunoglobulin-like extracellular domains. IgSF receptor-mediated binding is divalent cation-independent and may be heterophilic (e.g. endothelial ICAM-1,-2,-3 binding to leukocyte integrins) or homophilic (e.g. N-CAM). The IgSF is the most abundant family of leukocyte cell surface molecules and are present on other cell types, including endothelial and epithelial cells. Firm adherence of leukocytes to blood vessels during inflammation involves ICAM-1 and VCAM-1. ICAM-2 is involved in lymphocyte recirculation through uninflamed endothelium. ICAM-1 and ICAM-2 are ligands for LFA-1, whereas VCAM-1 is a ligand VLA-4 (Springer, 1994).

ICAM-1 has 5 Ig-like domains (Dustin et al, 1986). As well as binding LFA-1 and Mac-1, ICAM-1 is also a receptor for the major group of rhinovirus (Greve et al, 1989). ICAM-1 function is regulated by increased expression on most cell types by the proinflammatory cytokines IL-1β, TNF-α and IFN-γ. Increased expression on HUVEC is protein synthesis-dependent and generally detectable after ~4h and maximal by 24h. ICAM-1 has been implicated in leukocyte migration, lymphocyte homing, cytotoxic T-lymphocyte function, antigen presentation and thymocyte maturation. ICAM-2 has 2 Ig-like domains that are most homologous (34%) to the two amino-terminal domains of ICAM-1. ICAM-2 is constitutively expressed on vascular endothelial cells, and is not increased by cytokine activation. ICAM-2 is involved in lymphocyte trafficking to uninflamed tissues (Staunton et al, 1989). It is also expressed on monocytes, and platelets, but not neutrophils. ICAM-3 has 5 Ig-like domains and is well-expressed on leukocyte, but not endothelium (de Fougerolles and Springer, 1992).

VCAM-1 has 6 Ig domains and is expressed by endothelial cells, although its expression on epithelial cells remains controversial (Atsuta et al, 1997). HUVEC VCAM-1 expression is induced by TNF-α and IL-1 with a similar time course to ICAM-1. Both IL-4 and IL-13 selectively upregulate VCAM-1 (Bochner et al, 1995). VCAM-1 is important in eosinophil adhesion to HUVEC and is a $\alpha_4\beta_1$ (VLA-4) ligand and binds weakly to $\alpha_4\beta_7$ (Walsh et al, 1991a; Bochner et al, 1991; Dobrina et al, 1991; Weller et al, 1991). Neutrophils do not express VLA-4 (Hemler, 1988) and do not bind to VCAM-1.

(iii) Cadherin

Cadherins are transmembrane Ca^{2+}-dependent homophilic adhesion receptors (Takeichi, 1990). The main cadherins are E-cadherin (epithelial), N-cadherin (neuronal) and P-cadherin (placental). Desmosomal cadherins are members of the cadherin family localised at epithelial desmosomes and provide anchorage for actin filaments. The mammalian families of desmosomal cadherins (desmocollins and desmogleins) are involved in desmosomal plaque formation.

Epithelial adhesion junctions and interactions are shown in Figure 1.4 (Garrod, 1986). Adult E-cadherin is present on the lateral epithelial cell surface and is concentrated in intercellular junctions known as the zonulae adherens in a ring around the epithelial cell apicolateral region. The zonula adherens is characterised by a cortical ring of cytoskeleton, the major component of which is actin.

Cadherin extracellular interactions are subclass-specific. Cadherins mediate homotypic adhesion via their extracellular regions, which typically contain five repeated subdomains, EC1 to EC5. The homophilic adhesive binding region on the N-terminal EC1 domain is dependent on the presence of extracellular Ca^{2+} ions. The presence of Ca^{2+} stabilises the functional architecture of the extracellular region rather than direct participation in the intercellular adhesive interface. Cadherin dimers are arranged as a linear "zipper" at the intercellular contact zone (Shapiro et al, 1995).

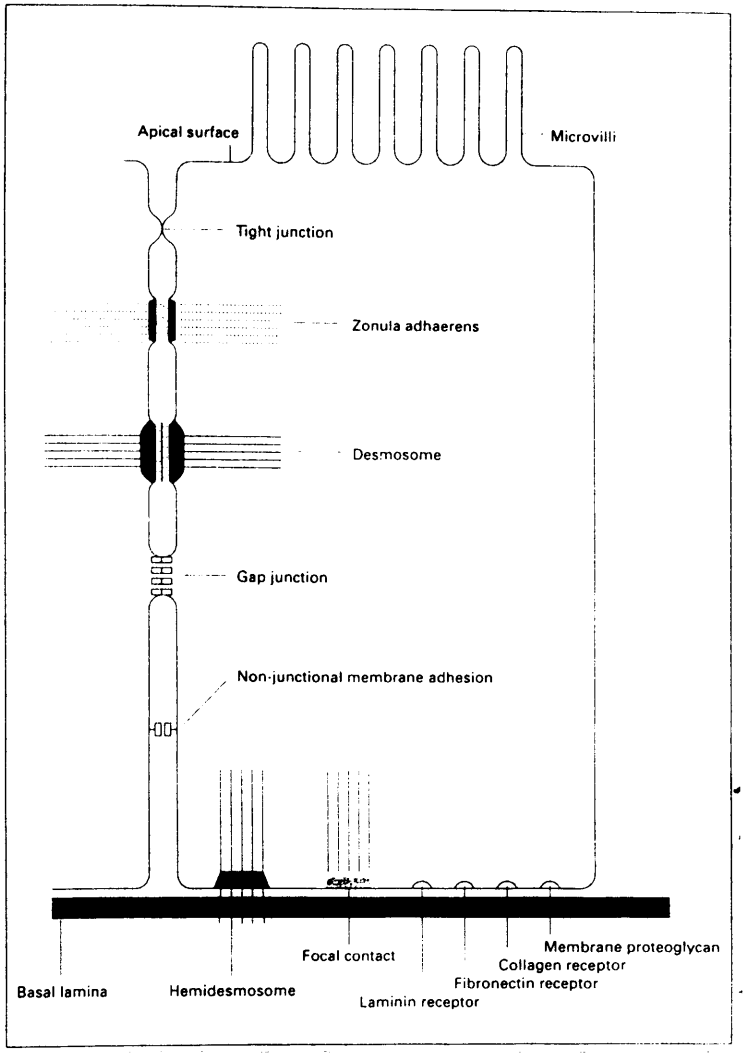


Figure 1.4 **Diagram showing junctional and non-junctional adhesion mechanisms of simple epithelia (from Garrod, 1986).** Bronchial epithelium is psuedostratified, and basal epithelial cells are not shown above. Adhesion mechanisms involved are identical.

The cytoplasmic domains of cadherins interact with catenins. Catenins link cadherin molecules to the actin cytoskeleton and are crucial for cadherin function. α-catenin is required for cadherin-mediated cell adhesion and links the cadherins to the actin cytoskeleton. β-catenin is also required for adhesion (as a necessary intermediate in the linkage of α-catenin to the cadherin cytoplasmic domain) and probably regulates complex formation, coupling physical adhesion to signalling events during morphogenesis (Takeichi, 1990).

Desmosomes are the other major intercellular junctions of epithelia, and are also adhesive glycoproteins of the cadherin family. Their extracellular domains are similar to those of cadherin but their cytoplasmic domains are specialised for forming desmosomal plaques and are attached to the cytokeratin intermediate filament cytoskeleton rather than to actin. Together the desmosomes and intermediate filament cytoskeleton form a contiguous network throughout the tissue and contribute to the high tensile strength of the epithelium. The adhesion receptors of the desmosomes (desmogleins and desmocollins) exist in a variety of isoforms with distinct tissue-specific patterns of expression. They are linked to the intermediate filament network by several cytoplasmic plaque proteins (including the desmoplakins and plakoglobin) (Garrod, 1986). Desmoplakin shares sequence similarity with intermediate filament proteins and appear to interact directly with them. Plakoglobin binds to the cytoplasmic tails of certain desmogleins and desmocollins and may be essential for desmosomal plaque formation and cytokeratin filament attachment.

Epithelial cells adhere to extracellular matrix via integrins in hemidesmosomes. Like the desmosome, the hemidesmosome is linked to the cytokeratin intermediate filament network, but has a different composition and structure. The main adhesion receptor is the integrin $\alpha_6\beta_4$, which binds to laminin in the basement membrane (Albelda, 1991). The cytoplasmic plaque proteins that link the hemidesmosome to the intermediate filaments are related to the desmosomal proteins, but are unique. This network of intermediate filament-basement membrane attachments is crucial for the maintenance of mechanical integrity of the epithelium.

1.2.3 Lectin-Carbohydrate interactions

Although most well-characterised cellular adhesive interactions appear to be (homophilic or heterophilic) protein-protein mediated, carbohydrates present on glycoproteins (e.g. selectin ligands) and on proteoglycans and glycosaminoglycans of the extracellular matrix (heparin and hyaluronic acid) may also be involved in adhesion (Lasky, 1991).

Lectins are implicated in intercellular recognition and adhesion. Lectins are non-enzymatic proteins that bind reversibly with high specificity to mono- or disaccharides on carbohydrate moieties present on glycoproteins. They may be soluble or cell-associated and are classified based on the specificity of their carbohydrate recognition domains (CRDs) (Ni and Tizard, 1996). Lectins may recognise mannose, galactose, N-acetylglucosamine, N-acetylgalactosamine, L-fucose or N-acetylneuraminic acid and are routinely used to identify glycoconjugate membrane receptors with high resolution. Several lectin families have been described based on their CRD specificities, including the Ca^{2+}-dependent C-type lectins (e.g. selectins), S-type lectins (e.g. galectins) and I-type lectins (e.g. sialadhesins). C-type CRDs are characterised by their Ca^{2+}-dependent adhesion, S-type CRDs (galectins) selectively bind β-galactosides and P-type CRDs bind mannose-6-phosphate (Lasky, 1991; Barondes et al, 1994).

(i) C-type lectins

C-type CRDs function at cell surfaces (e.g. selectins) or in the extracellular environment. Selectins also contain epidermal growth factor-like modules and 2 to 9 complement homology repeats. The selectins mediate initial interactions between circulating leukocytes and endothelial cells at sites of inflammation and in lymph nodes. The selectin CRDs recognise fucosylated and sialylated carbohydrate moieties (Symon and Wardlaw, 1996).

(ii) S-type lectins

Galectins are Ca^{2+}-independent β-galactoside-binding lectins (29-35kDa), and ten members of this family have been discovered so far. Galectins are involved in cell adhesion and growth regulation, immunomodulation, apoptosis, inflammation, embryogenesis, metastasis and pre-mRNA splicing (Perillo et al, 1998). Galectins are nonglycosylated cytosolic proteins, some of which are localised in the nucleus or at cell surfaces (in the extracellular matrix and in cell secretions). They are found in many cell types including mast cells and epithelium. Galectins are defined by their affinity for poly-N-acetyllactosamine. Galectins may display multivalent sugar binding, due to multiple CRDs (in one polypeptide or through oligomerisation) (Barondes et al, 1994).

Galectin-1 is a soluble noncovalent homodimer and is expressed with a broad tissue distribution. Galectin-1 is abundant in smooth, skeletal and cardiac muscles, neurons, thymus, kidney and placenta. Galectin-1 regulates cell growth and adhesion and induces apoptosis in activated human T cells. Human thymic epithelial cell galectin-1 mediates binding to thymocytes and T lymphoblastoid cells (Baum et al, 1995).

Galectin-3 (εBP, IgE-binding protein, Mac-2) lacks a transmembrane domain and is found in haematopoietic cell lineages (basophils, macrophages and mast cells), and a variety of epithelia. Mast cells express galectin-3, intracellularly and on their cell surface (Craig et al, 1995). Galectin-3 has also been associated with cellular transformation and metastasis in brain, thyroid and colonic mucosa. It is localised in the nucleus associated with ribonuclear protein particles and/or cytoplasm, on the cell surface or in the extracellular environment, and plays an important role in pre-mRNA splicing, cell-cell and cell-matrix interactions, cell growth, tumour metastasis and inflammation (Barondes et al, 1994).

(iii) Cell surface proteoglycans

The attachment of cells to the extracellular matrix is crucial for the maintenance of tissue integrity. Most extracellular matrix attachment proteins possess domains that bind to either the heparan sulphate or chondroitin sulphate glycosaminoglycans found on most proteoglycans. Cells may adhere to the extracellular matrix through cell surface proteoglycans (e.g. CD44 and syndecans).

Proteoglycans are chains of glycosaminoglycans (GAGs) covalently attached perpendicularly to the core protein (in a bottlebrush-like structure) through Ser/Thr (O-glycosidic) bonds. Glycosaminoglycans are long (100-200 monosaccharide) unbranched molecules containing repeating disaccharide units. One sugar is usually a uronic acid (D-glucuronic or L-iduronic) and the other is alternate 1,4- and 1,3-linked GlcNAc or GalNAc. One or both sugars contain sulphate groups (except hyaluronic acid). GAGs are highly negatively charged due to the presence of these anionic residues. GAGs include hyaluronic acid and heparan sulphate.

CD44 and Hyaluronic acid

Hyaluronic acid (alternate β1,4-D-glucuronate + β1,3-GlcNAc) is a unique GAG since it does not contain sulphate and is not found covalently attached to proteins. Hyaluronic acid may be hydrolysed by hyaluronidase at β1,4-linkages.

Hyaluronan-binding proteins (HABPs) such as the type I transmembrane receptor CD44 mediate cell adhesion and migration. CD44 is involved in lymphocyte homing to mucosal lymph nodes, haematopoiesis, tumour progression, signal transduction and inflammation. CD44 is the main receptor for the extracellular matrix GAG hyaluronan and is widely distributed (Aruffo et al, 1990). The CD44 family are generated by alternative splicing of a single gene. The CD44 gene consists of 20 exons, of which ten are normally expressed and encode CD44H (Lesley et al, 1993). The additional 10 exons (v1-v10) combine in a limited manner by alternative splicing to encode extracellular regions. Post-translational modifications affect activation- or differentiation-dependent ligand specificity and affinity.

Syndecans and heparan sulphate

Heparin and heparan sulphate are similar in structure (D-glucuronate sulphate + N-sulpho-D-glucosamine), although heparans have less sulphate groups than heparins. Heparan sulphate is found in basement membranes and as a component of cell surfaces. The syndecan family are transmembrane heparan sulphate proteoglycans (HSPGs) which mediate cellular adhesion and regulate growth factor activities. Heparinase (Flavobacterium heparinum) catalyses hydrolysis of αN-acetyl-D-glucosamidic linkage in heparan sulphate and heparin proteoglycans.

Syndecans are integral single membrane-spanning HSPGs that provide the major cell surface source of heparan sulphate. There are four (syndecan-1, -2, -3, -4) discovered so far, transcribed from four different genes. The GAG chains are predominantly heparan sulphate. The heparan sulphate chains on syndecan-1 are composed of highly sulphated (heparin-like) domains. Extracellular domains may be lost by regulated shedding, occurs at a conserved site adjacent to the plasma membrane and is susceptible to trypsin-like proteases. Shedding may be induced by cell stress, activation (EGF or thrombin) or phorbol esters (Jalkanen et al, 1987).

Syndecan-1 is absent from most terminally differentiated cells, and is present on basolateral surfaces of epithelia and plasma cells and syndecan-4 in most tissues (Sanderson and Bernfield, 1988). Syndecan-1 and –4 are induced and lost in response to tissue injury. Syndecan-1 reduction is associated with malignant transformation, invasion and metastasis. Syndecan-1 is a matrix co-receptor and allows mammary epithelial cells to bind fibronectin, fibrillar collagens, tenascin and thrombospondin. Syndecan-4 is involved in cell spreading and is an integrin co-receptor involved in microfilament assembly and focal adhesion formation (Couchman and Woods, 1996).

1.3 Mucosal inflammation

The mucosal surface is the primary target of inhaled allergen and is an important first line of defence, forming a barrier between the internal and external environments. Mucosal immunity may differ from other sites since there is a high level of IgA production, areas of organised lymphoid follicles – bronchus-associated lymphoid tissue (BALT) are found, and systemic T cell tolerance (rather than activation) may occur upon immunisation (oral mucosa). In addition the functional relevance of the greater number of γδ T cells (10% intraepithelial lymphocytes) compared to other sites is not yet known.

1.3.1 Asthma is a T_H2 lymphocyte disease

T cells are pivotal in orchestrating the inflammatory response through controlled synthesis and release of immunoregulatory cytokines (Robinson et al, 1992; Azzawi et al, 1990). BAL fluid of asthmatic subjects contained increased IL-4 and decreased IFN-γ levels, consistent with a T_H2 humoral response. In addition, IL-3, IL-5, GM-CSF, IL-6, IL-9, IL-10 and IL-13 production has also been observed (Robinson et al, 1992; Kay et al, 1991). Activated T_H2 cells expressed CD40 ligand (CD40L) and CD23 (the low affinity IgE receptor), which binds to CD40 and CR2 on B lymphocytes and results in class switching to IgE production and B cell proliferation. This IgE response may be amplified by mast cells or activated eosinophils within the bronchial mucosa, which also express CD40L and produce IL-4.

T_H2-derived IL-4 (Gauchat et al, 1990) and IL-13 (Punnonen et al, 1994; Punnonen et al, 1997) play a critical role in T_H2 development and IgE isotype switching. IL-4 enhanced IL-3-dependent mast cell line growth. IL-4 and IL-13 upregulate endothelial VCAM-1 and P-selectin expression, providing a mechanism of selective eosinophil infiltration (Moser et al, 1992; Bochner et al, 1995; Patel, 1998; Woltman et al, 2000). IL-5, an eosinophil growth and differentiation factor, also promoted vascular adhesion, chemotaxis, survival and activation of eosinophils (Rothenburg et al, 1989; Lopez et al, 1988). IL-3 (Rothenburg et al, 1988) and GM-CSF (Owen et al, 1987) were not as eosinophil-specific as IL-5. An overview of the bronchial mucosal immune response is shown (Figure 1.5).

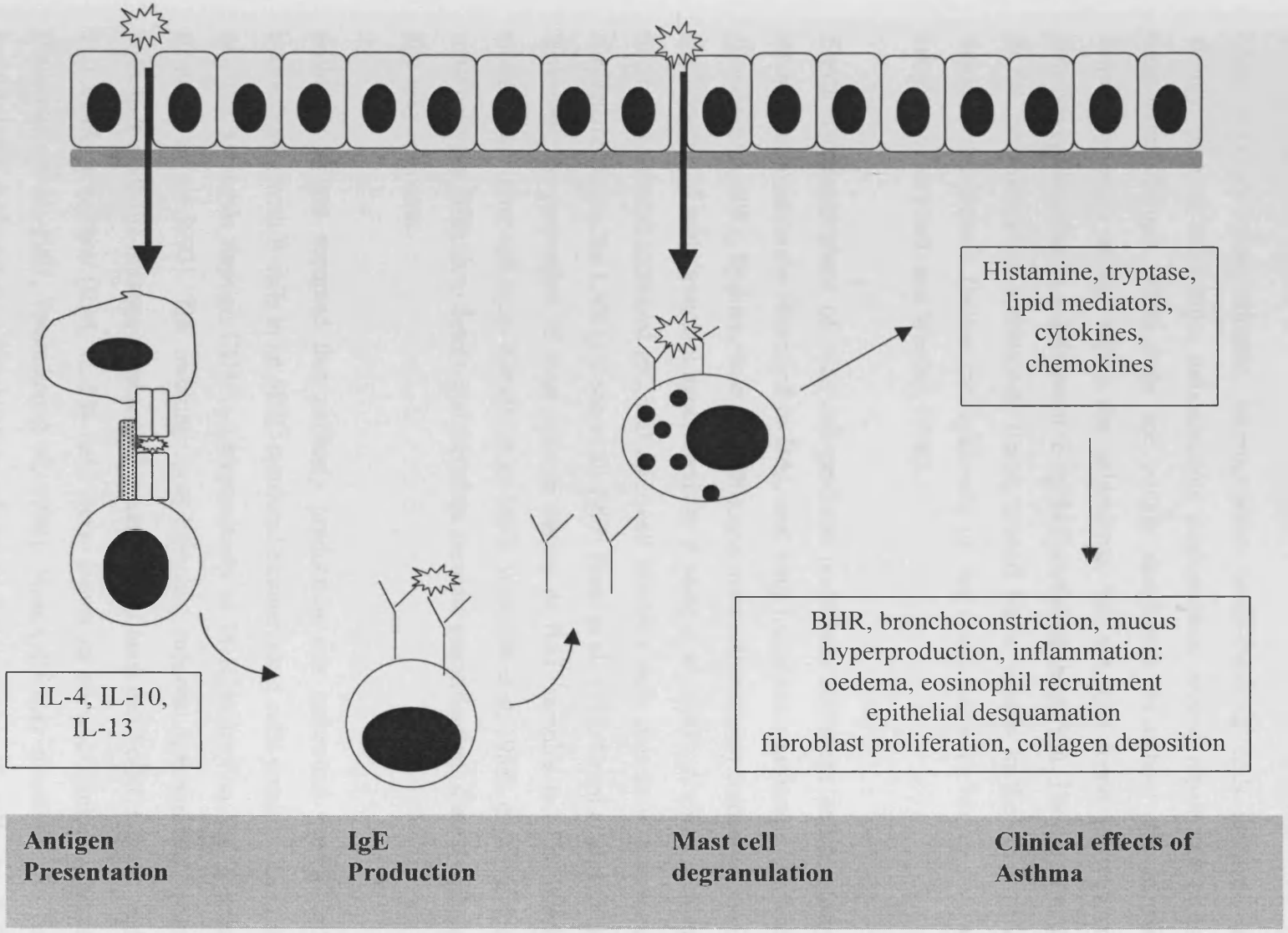


Figure 1.5 Overview of the bronchial mucosal immune response in asthma

1.3.2 Mast cell

1.3.2.1 Role

Mast cells are metachromatic, heterogeneous tissue-dwelling cells involved in allergy, parasite infestation, inflammation, angiogenesis, wound repair and tissue remodelling/fibrosis. Mast cells are widely distributed throughout the human respiratory tract, mostly within the submucosa, but are also found intercalated between epithelial cells and adjacent to epithelium facing the lumen. They are found in the gastrointestinal submucosal tissue, around blood vessels in the connective tissue of the dermis (below the epidermis of the skin), close to nerves and at neoplastic foci (Galli and Wershil, 1996).

Elevated concentrations of mast cell products (especially histamine and tryptase) have been consistently identified in BAL and lung tissue from asthmatic subjects (Broide et al, 1991; Djukanovic et al, 1990), and mast cell mediator content of BAL fluid correlated with bronchial hyperreactivity (Casale et al, 1987). A small number of studies showed increased mucosal mast cell numbers were present in asthmatic subjects or during the LAR (Gibson et al, 1993; Pesci et al, 1993; Crimi et al, 1991). However, the proportion of mast cells in biopsy or BAL samples is often much smaller than other cell types (Ohashi et al, 1992; Wardlaw et al, 1988), degranulated mast cells are difficult to detect, and therefore accurate quantification of mast cells in situ may not occur.

Although it was accepted that antibody production was controlled by T_H cell interaction(s) with B cells in an MHC-restricted manner, mast cells possibly direct B cell IgE synthesis through CD40L, independently of MHC restriction and T cells (Gauchat et al, 1993). The resulting local bronchial mucosal IgE synthesis may potentiate systemic immune responses. Increased nasal mast cell FcεRI, CD40L and T_H2 cytokine release (IL-4, IL-13) have been shown in allergic rhinitis subjects (Pawankar et al, 1997; Pawanker et al, 1998). Mast cells may therefore also be involved in preferential differentiation of naive and memory T cells to T_H2 phenotype (IL-4) prior to development of allergy and bronchial inflammation. Mast cell-derived TNF-α, IL-4 and IL-5 may control inflammatory cell infiltration through

endothelial cell adhesion modulation and activation. Mast cell surface adhesion receptors required for intraepithelial homing and localisation are thought to be similar to those found on T cells (Smith and Weis, 1996).

1.3.2.2 Development and maturation

Mast cells are derived from committed progenitor haemopoietic stem cells in the bone marrow, and enter the circulation as undifferentiated $CD34^{+}$*c-kit*$^{+}$ mast cell precursors (Agis et al, 1993). Circulating mononuclear precursors migrate into tissues where they differentiate under the influence of local tissue-derived microenvironmental signals and growth factors (including stem cell factor (SCF) and T_H2 lymphocyte cytokines such as IL-3) to become morphologically and functionally mature mast cells (Galli, 1990; Kitamura, 1989; Lantz et al, 1998). SCF has been shown to promote mast cell development, proliferation, survival, chemotaxis, promoted *c-kit*-dependent mast cell degranulation and mediator release, and enhanced FcεRI-dependent mediator release (Wershil et al, 1992; Bischoff and Dahinden, 1992; Galli et al, 1994).

1.3.2.3 Mast cell heterogeneity

Mast cell populations were found to vary in morphology, immunohistochemistry, mediator content, functional response to drugs and mode of activation (Pearce, 1986). Mast cell heterogeneity has been observed in rodents (mucosal and connective-tissue) and humans (tryptase- and tryptase/chymase-containing) (Irani et al, 1986). Mast cell heterogeneity was first noted when mucosal mast cells (lung, gastric mucosa, uterus) were found to be predominantly sensitive to formalin fixation, in contrast to connective tissue-type mast cells. Mucosal mast cells were also readily identified morphologically by the characteristic scroll structures present in their granules (Craig et al, 1988).

Environmental signals were found to determine mast cell phenotype. This was demonstrated when wild-type bone marrow-derived cultured mast cells were adoptively transferred into genetically mast cell-deficient mice (Kit^{W}/Kit^{Wv}), which were virtually devoid of mast cells in tissues due to lack of SCF receptor (Nakano et

al, 1985). Mast cells of donor origin assumed the characteristics of specific tissue mast cells when they homed to skin and the peritoneal cavity or the gastric mucosa, and provided convincing evidence that mast cell phenotype was dependent upon and reflected the action of the local microenvironment.

Immunocytochemical studies distinguished mast cell phenotypes by their neutral protease content (MC_T contained only tryptase and MC_{TC} contained both tryptase and chymase) (Irani et al, 1986). These subtypes were suggested as equivalent to rodent mucosal and connective tissue phenotypes respectively. The majority of mast cells present in the epithelium and submucosa of normal and asthmatic airways were tryptase-only MC_T type mast cells (>90%) (Djukanovic et al, 1990; Irani et al, 1989; Bradley et al, 1991; Irani and Schwartz, 1989).

Functional heterogeneity between mast cells from different tissues was demonstrated by *in vitro* studies comparing mast cells recovered from various tissues and organs both in rodents (lung, gut, peritoneal cavity) and humans (lung, skin, tonsil, intestine). Dermal, but not mucosal mast cells responded to nonimmunologic secretagogues such as basic polyamines, neuropeptides and drugs (e.g. morphine) (Lawrence et al, 1987; Church et al, 1982). Mucosal mast cells are therefore activated exclusively by IgE-dependent mechanisms, in contrast to connective tissue cells (such as skin mast cells) which released histamine in response to immunological (antigen and IgE) and non-immunological (substance P, C5a) activation (Dvorak et al, 1991; Lawrence et al, 1987; Columbo et al, 1996).

MC_T are preferentially located at mucosal surfaces and are increased in numbers in allergic disease. Mucosal mast cells appear to be T cell dependent, and are more sensitive to cytokine regulation (e.g. increased mast cell number in parasite infestation or decreased numbers in T-cell deficiency) (Irani et al, 1987).
Migration of mast cells into the epithelium has been observed in allergic rhinitis *in vivo* (Enerbäck et al, 1986). Mucosal mast cells were increased in a seasonal manner within nasal mucosal epithelium of grass pollen allergic subjects, but total mast cell numbers were not altered, suggesting mast cell migration occurred (Enerbäck et al,

1986). In allergic rhinitis, approximately 90% of intraepithelial mast cells were mucosal mast cells (Okuda et al, 1985). In addition, increased intraepithelial MC_T cells were observed in the nasal mucosa of birch pollen allergic subjects, even during asymptomatic periods (Juliusson et al, 1995).

1.3.2.4 Involvement in asthma

The regular occurrence of degranulated intraepithelial mast cells in acute and chronic allergic reactions such as asthma, has suggested their involvement in immunoregulation at mucosal surfaces during host defence (Crimi et al, 1991). Although postmortem and biopsy studies suggested that total mast cell numbers may increase in the airways of severe asthmatic patients, migration and activation of mast cells following exposure to allergen is thought to be more important. Migration from the submucosa into the epithelium may increase mast cell contact with inhaled allergen and other noxious stimuli. BAL mast cells make up 0.04 to 0.6% of the total nucleated cell population. Increased numbers of degranulated mast cells have been observed in the bronchial epithelium of asthmatics and may be identified morphologically by electron microscopy (Cutz et al, 1978; Djukanovic et al, 1990). A significant increase in the percentage (3- to 5-fold) and activation of mast cells has been observed in asthmatics compared with healthy controls, although this was not specific to asthma, and occurred in sarcoidosis and fibrosing alveolitis (Wardlaw et al, 1988; Flint et al, 1985; Tomioka et al, 1984).

1.3.2.5 Mast cell activation

Antigen-specific IgE binds via its Fc portion to the high affinity immunoglobulin superfamily receptor FcεRI on mast cells, basophils and activated eosinophils. The high level of IgE found in allergic diseases may upregulate FcεRI expression, probably due to new receptor synthesis and suppression of cell surface FcεRI internalisation (Furuichi et al, 1985). Increased mast cell sensitivity will lead to activation in response to low concentrations of specific antigen and a marked increase in immunologic release of mediators and cytokines (Yamaguchi et al, 1997).

Elevated histamine levels were observed in bronchoalveolar lavage fluid obtained from patients with active asthma (Wenzel et al, 1988) and BAL mast cells from atopic asthmatic subjects exhibited increased spontaneous and IgE-mediated histamine release (Pearce et al, 1987; Flint et al, 1985), providing further evidence of mast cell activation in asthma. In addition, other mast cell derived mediators, including prostaglandin D_2, leukotriene C_4 and tryptase were also increased (Wenzel et al, 1988; Broide et al, 1991).

Mast cells (and basophils, activated eosinophils, monocytes/macrophages) express high affinity IgE receptors (FcεRI) and are usually stably associated with bound IgE (association constant $10^{10}M^{-1}$). Aggregation of FcεRI when IgE is crosslinked by specific multivalent antigen results in granule exocytosis within seconds, and release of a broad spectrum of biologically active preformed granule contents (histamine, tryptase and other serine esterases), lipid mediators and cytokines.

Triggering of mast cells upon receptor aggregation leads to altered membrane fluidity (phospholipid methylation), transient intracellular cAMP increase, and Ca^{2+} ion influx. Microfilament-mediated granule movement to the cell surface results in membrane fusion and release of mediators by exocytosis (piecemeal degranulation) (Dvorak et al, 1996; Dvorak and Morgan, 1997; Dvorak et al, 1991a), which is distinct from anaphylactic degranulation characterised by classic granule extrusion (Dvorak et al, 1991b).

1.3.2.6 Proinflammatory mediator production

Mast cell mediators may be described as preformed or newly synthesised. Preformed proteoglycans such as heparin and chondroitin sulphate regulate packaging, storage, and activity of other preformed secretory granule mediators. Histamine is a short-lived vasoactive amine that causes an immediate rise in local blood flow and vessel permeability. The neutral serine endopeptidase tryptase is the major mast cell granule protein and is stored as an active tetramer (each 31-35kDa subunit possesses an active site) (Caughey, 1997). The effects of tryptase have been studied in a number of cell types, including epithelium (mitogenesis, ICAM-1 expression and IL-8

induction), fibroblasts (mitogenesis and collagen synthesis), and tracheal smooth muscle cell (mitogenesis) (Cairns and Walls, 1996; Hartmann et al, 1992; Gruber et al, 1997).

Unactivated and IgE-activated purified or cultured human mast cells have been assessed for mediator production. Synthesis and release of lipid mediators (PGD_2, LTC_4, LTD_4, LTE_4, PAF), growth factors (SCF, GM-CSF, bFGF, TGF-β, VEGF), cytokines (IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, TNF-α, MIP-1α) have been found (Galli et al, 1991; Ohkawara et al, 1992; Okayama et al, 1995; Jaffe et al, 1995; Kobayashi et al, 2000). Mucosal mast cells may synthesis a different profile of mediators to connective tissue-derived mast cells. IgE-mediated activation of human lung and gut mast cells resulted in PGD_2 and LTC_4 synthesis (Triggiani et al, 1995; Patella et al, 1995). Skin mast cells produced PGD_2, but little or no LTC_4 (Lawrence et al, 1987). Preformed tumour necrosis factor-α (TNF-α) and VEGF were stored in mast cell granules and may also be newly synthesised upon mast cell activation (Boesiger et al, 1998). TNF-α influenced endothelial cell adhesion molecule expression, thereby promoting inflammatory leukocyte influx (Walsh et al, 1991b).

Mast cells are the primary resident effector cells of allergic inflammation, and are thought to trigger blood leukocyte recruitment. Dermal mast cell histamine, PAF, IL-4 and TNF-α may contribute to endothelial adhesion molecule upregulation (P-selectin, E-selectin, ICAM-1, VCAM-1) (Teixido et al, 1992; Walsh et al, 1991b; Gaboury et al, 1995; Gaboury et al, 1996). TNF-α present in mast cell conditioned medium increased fibroblast ICAM-1 and VCAM-1, and increased T cell adherence (Meng et al, 1995). Histamine present in immunologically activated mast cell supernatant decreased PMA-induced T-cell adhesion to both fibronectin and laminin (Hershkoviz et al, 1994). Immunohistochemical staining showed histamine increased ICAM-1 and HLA-DR expression, and fibronectin production of non-asthmatic, bronchial brushing-derived epithelial cells by increased protein synthesis (Vignola et al, 1993a).

1.3.2.7 Mast cell adhesion

Cell surface receptors may be divided into immune response receptors, receptors involved in growth and differentiation, and receptors involved in cell trafficking and distribution (Gumbiner, 1996; Valent and Bettelheim, 1992). Mast cell differentiation, migration and localisation may be affected by their interactions with extracellular matrix (fibronectin, laminin, vitronectin) and other tissue-resident cells present (e.g. fibroblasts, epithelium) (Hamawy et al, 1994a; Kinashi and Springer, 1994).

Cell adhesion is critical for the differentiation, recruitment and activation of all immune cells (Springer, 1994). Mast cell precursors are released from bone marrow through disengagement of adhesion receptors and travel through bloodstream. Mast cell precursors are stimulated to adhere and transmigrate through endothelium, migrate through connective tissue and matrix and are retained at the mucosal surface. This sequence of events is regulated by adhesive interactions and modulated by local SCF production (Dastych and Metcalfe, 1994). Matrix receptors would therefore be expected to vary depending on stage of mast cell development. Composition of the extracellular matrix may control mast cell proliferation (Bianchine et al, 1992), ability to respond to degranulating agents, and cytokine synthesis (Dastych and Metcalfe, 1994). Cell-cell and cell-extracellular matrix adhesion interactions have been shown to control differentiation, migration, retention, survival, priming and activation of mast cells (Hamawy et al, 1994a). Recent mast cell research in this area has concentrated on integrin expression and regulation by activation (immunological, non-immunological, SCF-*c-kit*, TGF-β) in rodent and human mast cells.

1.3.2.8 Mast cell interaction with extracellular matrix

Mast cells are able to migrate and localise at specific sites within the microenvironment. When stimulated, mast cells will engage specific components of the connective tissue matrix, including laminin, fibronectin and vitronectin, in particular, through integrin receptors on the mast cell surface (Vliagloftis and Metcalfe, 1997; Columbo et al, 1995). Increased adhesion has been shown to usually follow activation of mast cells through FcεRI (Thompson et al, 1990) or exposure to

growth factors, such as *c-kit* ligand (SCF) expressed by stromal cells within tissues (Bianchine et al, 1992; Dastych and Metcalfe, 1994).

The adhesion of mast cells to matrix components is both temperature and calcium dependent and may be increased by transforming growth factor β_1 (Thompson et al, 1990). Mast cell adhesion may be partially inhibitable by protein kinase C or tyrosine kinase inhibitors (Thompson et al, 1990; Kinashi and Springer, 1994). In most instances, mast cell adhesion is RGD-dependent, suggesting integrin involvement.

Bone-marrow-cultured mast cells showed high spontaneous adhesion to vitronectin (Bianchine et al, 1992) or entactin (Bhattacharyya et al, 1999), but only adhered to fibronectin (Dastych et al, 1991) or laminin (Thompson et al, 1989a; Thompson et al, 1990) after exposure to phorbol myrisate acetate or *c-kit* activation (Thompson et al, 1993; Dastych and Metcalfe, 1994; Wyczolkowska et al, 1994). Mast cells also adhered to unpurified extracts of cell cultures which resembled basement membrane (such as matrigel matrix), but did not adhere to collagen (Thompson et al, 1989; Thompson et al, 1993).

Adhesion of IL-3 cultured bone marrow derived mast cells to fibronectin may be promoted by SCF at concentrations in the range found in plasma (Kinashi and Springer, 1994). Metachromatic *c-kit*+, tryptase+ mast cells derived from human fetal liver in the presence of rSCF spontaneously adhered to vitronectin through $\alpha_v\beta_3$ (Shimizu et al, 1995; Bianchine et al, 1992), adhered to fibronectin through $\alpha_5\beta_1$ (Dastych et al, 1994), and adherence to laminin was mediated by at least one α_6-containing integrin. Preincubation with TGF-β_1 significantly increased FcεRI-dependent mast cell adhesion to laminin, with maximal effect with 2ng/ml TGF-β for 24 hours (Thompson et al, 1990).

1.3.2.9 Mast cell interaction with fibroblasts

Cell-cell interactions may affect mast cell phenotype and biological function. Mast cell co-culture with fibroblasts promoted mast cell differentiation to connective tissue-type mast cells (CTMC), assessed by their synthesis of heparin and specific proteases (Bland et al, 1982; Levi-Schaffer et al, 1986). This was dependent upon direct contact of mast cells with the fibroblast monolayer. The *c-kit* ligand (stem cell factor, SCF; mast cell growth factor; steel factor) is the major growth and differentiation factor for murine and human mast cells. Stem cell factor may exist in two forms, either as a soluble molecule or, due to alternative splicing, as an integral cell-surface protein on fibroblasts. *c-kit* (W locus) is a receptor tyrosine kinase present on haematopoietic progenitor cells and mast cells (Adachi et al, 1992). The $S1^d$ mutation in mice removed transmembrane and intracellular portions of SCF, together with 9 amino acids of the extracellular segment. Mast cells adhered to COS cells transfected with normal, but not $S1^d$ cDNA. $S1/S1^d$ mice lacked membrane-asociated SCF, had normal amounts of soluble SCF, but only 1% normal number of tissue mast cells (Flanagan et al, 1991).

The adhesive interaction between mast cells and fibroblasts involved mast cell *c-kit* receptor and fibroblast surface-bound *c-kit* ligand (Adachi et al, 1992; Adachi et al, 1995). Therefore the *c-kit* receptor tyrosine kinase may function as an adhesion receptor on mast cells (Kinashi and Springer, 1994; Kaneko et al, 1991). This adhesive interaction may be important for the differentiation and survival of mast cells in tissues.

Mast cell adherence to fibroblasts regulated secretory function (Galli, 1990). Activation of BMMC induced leukotriene production, but cells co-cultured with fibroblasts, showed a shift to increased prostaglandin D_2 production (Levi-Schaffer et al, 1986; Levi-Schaffer et al, 1987). Co-culture of BMMC with fibroblasts resulted in increased FcγRIII expression and enhanced degranulation upon FcγR activation (Katz et al, 1990; Katz et al, 1992). Coculture with fibroblasts for two weeks was required for compound 48/80 and substance P-activated RBL-2H3 histamine release.

rSCF or other fibroblast-derived soluble factors did not induce RBL-2H3 histamine release (Swieter et al, 1993).

1.3.2.10 Mast cell interaction with bronchial epithelium

In asthma, increased numbers of mast cells may be recruited to the epithelium, but were scarce in BAL fluid and sputum compared to eosinophils (Pesci et al, 1993; Gibson et al, 1993; Wardlaw et al, 1988; Flint et al, 1985). Mast cell retention was thought to involve specific cell adhesion molecule interaction(s).

Changes in murine mast cell integrin expression (α_4, β_1 and β_7) were observed upon culture with different growth factors, suggesting modulation of integrin expression may be controlled in this manner during mast cell differentiation (Gurish et al, 1992; Ducharme and Weis, 1992). Bone marrow-derived mast cells cultured in SCF transcribe α_4, but not β_7 integrins and when cultured in IL-3, induced β_7 but abrogated α_4 transcription (Ducharme and Weis, 1992). The integrin chain α_{M290} (the murine homolog of human α_E) was induced upon murine mast cells following exposure to TGF-β or IgE-mediated crosslinking (Smith et al, 1994). The $\alpha_E\beta_7$ integrin was an unexpected E-cadherin ligand which may have a role in intraepithelial lymphocytes homing and retention within the mucosa (Cepek et al, 1993; Cepek et al, 1994). Mucosal mast cells and lymphocytes have similar tissue distribution. Retention of intraepithelial mast cells was thought to also involve $\alpha_E\beta_7$ interaction with epithelial E-cadherin (Smith and Weis, 1996).

Mast cell adhesion to airway epithelium may be important for development of allergic symptoms since it may lead to higher numbers of intraluminal mast cells, and therefore inflammatory mediators released from activated mast cells may reach high local concentrations. Canine mastocytoma mast cells adhered to cultured tracheal epithelial cells (35±13%), but adhered poorly to types I and IV collagen, or fibronectin (less than 7.5% mean adhesion) (Varsano et al, 1988). In tracheal tissue sections, mast cells adhered preferentially to epithelial cells in surface epithelium or submucosal glands, but not to basal membrane or connective tissue. Adhesion to cultured epithelial cells was characteristic of a subpopulation of mast cells, could

persist for more than 48hr, did not require energy or the presence of divalent cations, and was not mediated by a known family of leukocyte-associated adhesion glycoproteins (CD18) (Varsano et al, 1988).

1.3.2.11 Mast cell adhesion receptors

Adhesion receptor superfamilies include integrin, immunoglobulin, cadherin, selectin, and proteoglycan. Cell adhesion receptors function by formation of multiprotein complexes made up of cell surface adhesion receptor and cytoplasmic plaque intermediate filament or actin-binding proteins, linked extracellularly to ECM or the adjacent cell surface ligand (Gumbiner, 1996). Mast cell surface adhesion molecule expression has been investigated by a number of laboratories, and mast cell integrin/ECM interactions have been studied in most detail. Human mast cell integrin expression has been studied using enzyme-dispersed tissue mast cells dispersed or cultured mast cells. The human mast cell line HMC-1 derived from a patient with mast cell leukaemia (Butterfield et al, 1988) has also been investigated for comparison. HMC-1 cells were representative of immature mast cells, and expressed tryptase, histamine and *c-kit* receptors, but did not express FcεRI, and were therefore not typical of mature tissue-derived mast cells. In general, mast cells expressed integrins of the β_1, β_2, β_3 and β_7 families.

Human lung mast cells (HLMC) and cultured mast cells express a number of other integrins and immunoglobulin superfamily adhesion receptors which may be involved in mast cell interactions with extracellular matrix, submucosa or epithelium. Cell adhesion receptors observed included the β_1 integrins CD49c/CD29 (very late antigen-3, VLA-3; $\alpha_3\beta_1$), CD49d/CD29 (VLA-4; $\alpha_4\beta_1$) and CD49e/CD29 (VLA-5; $\alpha_5\beta_1$), which enable interactions with laminin and fibronectin, and the vitronectin receptor ($\alpha_v\beta_3$; CD51/CD61) (Agis et al, 1996; Valent, 1995; Columbo et al, 1995; Hamawy et al, 1994a; Toru et al, 1997; Sperr et al, 1992). Ligands for these integrins are: CD49d/CD29 (fibronectin and VCAM-1), CD49e/CD29 (fibronectin) and CD51/CD61 (vitronectin, fibronectin, thrombospondin and fibrinogen). Human lung or cultured mast cells did not express VLA-1, -2, or –6 and did not adhere to collagen type I or IV (Sperr et al, 1992; Valent, 1994). Lung mast cells also did not

express selectins or their ligands (L-, E- and P-selectin, sLe^x, PSGL-1), a number of integrins ($\alpha_{IIb}\beta_3$, β_4 and $\alpha_E\beta_7$), CD138 (syndecan-1), CD144 (VE-cadherin), CD146 (MUC18), CD164 (MGC-24) and CD166 (ALCAM) (Wimazal et al, 1999).

Evidence for mast cell CD18 expression was conflicting, since phenotypic differences of mast cells from different organisms and tissues have been observed. The same group has stated human lung mast cells did not express CD11/CD18 (Valent, 1994) or expressed small amounts of CD18 (6-47% expression by flow cytometry (Wimazal et al, 1999). Contrasting results may have been due to differences in mAb or increased sensitivity of equipment. Human skin and uterine mast cells have shown cell surface CD18 expression (Weber et al, 1997; Guo et al, 1992). Human cultured mast cells expressed CD11a, CD11b and CD18 (Toru et al, 1997). Murine peritoneal mast cells expressed CD11b/CD18 (Mac-1), it was present on a subpopulation of cells (30-40%) and may be important for mast cell development, homing, proliferation or survival (Rosenkranz et al, 1998). Increased CD11a was expressed by a more differentiated HMC-1 subclone (5C6) (Weber et al, 1996a).

Non-integrin adhesion molecules expressed by human mast cells included intercellular adhesion molecule (ICAM)-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50), leukosialin (CD43), Pgp-1 (CD44), leukocyte function associated antigen (LFA)-3 and *c-kit* (Agis et al, 1996; Hamawy et al, 1994a; Valent, 1994; Valent, 1995; Toru et al, 1997; Nilsson et al, 1994a; Sperr et al, 1992). Human lung and uterine mast cells expressed low levels of ICAM-1 (CD54) (Valent et al, 1990). HMC-1 mast cells constitutively expressed mRNA and showed surface staining for ICAM-1 (Valent et al, 1991). ICAM-1, α_L (CD11a) and β_2 (CD18) expression were upregulated after IL-4 treatment (Toru et al, 1997). IL-13 increased HMC-1 ICAM-1, but not integrin expression (Nilsson and Nilsson, 1995), unlike increased monocyte CD11b, CD11c, CD18, CD29 and CD49e expression observed upon IL-13 treatment (de Waal Malefyt et al, 1993). HMC-1 treatment with IFN-γ or TNF-α increased ICAM-1, but not CD11a, CD11b or CD11c expression. IFN-γ increased CD18 expression (Weber et al, 1995).

Cultured human foetal liver mast cells (cultured with stem cell factor) expressed CD51/CD61 ($\alpha_v\beta_3$), a receptor for vitronectin (Shimizu et al, 1995). These mast cells also expressed surface CD29 (β_1), CD49d (α_4), and CD49e (α_5) integrins when measured by flow cytometry. Low levels of CD18 (β_2) and CD54 (ICAM-1) were also observed.

Human skin mast cells were reported to express VLA-3, VLA-4 and VLA-5, but not VLA-1, VLA-2 or VLA-6 (Columbo et al, 1995). Adhesion to fibronectin was inhibited by β_1 integrin mAbs to VLA-3, VLA-4 and VLA-5. Adhesion to laminin was only inhibited by antibodies against VLA-3 (Columbo et al, 1995). Human skin mast cells expressed α_6 integrin, when determined by immunohistochemistry. These dermal mast cells were closely associated with microvascular and neural basement membrane laminin in vivo and pericellular laminin complexes in vitro (Walsh et al, 1991c). Upon morphine sulphate or ionophore A23187 induced degranulation *in vitro*, mast cell-laminin complexes dissociated and mast cells were not adherent to laminin substrates. CTMC-laminin interactions may be important determinants of mast cell localisation in tissue compartments (Thompson et al, 1989b).

Human cardiac mast cells expressed ICAM-1, CD44, CD45 and CD9. The β_2 integrin subunits CD11a, CD11b and CD11c were not present (Sperr et al, 1994).

1.3.2.12 Regulation of mast cell adhesion

Basophil activation enhanced adhesion molecule expression and/or increased adhesion, both to other cells (endothelium) and to ECM proteins (Bochner and Sterbinsky, 1991; Bochner et al, 1990; Bochner et al, 1989; Bochner et al, 1988). Integrin adhesion and function may be upregulated through 'inside-out' signalling by growth factor, cytokine or chemokine treatment. Cell activation-induced changes in adherence may involve redistribution of adhesion receptors on the cell surface, enhancement of expression and/or modulation of affinity for ligands. Physiologic stimuli that have resulted in increased mast cell adhesion included FcεRI aggregation (Wyczolkowska et al, 1994, Thompson et al, 1990) or SCF treatment (Dastych and Metcalfe, 1994). Homotypic aggregation of cultured mast cells was enhanced in the

presence of IL-4. IL-4 increased ICAM-1 and LFA-1 expression, (but did not affect VCAM-1 or the VLA family (Toru et al, 1997).

Many integrins, although constitutively expressed on the mast cell surface, did not function unless cells were activated (Bianchine et al, 1992; Thompson et al, 1989b) – with FcεRI cross-linking (Thompson et al, 1993) exposure to phorbol ester PMA (Dastych et al, 1991), the ionophore A23187 (Thompson et al, 1990) or stem cell factor (SCF)(Dastych and Metcalfe, 1994). Integrin (VLA-5)-mediated mast cell adhesion to fibronectin following FcεRI aggregation occurred in the absence of increased surface integrin expression and increased integrin avidity was thought to result from 'inside-out' integrin signalling (Wyczolkowska et al, 1994). Cell adherence and FcεRI aggregation synergistically regulated tyrosine phosphorylation of paxillin, pp105-115, and $pp125^{FAK}$ (Hamawy et al, 1994b). The tyrosine kinase Lyn was required for fibronectin-mediated RBL-2H3 cell motility and filamentous actin assembly (Suzuki et al, 1998).

SCF is a primary growth factor for mast cells and induced BMCMC and MCP5/L mast cells to adhere to fibronectin and laminin (Dastych and Metcalfe, 1994; Kinashi and Springer, 1994). Adhesion was enhanced at 1-2ng/ml, a concentration of SCF found in serum. *c-kit* regulated murine mast cell-ECM adhesion through phosphatidylinositol 3 kinase and phospholipase C-γ1 pathways (Kinashi et al, 1995). Adhesion was extracellular calcium-dependent and inhibited by the tyrosine kinase inhibitor genistein (Dastych and Metcalfe, 1994). Genistein did not inhibit PMA-induced mast cell adhesion.

1.3.2.13 Biological effects of adhesion

(i) Migration

Murine mast cells adhered to and exhibited random migration on laminin, fibronectin or matrigel, and adhesion was enhanced by FcεRI-mediated activation, PMA or A23187 treatment (Thompson et al, 1993). SCF-induced migration of cultured human mast cells or HMC-1 required mast cell adhesion to extracellular matrix protein (especially fibronectin), and mast cells did not undergo chemoattraction through uncoated filters (Nilsson et al, 1994b).

(ii) Degranulation

The mechanism by which cell adhesion regulates secretion is not fully understood. However, cell attachment results in reorganisation of the cytokeleton, redistribution of secretory granules and protein tyrosine phosphorylation, which are all important for degranulation (Hamawy et al, 1992a).

Protein tyrosine phosphorylation is known to be important in integrin-dependent signalling (Warner et al, 1995) and FcεRI signalling, and was required for degranulation (Benhamou and Siraganian, 1992; Hamawy et al, 1993a). Several studies have investigated intracellular protein phosphorylation after adhesion to extracellular matrix proteins. RBL-2H3 adhesion to fibronectin or vitronectin resulted in $p125^{FAK}$ phosphorylation (Hamawy et al, 1993a). Adhesion to fibronectin and FcεRI aggregation resulted in paxillin phosphorylation (Hamawy et al, 1994b). Spontaneous adhesion to vitronectin resulted in phosphorylation of multiple intracellular proteins, including focal adhesion kinase (Bhattacharyya et al, 1999, Hamawy et al, 1993a; Hamawy et al, 1993b). Aggregation of FcεRI-bound IgE by multivalent antigen resulted in tyrosine phosphorylation of the β and γ subunits of the FcεRI, $p72^{syk}$, $p53/56^{lyn}$, $pp60^{c\text{-}src}$, phospholipase C-γ, $p95^{vav}$, paxillin, pp105-115 and $pp125^{FAK}$.

Adherence of RBL-2H3 cells to fibronectin, in the absence of FcεRI aggregation, resulted in tyrosine phosphorylation of paxillin, pp105-115 and $pp125^{FAK}$ (Hamawy et al, 1993a; Hamawy et al, 1993b). The aggregation of FcεRI in fibronectin-

adherent cells markedly enhanced the tyrosine phosphorylation of these proteins (in contrast to low tyrosine phosphorylation levels in non-adherent cells), indicating synergistic regulatory pathways of protein tyrosine phosphorylation by cell adherence and FcεRI aggregation. Adherence to fibronectin modulated the extent of cell degranulation. IgE-dependent degranulation was enhanced by adhesion of the rat basophilic-leukaemia cell line (RBL-2H3) to immobilised fibronectin (Yasuda et al, 1995). Binding of RBL-2H3 cells to immobilised fibronectin resulted in enhanced FcεRI-and A23187-mediated histamine release compared to non-adherent cells (Hamawy et al, 1992b). Adherence alone did not result in secretion. The mechanism involved adhesion receptor aggregation since immobilised fibronectin was required. Signalling pathways after ligand binding to HLMC CD49/CD29 may be different to rodent mast cells. The tyrosine kinase inhibitor genistein inhibited anti-IgE-induced histamine release of HLMC (by 18+/-6% at 10μM) (Lavens et al, 1992). Histamine release did not occur after CD29 or CD49d crosslinking of HLMC, in agreement with RBL-2H3 cells (Warner et al, 1995; Lavens et al, 1996). However, IgE-dependent HLMC degranulation was significantly reduced after 60 minutes pre-clustering of CD29 or CD49d (Lavens et al, 1996).

Mast cell adherence to fibroblasts through *c-kit* also resulted in changes in response to activation. Immunologic or A23187-induced activation of fibroblast-adherent bone marrow cultured mast cells (3 weeks) caused a 2-3-fold increase in histamine, LTB_4 and PGD_2 release (Levi Schaffer et al, 1987). Mast cell activation in the presence of epithelium has not been studied.

(iii) Cell proliferation and differentiation

Adhesion to fibroblasts induced proliferation and differentiation of IL-3-cultured bone marrow cultured mast cells, measured by heparin and histamine production (Levi-Schaffer et al, 1986). Spontaneous adhesion of mast cells to vitronectin (via $\alpha_v\beta_3$), but not entactin, in the presence of IL-3 enhanced mast cell proliferation rate over at least 72 hours (Bianchine et al, 1992).

1.3.3 Eosinophil

1.3.3.1 Development and maturation

Eosinophils develop from $CD34^+$ pluripotent progenitor cells, which proliferate and differentiate within the bone marrow in response to IL-3, IL-5 and GM-CSF. Terminal differentiation of committed precursors occurs within the bone marrow under the influence of IL-5. Bone marrow-derived mature eosinophils are released into the peripheral blood, transmigrate between lung microvascular endothelial cells, and are recruited to the respiratory mucosa under the influence of chemotactic signals.

Selective eosinophil infiltration is observed in both mild atopic and non-atopic asthma (Beasley et al, 1989) and large numbers of bronchial eosinophils are seen in postmortem examination of asthma-related death (Dunnill, 1960; Cutz et al, 1978). Eosinophils are a consistent feature of the airway mucosa and BAL fluid during the late-phase asthmatic response (LAR) to inhaled allergens (Durham et al, 1989; Frigas and Gleich, 1986) and are usually activated.

Increased proportions of activated (hypodense) eosinophils in the peripheral blood (Fukuda et al, 1985) and increased numbers of $EG2^+$ eosinophils in the bronchial mucosa of asthmatics have been observed (Azzawi et al, 1990; Bradley et al, 1991). Although EG2 was initially thought to relate to activated, degranulating eosinophils, it has more recently been found in both resting and activated cells by immunocytochemistry (Jahnsen et al, 1994). Examination of repeated bronchial biopsy specimens has demonstrated that, after allergen challenge, activated eosinophils migrate from the submucosa through the basement membrane into the epithelium (Aalbers et al, 1993). Their presence and activation state correlates well with the activity and severity of asthma (Wardlaw et al, 1988; Bousquet et al, 1990).

In chronic allergic reactions such as asthma, the persistence of eosinophils is thought to be responsible for the observed tissue damage. Deterioration of asthma symptoms has often been associated with increased eosinophil infiltration into the airway mucosa (Laitinen et al, 1991; Tomioka et al, 1984; Flint et al, 1985).

1.3.3.2 Eosinophil activation

Antibody-dependent eosinophil-mediated damage to Schistosomula *of Schistosoma mansoni in vitro* occurs by degranulation of highly basic eosinophil proteins onto its surface (Butterworth et al, 1979). Eosinophils may target and damage the bronchial mucosa in asthma, and cause epithelial fragility and desquamation through the action of histotoxic granule proteins (major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN)) (Frigas and Gleich, 1986). Respiratory epithelial damage during fatal asthma attacks may be particularly associated with major basic protein (MBP) release. The amount of released MBP correlates with eosinophil activity rather than number (Wardlaw et al, 1988). Immunofluorescent staining of specimens from fatal asthma show that MBP is deposited at sites of bronchial epithelial damage, in mucus plugs and beneath the epithelium (Filley et al, 1982). MBP causes guinea pig tracheal epithelial cell ciliostasis at low concentrations (10μg/ml) and extensive epithelial damage at higher concentrations (50μg/ml). In addition eosinophil granule proteins (MBP and ECP) upregulate cultured human nasal epithelial cell ICAM-1 (Altman et al, 1993), which may contribute to cell damage.

In addition, because of their capacity to release significant amounts of pro-inflammatory mediators such as PAF (Lee et al, 1984), leukotrienes (especially LTC_4) (Weller et al, 1983; Shaw et al, 1985; Jörg et al, 1982) and cytokines (e.g. GM-CSF, TGF-β) (Lee et al, 1984; Moqbel et al, 1991), eosinophil activation may be central to the development and extent of tissue damage and fibrosis in asthma.

1.3.3.3 Eosinophil adhesion to endothelium

In asthma selective rolling, adhesion and transmigration of eosinophils expressing VLA-4 ($\alpha_4\beta_1$) occurs (Sriramarao et al, 1994; Kitayama et al, 1997). Firm adhesion independent of shear forces, is integrin-mediated (LFA-1, Mac-1 and VLA-4), and depends partly on the activation state of the cell (Bochner et al, 1994; Schleimer et al, 1992). The principal integrin counterligands are ICAM-1 and VCAM-1 respectively (Springer, 1994). Extravasation through endothelial intercellular junctions involves PECAM-1 (CD31). Integrin molecules (particularly β_1 integrins)

mediate cell-matrix adhesion, priming and survival (Anwar et al, 1993; Anwar et al, 1994). In addition to β_1 and β_2 integrins, eosinophils express $\alpha_4\beta_7$, a counterligand for MAdCAM-1 (Walsh et al, 1996).

1.3.3.4 Eosinophil adhesion to extracellular matrix

Calcium ionophore-stimulated LTC_4 generation was enhanced in eosinophils adherent to fibronectin compared with BSA coated surfaces (Anwar et al, 1994). Increased fMLP-induced eosinophil degranulation upon VLA-4-mediated interaction with fibronectin occurred (Neeley et al, 1994). In contrast, adherence to fibronectin and laminin inhibited eosinophil-derived neurotoxin (EDN) release stimulated by PAF, C5a and IL-5 but not by PMA (Kita et al, 1996). When eosinophils were cultured for several days on plasma fibronectin, their increased survival (compared to those cultured on BSA or plastic) was thought to be caused by autocrine generation of GM-CSF and IL-3. Eosinophil survival on matrix proteins was inhibited by glucocorticoids possibly by the inhibition of autocrine GM-CSF synthesis (Walsh et al, 1997). Cytokine release and survival was inhibited by anti-VLA-4 mAb (Anwar et al, 1993). Tissue fibronectin was more effective than plasma fibronectin at supporting eosinophil survival, since tissue fibronectin contained increased alternatively spliced IIICS regions that binds to VLA-4. Activated eosinophils can also adhere to fibronectin through $\alpha_4\beta_7$ (Walsh et al, 1996). Eosinophils can adhere to laminin through $\alpha_6\beta_1$ (Georas et al, 1993) and laminin also promotes eosinophil survival (Tourkin et al, 1993).

1.3.3.5 Eosinophil adhesion to epithelium

Mac-1 (CD11b/CD18) is upregulated on activated eosinophils (Neeley et al, 1993). Sputum eosinophils show increased expression of CD11b, compared with eosinophils in blood suggesting upregulation has occurred during the passage from blood to airway (Hansel and Walker, 1992). In addition, eosinophil expression and/or activation of CD11b/CD18 may be increased by inflammatory mediator (C5a, f-MLP, PAF), chemokine (eotaxin and RANTES), or non-physiological (phorbol myristate acetate) treatment (Alam et al, 1993; Weber et al, 1996b). Eosinophil degranulation induced either by immobilised IgG, recombinant GM-CSF or PAF

occurred through a CD11/CD18-dependent mechanism (Kaneko et al, 1995; Horie and Kita, 1994). Phorbol ester-induced degranulation in adherent human eosinophils was also CD11/CD18 dependent (Egesten et al, 1993). These studies illustrate that β_2 (CD18)-mediated adhesion and signalling may be important in mediating degranulation of activated eosinophils.

Eosinophils express LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) which are counterreceptors for ICAM-1 (Walsh et al, 1990). The most likely adhesive interaction of activated eosinophils with inflamed bronchial epithelium was thought to be eosinophil CD18 integrin(s) and epithelial ICAM-1, since ICAM-1 is an important ligand for the leukocyte β_2 integrins and is upregulated in asthma (Look et al, 1992; Altman et al, 1993). Support for a role of both ICAM-1 and the CD18 integrins in asthma was shown in *in vivo* primate studies that showed ICAM-1 expression was upregulated on inflamed airway epithelium (Wegner et al, 1990) and blocking antibodies against CD18 and/or ICAM-1 inhibited airway eosinophilia and bronchial hyperresponsiveness in animal models of asthma (Richards, 1996; Wegner et al, 1990; Milne and Piper, 1994). Adhesion may therefore be involved in eosinophil-mediated epithelial damage in asthma.

1.3.4 Bronchial epithelium

The conducting airways of the lungs are lined by pseudostratified, ciliated, columnar epithelium. Intraepithelial lymphocytes are normally observed, and increased numbers of activated intraepithelial T lymphocytes, mast cells and eosinophils may be observed in asthmatic subjects. Ciliated cells propel tracheobronchial secretions toward the pharynx and may also be involved in transepithelial electrolyte transport. Goblet and serous cells synthesise mucin. Basal cells are involved in the attachment of superficial cells to the basement membrane and may act as precursors to other airway epithelial cell types. Secretory cells may also act as progenitor cells for epithelial regeneration since they have the capacity to divide and differentiate. Ciliated cells are end-stage cells and are incapable of cell division (Campbell, 1997).

1.3.4.1 Role of the normal bronchial epithelium

Bronchial epithelial function has traditionally been viewed as a physical protective barrier against exogenous inhaled allergens and irritants, with the additional mechanical function of propelling tracheobronchial secretions toward the pharynx. It is now clear that the bronchial epithelium also initiates and augments pulmonary host defence mechanisms during inflammation or injury through proinflammatory mediator synthesis and release. Inflammatory cell infiltration and activation, and structural cell proliferation and fibrosis may be controlled by epithelial cell mediator release (Djukanovic et al, 1990; Corrigan and Kay, 1991). Activated epithelial cells in asthma may display increased mediator production when activated by IgE (low affinity IgE receptor FcεRII, CD23) (Campbell, 1997), viruses, pollutants and oxidants (such as NO_2, SO_2 and ozone), changes in osmolarity, or proinflammatory cell mediators such as mast cell-derived histamine (Vignola et al, 1993b).

1.3.4.2 Products of inflamed epithelium

Human bronchial epithelial cells constitutively and inducibly synthesise and release IL-1β and TNFα, which increases cell adhesion molecule expression and IL-6, IL-8 and GM-CSF expression (Abdelaziz et al, 1995; Cromwell et al, 1992). Airway epithelium has also been shown to produce IL-1, IL-6 (Mattoli et al, 1991; Marini et al, 1992), IL-8 (Nakamura et al, 1991; Marini et al, 1992; Cromwell et al, 1992),

GM-CSF (Sousa et al, 1993; Marini et al, 1992; Cromwell et al, 1992), RANTES (Wang et al, 1996a) TGF-β and SCF (Sacco et al, 1992; Magnan et al, 1994; Wen et al, 1996). Cultured and inflamed human bronchial epithelial cells promote eosinophil survival and activation by GM-CSF production (Cox et al, 1992). Inflamed airway epithelium has also been shown to express G-CSF (Ohtoshi et al, 1991). In asthmatic subjects, the bronchial epithelium demonstrates increased expression of GM-CSF and MIP-1α compared to non-atopic non-asthmatic epithelium (Sousa et al, 1993; Sousa et al, 1994). IL-8 and RANTES are produced by inflamed pulmonary epithelium (Bédard et al, 1993; Stellato et al, 1995; Abdelaziz et al, 1995; Cox et al, 1992).

1.3.4.3 Epithelial integrity and adhesion molecule expression

Bronchial hyperresponsiveness (BHR) is a prominent feature of asthma and is commonly associated with epithelial injury and fragility. A marked shedding of areas of airway columnar epithelial cells (desquamation), with basal cells remaining adherent has been observed in the pathology of asthma (Dunnill, 1960). Loss of columnar epithelial cells may cause increased BHR due to physical and metabolic diffusion barrier loss or increased nerve ending exposure. The sloughed epithelial cells retain tight junctions and have preserved ciliary function (Djukanovic et al, 1990). Epithelial cells from asthmatics are activated (increased cell surface markers and proinflammatory mediator production) (Vignola et al, 1993a).

Normal and inflammatory function and integrity of the epithelium is dependent upon an organised distribution of cell surface adhesion molecules (Montefort et al, 1993; Albelda, 1991). Basolateral adhesion molecules are involved in epithelial cell-matrix and epithelial cell-cell adhesion (e.g. integrins and E-cadherin). Luminal adhesion molecules may be involved in leukocyte retention during inflammation (e.g. ICAM-1). The epithelium may also participate in antigen presentation through its increased expression of the MHC class II antigen HLA-DR in asthmatic subjects (Vignola et al, 1993a; Vignola et al, 1994).

(i) Integrins

Bronchial epithelial cells express a number of well-characterised integrins involved in maintaining normal epithelial structure by direct adhesion to extracellular matrix and via hemidesmosomes, which may be up-regulated by TGF-β (Sheppard, 1993; Wang et al, 1996b). These include the collagen-laminin α_2, α_3, α_6, β_1, the fibronectin α_v and the hemidesmosome $\alpha_6\beta_4$ integrin receptors (Albelda, 1991). Columnar epithelial cells do not form hemidesmosomes with basal lamina. Epithelial cell β_1 integrins were not altered in mild asthma (Manolitsas et al, 1994).

(ii) E-cadherin

Epithelial cells also express the calcium-dependent (1-2mM) homotypic and homophilic adhesion molecule E-cadherin which mediates binding between the lateral membranes of adjacent cells and involves the cytoskeleton and catenins (Takeichi, 1990). Epithelial E-cadherin is also able to specifically interact with intraepithelial T lymphocyte $\alpha_E\beta_7$ (CD103) (Shimoyama et al, 1989; Cepek et al, 1994; Higgins et al, 1998). These T cells are located at mucosal surfaces (Cerf-Bensussan et al, 1987) and this interaction has been demonstrated during murine T cell adhesion to breast and intestine epithelium (Cepek et al, 1993).

(iii) Other epithelial adhesion molecules

In contrast to endothelial cells, neither P- nor E-selectin are expressed by airway epithelial cells under inflamed or non-inflamed conditions. Primary cultures of airway epithelial cells have been shown to express ICAM-1, CD44 and LFA-3 (Tosi et al, 1992a; Bloemen et al, 1993, Bloemen et al, 1997). In addition, cytokine stimulation of bronchial epithelium may induce VCAM-1 expression (Atsuta et al, 1997), and CD44 expression is increased at sites of regeneration of bronchial epithelium in asthma, although it is also found on normal epithelium (Peroni et al, 1996; Lackie et al, 1997).

ICAM-1

ICAM-1 is a member of the immunoglobulin gene superfamily, and is a counter-ligand for LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18). In recent years, since

its initial study in primates, much attention has been focused on bronchial epithelial ICAM-1 and its upregulation during asthmatic inflammation (Wegner et al, 1990). Immunohistochemistry has been used to investigate epithelial ICAM-1 expression upon allergen challenge or allergic inflammation in atopic asthmatic nasal and endobronchial mucosal biopsies. Increased bronchial epithelial ICAM-1 in asthma has been observed by a number of groups (Bentley et al, 1993; Vignola et al, 1993a; Manolitsas et al, 1994). Expression was observed generally on basal epithelium adjacent to the basement membrane, and the degree of expression may correlate with clinical severity. Since ICAM-1 is a receptor for the major group of rhinoviruses, asthmatic epithelium may be more vulnerable to viral infection. Cultured human bronchial and tracheal epithelial cells express ICAM-1, which is increased upon IFN-γ, TNF-α or IL-1β treatment (Look et al, 1992; Tosi et al, 1992a; Tosi et al, 1992b; Jagels et al, 1999; Bloemen et al, 1993; Godding et al, 1995). Significant basal expression of ICAM has been observed on the bronchial epithelial cell line BEAS-2B, and is also increased further by IFN-γ, TNF-α and IL-1β (Look et al, 1992). Since increased concentrations of IL-1β, TNF-α and IFN-γ have been found in asthmatic BAL fluid and culture supernatant from BAL leukocytes, these cytokines may act locally *in vivo* to cause epithelial stimulation and ICAM-1 upregulation (Broide et al, 1992; Barnes, 1994; Cembrzynska-Nowak et al, 1993). IFN-γ has been shown to potentiate TNF-α- or IL-1-induced ICAM-1 expression on airway epithelial cells and may be involved in enhancing epithelial ICAM-1 expression in bronchial mucosa during viral exacerbation of asthma (Bloemen et al, 1993; Vignola et al, 1993b; Manolitsas et al, 1994).

VCAM-1

VCAM is the counterligand for VLA-4 ($\alpha_4\beta_1$) and $\alpha_4\beta_7$ (Bochner and Schleimer, 1994). There is conflicting evidence for VCAM-1 expression by human bronchial epithelial cells, but expression has been newly induced on the epithelial cell line BEAS-2B after stimulation with a combination of cytokines (Bloemen et al, 1997; Godding et al, 1995; Atsuta et al, 1997). Recently bronchial epithelial cells were shown to express mRNA and protein for VCAM-1, and an antibody against VCAM-1, but not ICAM-1, inhibited eosinophil adhesion to TNFα-stimulated BEAS-2B

(Atsuta et al, 1997). This was in contrast with a previous report that did not observe VCAM-1 expression on BEAS-2B cells, and showed that VCAM-1 was not present on resting human bronchial epithelial cells and was not upregulated after stimulation with IFN-γ, IL-1, IL-4 or TNF-α (Bloemen et al, 1993). This contrasts with the effects of these cytokines on endothelial cells, which express both ICAM-1 and VCAM-1, and which respond strongly to IL-1, TNF-α and IL-4, but not IFN-γ (Look et al, 1992; Dustin et al, 1986).

1.4 Summary and Aims

Cell adhesion molecule expression, function and interaction in normal development and disease is currently a rapidly expanding subject. However, leukocyte-epithelial interaction has not been investigated in detail (compared to leukocyte-endothelial adhesion). Asthma is a localised inflammatory condition of the bronchial mucosa, and mast cells and eosinophils perform their immunomodulatory function at this interface with the external environment. Therefore the adhesion mechanism(s) of mast cells and eosinophils to bronchial epithelium (which may be critical in subsequent cell function) was investigated.

The main aims of this project were to:

1. Assess sources and suitability of human primary epithelial cells for use in adhesion assays and establish accurate *in vitro* adhesion assays for human lung mast cell and eosinophil adhesion to bronchial epithelium
2. Assess human lung mast cell adhesion to bronchial epithelium and the effect of activation on baseline adhesion
3. Investigate whether known families of cell adhesion molecules were involved in the adhesion mechanism(s) of mast cell and eosinophil adhesion to bronchial epithelium
4. Investigate the effect of adhesion to bronchial epithelium on human lung mast cell function
5. Compare eosinophil adhesion to primary, alveolar and bronchial epithelial cells; Assess eosinophil adhesion to bronchial epithelium and the effect of cell activation on baseline adhesion; Investigate whether known cell adhesion molecules were involved in eosinophil adhesion to bronchial epithelium

The results obtained may contribute to current knowledge of the mechanism of inflammatory cell-mediated epithelial damage during asthma pathogenesis.

Chapter 2: Assessment of epithelial suitability

2.1 Introduction

The human bronchial epithelium is described as pseudostratified, columnar and ciliated. The mucociliary escalator removes entrapped particles and aeroallergens when they adhere to the surface mucus layer. Epithelia display polarity in terms of structure and function, and the apical surface is separated from basolateral surfaces at tight junctions. Routinely cultured epithelial cells may not reflect *in vivo* phenotype, and therefore primary human epithelial cells from a number of sources were compared for their suitability in adhesion assays. Primary epithelial cells were investigated first since they would be expected to retain similar features to those found *in vivo* (differentiation, normal and proinflammatory mediator production, cell adhesion molecule expression). Primary bronchial epithelial cells from explants and commercially available epithelial cells were cultured and compared with cell lines (alveolar A549 and bronchial BEAS-2B). Cells were grown on different extracellular matrix proteins and on transwells to attempt to retain differentiation. Culture of primary epithelial cells from bronchoalveolar lavage was also attempted, for comparison.

During airway inflammation, increased epithelial ICAM-1 expression may be caused by IFN-γ, TNF-α and/or IL-1β (Stark et al, 1992; Bloemen et al, 1993; Godding et al, 1995; Look et al, 1992). These cytokines have been found *in vivo* in asthmatic BAL fluid and are released by cultured BAL leukocytes (Broide et al, 1992; Barnes, 1994; Cembrzynska-Nowak et al, 1993). IFN-γ enhanced TNF-α or IFN-γ-induced airway epithelial ICAM-1 expression (Bloemen et al, 1993), and may contribute to exacerbation of asthma during viral infection. ICAM-1 expression of basal and cytokine stimulated primary and cell lines was also investigated.

2.2 Materials

Small airway growth medium (SAGM), trypsin, trypsin neutralising solution (TNS) and Hanks' balanced salt solution (HBSS) were purchased from Biowhittaker (Wokingham, UK) or TCS Biologicals; Dulbecco's modified Eeagle medium (DMEM) (with L-Glutamine and D-glucose, without sodium pyruvate, DMEM/F12 medium, foetal calf serum (FCS) and trypsin/EDTA solution were obtained from Life Technologies (Paisley, Scotland); matrigel matrix and human extracellular matrix were bought from Becton Dickinson (Bedford, MA, USA); human plasma fibronectin (1mg/ml, 0.05M TBS pH7.5), collagen IV, laminin, phosphate buffered saline (PBS, 10x concentration), cell dissociation fluid, protease XIV, trypan blue, bovine serum albumine (BSA), formaldehyde, propidium iodide (PI), citric acid, *o*-phenylenediamine (OPD) dihydrochloride, Na_2HPO_4, H_2O_2, calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) were purchased from Sigma (Poole, UK); tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interferon-γ (IFN-γ) were purchased from R&D (Abingdon, UK); sulphuric acid (H_2SO_4) was purchased from Fisher Scientific (Loughborough, UK).

Antibodies

MoIgG, rabbit anti-mouse peroxidase conjugated immunoglobulins, swine anti-rabbit peroxidase conjugated immunoglobulins and FITC-conjugated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulins were purchased from Dako (Ely, Cambridge, UK); BBIG-I1 (ICAM-1), BBIG-V1 (VCAM-1) and HECD-1 (E-cadherin) were purchased from R&D (Abingdon, UK); annexin V-FITC kit was obtained from Becton Dickinson (Bedford, MA, USA). Mouse primary antibody IgG isotype control, IA4 (smooth muscle actin, IgG_{2a}), CD34, AE1/AE3 (cytokeratin, IgG_1), V9 (vimentin, IgG_1) were bought from Zymed laboratories (San Francisco, CA, USA).

Equipment

Conical plastic tubes (50ml and 15 ml), eppendorfs, sterile scalpels and aluminium foil were obtained from Leicester university bulk store; tissue culture flasks ($25cm^2$ and $75cm^2$) and transwells (3μm) were purchased from Costar (High Wycombe, UK). Gelaire, ICN (Thame, UK) class two laminar flow tissue culture cabinets, Heraeus

(Brentwood, UK) Megafuge 1.0R bench top centrifuge, Becton Dickenson (Oxford, UK) FACScan flow cytometer and plate reader spectrophotometer were used.

Epithelial cells

The A549 and BEAS-2B epithelial cell lines were purchased from the European Collection of Animal Cell Cultures (ECACC, Porton Down, Wiltshire, UK); primary normal human bronchial epithelial cells (NHBE) were purchased from TCS Biologicals Limited (Buckingham, UK).

Methods

(A) Primary Epithelium

2.2.1 Primary epithelial culture

Human bronchial tissue was obtained from 6 patients (5 male, 1 female) undergoing surgery for lung cancer (mean age 61.6±10.3 years). Bronchial rings from the 3rd to the 5th generation bronchi were cut into 5mm lengths and placed in DMEM/F12 (with 2% FCS, penicillin and streptomycin) or SAGM (with gentamycin) within 2 hours of lung or lobe resection. The antibiotics were added throughout culture, since fresh tissue was stored in a non-sterile environment after excision from the patient. All procedures were carried out in a class II laminar flow cabinet using aseptic technique and pre-warmed medium (37^{o}C).

2.2.2 Primary epithelial culture from enzyme-digested bronchial sections

Culture of primary epithelium (from bronchial explants) was compared using the culture media DMEM, DMEM/F12 or SAGM. Epithelium was disected from the bronchus, and/or digested with protease XIV, washed and grown on matrix-coated 25cm^2 flasks and/or transwells. Immunostaining was used to verify the epithelial status of cells grown from human bronchial explant cultures.

2.2.3 Culture of epithelial cells on matrix-coated transwells

Epithelial cells grow *in vivo* at an air-liquid interface. Cell culture systems containing microporous membranes (especially those pre-coated with extracellular matrix) promote epithelial morphological and functional differentiation *in vitro*. Extracellular matrix (ECM) coated transwells were thought to provide a more physiological

environment for epithelial growth compared to ECM-coated tissue culture flasks. ECM has been shown to promote and regulate growth, differentiation and function of cells *in vitro*.

Transwells are polycarbonate microporous cell culture inserts for tissue culture plates. They were used in 24 well plate size (6.5mm membrane diameter, 0.33cm^2 area) and at 3.0μm pore size. Inserts were coated with extracellular matrix under aseptic conditions and were manipulated using sterile forceps. Transwells were coated with human plasma fibronectin (20μg/cm^2), collagen IV (50μg/cm^2), laminin (50μg/cm^2), human extracellular matrix (50μg/cm^2) and matrigel matrix (100μg/cm^2) as recommended by Becton Dickenson. The optimal seeding density of epithelial cells was investigated prior to these experiments. Epithelial medium (0.9ml/well) was added to appropriate wells of the 24 well plate and placed in the 37°C incubator in 5% CO_2 to allow equilibration. Cells were added in 0.35ml at 3x10^5/cm^2 and were fed every two days by removing media from above and below membrane. Cells were visualised directly by light microscopy.

2.2.4 Primary epithelial culture from explant outgrowth

Bronchial epithelium was dissected into 1-3mm^2 pieces and placed in 25cm^2 tissue culture flasks containing 3ml SAGM medium. Cells were initially fed by replacing with 1ml fresh medium every 3-5 days (depending on cell growth rate). Once epithelial colonies were established, explants were removed and cells were fed by a total replacement of medium (5ml) every 2 days.

2.2.5 Subculture of explant outgrowth epithelial cells

Epithelial cells grown from explants were subcultured using trypsin, dispase and non-enzymic methods (cell dissociation fluid and physical scraping). Cells were seeded (at the same density as recommended for NHBE cells) in SAGM. The method used for enzymatic removal of epithelial cells was identical to cell line subculture. Cell dissociation fluid was non-enzymatic (EDTA solution, 0.02% w/v) and was added after washing cells twice with PBS. Cells were placed in the incubator and viewed at 2 minute intervals until detachment of >85% of cells. Cells detached within 10 minutes and flasks were gently tapped to ensure total removal of cells into solution. Cells were centrifuged, counted and placed in fresh fibronectin-coated tissue culture

flasks (as described later for cell line subculture). Physical removal of cells was attempted using plastic cell scrapers from areas of epithelial cell growth. Cells were directly transferred into fresh tissue culture flasks containing 3ml SAGM medium.

2.2.6 Confirmation of epithelial phenotype

Bronchial explants were placed in 1ml/well SAGM medium in 24-well tissue culture plates containing fibronectin-coated sterile glass coverslips. Medium was replaced every 2 days. Epithelial cells were grown to confluence (9-14 days) and were washed gently three times with PBS prior to indirect immunofluorescence staining as follows: a 1:1 mixture of acetone:methanol was added (50µl/well) for 2 minutes at room temperature, cells were air-dried and 100µl PBS-diluted primary mAb was added for 2 hours at 4°C, with gentle rocking. Excess mAb was removed and monolayers were gently washed three times with PBS. PBS-diluted FITC-conjugated secondary Ab was added (100µl/well) and incubated with gentle rocking for 60 minutes at room temperature. Excess unbound Ab was removed and monolayers were washed three times with PBS. Coverslips were mounted in glycerol (100mg para-phenylenediamine in 10ml PBS, added to 90ml glycerol, and adjusted with NaOH to pH 8.0), stored in the dark and analysed within 2 days using a fluorescence microscope.

2.2.7 Primary epithelial culture from BAL fluid

Epithelial culture was attempted using three BAL fluid samples. Bronchoalveolar lavage was carried out by Professor Wardlaw. Cell viability and number was immediately assessed using trypan blue and Kimura stain. Percentage epithelial cells was also calculated, cells were washed into SAGM medium and placed in ECM-precoated tissue culture flasks or transwells at $1x10^6$/ml. Cells were cultured on uncoated, BSA, laminin, collagen, human plasma fibronectin, human extracellular matrix and matrigel matrix. Medium was replaced, and cell viability and growth were assessed at 2 day intervals for at least 2 weeks.

(B) Epithelial cell lines

Manipulations were carried out in a class two laminar flow tissue culture cabinet under sterile conditions, with pre-warmed reagents. Epithelial cells were cultured in human plasma fibronectin-coated $25cm^2$ and $75cm^2$ flasks and placed in an atmosphere of 5% CO_2 at 37°C in a humidified incubator. Mycoplasma testing of actively growing cells was carried out at 12 month intervals (at Leicester university). No cell lines used in this project tested positive for mycoplasma.

Alveolar epithelial cells

Alveolar epithelial cells (A549 human lung carcinoma cell line) were cultured using Dulbecco's Modified Eagle Medium (DMEM) (with L-Glutamine and D-glucose, without sodium pyruvate) with added 10% FCS. The A549 cell line was originally derived from type II alveolar cells from a Caucasian male, aged 58 years. Confluent epithelial cell monolayers had a typical cobblestone appearance and cytoplasm inclusion bodies resulted in a granular appearance (ECACC catalogue, 1995).

Bronchial epithelial cells

Bronchial (BEAS-2B, NHBE) epithelial cells were grown in small airway growth medium (SAGM). Serum-free SAGM was based on LHC-9 medium optimised for maintenance of epithelial cell lines or establishing primary bronchial epithelial cells. Medium was used within 2 months of reconstitution. Each 500ml bottle of SAGM basal medium was supplemented with the following provided growth and differentiation-promoting factors: human recombinant epidermal growth factor (0.5ng/ml), insulin (5μg/ml), hydrocortisone (0.5μg/ml), transferrin (10μg/ml), epinephrine (0.5μg/ml), triiodothyronine (6.5ng/ml), bovine pituitary extract (BPE) (concentration not stated), retinoic acid (0.1ng/ml), gentamycin (50μg/ml) and amphotericin (50ng/ml).

BEAS-2B

The human bronchial epithelial SV-40 virus transformed cell line BEAS-2B was developed at the National Cancer Institute (Bethseda, Maryland) (Reddel et al., 1988). Normal human bronchial epithelial cells from autopsy of non-cancerous individuals, were infected with an adenovirus 12-SV40 virus hybrid (Ad12SV40) and cloned.

These cells retained the ability to undergo squamous differentiation in response to serum, may be used to screen chemical and biological agents for the ability to induce or affect differentiation and/or carcinogenesis. Cells stained positively for keratins and SV40 T antigen (ECACC catalogue, 1995). Cells were used at passages 44 to 64 for experiments described in these studies.

NHBE

Normal Human Bronchial Epithelial cells (NHBE) (Clonetics) were pooled from a number of donors by Clonetics and were recommended for use upto the 3^{rd}-5^{th} passage, after which the cells appeared more squamous and did not divide as readily.

2.2.8 Epithelial subculture

Confluent cultures were routinely split every 48 hours. After removal of nutrient-depleted media, cells were washed twice with pre-warmed sterile PBS and incubated at 37°C for approximately 3 minutes with 1ml/25cm^2 trypsin/EDTA solution. The detached cells were checked microscopically and an equal volume of fresh medium (containing FCS) or trypsin neutralising solution was added. The cell suspension was centrifuged at 250g for 8 minutes, the cell pellet was resuspended in fresh pre-warmed medium, counted and added to new flasks at a seeding density of 3-6x10^4 cells per cm^2.

2.2.9 Assessment of epithelial cell number and viability (Trypan blue)

Cell counts were performed using a haemocytometer slide. Active exclusion of 0.4% trypan blue differentiated living, viable cells from non-viable cells. Cells were mixed with trypan blue (1:1) and counted immediately. When the haemocytometer coverslip was firmly in place, the 9 large squares represented a volume of 10^{-4} cm^3.
Cell number and viability were calculated as follows:

cells/ml = mean count of 4 large squares x dilution factor of stain x 10^4

$$\% \text{ cell viability} = \frac{\text{total unstained cells}}{\text{total cells}} \times 100$$

2.2.10 Preparation of stock frozen cells

A frozen cell bank was routinely created from established log phase cells. Cells were resuspended at 1-5x10^6/ml by adding drop-wise freshly prepared ice-cold freezing medium (80% growth medium, 10% FCS and 10% dimethyl sulphoxide (DMSO)). Cells were transferred to cryotubes (1ml/tube), placed in a well-insulated polystyrene box and cooled slowly overnight at –80°C. Cells were stored in the vapour phase of liquid nitrogen (-196°C).

2.2.11 Retrieval of cells from liquid nitrogen storage

Cells were removed from storage (using long-armed forceps and face protection), placed within a sealed container and thawed rapidly at 37°C. Cryotubes were removed to room temperature prior to complete thawing, swabbed with 70% alcohol and placed in the laminar flow cabinet. The appropriate cell number was added slowly, with mixing to 25cm^2 flasks containing 5ml pre-warmed and pre-equilibrated tissue culture medium, and immediately placed in the 37°C incubator at 5% CO_2. Medium was replaced from adherent cells to remove DMSO (suspension cultured cells were centrifuged prior to culture).

2.2.12 Preparation of epithelial monolayers

Monolayers were grown to confluence on fibronectin-coated 96-well tissue culture plates. Fibronectin was used to enhance epithelial cell adherence and phenotype. Cell adherence promotion by matrix proteins generally occurs at 1-5μg/cm^2. Human plasma fibronectin (1mg/ml in 0.05M TBS, pH7.5) was diluted with sterile PBS and 5μg/cm^2 was added to coat 96-well plate(s). Plates were incubated for 1hr at 37°C (or overnight at 4°C), excess fibronectin was removed from each well and the plate was air-dried, foil-wrapped and stored at room temperature before use within one week of coating. After routine subculture, 100μl resuspended cells (at 0.2x10^6 cells/ml), was added per well. Confluent monolayers were obtained after two days and were treated as required with cytomix (50ng/ml TNF-α, IFN-γ and IL-1β), for the last 24 hour period.

2.2.13 ICAM-1 expression of alveolar and bronchial epithelial cells

A549

Intercellular adhesion molecule-1 (ICAM-1) expression on untreated and cytokine-treated A549 epithelial monolayers was measured using ELISA (enzyme-linked immunosorbant assay). Monolayers (stimulated with cytokines as required) were washed three times and 50µl/well 5% formyl saline at room temperature was added for 10 minutes. Monolayers were washed three times with PBS and 100µl antibody (appropriately diluted) was added for 60 minutes at 37°C. Unbound antibody was removed with three washes of PBS, and 50µl/well diluted (1:500 in PBS) rabbit anti-mouse peroxidase conjugated immunoglobins were added for 30 minutes at 37°C. Excess antibody was washed off (as before) and swine anti-rabbit peroxidase conjugated immunoglobulin was added to further amplify the signal from the original bound antibody. The bound antibody was visualised by peroxidase chimera: 0.05M phosphate-citrate buffer (pH 5.0) was prepared (25mls 0.05M citric acid/0.1M Na_2HPO_4, 10mg OPD and 10µl H_2O_2), 50µl/well was added and the chromogenic reaction was allowed to proceed in the dark for 15 minutes at room temperature. The reaction was stopped by the addition of 50µl/well 3M H_2SO_4, the plate was read in a spectrophotometer at 492nm (reference filter 620nm), and expressed as optical density (OD). Internal standards were performed for each assay (primary ICAM-1 and/or secondary antibodies were omitted) and background staining was taken into account.

BEAS-2B

ICAM-1 expression of BEAS-2B cells was assessed using flow cytometry. Individual cells were passed in a stream through a laser beam and were detected and counted. Sensitive photomultiplier tubes detected light scatter (forward scatter was a measure of cell size and side scatter, granularity) and fluorescence (cell surface protein expression). BEAS-2B cell antigen expression was studied by indirect immunofluorescence labelling using FITC labelled secondary anti-immunoglobulin antibody to detect cell-bound specific primary antibody.

BEAS-2B cells were grown to confluence in $25cm^2$ flasks, treated for 24 hours with cytomix (a mixture of 50ng/ml TNF-α, IL-1β and IFN-γ) as required. Monolayers were washed twice with 2.5ml PBS and incubated with 1ml cell dissociation fluid at

37°C for 15 min at 37°C, until >95% cells were in suspension. All procedures were carried out at 4°C. Cells were washed into FACS buffer (PBS, 1mM $CaCl_2$, 1mM $MgCl_2$, 1% BSA), resuspended at 2-5x10^6/ml and 100µl cell suspension was placed in each tube. Previously determined optimal concentrations of primary (mouse) mAb was added to cells for 15 minutes at 4°C: MoIgG, ICAM-1 (BBIG-I1), BBIG-V1 (VCAM-1) and E-cadherin (HECD-1). Controls were performed with multiple isotype mouse IgG and by staining with secondary antibody only. Cells were washed from unbound antibody by the addition of 1ml FACS buffer and centrifuged at 4°C, 250g for 7 minutes. Supernatant was removed by inversion of each tube onto tissue. The cell pellet was resuspended in optimal, previously titrated dilution (1:10) FITC-conjugated $F(ab')_2$ fragments of rabbit anti-mouse Ig in FACS buffer and incubated at 4°C for 15 minutes. Cells were washed as before and resuspended in 300-500µl buffer. Cells were analysed by FACScan using Lysys II version 1.1 software.

Early apoptotic cells have increased exposure of the membrane phospholipid phosphatidylserine (PS) on their surface, which occurs prior to nuclear changes such as DNA fragmentation. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with a high affinity for PS. Cells were stained with the vital dye propidium iodide (PI) to distinguish early apoptotic cells (annexin positive, PI negative) from necrotic cells (annexin positive, PI positive). PI intercalated with double-stranded DNA and generated a red fluorescent signal.

Annexin V staining of BEAS-2B was used to confirm that cytomix-stimulation and removal of cells from adherence did not cause or increase apoptosis. Annexin V-FITC was added to cell suspension (1.5µl/sample) and incubated for 8 minutes at room temperature to directly stain cells with annexin V (green fluorescence FL1). Propidium iodide (PI) was added (5µl of a 50µg/ml solution in PBS, stored at 4°C in the dark) and cells were incubated for 2 minutes at 4°C prior to analysis.

2.3 Results

Bronchial explants were originally used as a source of epithelial cells (Figure 2.1a). Growth media used were DMEM, DMEM/F12 or SAGM. SAGM was most successful in maintaining viable epithelium for extended periods of time (>4 weeks). Cells detached from the explant and adhered to fibronectin-coated tissue culture-treated plastic (Figure 2.1b and c). The disadvantage of this method was that long term culture resulted in growth of contaminating cell types (Figure 2.2), and therefore explants were removed after assessing establishment of cell growth.

Epithelium was disected from submucosa or removed by treatment with protease XIV (Figure 2.3a). Epithelium after both treatments appeared identical and were viable (>95%) as assessed by trypan blue. However, protease treatment (>5 minutes) resulted in non-adherent epithelial cells. Cells treated for less than 5 minutes adhered to fibronectin-coated plastic, but most adherent cells did not spread or divide (Figure 2.3b). Less than 5 minutes treatment with protease resulted in a lower cell yield. Physical disaggregation of epithelium resulted in adherent, spread and dividing epithelial cells (Figure 2.3c). However, after subculture using trypsin/EDTA, cells appeared squamous in phenotype (Figure 2.4).

Cells were grown at an air-liquid interface in an attempt to maintain differentiation. Transwell membranes were coated with extracellular matrix prior to addition of epithelial cells. Cell phenotype was assessed visually at 2-3 day intervals (Table 2.1). Extracellular matrix maintained cell viability for at least 2 weeks. Cells maintained normal phenotype when adherent to human extracellular matrix and matrigel matrix. Cells attached and spread when cultured on laminin, and to a lesser extent on human plasma fibronectin alone. These results were reflected in cells cultured in tissue culture flasks.

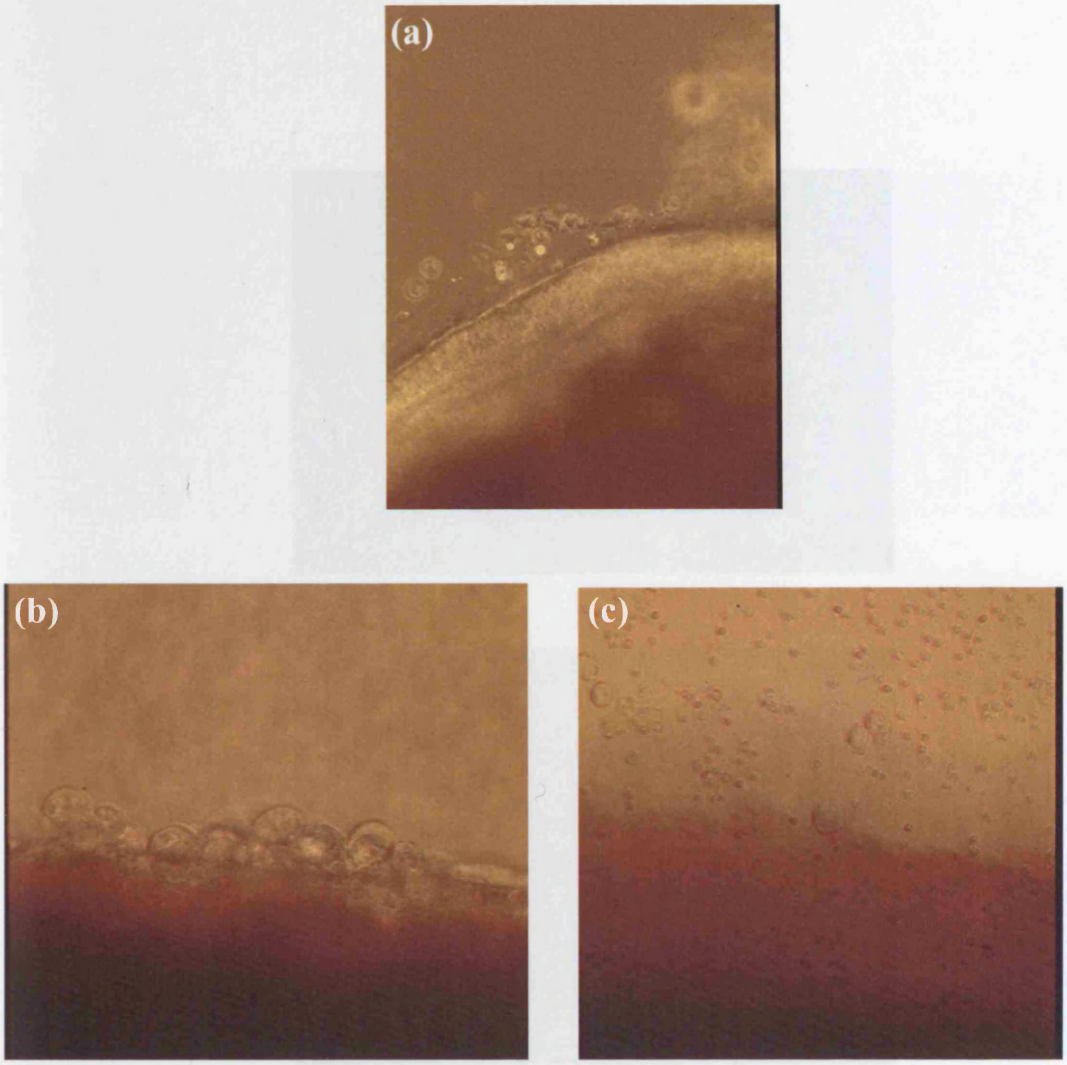


Figure 2.1 **Bronchial epithelial explant culture**
Sections of bronchus were placed in fibronectin-coated tissue culture flasks containing SAGM (a) fresh explant showing columnar epithelium with active cilia (x250) (b) explant (after 1 week) showing epithelial cells detaching from explant surface (x400) (c) explant culture (identical to b) showing explant-detached epithelial cells on surface of culture flask (x400).

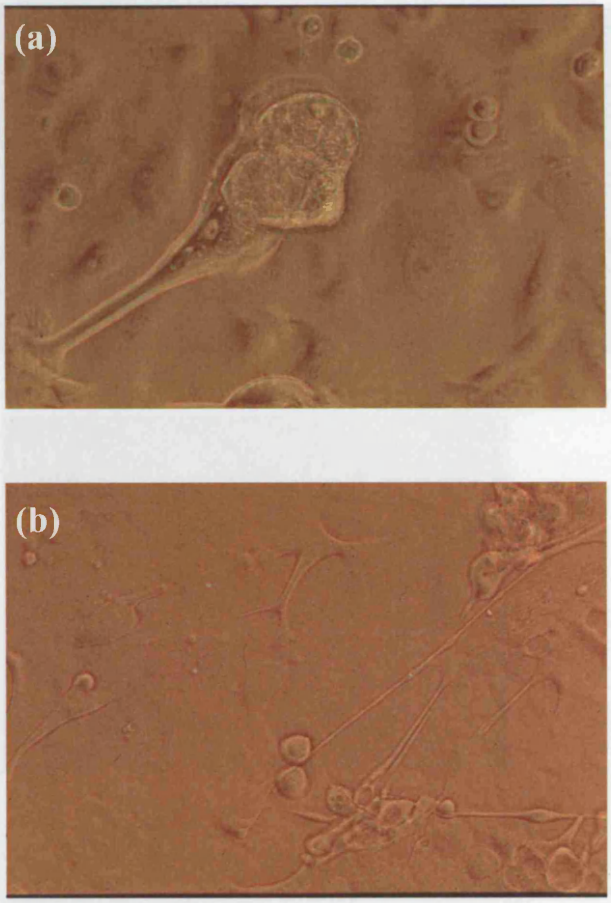


Figure 2.2 **Long-term explant culture without removal of explant (x400)**
Culture (>4 weeks) in the presence of explant showing examples of non-epithelial 'contaminating' cells
(a) epithelial gland (b) fibroblast-like cells
Cultures were discarded if these cell types were observed.

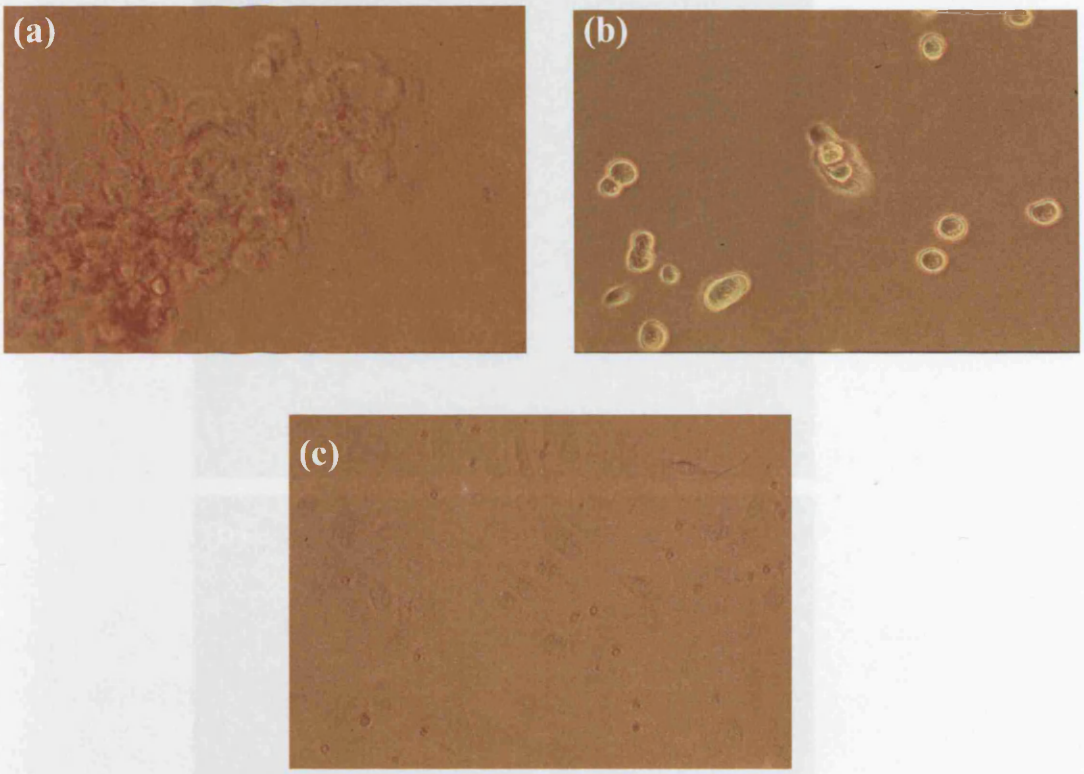


Figure 2.3 **Protease treatment of bronchial explant-derived sections (x400)**
(a) Epithelial cell aggregates post-enzyme digestion
(b) Non-adherent/non-spreading epithelial cells after enzyme digestion
(c) Adherent epithelial cells after physical removal

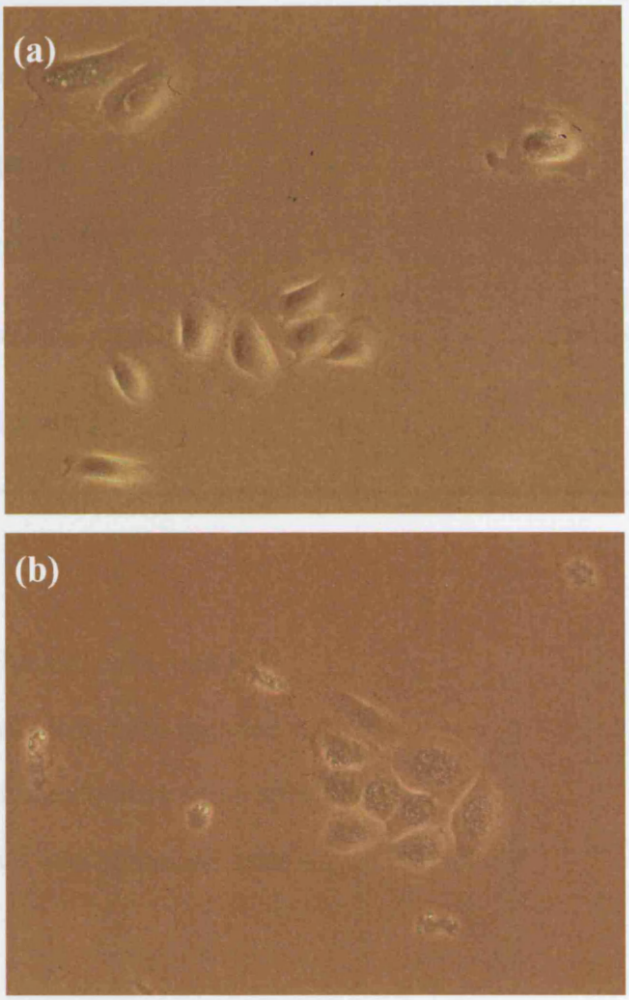


Figure 2.4 **Primary epithelial cells from explant outgrowth (x400)**
Sections of human bronchial mucosa were excised and cultured in bronchial epithelial growth medium (SAGM). Medium was replaced every 48 hours, and upon cell coverage of approximately 25% available area, explants were removed and medium replaced as before. Cells were passaged using trypsin/EDTA upon coverage of >75% available area. (a) cells directly from explant (b) cells passaged once (showing squamous phenotype)

Matrix protein	attachment	Morphology after 2 weeks
None	-	cells non-viable
Human plasma fibronectin	+	viable
Collagen IV	-	viable
Laminin	++	viable
Human extracellular matrix	+++	viable, cilia active
Matrigel matrix	++	viable

- no attachment, cells rounded
+ cell attachment, cells rounded
++ good cell attachment, some spreading of cells
+++ very good attachment, all cells spread maximally

Table 2.1 **Culture of primary explant-derived epithelial cells grown upon transwells precoated with various extracellular matrix proteins.** Cells were assessed visually for attachment and cilial activity, and by trypan blue exclusion for viability. Results shown were representative of 4 separate experiments from 4 different bronchial tissue donors.

The phenotype of cells grown after physical removal from explants were assessed and appeared to be 100% epithelial cells (from positive intracellular immunofluorescent cytokeratin staining) (Figure 2.5).

Culture of bronchoalveolar lavage fluid epithelial cells was also attempted (Figure 2.6a). Cells were added to human plasma fibronectin-coated tissue culture flasks, and observed every 2-3 days. Only one sample (from three attempts) was successful. Adherent cells after 3 hours were macrophages (2.6b), after 24 hours were macrophages and epithelial cells (2.6c). Non-adherent epithelial cells appeared to be damaged, and possessed only partial trypan blue exclusion.

Epithelial cells express basal ICAM-1 levels, which are raised after cytokine treatment. A549, BEAS-2B and NHBE epithelial cells expressed approximately equal basal ICAM-1 expression (corrected for MoIgG controls) (Figure 2.7). Figure 2.8 showed A549 ICAM-1 levels were greater than ICAM-2 or ICAM-3 levels.

Epithelial cells were removed using an EDTA-based cell dissociation solution for assessment of cell surface adhesion molecule expression, since trypsin treatment was found to reduce E-cadherin expression (Figure 2.9). BEAS-2B surface adhesion molecule expression upon treatment with cytomix was investigated (Figure 2.10). Cell viability (using trypan blue and PI/annexin) after treatment with increasing cytomix (0-50ng/ml TNF-α, IFN-γ, IL-1β) and removal using dissociation solution was analysed, and cells remained >95% viable. E-cadherin expression was unchanged after cytomix treatment, as expected. The high basal ICAM-1 expression was increased in a dose-dependent manner by cytomix, and a small amount of VCAM-1 was induced at concentrations >5ng/ml.

In addition, NHBE cells were confirmed to possess epithelial phenotype and ICAM-1 expression was measured using flow cytometry (Figure 2.11).

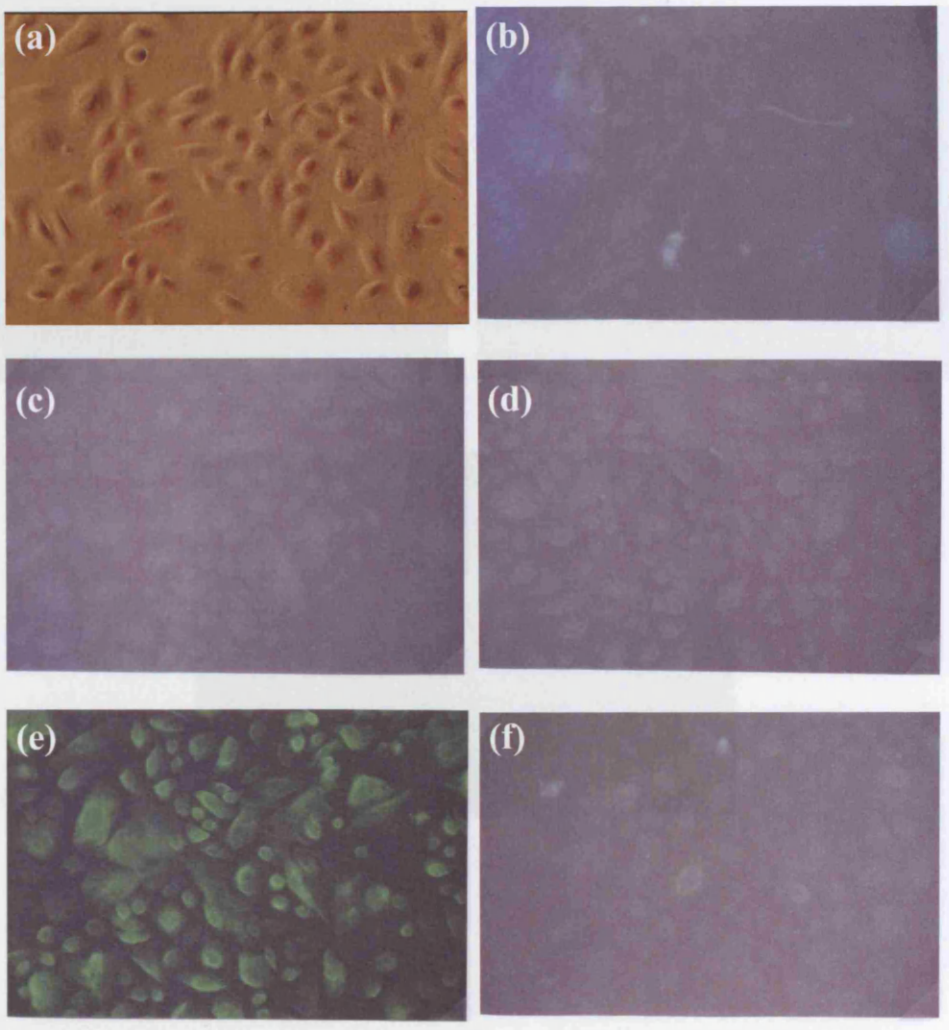


Figure 2.5 **Confirmation of epithelial phenotype (x400)**
Explant-derived primary epithelial cells were grown on fibronectin-coated glass coverslips and indirectly fluorescently labelled (FITC). (a) unlabelled cells (b) intracellular MoIgG (control), (c) smooth muscle actin (smooth muscle), (d) CD34 (endothelium), (e) cytokeratin (epithelium) and (f) vimentin (fibroblast). Results were representative of two individual experiments from two different patients.

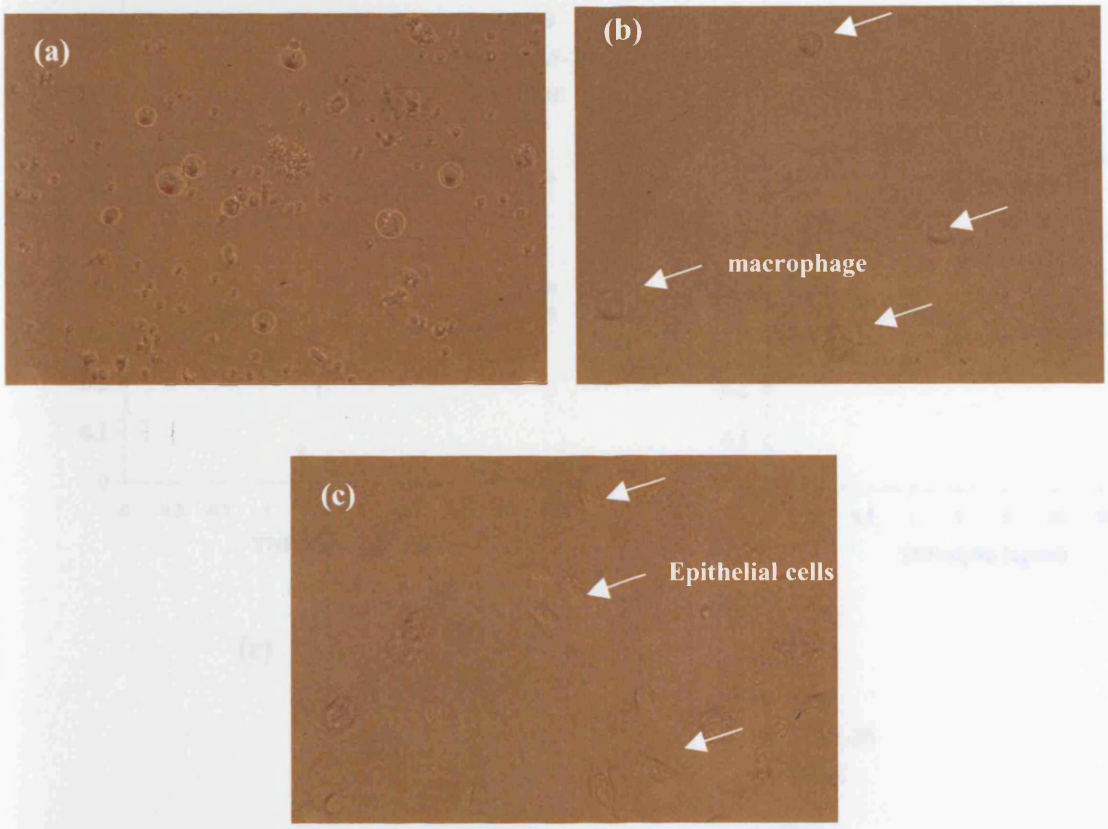


Figure 2.6 **Primary epithelial culture from BAL fluid (x400)**
BAL cells were cultured in tissue culture flasks (a) and adherent macrophages were observed within 3 hours (b). Adherent epithelial cells were observed after 48 hours and were distinguishable by their morphology (c).

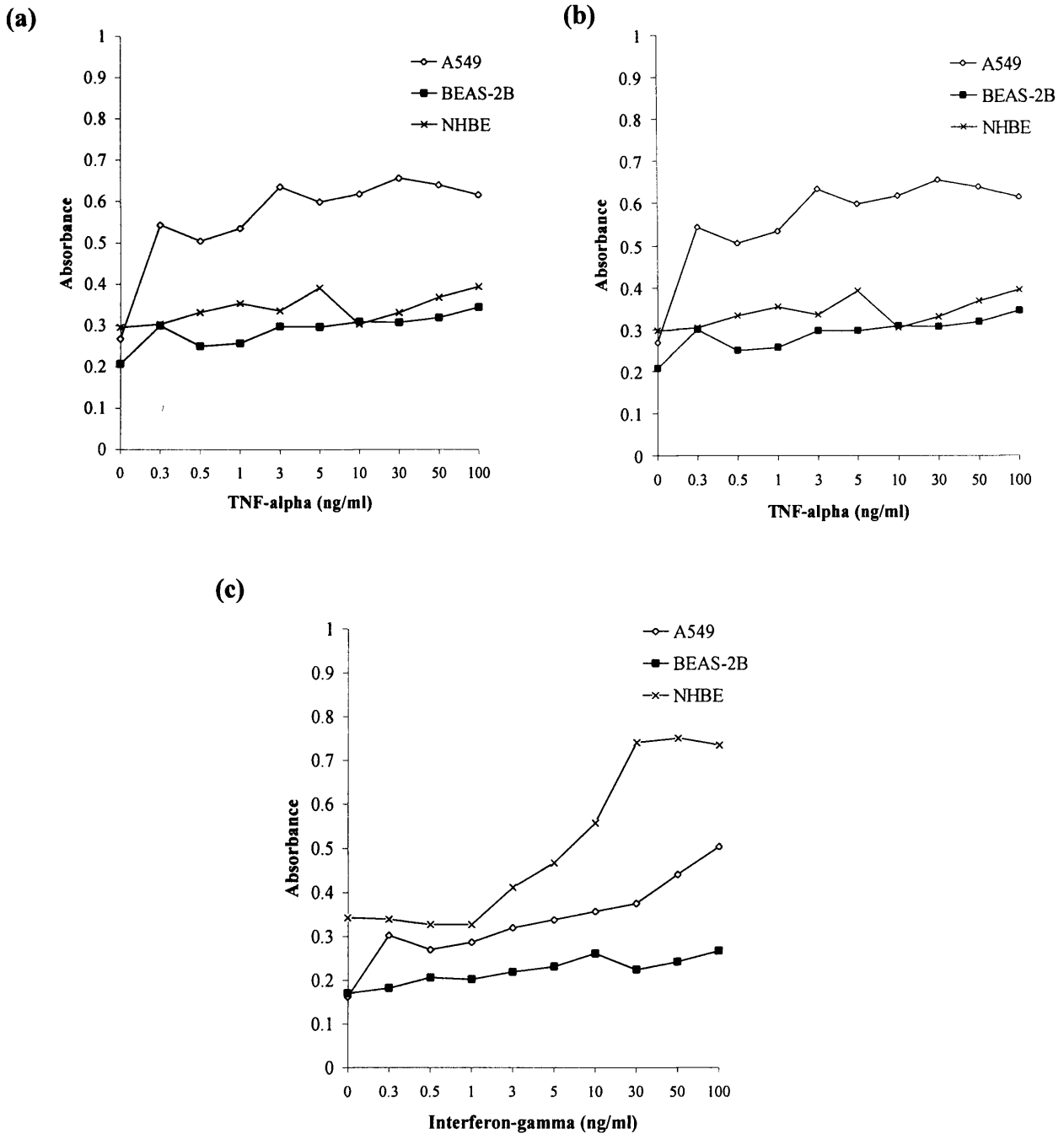


Figure 2.7 **Preliminary comparison of epithelial ICAM-1 expression by ELISA (n=1).** Epithelial monolayers (A549, BEAS-2B and NHBE) were stimulated for 24 hours with 0-100ng/ml cytokine as shown (a) IL-1β (b) TNF-α (c) IFN-γ. Values shown are corrected for MoIgG control, and are the average of four replicates.

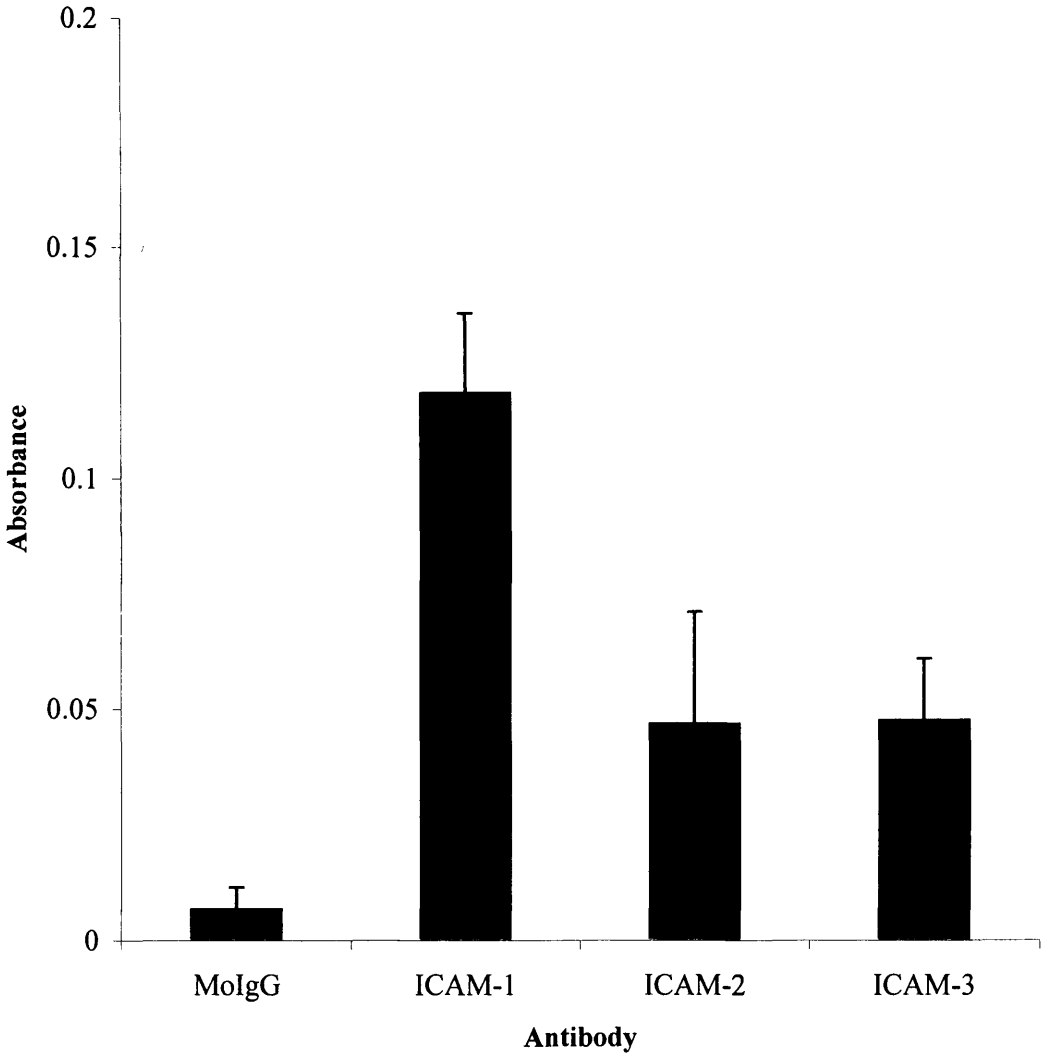


Figure 2.8 **Alveolar epithelial (A549) ICAM expression by ELISA (n=3)** Unstimulated A549 monolayer ICAM-1, ICAM-2 and ICAM-3 expression were compared. Results expressed as mean +/- sem, and are determined from the average of four replicates.

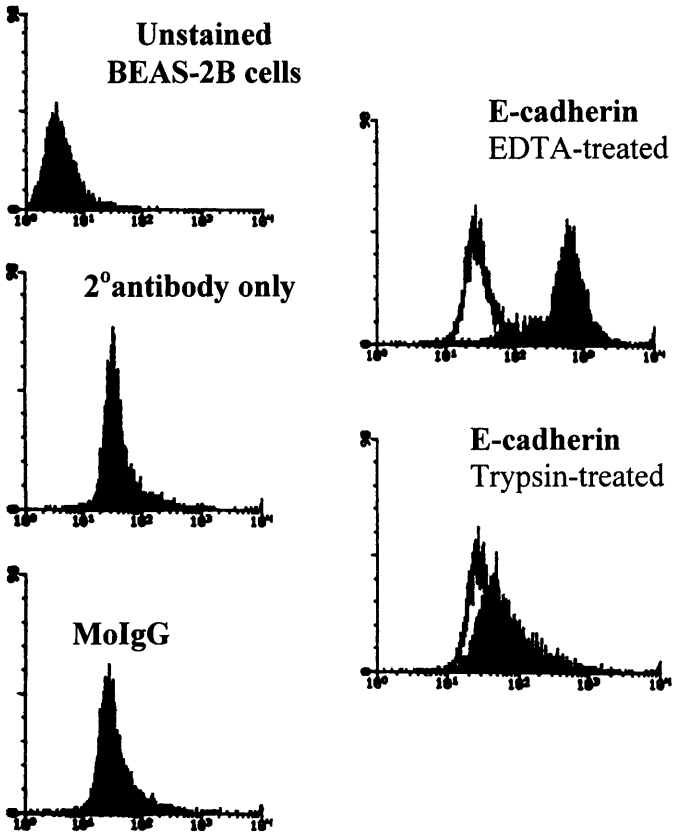


Figure 2.9 **Comparison of the effect on epithelial E-cadherin expression of either non-enzyme (EDTA) or enzyme (trypsin)-based methods for removing adherent BEAS-2B.** BEAS-2B monolayers were treated for 5 minutes at 37^{0}C. E-cadherin was assessed by indirect flow cytometry using FITC labelled secondary antibody
Results shown are representative of two individual experiments

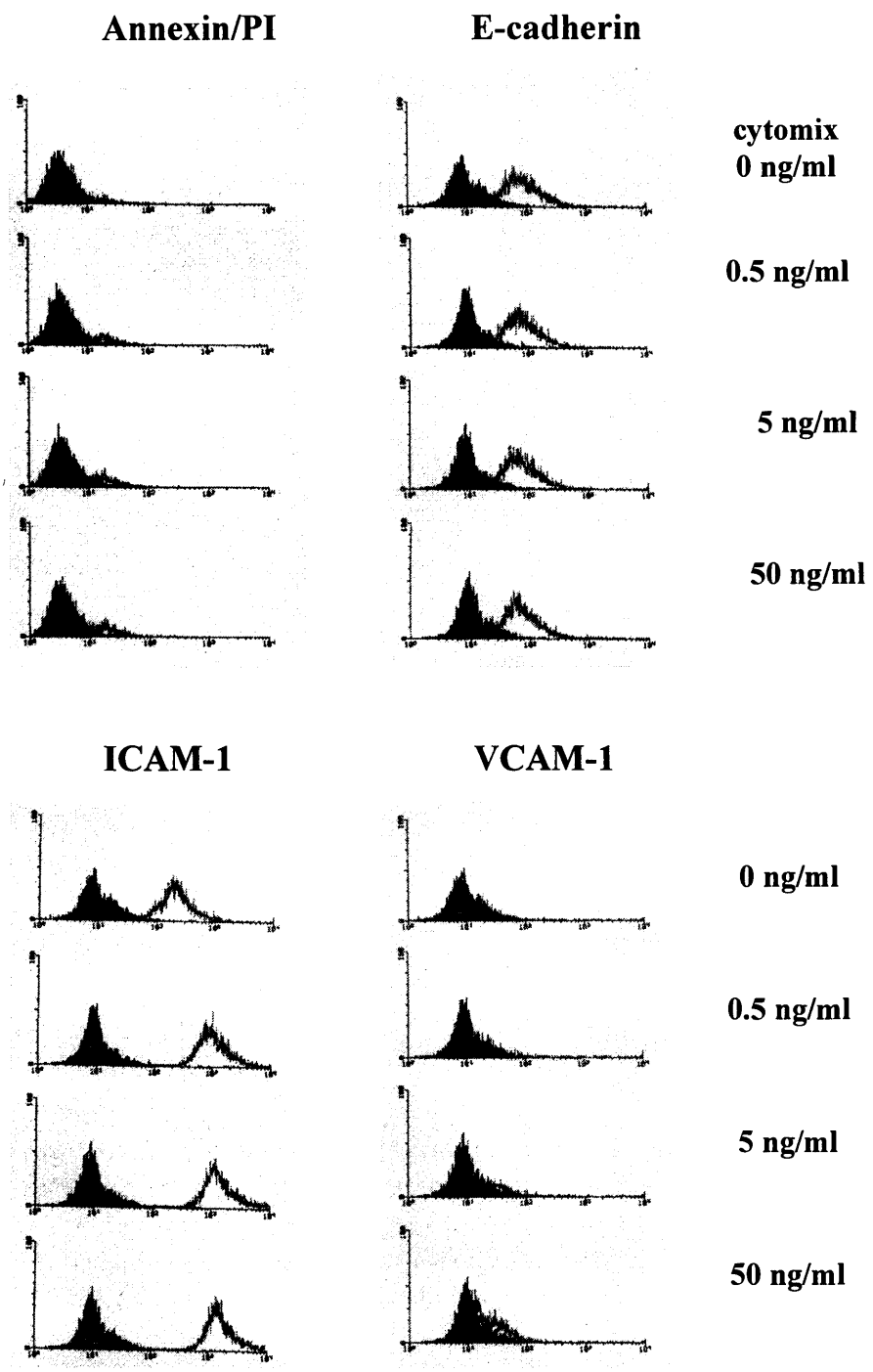


Figure 2.10 **BEAS-2B adhesion molecule expression upon increased cytomix stimulation** BEAS-2B epithelial cells were grown on fibronectin-coated tissue culture plastic, removed with EDTA and analysed by flow cytometry for (a) surface annexin V/intracellular PI (b) E-cadherin (c) ICAM-1 (d) VCAM-1
Results are representative of two separate experiments.

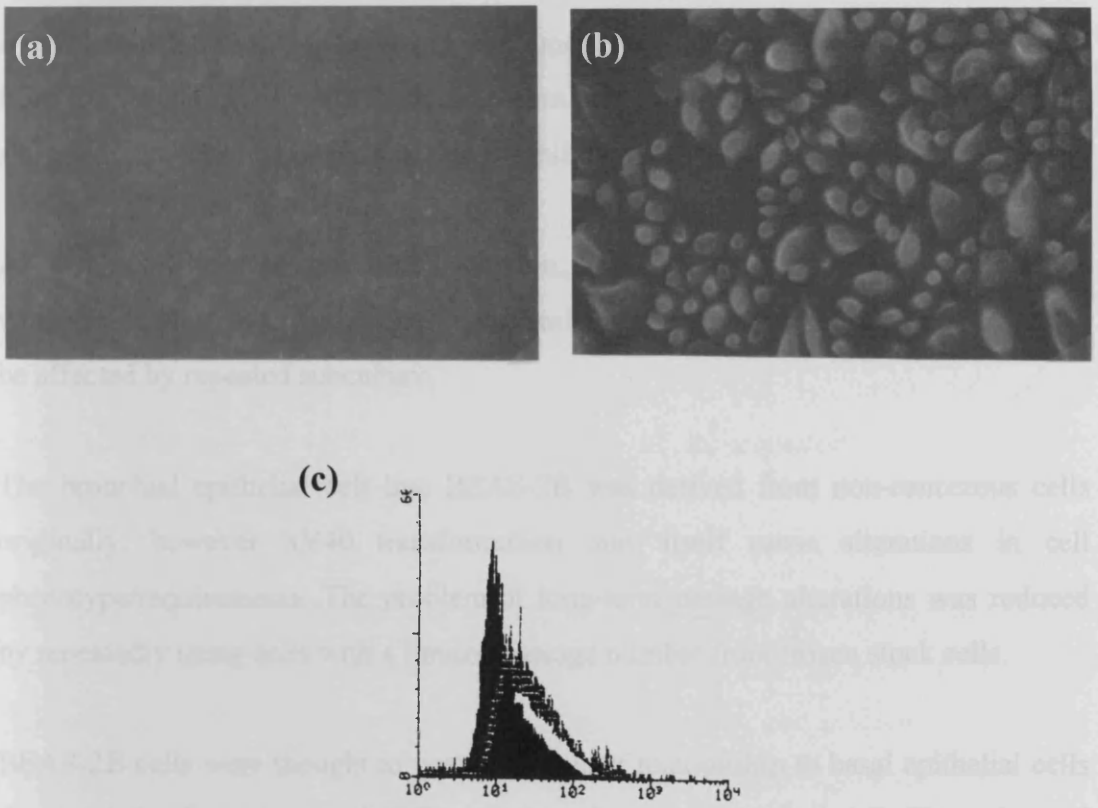


Figure 2.11 Confirmation of epithelial phenotype of normal human bronchial epithelial cells (NHBE) (Clonetics) (x400). Epithelial cells were grown on fibronectin-coated glass coverslips and stained with (a) intracellular MoIgG control and (b) cytokeratin. (c) NHBE ICAM-1 was assessed using flow cytometry (Epithelial cells were detached using EDTA).

2.4 Discussion

Although primary explant-derived epithelial cells were expected to be a good model of *in vivo* epithelium, they were problematic for long term use in cell adhesion assays for a number of reasons. They became squamous upon subculture, did not passage well and were not readily available in sufficient numbers.

A good substitute were thought to be commercially-available primary epithelial cells from Clonetics (NHBE), but these cells were only recommended to be subcultured for a limited number of passages and were prohibitively expensive for routine use.

Although cell lines passage well long term, many are derived from cancerous cells with altered phenotype with respect to adhesion molecule expression, which may also be affected by repeated subculture.

The bronchial epithelial cell line BEAS-2B was derived from non-cancerous cells originally, however SV40 transformation may itself cause alterations in cell phenotype/requirements. The problem of long-term passage alterations was reduced by repeatedly using cells with a limited passage number from frozen stock cells.

BEAS-2B cells were thought to possess a greater relationship to basal epithelial cells due to their phenotypic and differentiation characteristics (lack of cilia, enhanced Ca^{2+}-dependent adhesion to extracellular matrix proteins, and ability to divide). Migrating leukocytes involved in the pathogenesis of asthma may adhere between non-dividing columnar epithelial cells and basal cells *in vivo,* and therefore assessing adhesion to epithelial cells retaining the ability to divide may be physiologically relevant.

In summary, BEAS-2B bronchial epithelial cells were found to be most suitable for use in preliminary investigation(s) of mast cell and eosinophil adhesion to bronchial epithelium. However, in common with all studies using cell lines, it is recommended that primary epithelial cells should be used to confirm major findings.

Chapter 3: Mast cell adhesion to bronchial epithelium

3.1 Introduction

Mast cell accumulation within the bronchial mucosa and increased degranulation at this site may be observed in asthma (Djukanovic et al, 1990; Crimi et al, 1991; Gibson et al, 1993; Pesci et al, 1993). Mast cells contribute to asthma pathophysiology by proinflammatory mediator, immunoregulatory and chemotactic cytokine production in response to multiple stimuli, including IgE and SCF (Bischoff and Dahinden, 1992; Coleman et al., 1993; Columbo et al., 1992).

Mast cell mediators are preformed (histamine, tryptase, heparin) and newly synthesised (leukotrienes, PGD_2 and cytokines). The activated bronchial epithelium is also a source of proinflammatory mediators (particularly cytokines and growth factors) and may be responsible for control of inflammatory cell infiltration, subsequent tissue damage and ensuing fibrosis.

Mast cell interaction with bronchial epithelium may regulate mast cell activation, and thereby control epithelial activation and damage. Mast cell adhesion to bronchial epithelium was studied using function-blocking antibodies, cell activation and signal transduction modulation, soluble carbohydrates (as competitive ligands), protease and exoglycosidase treatment (of either cell type).

3.2 Materials

Antibodies

Mouse IgG control; MHM23 (CD18), 2LPM19c (CD11b), KB90 (CD11c), UCHT1 (CD3), P1/33/2 (CD9), (Dako, Cambridge, UK); P4C10 (β_1-chain), BBIG-I1 (ICAM-1), BBIG-V1 (VCAM-1), HECD-1 (E-cadherin) (R&D, Abingdon, UK); SHE78-7 (E-cadherin, IgG_{2a}), YB5.B8 (CD117) (Cambridge Bioscience, Cambridge, UK); IB4 (CD18, IgG_{2a}) (Alexis Corporation, Nottingham, UK); LM609 (CD51/61) (Chemicon international, Harrow, UK); polyclonal anti-stem cell factor antibody (cat.no. 2370-01) was purchased from Genzyme (West Malling, Kent, UK); fluorescein isothiocyanate (FITC)-labelled rabbit anti-mouse immunoglobulin, anti-tryptase and anti-elastase were purchased from Dako. Polyclonal anti-human IgE was obtained from Serotec (Oxford, UK).

The following mAbs were gifts:

CBL451 (CD11a) (Cymbus, Southampton, UK), LF61 ($\alpha_E\beta_7$) (leucocyte typing workshop 1996); Ber-Act-8 ($\alpha_E\beta_7$) (Dr H.Dürkop, Freie Universität, Berlin, Germany); 29C6 (FcεRI) (Dr J. Hakimi, Hoffmann-LaRoche, New Jersey, USA); HP1/2 (α_4-chain) (Dr F.Sánchez-Madrid, Hospital de la Princesa, Madrid, Spain); Act I ($\alpha_4\beta_7$) (Dr A.I. Lazarovits, University Hospital, Ontario, Canada); 4B9 (VCAM-1) (Dr R.Lobb, Biogen, Cambridge, MA, USA); R6.5 $F(ab)_2$ (ICAM-1) (Dr R.Rothlein, Boehringer Ingelheim, Connecticut, USA), B2C10, A3A12 (250μg at 1mg/ml in PBS, mouse anti-galectin 3) and anti-DNP-IgG_1 (250μg at 1mg/ml in PBS), human recombinant galectin 3 (100μg at 0.6mg/ml in PBS/10% glycerol) (Dr F-T Liu, La Jolla Institute for Allergy and Immunology, San Diego, CA, USA); Polyclonal galectin-1 rabbit antiserum (Dr L.G. Baum, UCLA School of Medicine, Los Angeles, CA,USA); galectin-1 (Dr E.P.Moiseeva, Leicester University, UK).

Cytokines and Reagents

Stem cell factor (SCF), transforming growth factor (TGF)-β, tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and interferon (IFN)-γ were purchased from R&D (Abingdon, Oxon, UK); pronase, proteinase k, heparinase I (heparinase from *Flavobacterium heparinum*), heparinase III (heparitinase I from *Flavobacterium heparinum*), hyaluronidase type I-S (from Bovine Testes), β-galactosidase (from

Bovine testis), endo-α–N-acetylgalactosaminidase (from *Diplococcus pneumoniae*), collagenase type 1A (from *Clostridium histolyticum*), hyaluronic acid (sodium salt from Bovine trachea), EDTA, EGTA, manganese, paraformaldehyde, pertussis toxin, cholera toxin, cytochalasin D, genistein, sodium azide, deoxyglucose, phorbol 12-myristate 13-acetate (PMA), leupeptin, calcimycin (A23187), galactose, mannose, mannose-6-phosphate, fucoidin, lactose and hyaluronic acid were purchased from Sigma (Poole, Dorset, UK); neuraminidase (from *Arthrobacter ureafaciens*) and human myeloma IgE were purchased from Calbiochem-Novabiochem (Nottingham, UK). Histamine and S-adenosyl-L-[*methyl*-^{3}H]methionine was purchased from Amersham Life Sciences (Little Chalfont, Bucks, UK); rat kidney histamine methyl transferase and ^{14}C-histamine were generous gifts from Dr. S. Harper and Dr K.Hallam (AstraZeneca Charnwood, Loughborough, UK).

Toluene, isoamylalcohol, optiphase hisafe III scintillation fluid, NH_4Cl and $KHCO_3$ were obtained from Fisher Scientific (Loughborough, UK), HBSS from Life technologies (Paisley, Scotland, UK), human myeloma IgE (from Calbiochem) and human IgG (from Sigma). Anti-mouse IgG_1 Dynabeads and rat anti-mouse IgG_1 CELLection kit were purchased from Dynal (Wirral, UK), and ^{51}Cr (sodium chromate) was purchased from Amersham.

Cell lines

The human mast cell line HMC-1 was a kind gift from Dr J.H.Butterfield (Mayo clinic, Rochester, MN, USA). Human bronchial epithelial cell line BEAS-2B was purchased from ECACC and cultured as previously described (chapter 2).

Cell culture and isolation reagents

Phosphate buffered saline (PBS), Hanks' balanced salt solution (HBSS), foetal calf serum (FCS), RPMI 1640 with glutamax and Hepes, minimal essential medium (MEM) non essential amino acids, antibiotic-antimycotic solution, horse serum, Iscove's modifed Eagle's medium (IMDM), iron-supplemented FCS were obtained from Life technologies (Paisley, Scotland, UK). Collagenase (type IA), hyaluronidase (type I), bovine serum albumin fraction V, human IgG, human myeloma IgE, sheep anti-human IgE, monothioglycerol and human plasma fibronectin were bought from

Sigma (Poole, UK). Anti-*c-kit* (CD117) mAb YB5.B8 was purchased from Cambridge Bioscience (Cambridge, UK). Rat anti-mouse IgG_1 magnetic dynabeads or rat anti-mouse IgG_1 CELLection^{TM} kit (Dynal, Wirral, UK).

Adhesion assay reagents

Human plasma fibronectin, phosphate buffered saline, proteinase k, pronase, heparin, hyaluronic acid, galactose, lactose, mannose, mannose-6-phosphate, fucoidin, genistein, pertussis toxin, cholera toxin, sodium azide, deoxyglucose, cytochalasin D, phorbol myristate actetate (PMA), EDTA, EGTA, paraformaldehyde, Mn^{2+}, leupeptin, A23187 and sheep serum were obtained from Sigma (Poole, UK). Trypsin was purchased from Life technologies (Paisley, UK). Heparinase I, heparinase III, hyaluronidase, neuraminidase, endo-α-N-acetylgalactosaminidase, β(1-3,4,6)-galactosidase were obtained from Calbiochem-Novabiochem (Nottingham, UK).

Histamine assay reagents

Histamine (Sigma, Poole, UK), Tyrode's buffer (Life Technologies, Paisley, UK).
Rat kidney histamine methyl transferase (HMT) was a generous gift from Dr S Harper (AstraZeneca, Loughborough, UK), [^{3}H]methyl-S-adenosyl methionine (SAM) was obtained from Amersham Life Sciences (Little Chalfont, Bucks, UK), NaOH, toluene, isoamylalcohol were obtained from Fisher Scientific (Loughborough, UK), optiphase hisafe III scintillation fluid (Wallac, UK).

Equipment

50ml and 15ml tubes, eppendorf tubes, petri dishes, 12-well and 96-well tissue culture plates (University bulk store), scissors and 100 μm gauze (Fisher Scientific, Loughborough, UK), Fahrenheit cell strainers were bought from Becton Dickenson (Oxford, UK), MPC-1 magnet (Dynal, Wirral, UK), mylar sealing tape was obtained from Sigma (Poole, UK). Capped sterile clustertube system was obtained from Costar (Cambridge, MA, USA), mini-scintillation vials were bought from Fisher Scientific (Loughborough, UK). The tri-carb liquid scintillation analyser (model 1500) was purchased from Packard (Pangbourne, Berks, UK).

Methods

3.2.1 Human lung mast cell (HLMC) purification

When large numbers of pure cells are required in a short time, paramagnetic beads coupled to cell-specific mAb may be used. CD117-coated beads may be mixed with enzyme-digested lung cells to rapidly positively select human lung mast cells.

Lung tissue donors

Resected lung tissue was obtained from operations for lung carcinomas performed at Glenfield hospital. Approval for this study was obtained from the Leicestershire research ethics committee. Tissue was obtained mainly from smokers (identified by pronounced presence of tar deposits in macrophages, Pathology department) who were 89% male, average patient age 67±7.1 years (n=72). Tissue was obtained within 1 hour of resection, immediately weighed and placed in HBSS/2% FCS prior to processing.

Human lung cell dispersal

This was achieved by positive selection with the monoclonal antibody YB5.B8 known to bind to the second domain of the *c-kit* proto-oncogene product on all human tissue mast cells. This antibody was used in conjunction with magnetic Dynabeads (Dynal, Wirral, UK) to purify mast cells from lung tissue that had been enzymically dispersed (Schulman et al, 1982; Okayama et al., 1994). The published method was modified with red cell lysis (later omitted) and sensitisation of cells with human myeloma IgE (3μg/ml) during the IgG blocking stage.

YB5.B8 has been shown to bind to the *c-kit* proto-oncogene product (Lerner et al., 1991) at the same receptor domain as stem cell factor (SCF). Mast cell function was not affected by the use of this antibody for purification purposes (Okayama et al., 1994). This procedure resulted in enrichment of mast cells from 3.8±2.5% to 99.7 ±0.5% purity with yields of approximately 67±8% (n=72), which was comparable to results obtained by Okayama et al (1994). Viability of the cells was 100% after purification and >97% after overnight culture with 10ng/ml SCF.

HLMC were purified from macroscopically normal human lung tissue (identified by the Pathology Department, Glenfield Hospital). The lung tissue was dissected free of major bronchi and blood vessels and was chopped into 0.5-2.0mm^3 pieces using sterile scissors within sterile petri dishes. The fragments were washed twice with HBSS/10% FCS through 100μm gauze, collected and placed overnight at 4°C in 50ml tubes in RPMI 1640 containing 10% FCS/Non-essential amino acids/antibiotic-antimycotic solution. The following day lung fragments were recovered and washed using HBSS/2% FCS by filtration through cell strainers. Tissue was subsequently placed in RPMI 1640 containing 10% FCS (1g tissue/4ml buffer) containing 1.5 mg/ml collagenase (type IA) and 0.75 mg/ml hyaluronidase (type I). Incubation at 37°C for 75 minutes with constant stirring (magnetic stirrer) resulted in almost complete digestion of tissue. Cells dispersed by this procedure were separated from undissociated tissue by filtration through fresh nylon gauze and washed twice with HBSS/FCS by centrifugation at 250g for 8 minutes at 20°C. Mast cell number and percentage were assessed using a Neubauer haemocytometer after metachromatic staining with Kimura stain (Kimura et al., 1973) and viability was assessed by Trypan blue exclusion. At this stage mast cell percentage ranged from 1.2 to 8.1%, mean 3.7±1.8% (n=72). Other cells present in the lung digest were alveolar macrophages (82.7±3.1%), epithelial cells, T and B lymphocytes (8.4±1.9), neutrophils (5.3±1.5%), and occasionally eosinophils (3.6±0.3%).

Immunomagnetic affinity selection of HLMC

The dispersed cell suspension was cooled slowly on ice to 4°C and kept at this temperature throughout the rest of the purification procedure. A previously described protocol was followed (Okayama et al., 1994) with the exception that red cells were removed with NH_4Cl lysis buffer at 4°C (rather than during centrifugation through a Percoll gradient) and washed twice with HBSS/2%FCS. The cells were resuspended in 2ml HBSS/2%FCS containing 10% horse serum, 1% bovine serum albumin (BSA, fraction V) (HBSS-protein) with 50μg/ml human IgG to block non-specific binding and pre-sensitised with 2.5μg/ml human myeloma IgE for 30 minutes with gentle rolling. The cells were centrifuged at 250g, resuspended in 1ml HBSS-protein containing 5μg/ml mouse anti-*c-kit* mAb YB5.B8 and incubated for 30 minutes with gentle rolling. Excess antibody was removed by washing the cells twice in

HBSS/FCS. The cell suspensions were incubated with pre-washed rat anti-mouse IgG_1 magnetic Dynabeads (3 Dynabead: 1 mast cell ratio) in a volume of 1ml for 90 minutes at 4°C with gentle rolling. Dynabeads may be linked to YB5.B8 mAb prior to separation, allowing cells to be incubated for less time. After incubation, the suspension was made up to 20ml with HBSS/FCS and the tube was placed within the magnetic field of an MPC-1 magnet for 3-5 min, depending on the cell number. Cells attached to Dynabeads and free dynabeads were adherent to the magnet side of the tube, while the remaining supernatant was magnetically separated again before being discarded. The purified cells were pooled and washed three times in 20ml HBSS by separation in the magnetic field. The last wash was carried out at room temperature. If cells with beads were required, the separated cells were resuspended in fresh culture medium (RPMI 1640/FCS with non-essential amino acids and antibiotic-antimycotic solution).

Spontaneous detachment of beads from mast cells occurred if mast cells were cultured overnight. The MPC-1 magnet was subsequently used to separate these cells. In later experiments, beadless mast cells were obtained using the Dynal rat anti-mouse IgG_1 CELLection kit in accordance with the manufacturers instructions. Mast cells were detached from beads using DNase digestion, which enabled FACS and chemotaxis to be performed. There was no difference in percentage adhesion of mast cells to epithelium when using mast cells purified using either of these methods (implying lack of direct involvement of *c-kit* in adhesion). Cells were resuspended in 200µl RPMI 1640/1% FCS (for up to $5x10^7$ beads) with 4µl (200U) DNase solution (for cells isolated using $1x10^7$ dynabeads). Cells were incubated at 37°C for 15-30 minutes and detached cells were removed into a fresh tube following magnetic separation. Cells/ beads were re-suspended in a further 200µl RPMI and separated as before. This was repeated three more times until released cells occupied a total volume of 1ml. Cells were centrifuged and resuspended in fresh culture medium. Purified cells were counted (using Kimura stain, described below), assessed for viability (0.02% Trypan blue dye exclusion) and resuspended at $1x10^6$ cells/ml. Final mast cell purity was 99.8±0.1%, and viability 99.7±0.2% (n=72).

3.2.2 CAM-positive selection of dispersed lung cells

The dynabead technology was used to confirm that epitopes were not digested by the enzymic dispersion method. Known cell surface receptors were selected for (including YB5.B8) on the same lung cell suspension, using the same method as above. CD18-, CD11a-, CD11b-, CD11c-, $\alpha_E\beta_7$-positive cells were selectively purified from dispersed human lung tissue in this manner. Cell number and mast cell percentage were quantified using Kimura staining.

3.2.3 Culture of purified human lung mast cells (HLMC)

Overnight culture of purified HLMC at $1x10^6$ cells/ml in RPMI 1640/Glutamax/Hepes containing 10% FCS, non-essential amino acids and antibiotic-antimycotic was carried out on 1% BSA-coated 12-well plates (in humidified 95% air/ 5% CO_2 at 37°C). HLMC were cultured in the presence or absence of SCF (10ng/ml), sheep anti-human IgE (1%, or as required) or TGFβ (5ng/ml). The following day cell number and viability were assessed as described above. No treatments were cytotoxic, and mast cell viability remained >97% in all experiments (cells were discarded if viability was low, purity was rarely less than 99%).

3.2.4 Human mast cell line (HMC-1) culture

The human mast cell line HMC-1 was derived from a patient with mast cell leukaemia and expressed a number of mast cell surface antigens (c-kit, ICAM-1). It has immature mast cell characteristics and the mast cell mediators histamine and tryptase are also present (Butterfield et al, 1988). A low proportion (5%) of cells stained positively for tryptase, low levels of histamine (<1pg/cell) were observed, and cells did not bind IgE. However, a more differentiated subclone 5C6 was stained more positively with tryptase antibody (73% compared to 39% HMC-1), expressed the FcεRI α chain (65% compared to 12%) and comparable c-*kit* (64% compared to 59%) (Weber et al, 1996). HMC-1 cells were cultured in Iscove's medium containing 10% defined iron-supplemented FCS and 1.2mM monothioglycerol. Cell cultures were split when cell density reached $1.0\text{-}1.5x10^6$ cells/ml by removing half the cell suspension and replacing with an equal amount of fresh medium at least once a week, or earlier as required.

HMC-1 suspension culture

Phase contrast microscopy was used to check cell suspension to judge requirement for feeding. Fresh medium was used to replace half the cell suspension every 3-4 days. After every third feeding, a complete medium change was carried out by centrifuging cells at 300g for 5 minutes, gently resuspending in fresh medium and placing in new tissue culture flasks.

3.2.5 Assessment of leukocyte cell number by Kimura stain

A toluidine blue-containing stain originally developed for basophils (Kimura et al, 1973) was used to differentially count white blood cells and to identify and calculate percentage mast cells and eosinophils. The cytoplasm of mast cells stained bright pink, while eosinophils stained a light green colour and the cytoplasm of other cells remained clear. The nuclei of all leukocytes stained dark purple. All reagents for the stain were supplied by Sigma (Poole, UK). Once made, aliquots of the stain had a bench life of approximately one month, after which time the mixture began to deteriorate. The stain was made by mixing and filtering the following solutions: 11mls toluidine blue (0.05g toluidine blue added to 50mls 0.3M NaCl), 5ml 1/15M phosphate buffer, pH6.4 (1:3 mixture of 0.08M Na_2HPO_4 and 0.06M KH_2PO_4), 0.8mls light green (0.03g in 100mls water) and 0.5mls saturated saponin in 50% ethyl alcohol.

3.2.6 Flow cytometry analysis of cell surface adhesion receptors

Unpurified HLMC surface receptors were initially measured using double labelling (mast cells were identified by RPE-labelled *c-kit*). Purified mast cell surface adhesion receptors were subsequently analysed by indirect immunofluorescence with a single label (FITC-labelled secondary antibody detected the presence of cell-bound primary antibody). HLMC were treated overnight at 37°C with αIgE (1%), TGF-β (5ng/ml) or SCF (10ng/ml) and washed by centrifugation prior to flow cytometry labelling and analysis.

All procedures were carried out at 4°C. Cells were washed into FACS buffer (PBS, 1mM $CaCl_2$, 1mM $MgCl_2$, 1% BSA) and resuspended at 2-5x10^6/ml. 100µl cell suspension was placed in each tube and previously determined optimal concentrations of primary (mouse) mAb was added to cells for 15 minutes at 4°C. Controls were

performed with appropriate multiple isotype mouse IgG. Cells were washed from unbound antibody by the addition of 1ml FACS buffer and centrifuged at 4°C for 7 minutes at 250g. Supernatant was removed by inversion of each tube onto tissue. The cell pellet was resuspended in 1:10 dilution of FITC-conjugated $F(ab')_2$ fragments of rabbit anti-mouse Ig in FACS buffer and incubated at 4°C for 15 minutes. Cells were washed as before and resuspended in 300-500µl buffer. Cells were analysed using Lysys II version 1.1 software.

3.2.7 Intracellular antigen analysis by flow cytometry

Cells were resuspended at 10^6 cells/100µl in PBS/1% FCS, fixed in 100µl 4% w/v paraformaldehyde, pH7.4 for 20 minutes at 4°C and washed twice in permeabilisation buffer (PBS/1%FCS/0.1% saponin, pH7.4). Primary mAb or isotype control were diluted in permeabilisation buffer and incubated with cells for 30 minutes at 4°C. Cells were washed and secondary antibody was incubated with cells in buffer for 15 minutes at 4°C. Cells were washed, resuspended in PBS/1%FCS, and analysed by flow cytometry.

3.2.8 Mast cell adhesion assay

(i) Epithelium

Epithelial monolayers were gently washed twice with appropriate mast cell medium prior to use. Mast cells were resuspended at $0.1x10^6$/ml (HLMC) or $0.3x10^6$/ml (HMC-1) and 100µl per well added to pre-washed confluent epithelial monolayers grown on fibronectin-coated 96-well tissue culture plates for 48 hours (with 24 hours stimulation with cytomix if required). Mast cells were allowed to adhere for 1 hour at 37°C (following initial time course studies). Supernatant was gently removed from separate control wells and stored for later histamine analysis (for measurement of histamine release during adhesion). The sample wells were filled completely with medium warmed to 37°C and the plates were sealed with Mylar sealing tape, inverted, and centrifuged at 300rpm (15g) for 5 minutes at room temperature to remove non-adherent cells. All medium was gently removed from the inverted plate, and the remaining adherent cells lysed in 100µl ice-cold sterile water. Plates were frozen at -20°C until histamine analysis within two weeks. Control tubes containing HLMC (10^4) or HMC-1 ($3x10^4$) were lysed in sterile water and stored frozen with the

appropriate experiment (in 96-well plates) for measurement of total histamine content. All experiment conditions were performed in triplicate. Percentage adhesion was calculated as the average amount of histamine remaining in each experimental well compared to the average total amount of histamine added originally. The method of assessing mast cell adhesion by ^{51}Cr-radiolabelled HMC-1 was directly compared to measurement of cell adhesion by histamine content and visual counting.

(ii) Adhesion to matrix protein (fibronectin or galectin)

Human plasma fibronectin (40μg/ml), galectin-1 or galectin-3 were used to coat 96 well plates for 1 hour at 37°C. Protein was removed and wells were washed once with sterile phosphate-buffered saline (PBS). Non-specific sites were blocked using 1% BSA (for 1 hour at 37°C), and wells were washed with mast cell medium prior to the adhesion assay.

3.2.9 Mast cell adhesion modulation assays

Triplicate control aliquots of mast cells for measurement of total histamine content were kept for each of the experimental conditions. All experiments and procedures were performed at 37°C unless otherwise stated. Mast cell viability was assessed pre- and post-treatment by trypan blue exclusion. No treatment was cytotoxic or caused histamine release (except anti-IgE or A23187, which did cause histamine release), and mast cell viability remained >97% in all experiments.

(i) Cell adhesion molecule blocking mAb

The following well-characterised adhesion function-blocking mAb were incubated with the epithelial cell monolayers at room temperature for 15 minutes prior to the adhesion assay: R6.5 (ICAM-1) 15 μg/ml (Smith et al, 1988); 4B9 (VCAM-1) 20 μg/ml (Weller et al, 1991); HECD-1 (E-cadherin) 100 μg/ml (Watabe et al, 1994); SHE78-7 (E-cadherin) 1 μg/ml (St Croix et al, 1998); B2C10 (galectin-3) 20 and 100μg/ml (Liu et al, 1996); polyclonal anti-SCF 10 μg/ml.

The following adhesion function-blocking mAb were incubated with HLMC at room temperature for 15 min prior to the adhesion assay: BBIGI1 (ICAM-1) 10 μg/ml; IB4 (CD18) 5 μg/ml (Berman et al, 1996); HP1/2 (α_4 chain) supernatant diluted 1:2 (Weller et al, 1991); Ber-Act-8 ($\alpha_E\beta_7$) supernatant diluted 1:40 (Cepek et al, 1993);

LM609 (CD51/CD61, vitronectin receptor) 10μg/ml (Gawaz et al, 1987); P4C10 (β_1 chain) 20 μg/ml (McNulty et al, 1999); B2C10 (galectin-3) 20 and 100μg/ml (Liu et al, 1996). Controls were MoIgG at appropriate concentrations (upto 100μg/ml). Antibodies remained present during the adhesion assay.

(ii) Proteolytic digestion

To assess whether protease-sensitive structures on the surface of mast cells were involved, mast cells were pre-treated with trypsin, proteinase K and pronase (0, 1, 2.5, 7.5, and 10 μg/ml) for 20 min at 37°C in serum-free medium. An equal volume of medium containing 10% FCS was added, cells were pelleted, washed once more (using the same volume of medium) and counted prior to the adhesion assay.

(iii) Carbohydrate and proteoglycan blocking

To determine whether cell surface carbohydrates or proteoglycans were involved in mast cell adhesion to epithelial cells, HLMC and HMC-1 were pre-treated with the following at room temperature for 30 minutes: heparin (1mg/ml), hyaluronic acid (1mg/ml), galactose (50mM), lactose (50 and 200mM; for 30 minutes and 2h), mannose (50mM), mannose-6-phosphate (3.3mM), and fucoidin (1mg/ml).
BEAS-2B were incubated with these sugars (with the exception of mannose-6-phosphate) under the same conditions. These carbohydrates bind competitively (Incardona et al, 1996; Yednock et al, 1987) and remained present during the adhesion assay.

The following enzymes were used to pre-treat mast cells at 37°C for 30 minutes: heparinase I (2U/ml), heparinase III (2U/ml), hyaluronidase (300U/ml), neuraminidase (1U/ml), endo-α–N-acetylgalactosaminidase (0.3 U/ml), and β(1–3,4,6)-galactosidase (0.5U/ml). Mast cells exposed to enzymes were washed prior to the adhesion assay. β-galactosidase was also used to treat BEAS-2B monolayers under the same conditions. Monolayers were washed prior to adhesion assay. The concentrations of these carbohydrates and enzymes used were based on previously published data (Incardona et al, 1996; Baum et al, 1995; Rochon et al, 1994; Colgan et al, 1995; Symon et al, 1996; DeLisser et al, 1993; Inohara et al, 1995; Bhavanandan et al, 1983).

(iv) Modulation of cell signalling and metabolism

HMC-1 and HLMC were pre-treated with the following agents at 37°C: genistein 5μM for 30 minutes; pertussis toxin 0.1μg/ml for 2 hours; cholera toxin 0.1μg/ml for 2 hours; sodium azide 0.01M for 1 hour; deoxyglucose/sodium azide 0.01M/0.01M for 1 hour; cytochalasin D 12μM for 30 minutes and PMA 0.05 μg/ml for 10 minutes.

(v) Requirement for divalent cations

The Ca^{2+} and Mg^{2+} chelating agent EDTA (5mM), and the Ca^{2+} chelator EGTA (5mM) were added to HLMC at 37°C for 10 min prior to the adhesion assay. Because epithelial monolayers were detached in the presence of these chelators, monolayers were fixed with paraformaldehyde (0.4% and 4%) at room temperature for 5 min prior to the adhesion assay and washed twice with mast cell medium before use. Mast cells were also incubated with Mn^{2+} (5mM) at 37°C for 10 min to assess the effect of integrin activation on adhesion.

(vi) Mast cell activation

HLMC were activated with the following overnight treatments: sheep anti-human IgE (in the presence or absence of 10 μg/ml serine protease inhibitor leupeptin) or calcium ionophore A23187 (500 nM). Cells were washed three times prior to the adhesion assay. Controls were performed with sheep serum (1%). To assess the effects of pre-formed mast cell contents on mast cell adhesion, $1x10^6$ mast cells were lysed in 900μl sterile water and the solution was freeze-thawed to ensure release of cell contents into solution. The lysate was reconstituted with 100μl 10x PBS buffer. After centrifugation (7085g, 8 min), various dilutions of the lysate were incubated with mast cells at 37°C for 20 minutes prior to the adhesion assay.

(vii) Cytokine activation

Mast cells were treated overnight with SCF (10ng/ml) or TGF-β (5ng/ml) prior to the adhesion assay. BEAS-2B monolayers were treated overnight with cytomix (50ng/ml each of TNFα, IL-1β and IFN-γ). NHBE monolayers were treated with individual cytokines prior to adhesion.

3.2.10 Histamine assay

The double isotope radio-immunoassay used to measure histamine was based on the ability of the enzyme histamine methyl transferase (HMT) to transfer tritiated methyl groups from [^{3}H]methyl-S-adenosyl methionine (SAM) to histamine. Methyl histamine is extracted and quantified by liquid scintillation counting.

Tissue contractility, or spectroscopy/ fluorometry was previously used to measure sample histamine concentration, but these methods were not sensitive or suitable for high sample number. One of the first radioenzymatic assays was described by Snyder et al (1966) and was a single isotope method which involved the enzymatic transfer of a [^{3}H]methyl group from *S*[^{3}H]adenosyl-methionine-([^{3}H]SAM) to histamine, forming [^{3}H]-methylhistamine. The enzyme responsible for this transfer, histamine *N*-methyltransferase, was not commercially available, and was purified from male guinea pig brains or rat kidneys. The sensitivity of this assay was <1ng/ml. The assay was later adapted by adding a trace amount of [^{14}C]histamine (double isotope method) to correct for variabilities in the extraction of [^{3}H]methylhistamine, and the final product was [^{3}H]methyl[^{14}C]histamine. A standard curve was determined by incubation of known amounts of histamine with the enzyme.

Histamine assays were carried out using a capped sterile polypropylene clustertube system using standard radioactive isotope safety procedures. Standard controls were prepared from stock 10mM histamine in Tyrodes buffer (pH 7.4, Life Technologies), diluted to yield standards over the range 0-75pMol histamine. Aliquots of rat kidney histamine methyl transferase (HMT) (1ml) were diluted with 3ml Tyrodes buffer and 54µl (1MBq) [^{3}H]methyl-S-adenosyl methionine (SAM) and mixed. Samples and controls (75µl) were incubated for 45 minutes at 37°C with 20µl of HMT/^{3}H-SAM. At this stage 25µl diluted stock [^{14}C]histamine (1µl 295mCi/mmol histamine, added to 6ml tyrodes buffer) was added as an internal standard in initial experiments. The enzyme reaction was halted by adding NaOH (20µl, of a 10M solution). [^{3}H]methyl histamine was extracted by adding 0.9ml toluene:isoamylalcohol (4:1) and mixing. Phases were separated by centrifugation at 391g for 1 minute at room temperature and 0.75ml of the upper organic phase was transferred to mini-scintillation vials containing 3ml optiphase hisafe III scintillation fluid. The [^{3}H]/[^{14}C] dpm ratios were

initially determined from each sample (later only [^{3}H] was used) using a Tri-carb liquid scintillation analyser with the [^{3}H] window set at 0-12MeV and the [^{14}C] window at 25-256MeV. Histamine concentrations (pmol) were calculated using linear standard curves generated at each assay over the range 0-75pmol, and fitted by linear regression. This accounted for background readings and minor day-to-day variations in measurements of standards and samples. Data was processed using an Excel spreadsheet, ratios of [^{3}H] dpm/[^{14}C] dpm were plotted against standard control pmol, sample histamine values were determined by reference to the standard curve, and percentage mast cell adhesion was determined by comparison with total mast cell histamine added.

3.2.11 Statistical analysis of data

Each assay condition was performed in triplicate and mean values were calculated. The number of separate experiments carried out on different donors or cell line passage number was n. Results from a number of experiments (n=3 or above) were expressed as the overall mean±standard error of the mean (sem). Experiments carried out twice were expressed as mean±range. Mast cell adhesion modulation was analysed using the student's paired *t* test (two-tailed). Adhesion assay data comparing mast cell with eosinophil adhesion was analysed using the student's unpaired *t* test (two-tailed). Significance occurred when $p<0.05$.

3.3 Results

Development of the mast cell-epithelial cell adhesion assay

Differential cell counts were performed on enzyme-dispersed human lung cells after Kimura staining. Other cell types present were distinguished morphologically (Figure 3.1). Mast cells were purified by positive immunomagnetic selection. Variable numbers of rat anti-mouse IgG_1 magnetic dynabeads bound to mast cell-attached mouse anti-human CD117 mAb (Figure 3.2). Activated mast cells were distinguished morphologically, and were positively selected equally well (Figure 3.3). To ensure mast cell surface markers were not affected by enzyme dispersal and that positive selection was specific, cells were selected for a number of surface antigens (CD117, CD18, CD11a, CD11b, CD11c, $\alpha_E\beta_7$)(n=1) (Figure 3.4a) and mast cell percentage within these selected populations was assessed (n=1) (Figure 3.4b). Cells purified in this manner were used in further adhesion studies.

Percentage mast cell adhesion was quantified by measurement of adherent cell histamine content. The dual isotope histamine radioassay was developed from modification of an earlier published single isotope method (Snyder et al, 1966). Histamine levels of samples were calculated from standard curves generated at each assay (representative standard curves, Figure 3.5). Cell adhesion was calculated by comparing histamine content of remaining adherent mast cells with histamine content of total cells added. Although highly pure HLMC were used, small variations in purity would not affect measurement of adhesion since mast cells were thought to be the only cell type containing histamine within dispersed lung cells.

The mast cell adhesion assay assessed by histamine content was highly reproducible (intra-assay coefficient of variation of 12.5%), and compared favourably with mast cell adhesion assessed by ^{51}Cr labelling (intra-assay coefficient of variation of 15.8%). Mast cell adhesion measurement using histamine, ^{51}Cr and microscopic assessment were compared directly using HMC-1 adhesion to BEAS-2B (n=3) (Figure 3.6). This result showed that mast cell adhesion assessed using cell histamine content provided an accurate measure of adhesion and was suitable for further experiments.

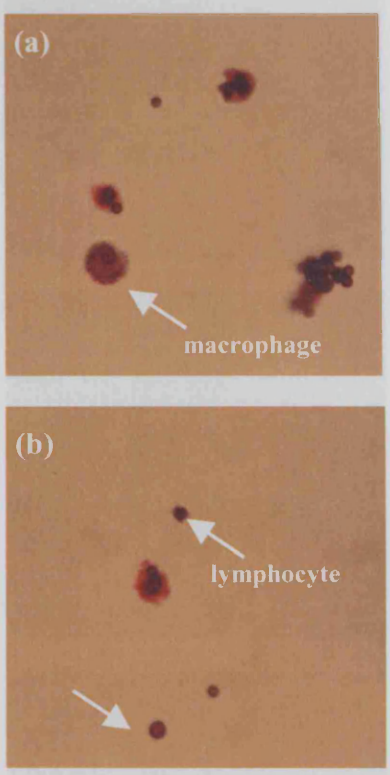


Figure 3.1 **Enzyme-digested human lung tissue, kimura stained (x400)**
Examples of cell types present directly after addition of *c-kit*-conjugated dynabeads, and prior to separation showing cells not attached to dynabeads (a) alveolar macrophage (b) lymphocytes. Also shown are HLMC attached to dynabeads and excess (unattached) dynabeads (distinguishable morphologically since they were slightly smaller than lymphocytes, were not stained by kimura and did not possess nuclei).

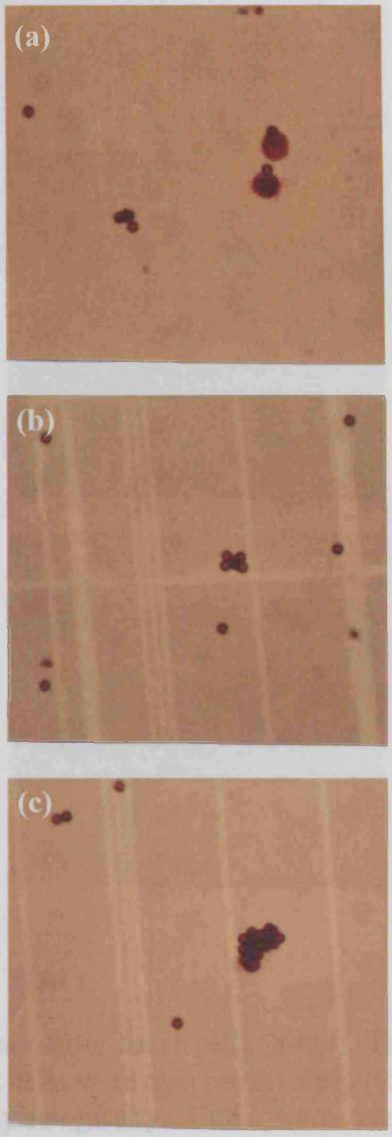


Figure 3.2 **Purified human lung mast cells attached to dynabeads via CD117 (*c-kit*) (x400)** Photograph of Kimura-stained human lung mast cells viewed on a haemocytometer, and showing a variable number of attached magnetic dynabeads (a) 1 bead (b) and (c) >1bead.

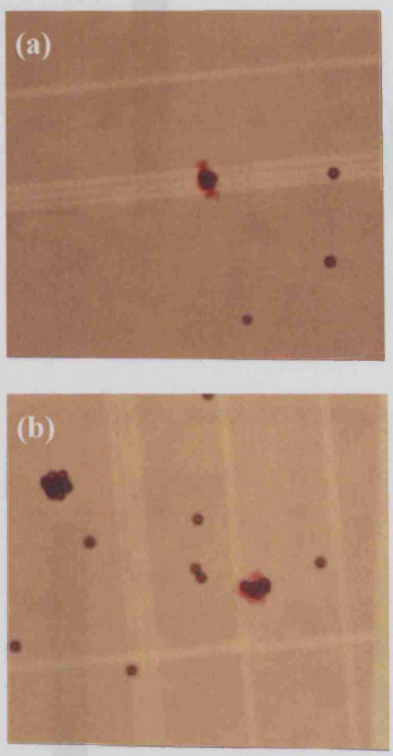


Figure 3.3 **Purified human lung mast cells (x400).** This photograph shows that activated mast cells were also positively selected and were identified as activated morphologically. Cells were viewed after addition to a haemocytometer. Photographs were taken:
(a) immediately after addition of dynabeads (prior to binding to cells)
(b) after 1.5 hours incubation with dynabeads

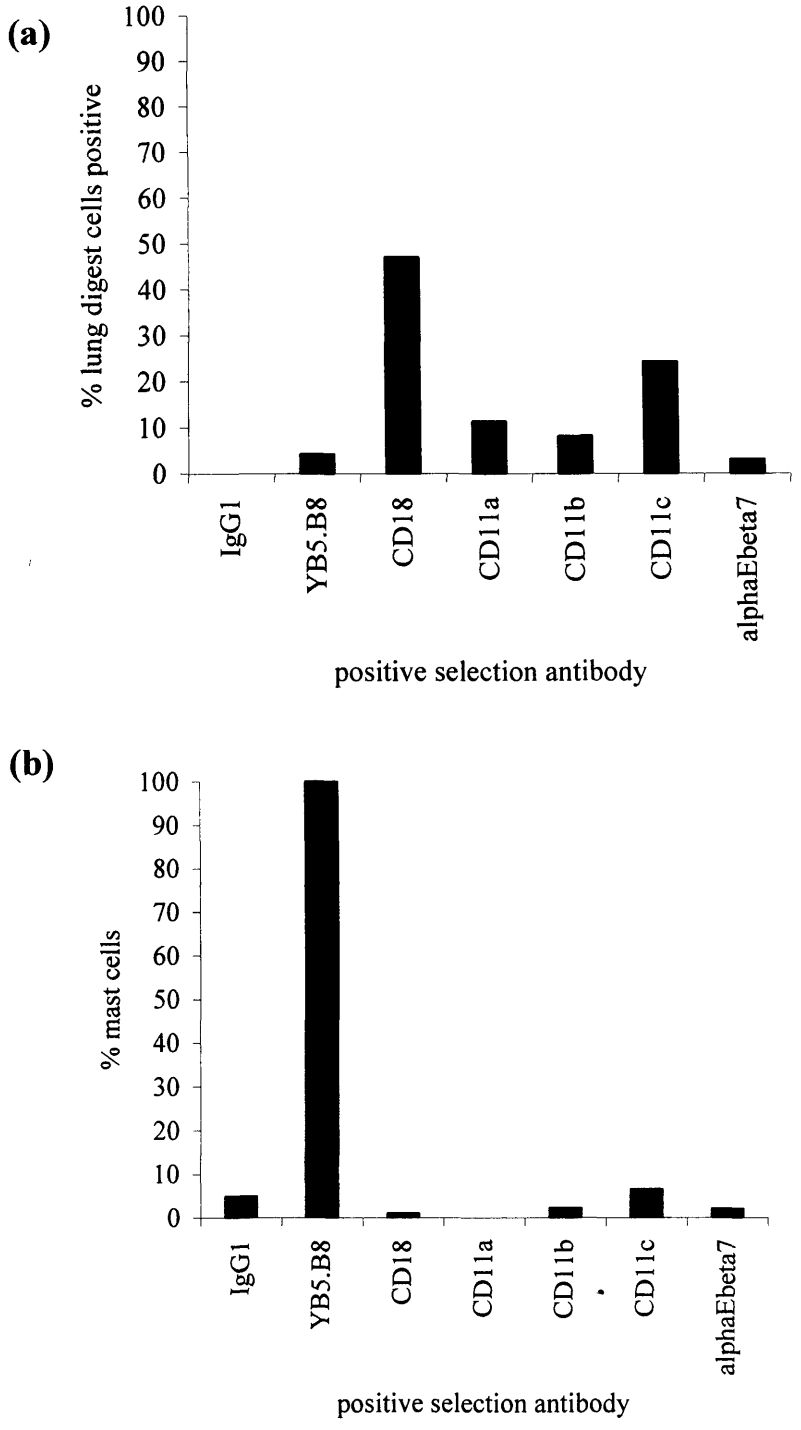


Figure 3.4 **Enzyme-dispersed human lung tissue cells selected positively for the presence of cell surface antigens (n=1)**
(a) percentage cells positive for indicated antigen
(b) percentage mast cells within positively selected cells from (a).
Therefore showing that adhesion receptors tested were not removed by enzyme-dispersion during cell separation procedure.

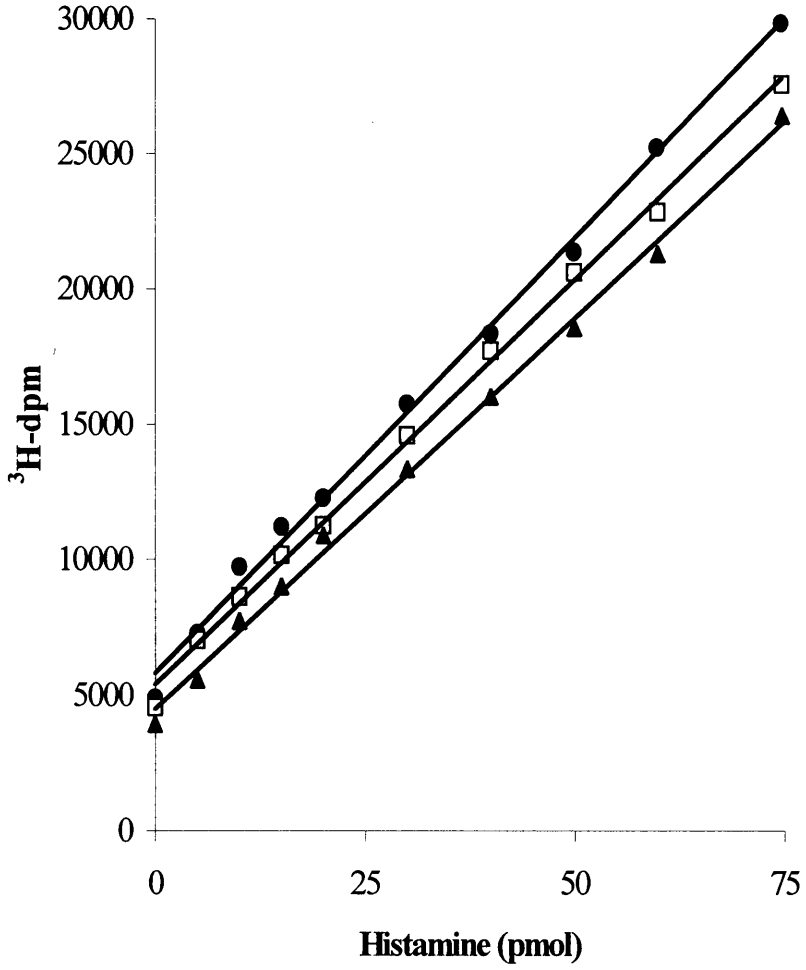


Figure 3.5 **Example histamine standard curves for three histamine assays.** Standard curves were established for each histamine assay. Each value was calculated from triplicate samples and the average dpm value at each concentration was plotted. If graphs were non-linear, their associated assays were not included, and the assays were repeated.

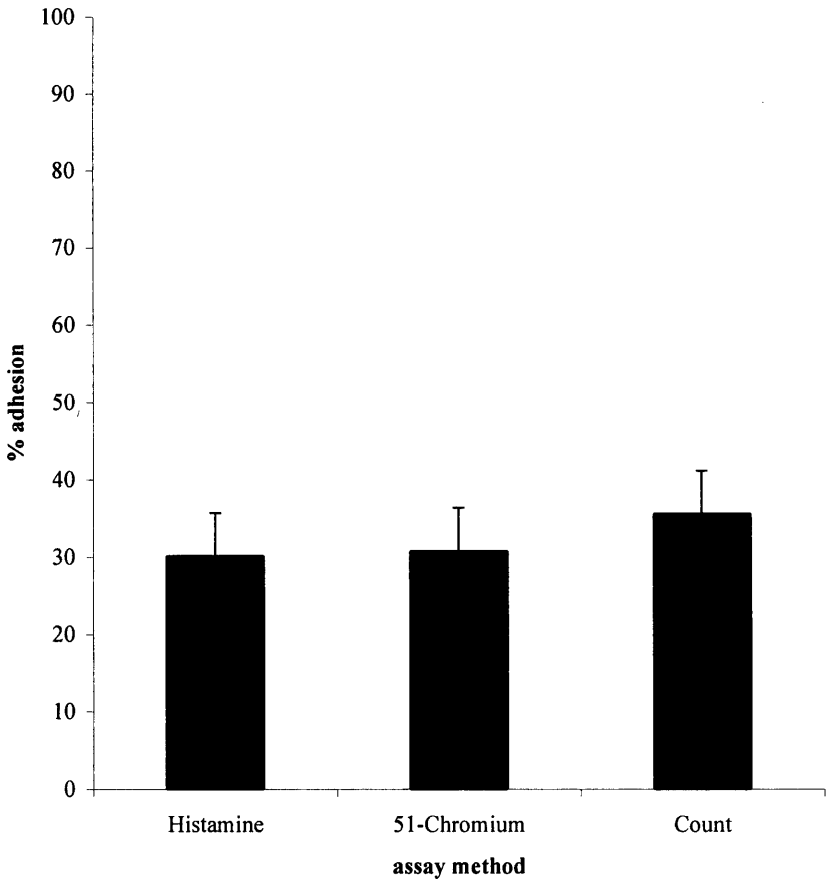


Figure 3.6 **Comparison of available methods that may be used to assess mast cell adhesion to bronchial epithelium (n=3).**
HMC-1 adhesion to BEAS-2B monolayers was assessed using mast cell histamine content, ^{51}Cr labelling or microscopic assessment by cell count (visual). Data is expressed as mean percent adherent cells±sem.

Histamine release was not altered compared to control cells by mast cell pretreatment, and no net histamine release occurred during adhesion assays, except degranulation-inducing treatments (table 3.1). Incubation of mast cells with epithelium did not evoke histamine release, in agreement with Varsano et al (1988). Appropriate controls were performed for 1% anti-IgE and calcium ionophore A23187 histamine release, and values were calculated with respect to total cell histamine content (table 3.1, n=3). Histamine was not detected when added and washed from epithelial cells, implying epithelial cells did not adsorb or internalise histamine (0 pmol, n=1). In addition, pretreatment of mast cells or epithelium with histamine did not affect adhesion (48.2% adhesion, untreated; HLMC histamine treatment, 38.7% adhesion; BEAS-2B histamine pretreatment, 44.2% adhesion, n=1).

Increased HLMC concentration had negligible effect on the proportion of mast cells adherent to bronchial epithelium (n=2) (Figure 3.7a). Accurate histamine measurement occurred within the range of sensitivity of the assay, HMC-1 cells contained less histamine than HLMC (since they are immature), and therefore a greater number of HMC-1 cells were added compared to HLMC during adhesion assays. In all subsequent experiments $1x10^4$/100µl HLMC and $3x10^4$/100µl HMC-1 were added to each well of the 96-well tissue culture plate. Initial time-course experiments indicated that adhesion reached a plateau by 15 minutes and remained stable for at least 1.5 hours (n=2) (Figure 3.7b). Adhesion was allowed to proceed for 1 hour in subsequent experiments to allow maximum adhesive interaction(s) to take place.

Treatment	Histamine release (%)
HR* after mast cell pre-treatment	3.8
HR* during adhesion assay	4.3
1% anti-IgE	23.7 ± 10.7
A23187	50.8 ± 19.2

* HR = histamine release in the absence of stimuli

Table 3.1 **Histamine release control experiment results (n=3)**
Histamine release after mast cell pretreatment and during adhesion are shown (and was less than 5% in all experiments). In addition, mast cell histamine release after degranulation treatments are shown for comparison. The optimal final concentration of anti-IgE (1%) was chosen from dose responses and the final A23187 concentration (500 nM) was determined from previously published HLMC studies (Peachell et al, 1988).

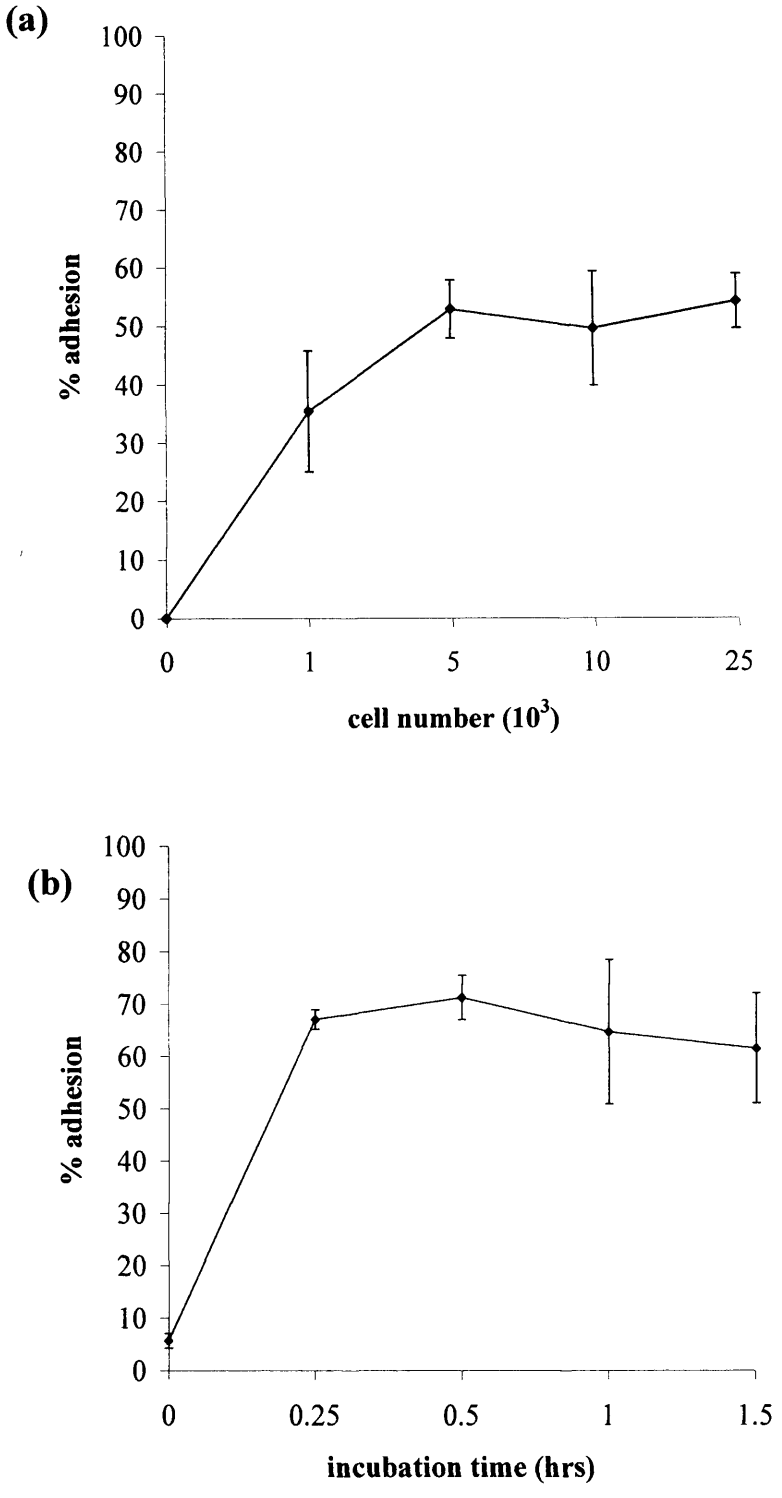


Figure 3.7 **Effect of increased HLMC concentration and incubation time on adhesion to BEAS-2B (n=2).**
Effect of (a) concentration (b) time course
Data is expressed as mean% adhesion ± range of values.

Basal levels of mast cell adhesion to bronchial epithelium

Adhesion of mast cells to epithelium was initially investigated by comparing adhesion of cell lines and primary cells. The bronchial epithelial cell line BEAS-2B was compared to primary epithelium NHBE (Clonetics). The percentage adhesion of HLMC to these cells was similar (n=3) (Figure 3.8a). Primary epithelial cells (NHBE) were not used routinely because they did not passage well long-term, and would have been prohibitively expensive. Tracheal explant-derived primary epithelial cells did not retain a normal phenotype after exposure to trypsin/EDTA (and became squamous). The bronchial epithelial cell line BEAS-2B was therefore used for subsequent investigation of the adhesion mechanism.

Adhesion of HLMC (n=45) or HMC-1 (n=13) to BEAS-2B were comparable and was significantly greater than eosinophil adhesion (n=7) (*p=0.0007) (Figure 3.8b). Intracellular flow cytometry confirmed that tryptase, but not elastase was present in these cells (Figure 3.9). Surface adhesion molecules present on HMC-1 included CD11a (LFA-1), CD54 (ICAM-1) and CD9 (Figure 3.10, 3.11). A small amount of CD18 and E-cadherin was also observed. HMC-1 adhesion was investigated for comparative purposes.

Involvement of known cell adhesion molecule families

Several potential integrin-immunoglobulin superfamily interactions could mediate adhesion between mast cells and epithelial cells. FACS analysis showed that HLMC expressed β_1, β_2 integrins and ICAM-1 (Figure 3.12, 3.13). A consistent number of HLMC expressed CD18, with a smaller proportion staining positive for CD11b and CD11c, in contrast to previous reports (Agis et al, 1996; Valent et al, 1989). Mast cell activation with anti-IgE resulted in reduced ICAM-1 expression (Figure 3.13). Murine mast cells expressed $\alpha_E\beta_7$ upon activation with anti-IgE or TGF-β (Smith et al, 1994). Activated HLMC did not appear to express $\alpha_E\beta_7$ (Figure 3.14) Mast cell treatment with function-blocking CD18, VLA-4, CD51/61 (vitronectin receptor) or epithelial treatment with function-blocking ICAM-1 or VCAM-1 mAb did not reduce adhesion (Figure 3.15). HLMC pretreatment with function-blocking anti-ICAM-1 (n=4) (Figure 3.16a) or β_1 mAb P4C10 (Figure 3.16b) did not inhibit adhesion.

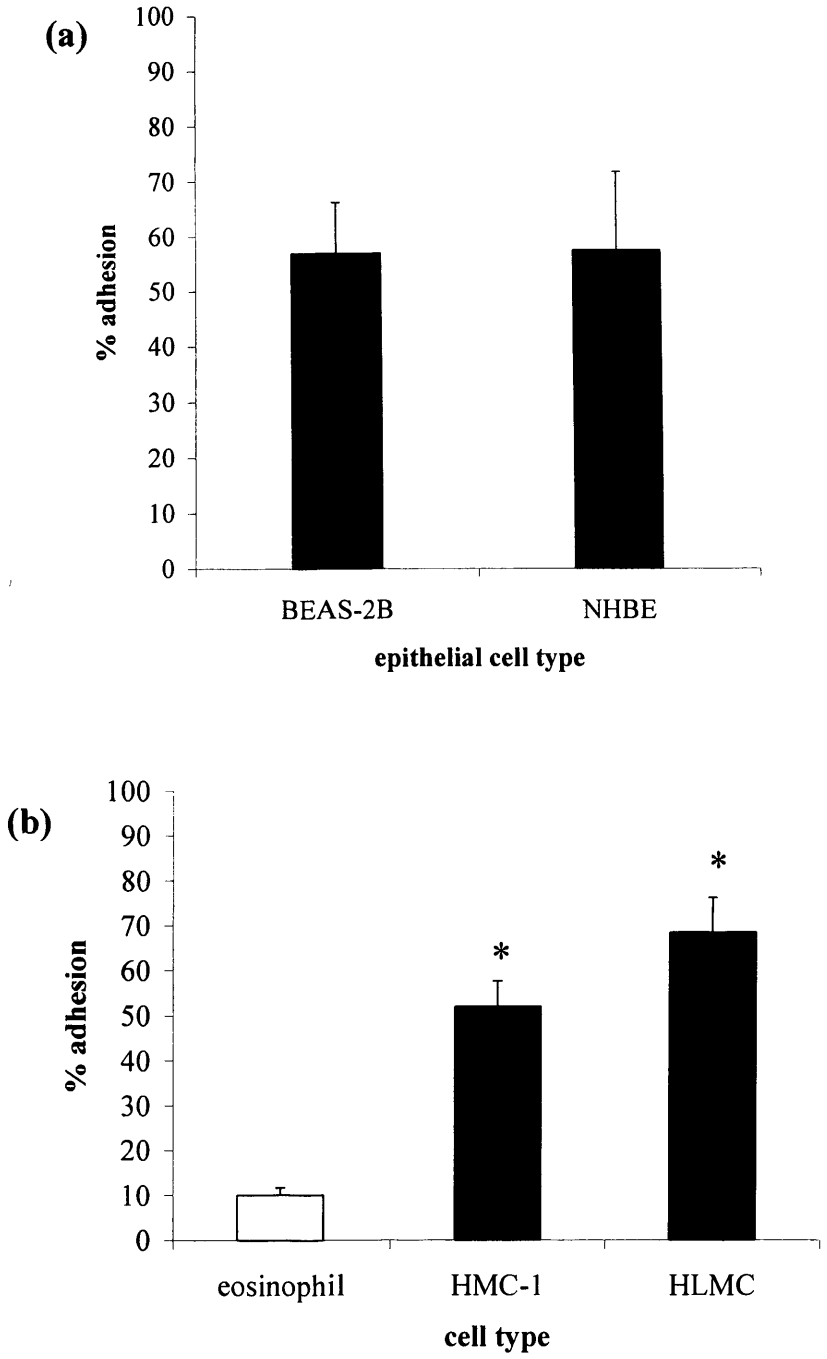


Figure 3.8 **Adhesion of human mast cell adhesion to bronchial epithelium: comparison of primary and cell lines**
(a) HLMC adhesion to BEAS-2B (SV-40 transformed bronchial epithelial cell line) and NHBE (primary bronchial epithelial cells) (n=3, identical HLMC donors, at the same time, for direct comparison)
(b) Eosinophil (n=7), HMC-1 (mast cell line, n=13) and HLMC (primary, n=45) adhesion to BEAS-2B.
Data is expressed as mean% adhesion ± sem.

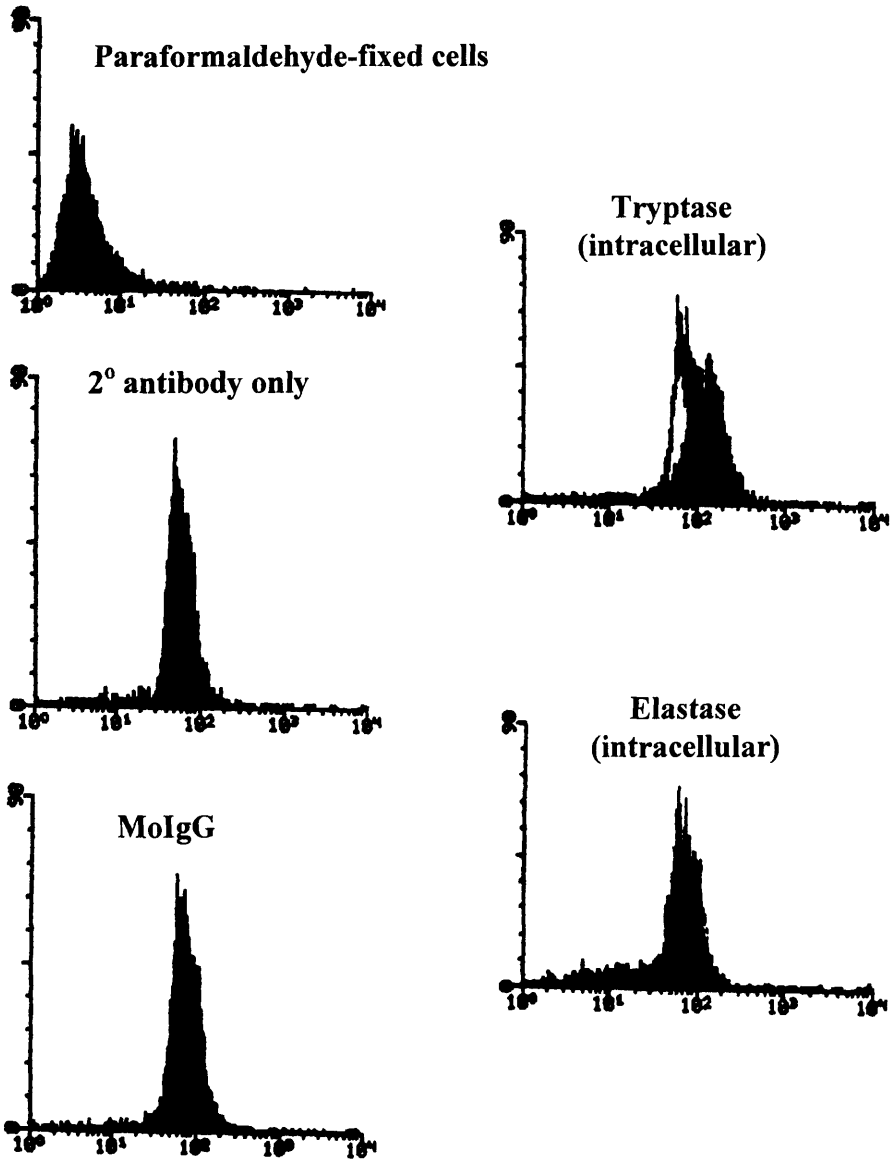


Figure 3.9 **Assessment of HMC-1 intracellular tryptase and elastase**
HMC-1 cells were analysed for intracellular tryptase and elastase by indirect flow cytometry. Cells were tested within two weeks of receipt of log-phase cells from Dr Butterfield (to ensure minimal changes from source cells). Results shown are representative of two experiments.

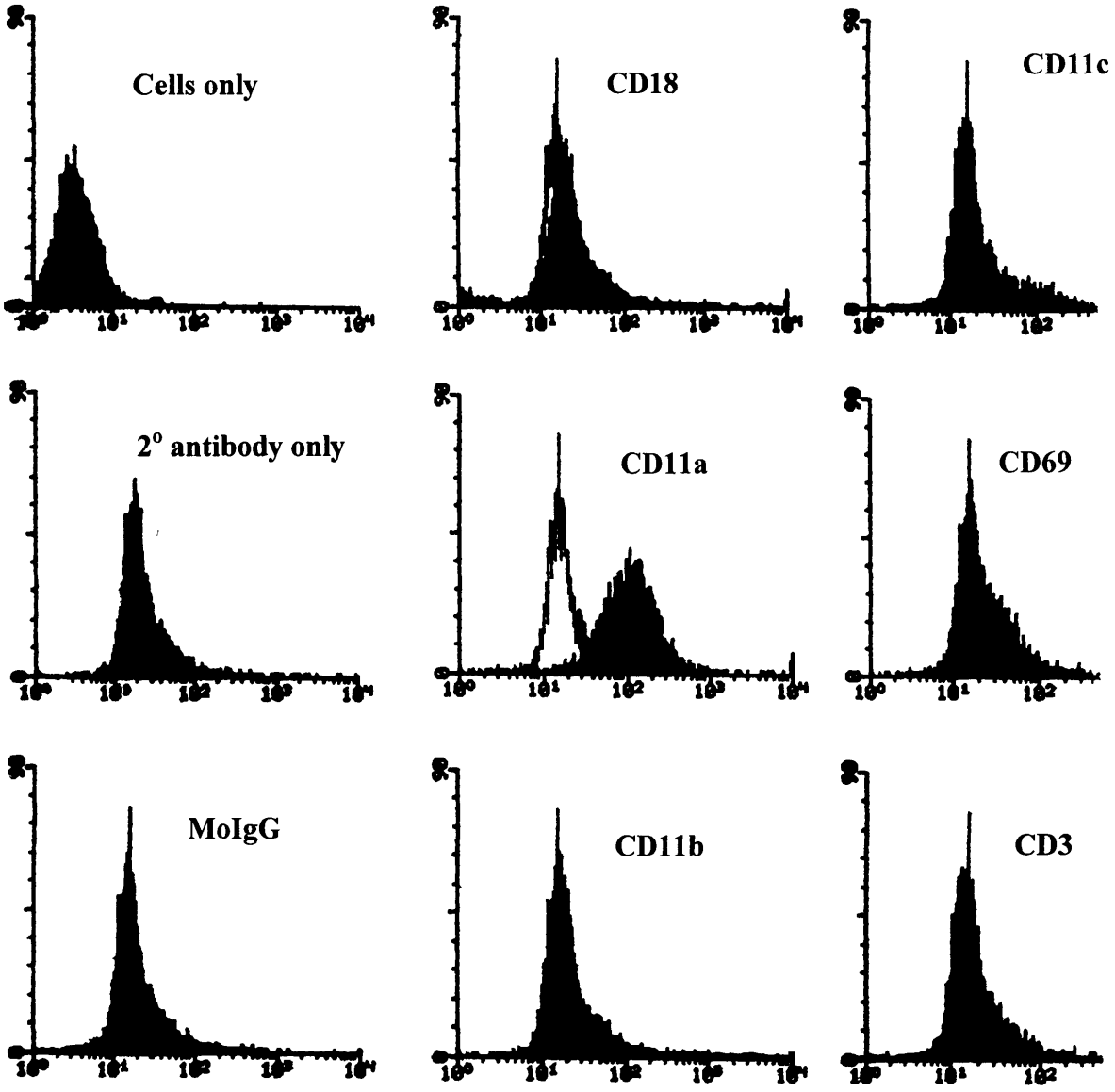


Figure 3.10 **Assessment of HMC-1 surface cell adhesion molecules by flow Cytometry (I).** Indirect labelling with FITC-labelled secondary antibody. Cells only, secondary antibody and MoIgE control apply to Figure 3.11 also. Cell surface CD18, CD11a, CD11b, CD11c, CD69 and CD3 were assessed. Cells were assessed within two weeks of receipt of cultured cells from Dr Butterfield. Results shown are representative of two experiments.

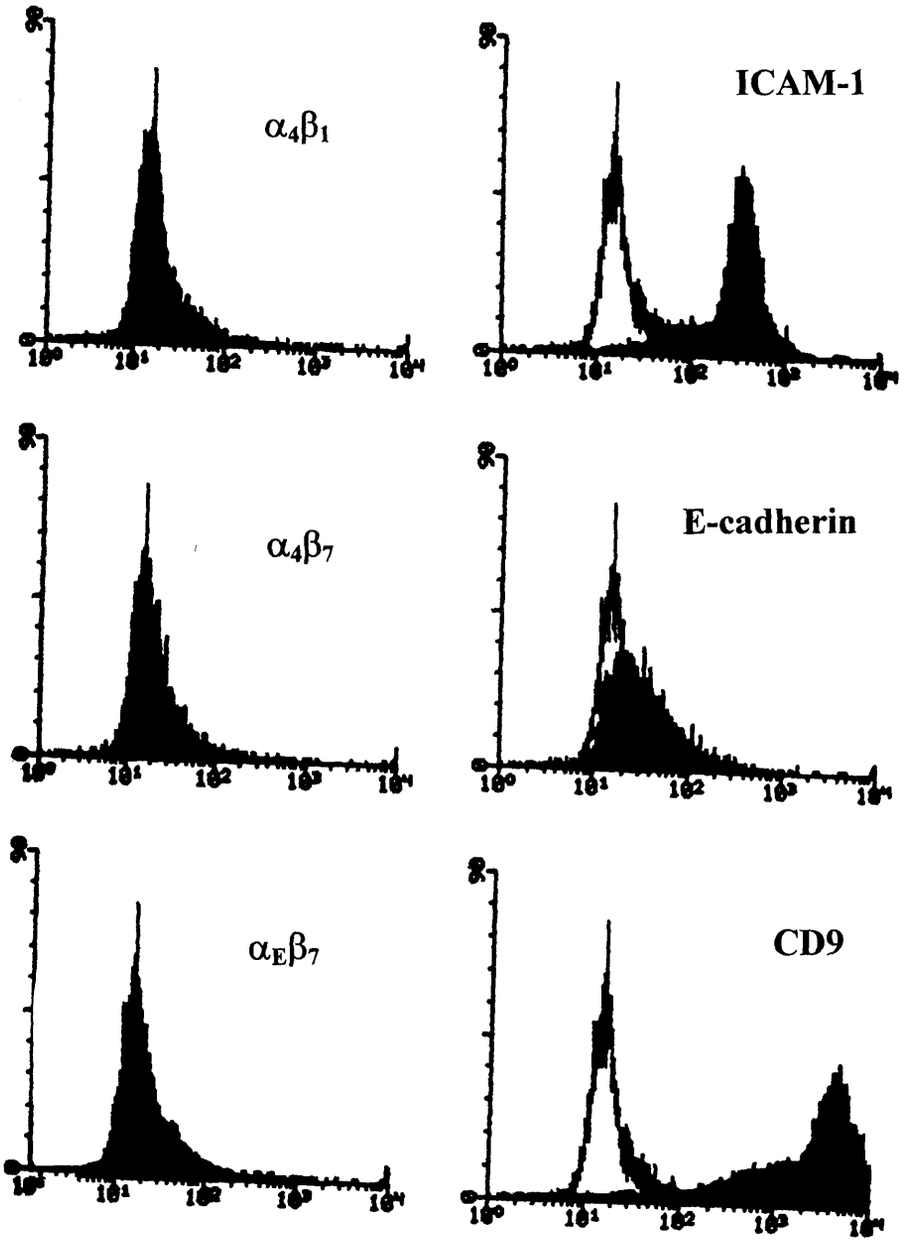


Figure 3.11 **Assessment of HMC-1 surface cell adhesion molecules by flow cytometry (II).** Indirect labelling with FITC secondary antibody. Cell surface $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_E\beta_7$, ICAM-1, E-cadherin and CD9 were assessed. Controls were shown in Figure 3.10. Cells were assessed within two weeks of receipt of cultured cells from Dr Butterfield. Results shown are representative of two experiments.

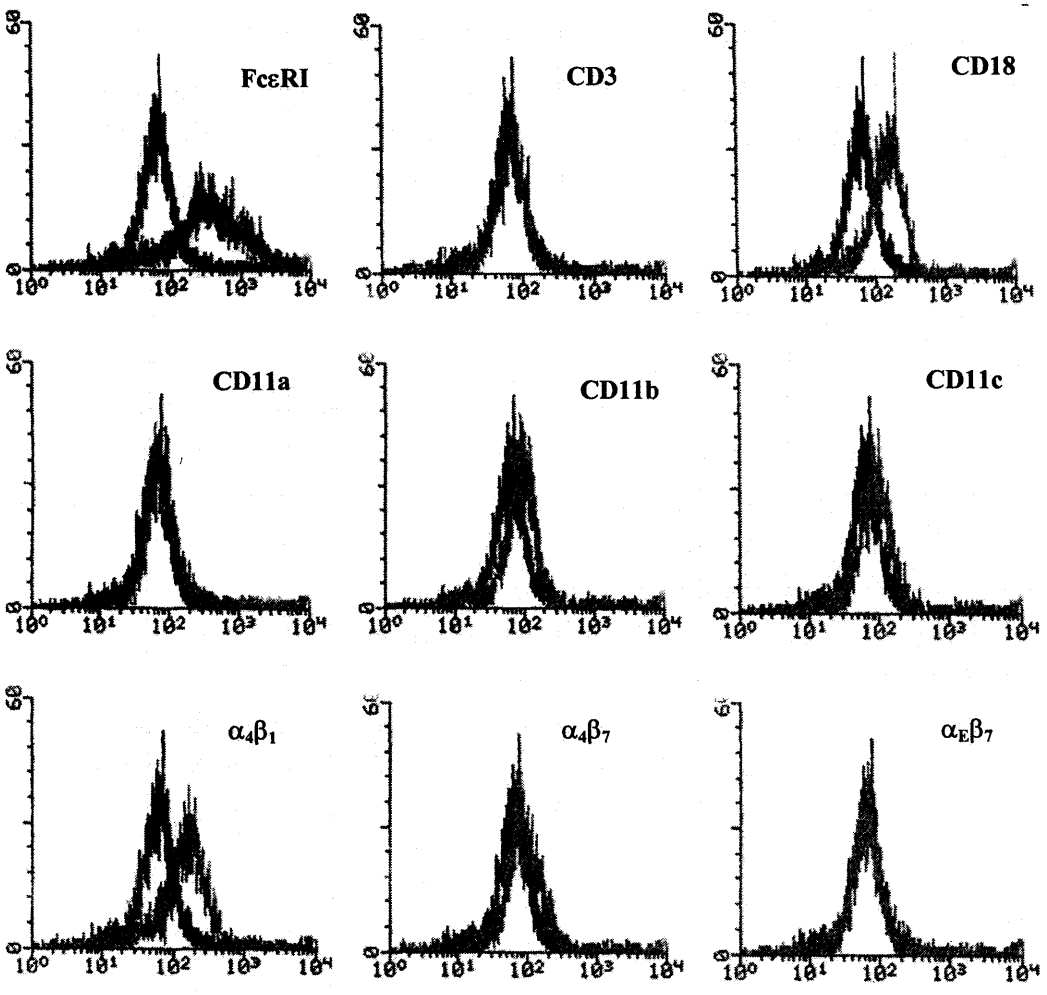


Figure 3.12 Assessment of human lung mast cell (HLMC) surface cell adhesion molecule expression. HLMC were cultured overnight in 10ng/ml SCF and adhesion molecule expression was assessed by indirect flow cytometry. MoIgG control is shown overlayed by specific cell adhesion molecule expression. Surface molecules measured were FcεRI, CD3, CD18, CD11a, CD11b, CD11c, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_E\beta_7$. Results shown are representative of three experiments.

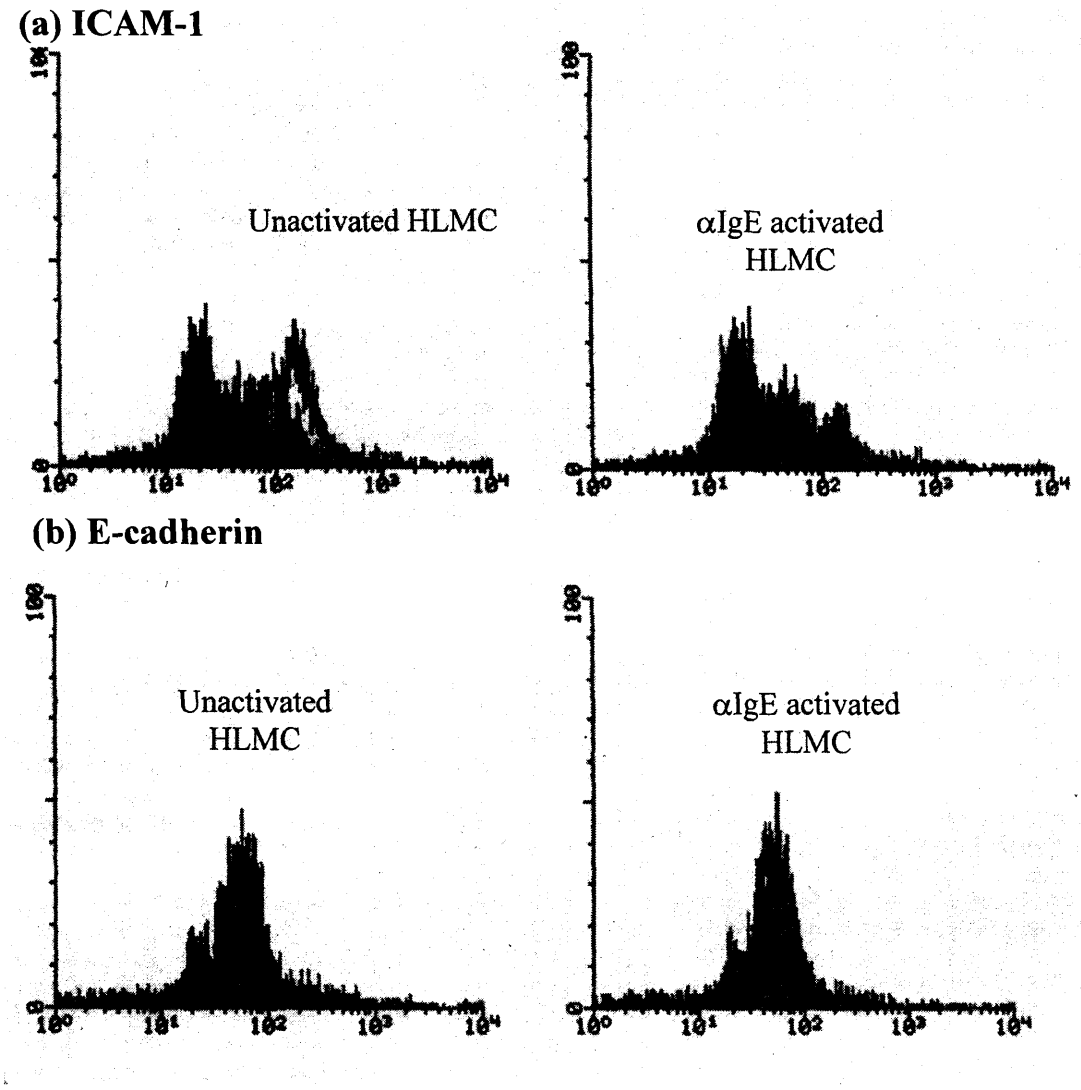


Figure 3.13 **Assessment of HLMC ICAM-1 and E-cadherin expression**
HLMC were treated for 24 hours with SCF alone (10ng/ml) or SCF (10ng/ml) and anti-IgE (1%). MoIgG is shown overlaid by adhesion molecule expression. (a) ICAM-1 (b) E-cadherin. Cells were assessed by indirect immunofluorescence using FITC-labelled secondary antibody and flow cytomety. Results shown are representative of two experiments

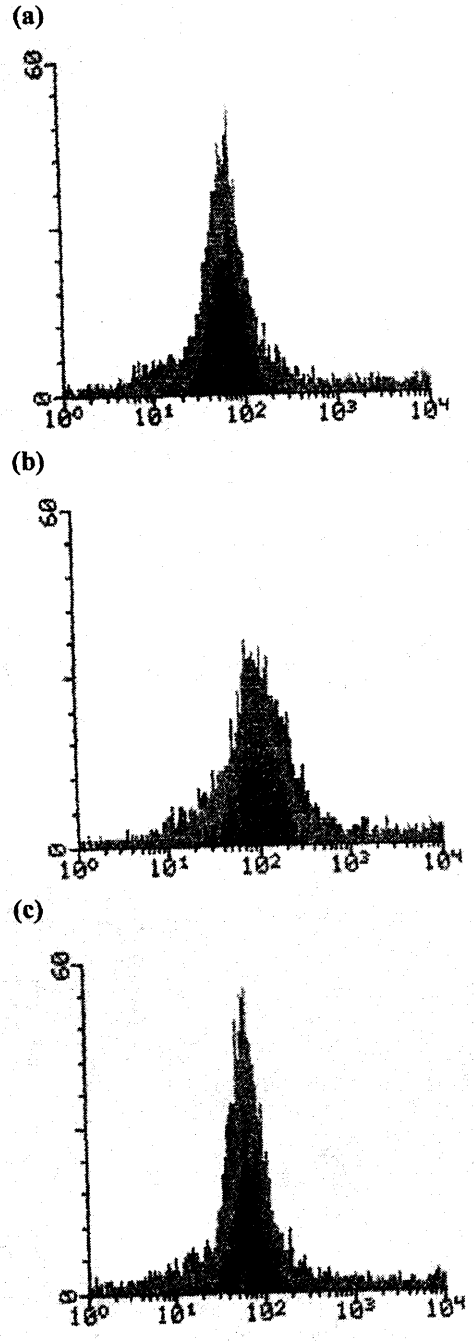


Figure 3.14 Assessment of HLMC $\alpha_E\beta_7$ expression
HLMC were treated for 24 hours with (a) SCF (10ng/ml) (a) SCF (10ng/ml) and anti-IgE (1%) (c) SCF (10ng/ml) and TGF-β (5ng/ml). MoIgG control is shown overlaid by $\alpha_E\beta_7$ expression. Results shown are representative of three experiments.

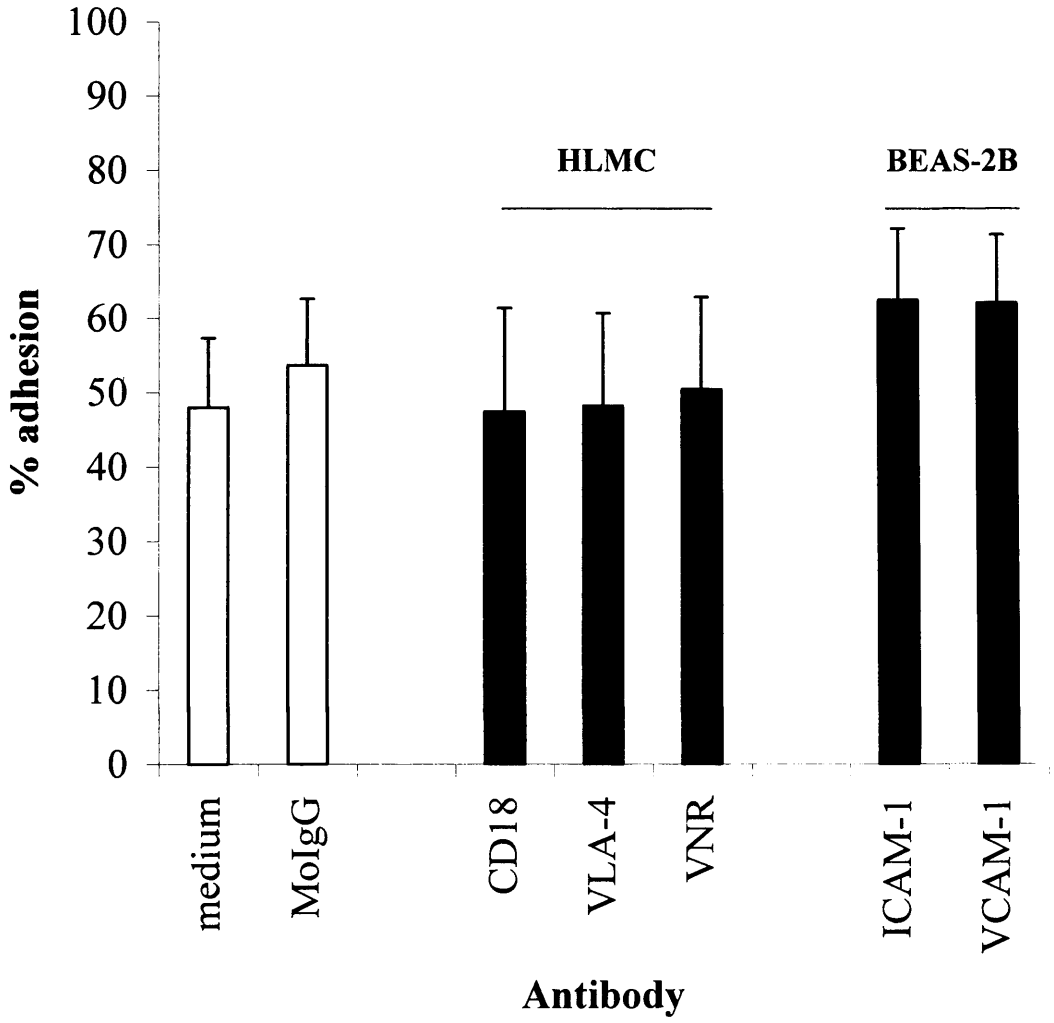


Figure 3.15 **Effect of cell adhesion molecule blockade on HLMC adhesion to BEAS-2B (n=4).** HLMC were pretreated with integrin mAb (CD18, β_2; VLA-4, CD49d/CD29, $\alpha_4\beta_1$; VNR, CD51/61, a_vb_3) or BEAS-2B epithelial monolayers were pretreated with IgSF mAb (ICAM-1, CD54; VCAM). Results are expressed as mean % adhesion ± sem.

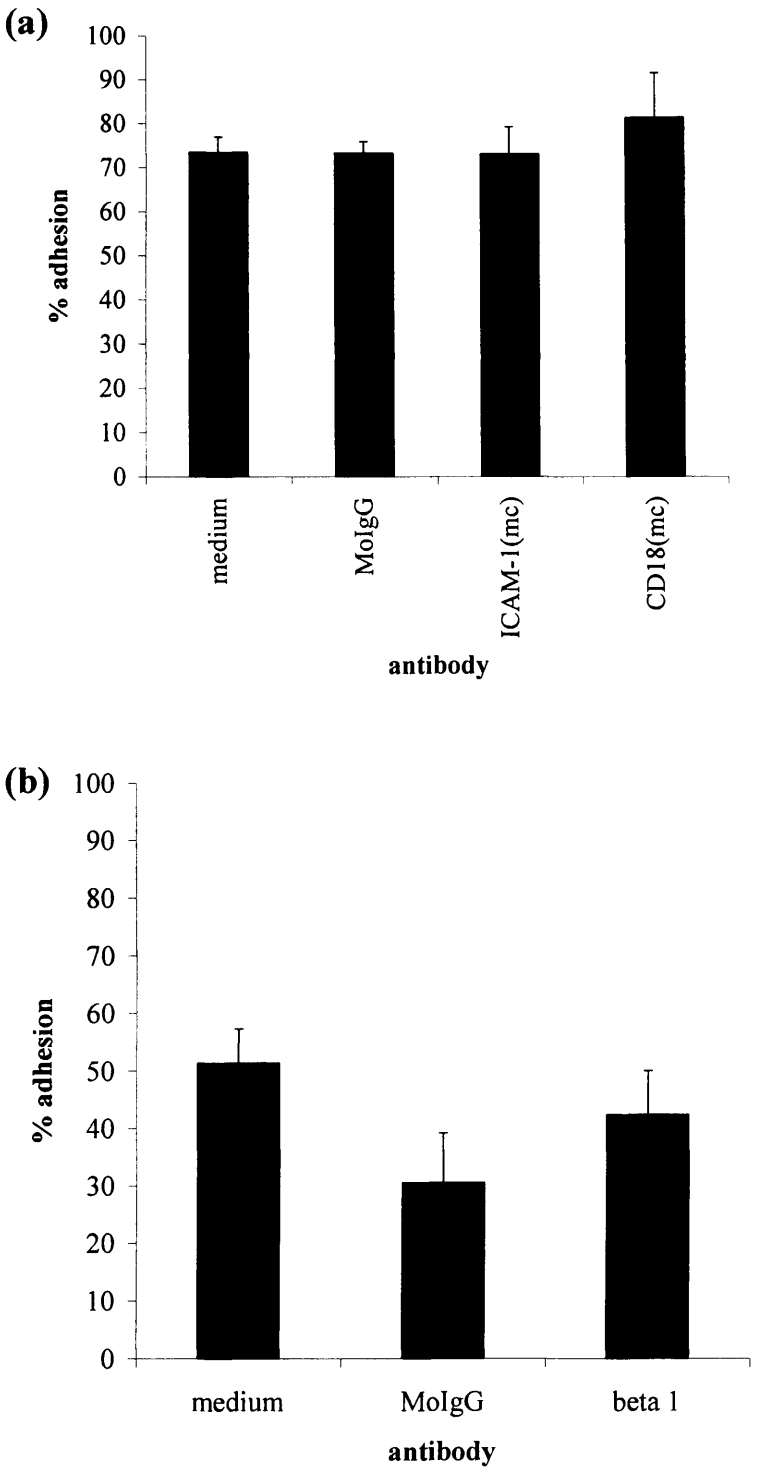


Figure 3.16 Adhesion blockade of HLMC adhesion to BEAS-2B.
HLMC were pretreated with the following function-blocking mAb
(a) ICAM-1 (CD54) or CD18 (β_2) mAb (n=4)
(b) CD29 (β_1) mAb (n=2)
Results are expressed as mean % adhesion ± sem (a) or range (b).

The blocking activity of the β_1 mAb was confirmed by attenuation of HLMC adhesion to human plasma fibronectin (n=2) (Figure 3.17a). Mast cell adherence to epithelial cells was not β_1-mediated and therefore the human plasma fibronectin required for epithelial cell anchorage did not contribute to adhesion. Epithelial cells used for adhesion assays were confluent when assessed microscopically. Adhesion to human plasma fibronectin was β_1-mediated, although adhesion was not through $\alpha_4\beta_1$ (Figure 3.17b). Other β_1 integrins may mediate adhesion to fibronectin (and is most likely to be $\alpha_5\beta_1$). Adhesion was not β_2-mediated.

Mast cell pretreatment with function-blocking $\alpha_E\beta_7$ mAb BerAct-8 did not block adhesion (n=2)(Figure 3.18a). Pretreatment of unstimulated or cytomix-stimulated epithelium with E-cadherin mAbs (which block homotypic E-cadherin adhesion) did not inhibit adhesion (n=4) (Figure 3.18b).

Extracellular calcium is required for integrin-, cadherin- and C-type lectin-mediated adhesion (Carlos and Harlan, 1994). EDTA (ethylenediaminetetraacetic acid), a Ca^{2+}/Mg^{2+} chelator and EGTA (ethyleneglycol-bis(β-aminoethyl)-N,N,N',N'-tetraacetic acid), a selective Ca^{2+}-chelator (which binds one cation per molecule) are generally used to investigate extracellular cation requirement. Epithelial cells detached in the presence of EDTA or EGTA, and therefore fixation with paraformaldehyde before addition of EDTA/EGTA-treated mast cells was required. Four-percent paraformaldehyde fixation reduced adhesion significantly and the remaining adhesion was not affected by HLMC cation chelation with 5mM EDTA or EGTA (n=2) (Figure 3.19a). Epithelial fixation with 0.4% paraformaldehyde resulted in a small, non-significant decrease in adhesion, which was also not reduced further in the presence of EDTA or EGTA (n=4) (Figure 3.19b). Mast cell integrin activation by Mn^{2+} had no effect on adhesion of untreated or SCF-treated HLMC to BEAS-2B (n=2) (Figure 3.20a), in contrast to HLMC adhesion to human plasma fibronectin. Adhesion to fibronectin was reduced by EDTA and EGTA, although these results were not significant (n=3) (Figure 3.20b).

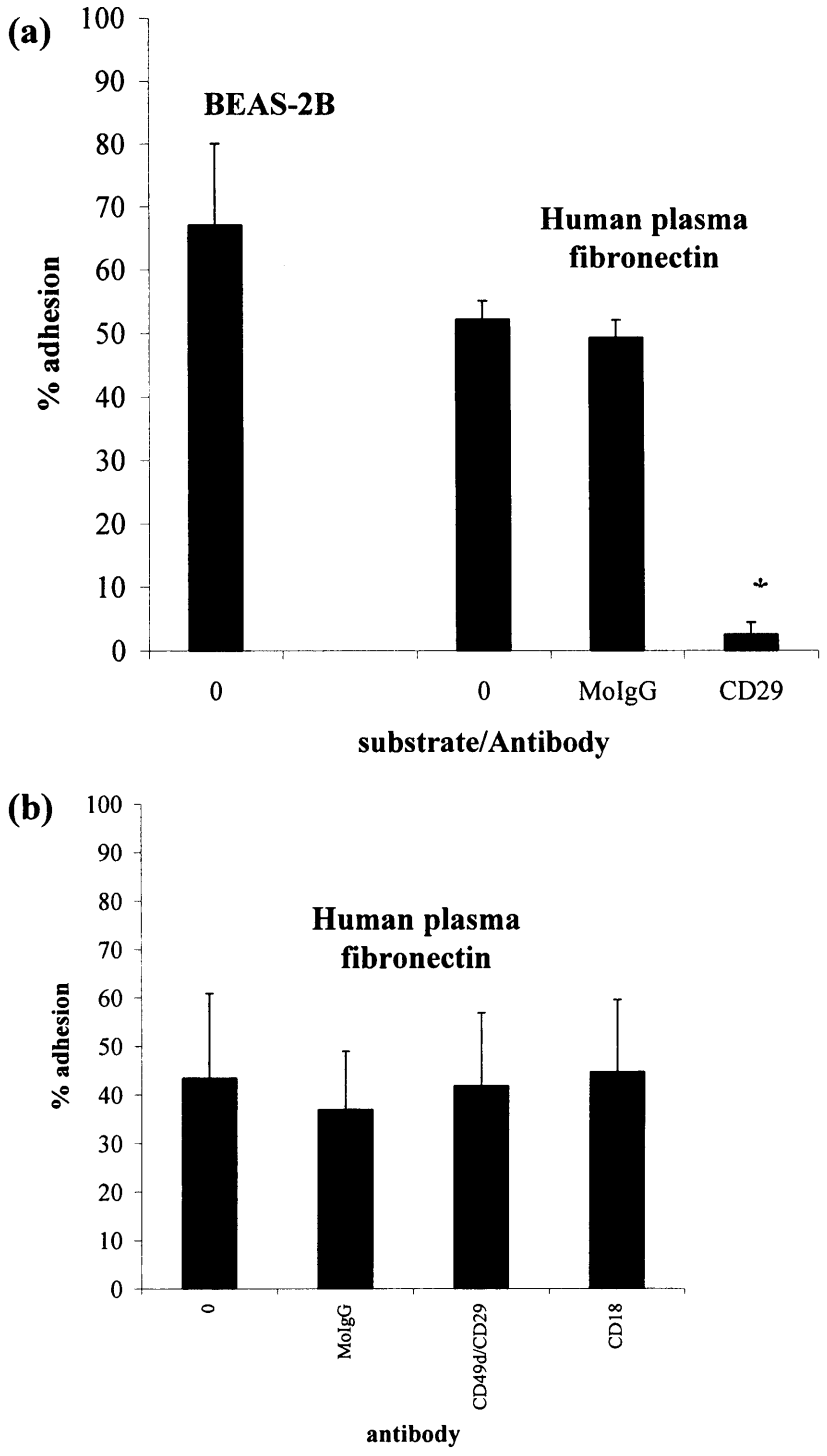


Figure 3.17 Treatment of HLMC with function-blocking mAb prior to adhesion To human plasma fibronectin (n=3)

(a) CD29 (β_1; P4C10) (b) CD49d/CD29 ($\alpha_4\beta_1$; HP1/2) and CD18 (β_2; IB4). Antibodies are shown as cell surface CD number (target cell adhesion molecule; mAb clone). Data is expressed as mean % adhesion ± sem. Large error bars indicate large differences in basal adhesion of HLMC to human plasma fibronectin.

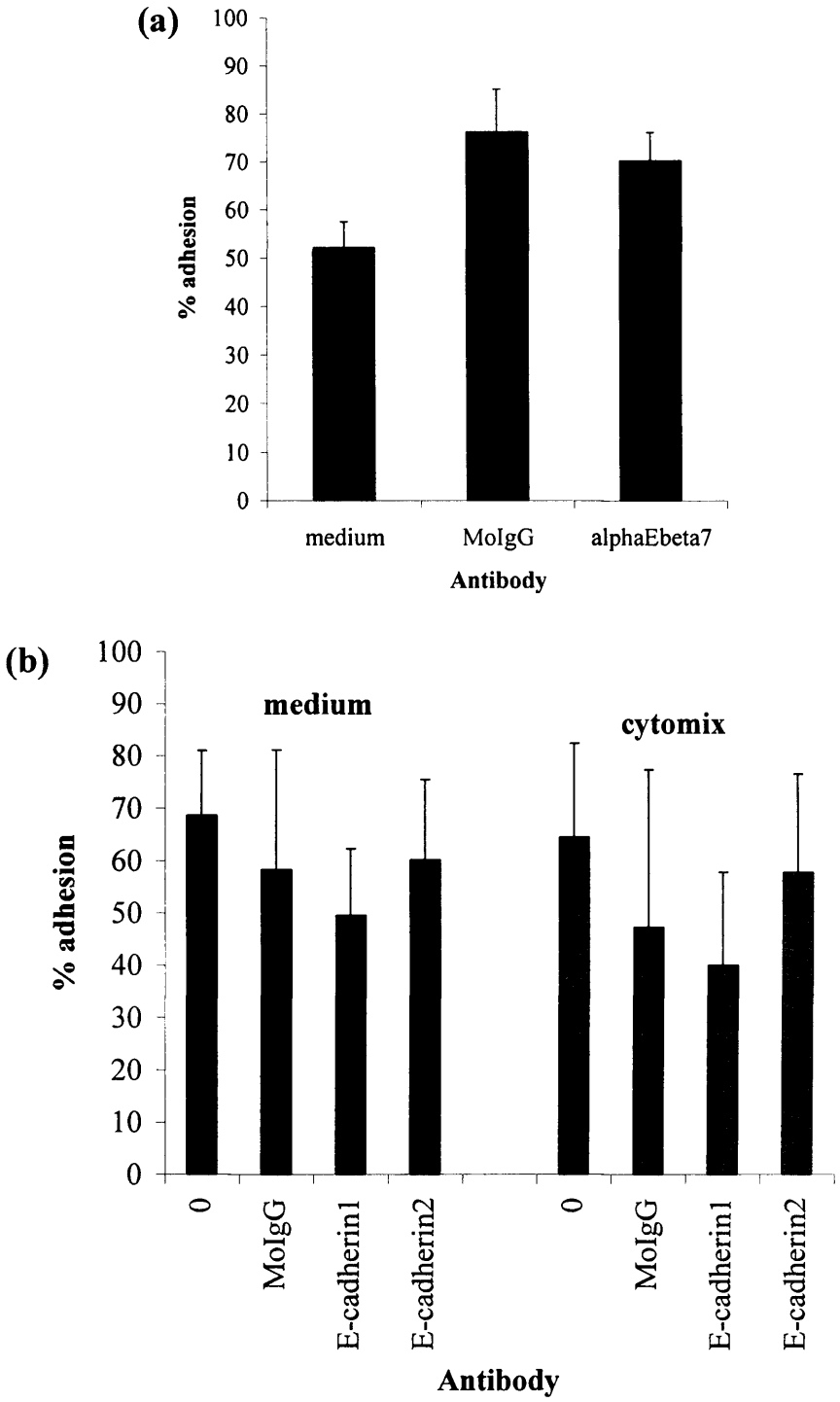


Figure 3.18 HLMC adhesion to BEAS-2B: effect of function-blocking mAbs
(a) HLMC pretreated with $\alpha_E\beta_7$ mab (BerACT8) (n=2)
(b) Unstimulated or cytomix-stimulated BEAS-2B pretreated with E-cadherin mAbs SHE-78 (1) and HECD-1 (2) (n=3)
Data is expressed as mean % adhesion ± range or sem

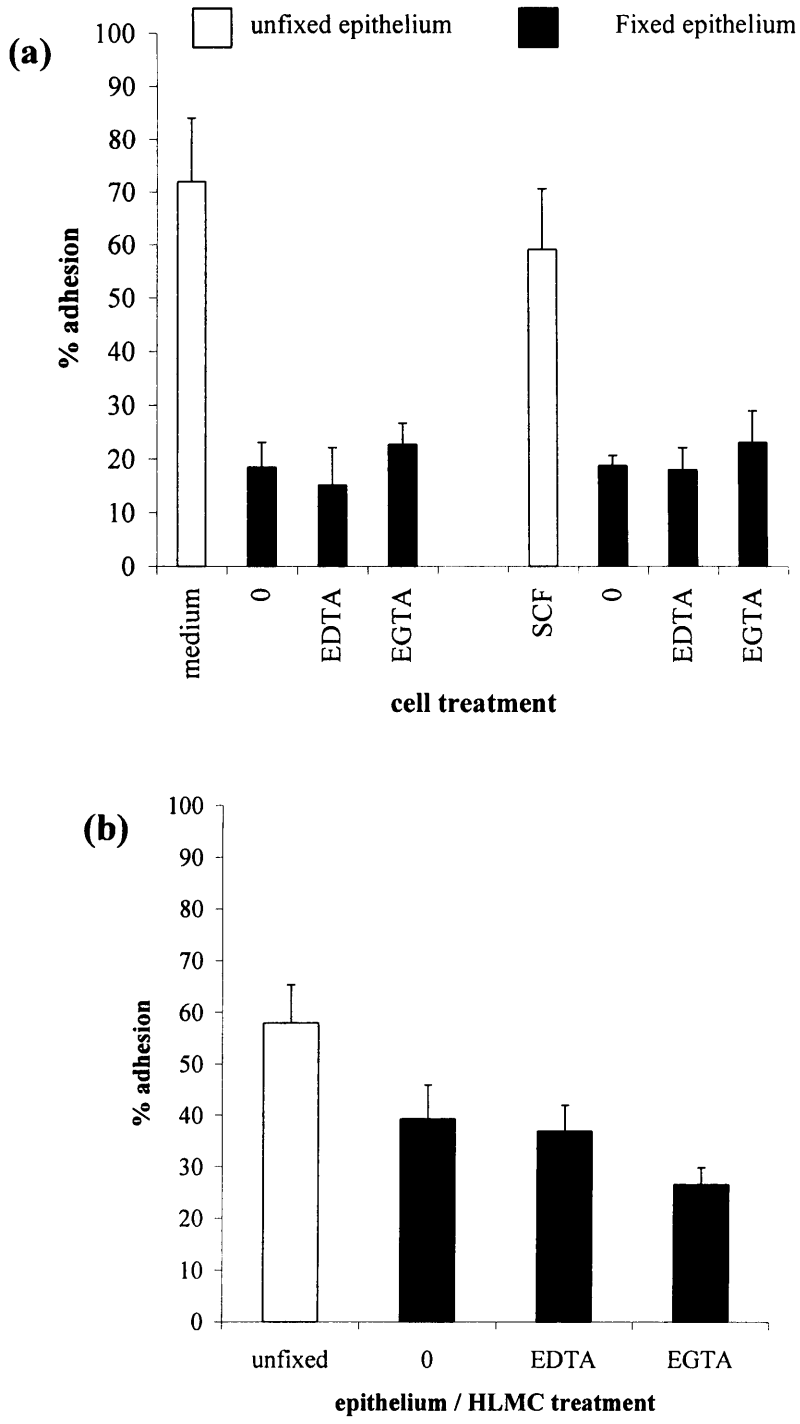


Figure 3.19 Effect of HLMC cation chelation by EDTA or EGTA prior to adhesion assay (a) Adhesion of untreated and SCF-treated HLMC to 4% paraformaldehyde-fixed BEAS-2B
(b) Adhesion of SCF-treated HLMC to 0.4% paraformaldehyde-fixed BEAS-2B. Results are expressed as mean % adhesion ± sem.

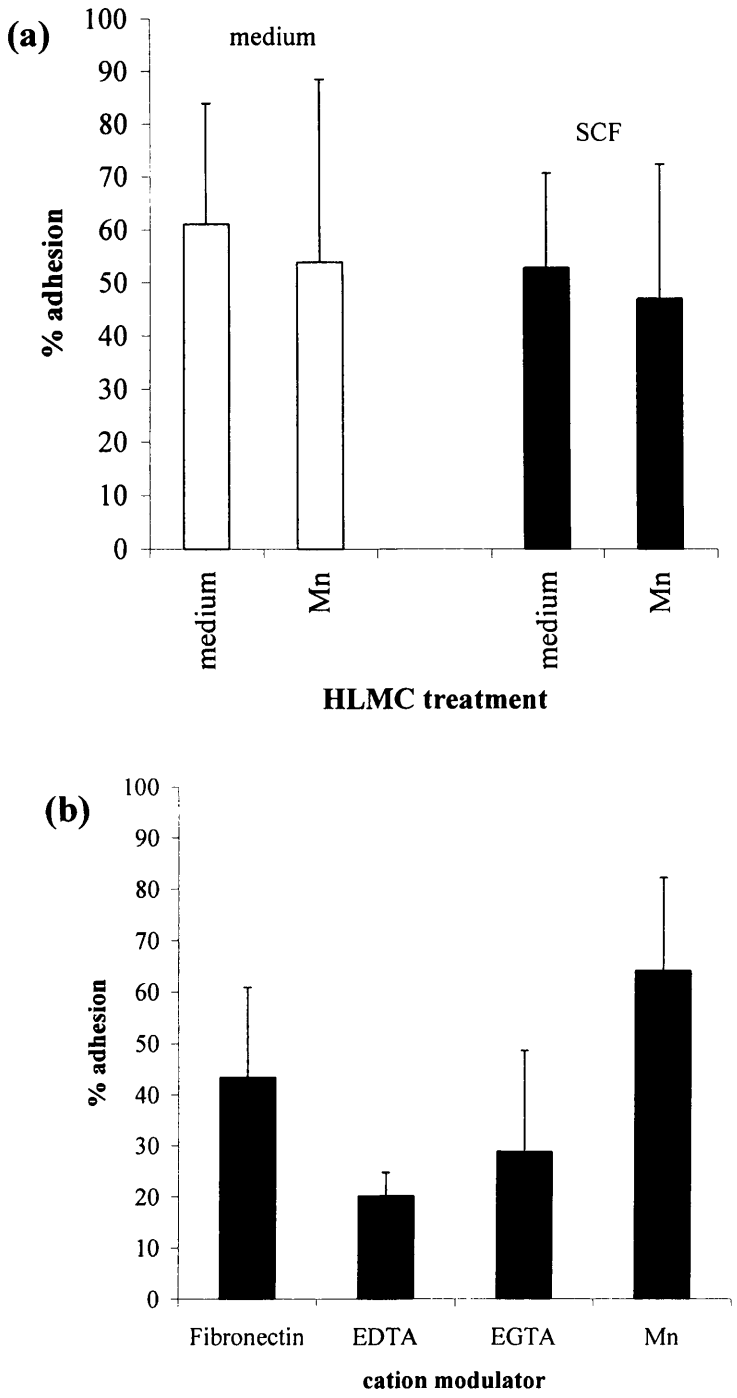


Figure 3.20 **Mn^{2+}-activated HLMC adhesion to BEAS-2B or fibronectin**
(a) Untreated and SCF-treated HLMC adhesion to BEAS-2B (n=2)
(b) adhesion to human plasma fibronectin (n=3)
Results are expressed as % adhesion ± range (a) or sem (b).
Errors bars are particularly large due to a large variation in basal adhesion to epithelium between individual donors used in these experiments.

Cytokine or growth factor treatment

The effect on adhesion of epithelial stimulation with proinflammatory cytokines alone or in combination (TNF-α, IFN-γ and IL-1β) was examined. BEAS-2B activation with cytomix (n=3) (Figure 3.21a) or NHBE activation with individual cytokines (n=1) (Figure 3.21b) did not influence mast cell adhesion despite enhanced BEAS-2B ICAM-1 expression and VCAM-1 induction by cytomix stimulation.

Mast cells express high levels of the SCF receptor *c-kit*. SCF plays a regulatory role in mast cell differentiation, function and survival. SCF promotes *c-kit*-dependent mast cell degranulation and mediator release, as well as enhancing FcεRI-dependent mediaor release. Airway epithelial cells produce SCF (Kim et al, 1997; Wen et al, 1996), although whether this is membrane-bound (fibroblast SCF) or secreted has not been confirmed. Murine mast cells adhere to fibroblasts through SCF receptor-SCF (Adachi et al, 1992). Whether mast cells adhesion to epithelial cells was affected by SCF-mediated signalling or involved membrane-bound SCF/*c-kit* was investigated. Overnight SCF treatment of HLMC failed to modulate adhesion (n=3) (Figure 3.22a) and incubation of epithelial monolayers with neutralising polyclonal goat anti-human SCF antibody did not reduce adhesion (n=3) (Figure 3.22b).

Overnight TGF-β pretreatment or anti-IgE activation has been shown to induce murine mast cell $\alpha_E\beta_7$ (Smith et al, 1994). Human T lymphocyte $\alpha_E\beta_7$ may interact with intestine epithelial E-cadherin. Mucosal T lymphocytes and mast cells are localised within epithelium, and were thought to ulitise this adhesion pathway. Unactivated or HLMC after 24 hour culture with TGF-β (5ng/ml) or anti-IgE (1%) did not express detectable levels of $\alpha_E\beta_7$. $\alpha_E\beta_7$ was not removed by lung tissue enzymatic dispersal since it was detected on lung T cells. Other surface markers were also not affected by mast cell activation (Table 3.2). Overnight HLMC culture with TGFβ (5ng/ml) did not affect adhesion to BEAS-2B (n=3, p=0.26), even in the presence of increasing epithelial TNF-α stimulation (n=3) (Figure 3.23a). Anti-IgE activation of HLMC significantly decreased adhesion, although no decrease in cell adhesion molecule expression was detected. There was no additional significant effect of epithelial TNF-α stimulation. Overnight pre-treatment did not cause loss of cell viability when assessed by trypan blue exclusion (n=1) (Figure 3.23b).

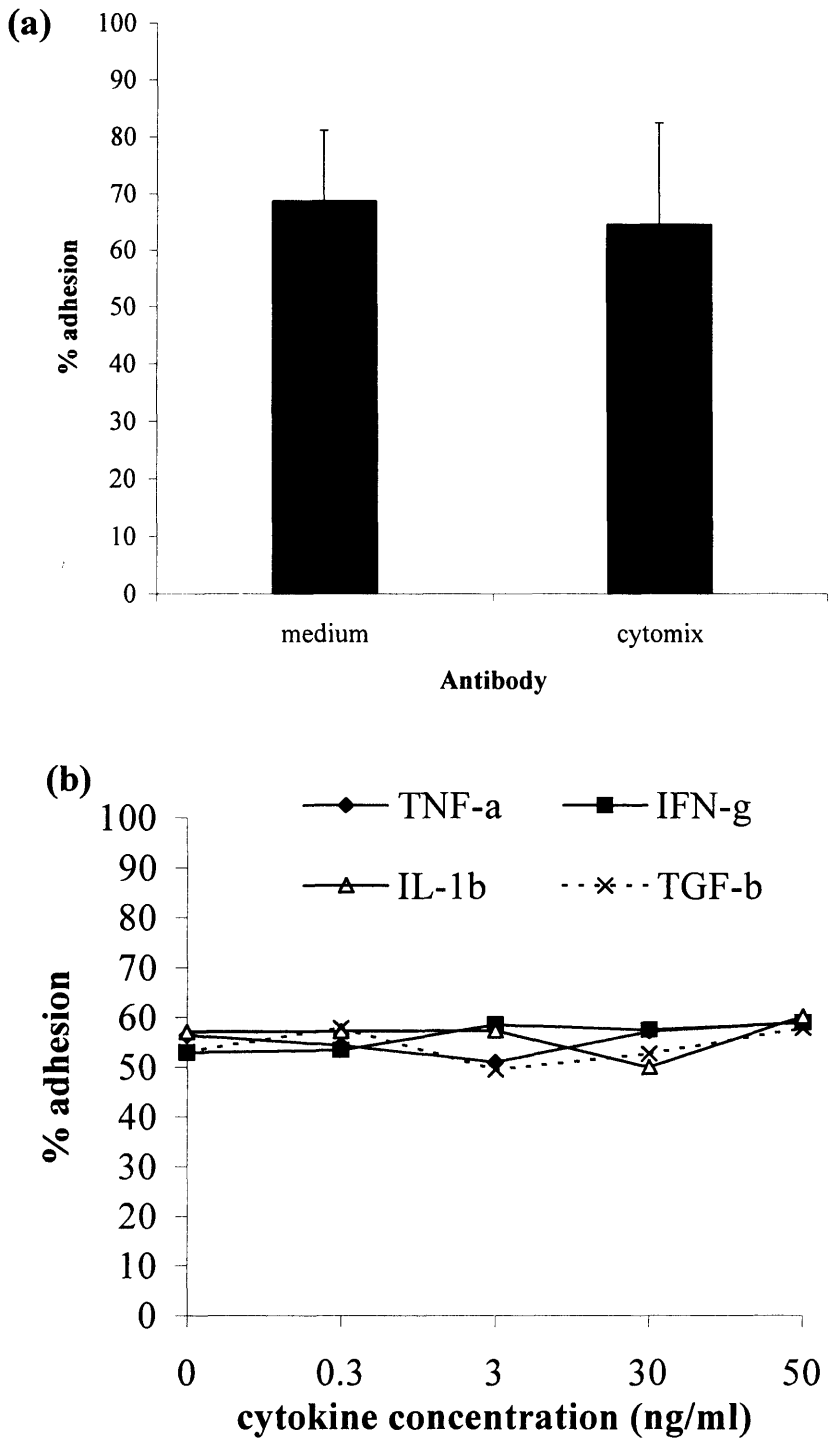
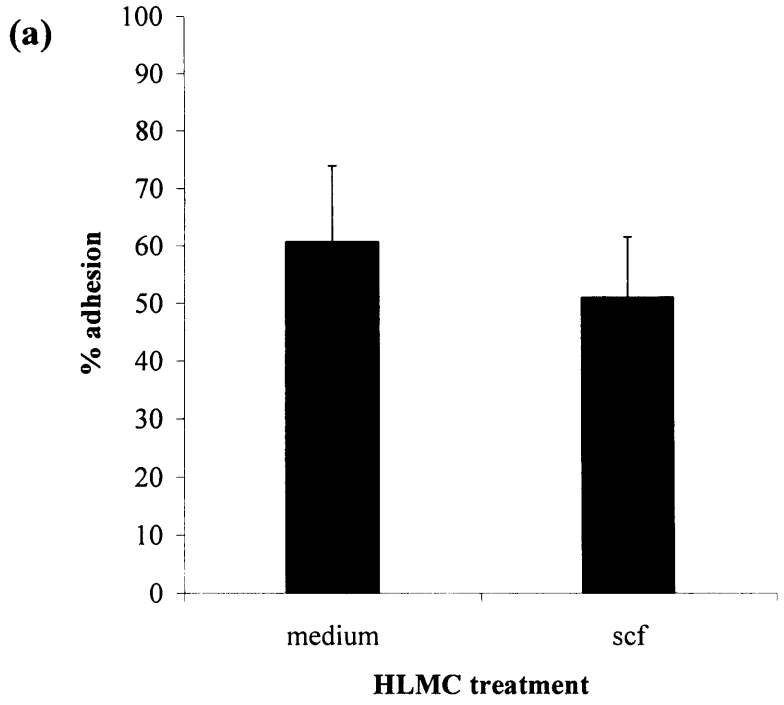


Figure 3.21 Effect of prior cytokine stimulation of epithelium (24hr) on HLMC adhesion
(a) BEAS-2B cytomix stimulation (n=3)
(b) NHBE stimulation with individual cytokines (n=1)
Results in (a) expressed as % adhesion ± sem



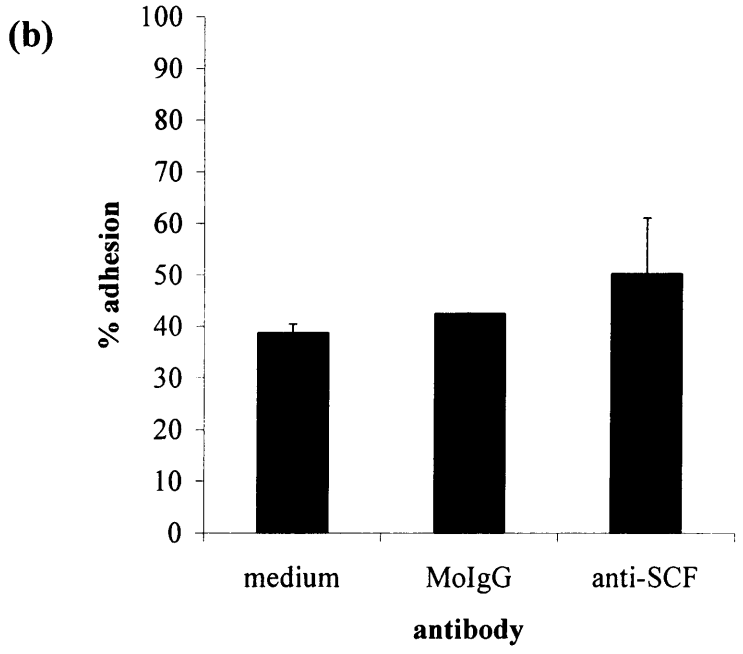


Figure 3.22 **Effect of signalling through *c-kit* or involvement of SCF-c-kit interaction on HLMC adhesion to BEAS-2B (n=3).**
(a) untreated or SCF-treated HLMC
(b) polyclonal anti-human SCF antibody pretreatment of epithelial monolayers Results expressed as % adhesion ± sem

mAb	SCF	SCF + anti-IgE	SCF + TGFβ
Mouse IgG control	44.3 ± 10.3	63.4 ± 18.5	43.9 ± 10.3
FcεRI	175 ± 104	80.1 ± 24.9	126.9 ± 73.1
CD18	105.8 ± 24.8	110.2 ± 25.2	114.5 ± 35.7
CD11a	58.7 ± 19.1	84.2 ± 20.4	62.4 ± 14.6
CD11b	72.3 ± 19.9	92.5 ± 33.9	71.7 ± 23.0
CD11c	70.4 ± 18.5	110.9 ± 27.3	66.9 ± 22.0
$\alpha_4\beta_1$	138.2 ± 10.0	118.1 ± 24.3	132.4 ± 25.2
$\alpha_4\beta_7$	59.6 ± 25.3	88.5 ± 32.3	56.0 ± 21.1
$\alpha_E\beta_7$	47.2± 8.6	66.0 ± 22.4	42.4 ± 9.43

Table 3.2 **HLMC surface marker expression analysed by indirect flow cytometry** after 24hrs pretreatment with SCF (10ng/ml), SCF (10ng/ml) and anti-IgE (1%) or SCF (10ng/ml) and TGF-β (5ng/ml) (n=3). Results expressed as average median fluorescence intensity (MFI).

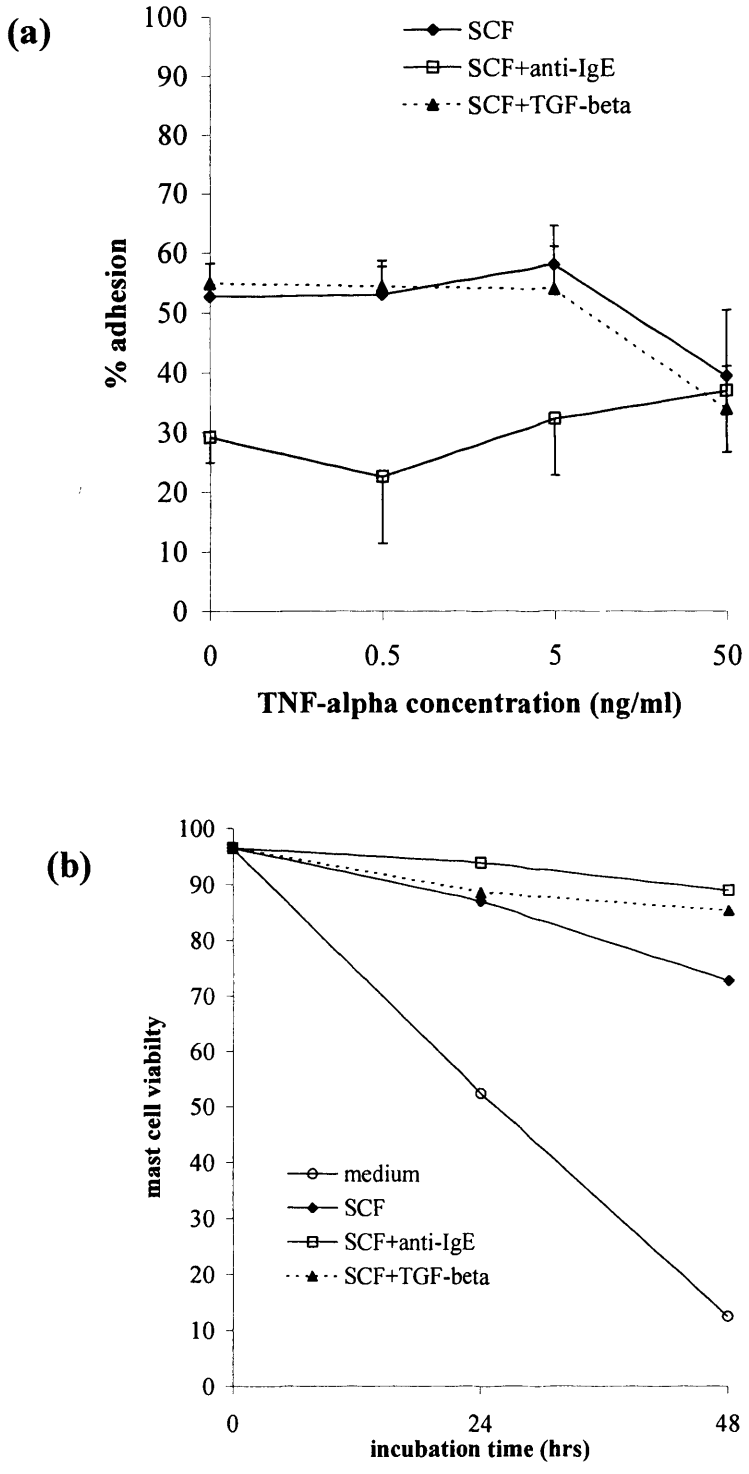


Figure 3.23 HLMC treated overnight with SCF alone (10ng/ml), SCF (10ng/ml and anti-IgE (1%) or SCF (10ng/ml) and TGF-β (5ng/ml)
(a) adhesion to unstimulated and TNF-α stimulated BEAS-2B (n=3)
(b) viability assessed by trypan blue exclusion (n=1)
Adhesion assay results expressed as mean % adhesion ± sem

Investigation of the effect of HLMC activation on adhesion

Mast cells initiate the inflammatory response when tightly bound surface high affinity FcεRI receptor-bound IgE is crosslinked by multivalent allergen, leading to mast cell activation. FcεRI is a tetrameric complex and consists of an IgE-binding α subunit, a β subunit and two disulphide-linked γ chains. FcεRI β and γ chains each contain two tyrosine residues within immunoreceptor tyrosine activation motifs (ITAMs) which allow SH_2-mediated association of the non-receptor tyrosine kinases lyn (with β) and syk (with γ) and subsequent phosphorylation, ultimately leading to a rise in $[Ca^{2+}]_i$ and degranulation. PI_3K is also phosphorylated upon FcεRI activation and regulates intracellular trafficking, cytoskeletal assembly and cell growth. Activation causes the immediate release of pre-formed inflammatory mediators and initiates synthesis of lipid-derived mediators (from arachidonic acid) and cytokines (by gene transcription). Preformed mediators are stored in granules and expulsion to the exterior of the cell involves regulated compound exocytosis or piecemeal degranulation (Dvorak et al, 1991a,b). Following anti-IgE stimulation, intracytoplasmic degranulation channels are formed, which allow passage of granules to the cell surface. The granule membrane docks with the plasma membrane, a fusion pore is formed which expands leading to complete integration of the granule membrane with the plasma membrane. The surface area of the cell is increased and the interior membrane of the granule is exposed to the exterior. Granule contents are released without compromising membrane integrity.

Overnight activation (18-24 hr) with 1% anti-IgE caused a consistent fall in HLMC adhesion to BEAS-2B (n=4) (*p=0.0008, Figure 3.24b). Further experiments showed that this effect was dose-dependent and that leupeptin (10μg/ml) did not prevent the anti-IgE-mediated decrease in adhesion (n=3) (Figure 3.24a). Tryptase is a major preformed mast cell product and its effects may be inhibited by leupeptin hemisulphate (Ac-Leu-Leu-arginal). Leupeptin is a reversible inhibitor of trypsin-like proteases and cysteine proteases. To reveal if decreased adhesion was non-specific (and not a direct result of IgE crosslinking), adhesion of sheep serum-treated HLMC was tested. No significant decrease in adhesion was observed (n=4) (Figure 3.24b).

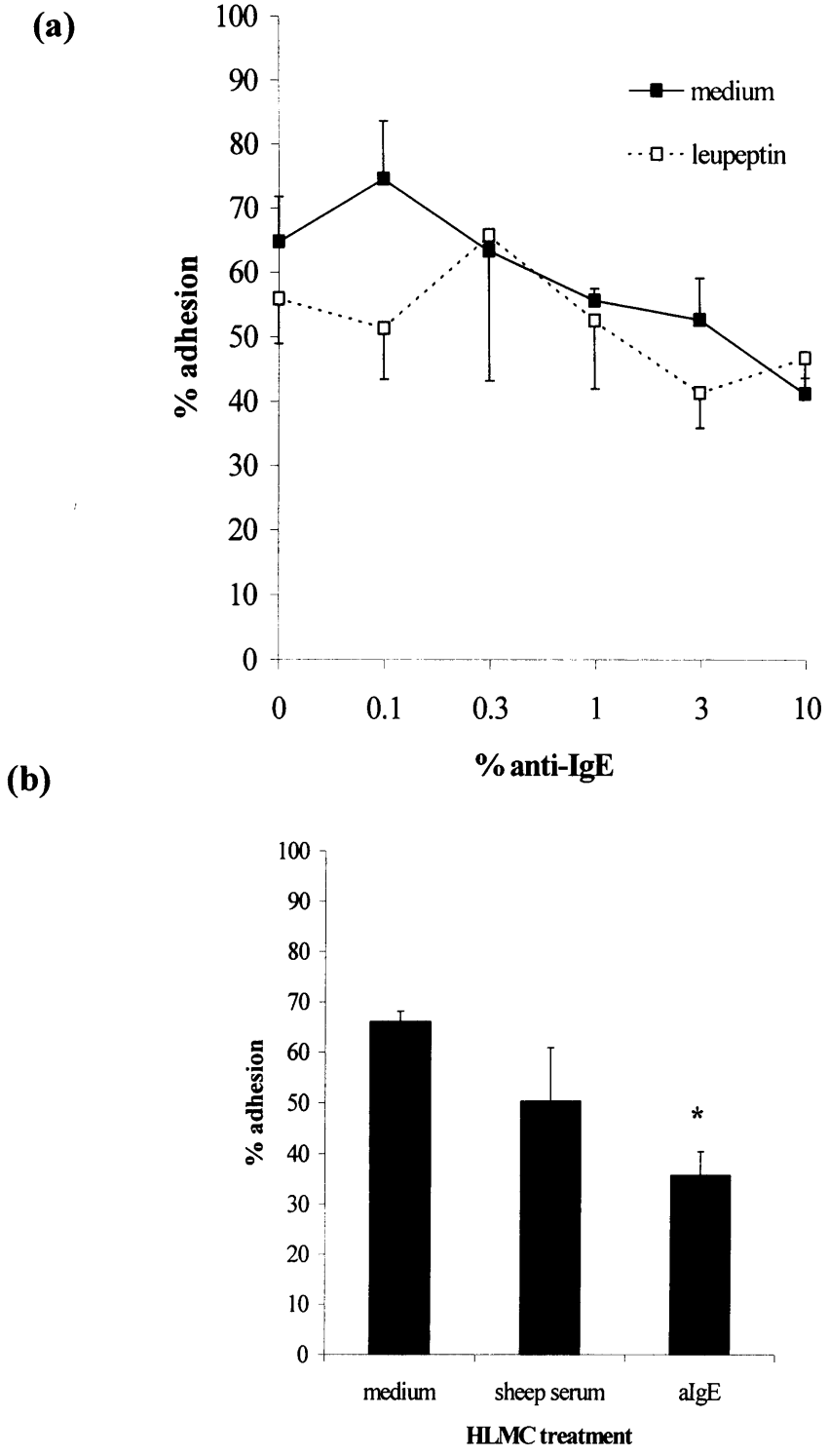


Figure 3.24 Effect of immunological activation (anti-IgE) of HLMC prior to adhesion to BEAS-2B.
(a) effect of the serine protease inhibitor leupeptin on decreased adhesion (n=3). (b) effect of sheep serum control (n=4)
Results expressed as mean % adhesion ± sem

Ionophores are small hydrophobic molecules that increase the membrane permeability of specific inorganic ions. They shield the charge of the ion, enabling it to be transported across the hydrophobic interior of the lipid bilayer. Ionophores may be channel-forming ionophores or mobile ion carriers. Ionophores also uncouple electron transfer from oxidative phosphorylation by creating electrical short circuits across the mitochondrial membranes. A23187 (calcimycin) is a mobile ion-carrier that forms stable complexes with divalent cations and may be used to increase intracellular Ca^{2+} levels (Pressman 1973). The Ca^{2+}-selective ionophore A23187 triggered the secretion of histamine from mast cells in a Ca^{2+}-dependent manner (Foreman et al, 1973; Cochrane and Douglas, 1974).

HLMC activation with calcium ionophore (A23187) was used to determine whether decreased adhesion was a consequence of increased intracellular Ca^{2+} and degranulation, or earlier IgE-mediated signalling (n=3) (Figure 3.25a). No decrease in adhesion was observed. To test if preformed mast cell products affected adhesion, the effect of 20 minutes HLMC pretreatment with HLMC lysate was measured, although no decrease in HLMC adhesion was observed (n=3) (Figure 3.25b).

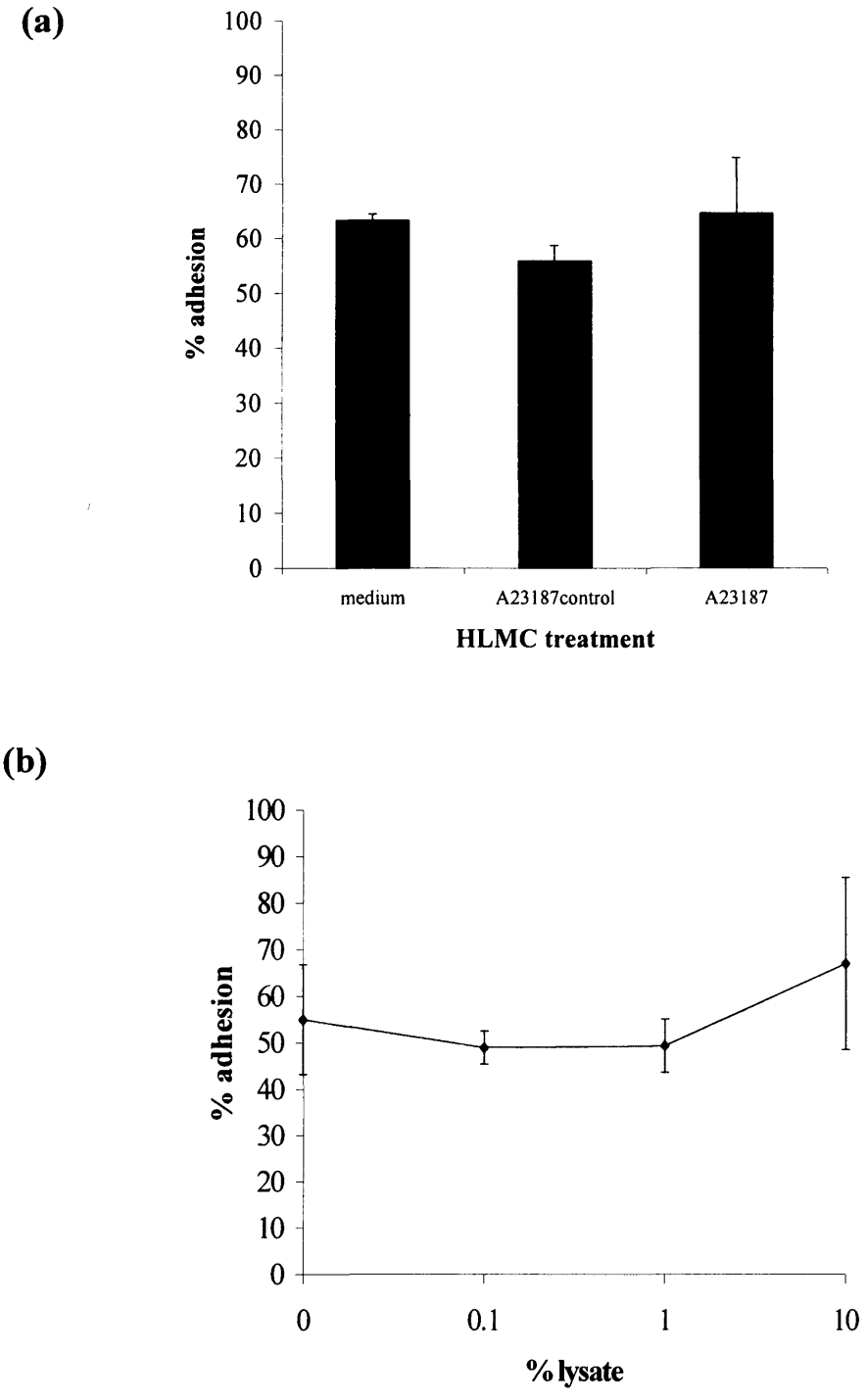


Figure 3.25 Effect of degranulation or preformed intracellular degranulation products on HLMC adhesion to BEAS-2B (n=3)
(a) A23187 activated HLMC (b) lysate-treated HLMC.
Results expressed as % adhesion ± sem

3.3.5 modulation of intracellular signalling

Inhibitors and activators of intracellular signal transduction were tested for their ability to modulate HLMC or HMC-1 adhesion to BEAS-2B. Metabolic energy is required for integrins to achieve the high avidity conformation required for maximum adhesive interaction. Other functions such as degranulation also require metabolic energy. Sodium azide is an inhibitor of oxidative phosphorylation. Adhesion did not alter following HLMC intracellular ATP depletion with a combination of 2-deoxyglucose and sodium azide (Figure 3.26).

Chemokines are a family of small highly basic proinflammatory chemotactic cytokines , and reversibly activate leukocyte integrins when presented upon extracellular matrix components. Chemokines act through serpentine GTP-binding protein (G-protein) -coupled transmembrane receptors. Heterotrimeric G proteins couple receptors to adenylate cyclase resulting in intracellular cyclic adenosine monophosphate (cAMP) elevation. Agonist binding alters receptor conformation and allows exchange of GDP for GTP on the G protein α-subunit. G-protein α-subunit conformational change results in receptor-G-protein complex dissociation, freeing α- and βγ-subunits to interact with their target effectors. Slow α-subunit intrinsic GTPase activity hydrolyses the bound GTP to GDP, ending the interaction of the α-subunit with its effector. The GDP bound α-subunit interacts strongly with βγ-subunits.

The G-proteins are classified by their α-subunit and are grouped into four general classes (α_s, $\alpha_{i/o}$, α_q and α_{12}). Several α-subunits are substrates for covalent modification by bacterial toxins, which have been used to identify whether cellular responses are modulated by adenylate cyclase activity via G-proteins. Pertussis toxin (*Bordetella pertussis*) ADP-ribosylates some members of the $\alpha_{i/o}$ family, uncouples heterotrimeric G proteins from receptors, and inhibits signalling. Cholera toxin (*Vibrio cholerae*) ADP-ribosylates members of the α_s family, stabilising the GTP-bound form and inhibiting intrinsic GTPase activity. This leads to constitutive activation of the A subunit and causes persistent activation of adenylate cyclase. HLMC adhesion to BEAS-2B was not modulated by pertussis or cholera toxin (Figure 3.27).

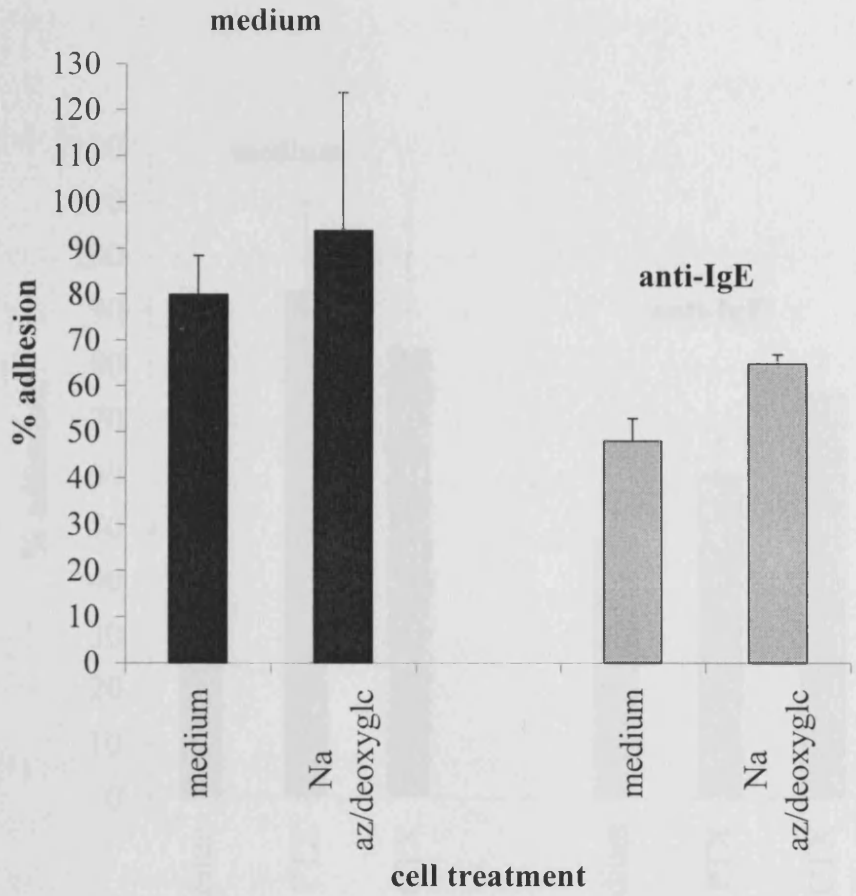


Figure 3.26 Effect of intracellular metabolic energy inhibition (n=3)
Unactivated and anti-IgE-activated (1%; 24hr) HLMC were pretreated with sodium azide/deoxyglucose (0.01M/0.01M; 1 hour) prior to adhesion to BEAS-2B. Results are expressed as percentage adhesion ± sem

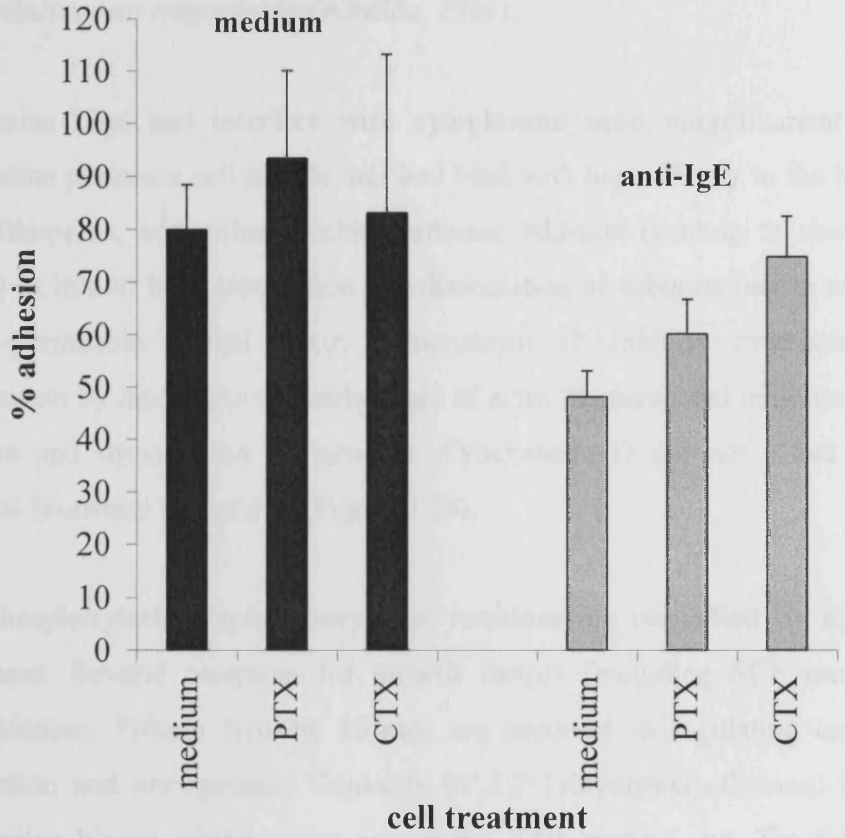


Figure 3.27 Effect of G protein-mediated intracellular cAMP modulation (n=3) Unactivated and anti-IgE-activated (1%; 24hr) HLMC were pretreated with pertussis toxin (PTX, 0.1μg/ml; 2hr) or cholera toxin (CTX, 0.1μgml for 2 hr) prior to adhesion to BEAS-2B. Results are expressed as percentage adhesion ± sem

The actin cytoskeleton is involved in cell shape, organisation, motility and intracellular transport. Cell adhesion molecule cytoplasmic domains are linked to the cytoskeleton. The cytoplasmic domains of both integrin subunits bind actin cytoskeleton-associated proteins such as talin, vinculin and α-actinin. Cadherins and CD44 are linked to the actin cytoskeleton via catenins/α-actinin and ezrin/radixin/moesin respectively (Albelda, 1991).

Cytochalasins bind and interfere with cytoplasmic actin microfilament function. Cytochalasins permeate cell membranes and bind with high affinity to the barbed end of actin filaments, and either inhibit monomer addition (leading to shortening of filaments) or inhibit both association and dissociation of subunits (act to cap fibrils). The cell-permeable fungal toxin cytochalasin D inhibits cytoskeletal actin polymerisation by binding to the barbed end of actin filaments and inhibiting both the association and dissociation of subunits. Cyochalasin D did not affect mast cell adhesion to bronchial epithelium (Figure 3.28).

Protein phosphorylation/dephosphorylation reactions are controlled by kinases and phosphatases. Several receptors for growth factors (including SCF receptor) are tyrosine kinases. Protein tyrosine kinases are involved in regulating cell growth, differentiation and oncogenesis. Genistein (4',5,7-Trihydroxyisoflavone) is a broad range tyrosine kinase inhibitor that acts at the ATP binding site. The inhibition is competitive with respect to ATP and non-competitive with respect to the phosphate acceptor. Genistein treatment of HLMC did not affect adhesion to BEAS-2B (Figure 3.29).

Ca^{2+}/phospholipid-dependent protein kinases (PKCs) play an important role in cell proliferation, differentiation, apoptosis and exocytosis. Phorbol esters activate PKC. PMA (phorbol-12-myristate-13-acetate) is the most commonly used phorbol ester and dramatically increase enzyme affinity for Ca^{2+} resulting in full activation. PMA did not affect HLMC adhesion to BEAS-2B (Figure 3.30).

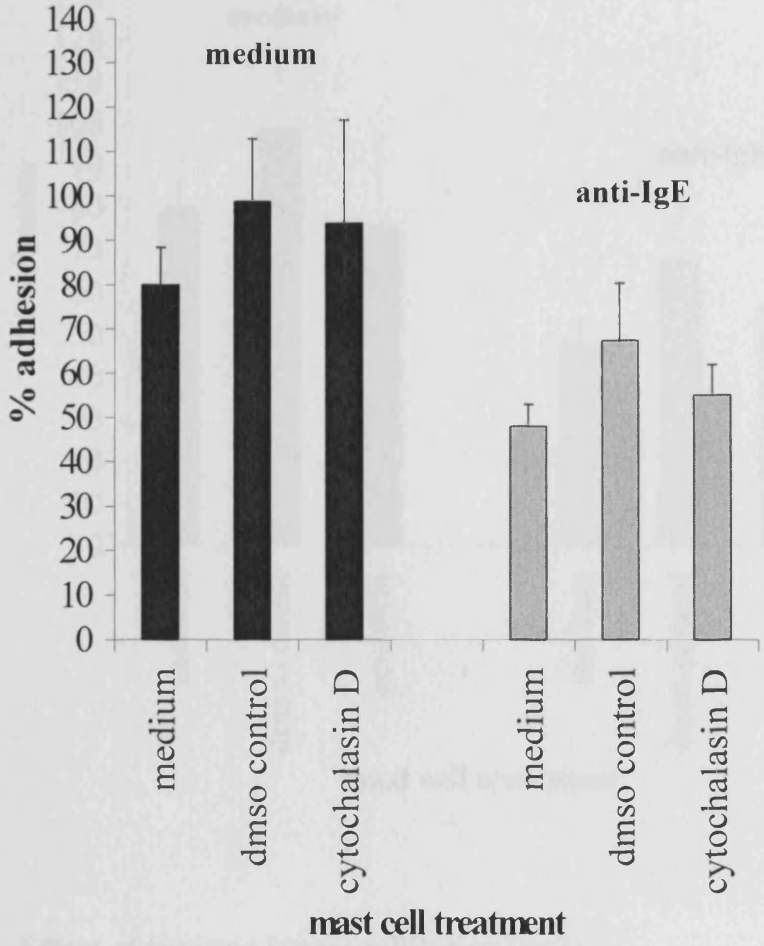


Figure 3.28 Effect of inhibition of actin cytoskeleton polymerisation (n=3)
Unactivated and anti-IgE-activated (1%; 24hr) HLMC were pretreated with cytochalasin D (12μM; 30 min) prior to adhesion to BEAS-2B. Results are expressed as percentage adhesion ± sem

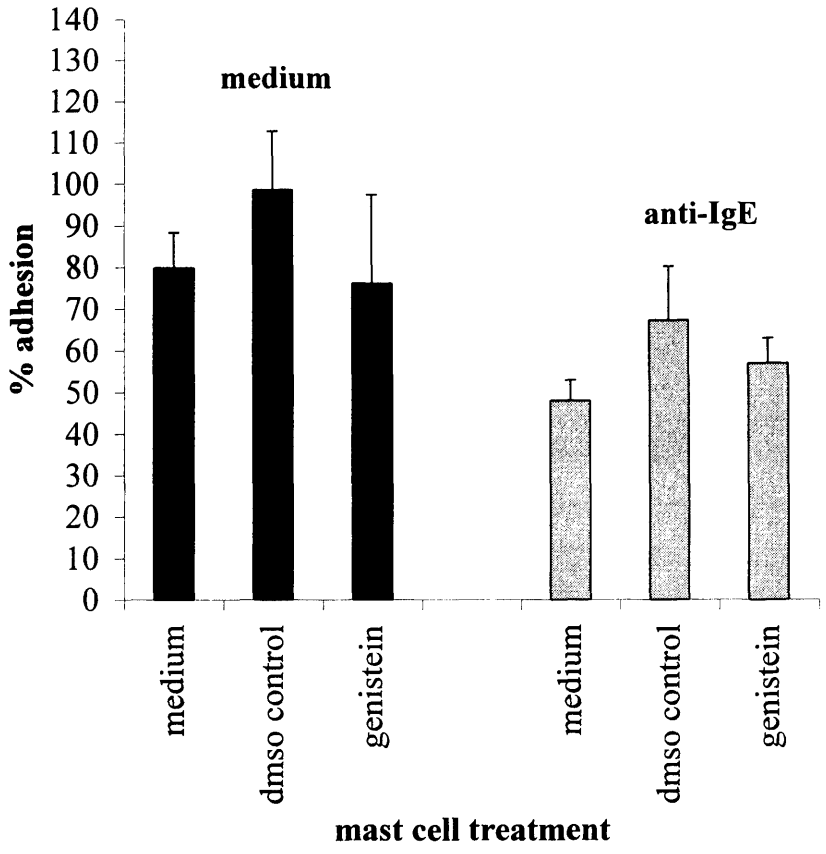


Figure 3.29 Effect of tyrosine kinase inhibition (n=3)
Unactivated and anti-IgE-activated (1%; 24hr) HLMC were pretreated with genistein (5μM; 30min) prior to adhesion to BEAS-2B.
Results are expressed as percentage adhesion ± sem

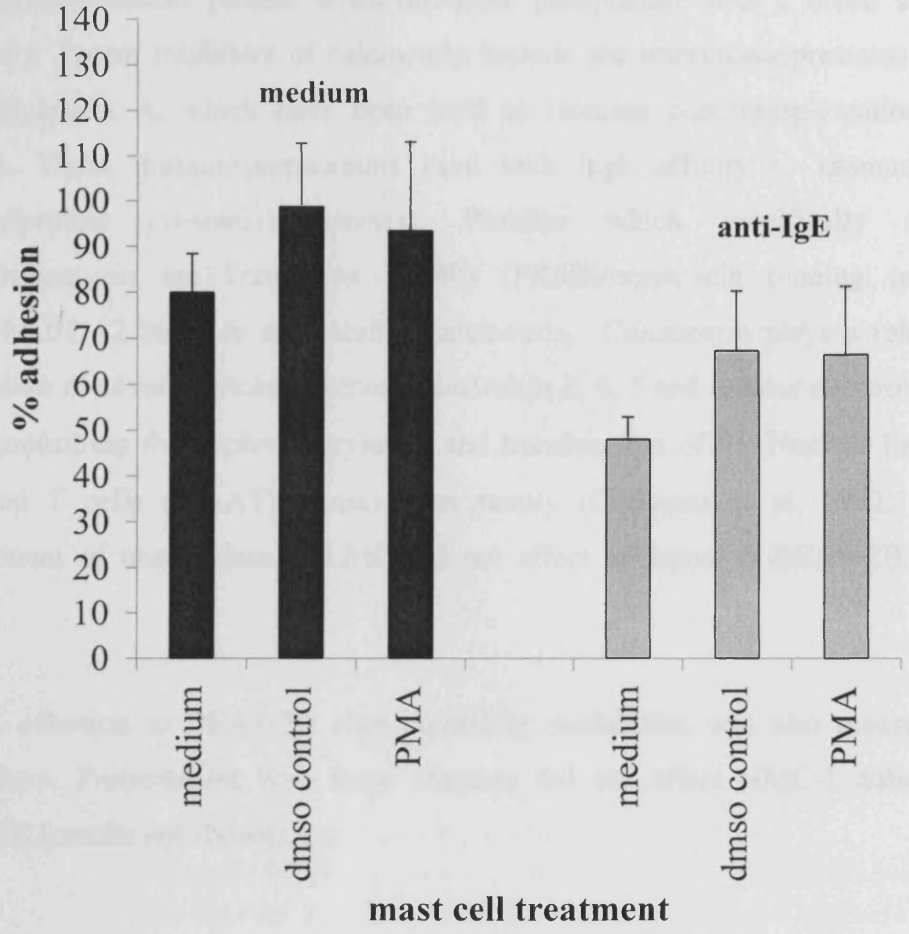


Figure 3.30 Effect of protein kinase C activation (n=3)
Unactivated and anti-IgE-activated (1%; 24hr) HLMC were pretreated with PMA (0.05μg/ml; 10min) prior to adhesion to BEAS-2B. Results are expressed as percentage adhesion ± sem

Protein phosphatases can be divided into three general categories: protein tyrosine phosphatases, protein serine/threonine phosphatases, and dual-specificity phosphatases. These categories have been further subdivided on the basis of cellular localisation, requirement for divalent metal cations and sensitivity to specific inhibitors. Protein Phosphatase 2B (calcineurin, PP2B) is a eukaryotic major Ca^{2+}-calmodulin-dependent protein serine/threonine phosphatase with a broad substrate specificity. Potent inhibitors of calcineurin include the immunosuppressants FK506 and cyclosporin A, which have been used to increase post-transplantation organ survival. These immunosuppressants bind with high affinity to immunophilins (peptidylproline cis-trans-isomerases). Proteins which specifically act on FK506/rapamycin are known as FKBPs (FK506/rapamycin binding proteins). FK506/FKBP 12 binds to and inhibits calcineurin. Calcineurin plays a role in the trancription of several cytokine genes (interleukin-2, 4, 5 and tumour necrosis factor-α) by controlling the dephosphorylation and translocation of the Nuclear Factor for Activated T cells (NF-AT) transcription family (Clipstone et al, 1992). FK506 pretreatment of unstimulated HLMC did not affect adhesion to BEAS-2B (Figure 3.31).

HMC-1 adhesion to BEAS-2B after signalling modulation was also measured for comparison. Pretreatment with these reagents did not affect HMC-1 adhesion to BEAS-2B (results not shown).

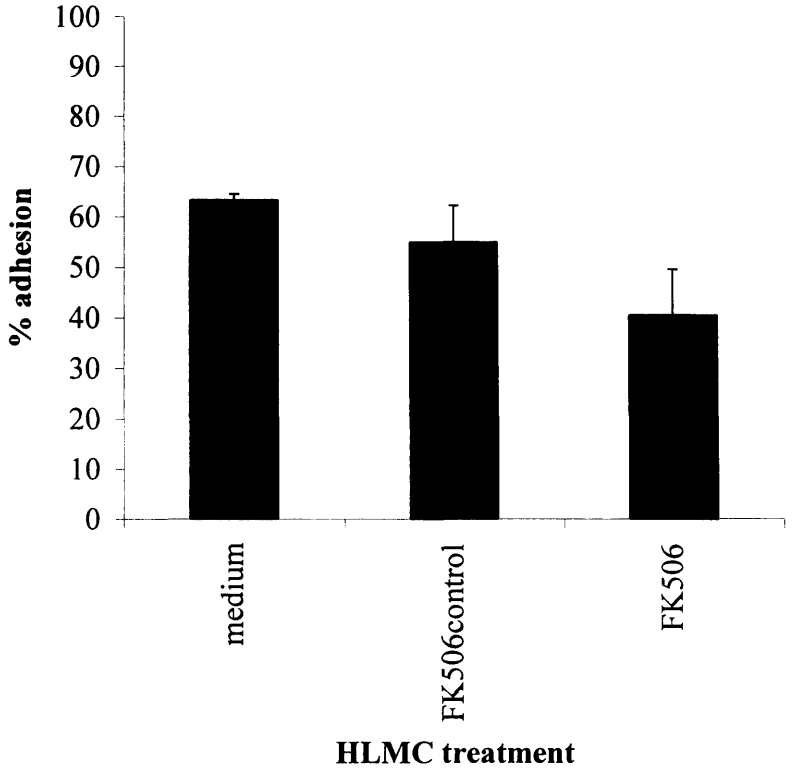


Figure 3.31 **Effect of calcineurin inhibition by FK506 (n=3)**
Unactivated HLMC were pretreated with FK506 (**M; hour**) prior to adhesion to BEAS-2B. Results are expressed as percentage adhesion ± sem

Effect of mast cell protease treatment on adhesion

HLMC pretreatment with the serine endoproteases trypsin or proteinase k did not affect adhesion to epithelium (Figure 3.32a,b). In addition, trypsin also generates intracellular signalling due to its effects on PAR-2. Trypsin cleaves PAR-2, the exposed tethered ligand binds and activates the cleaved receptor, leading to G-protein-mediated signal transduction and generation of inositol triphosphate and mobilisation of $[Ca^{2+}]_i$. Proteinase k has a broad spectrum of activity and cleaves peptide bonds at the carboxylic side of aliphatic, aromatic or hydrophobic amino acids.

Pronase (streptomyces griseus) is a mixture of several non-specific endo- and exo-proteinases with the ability to cleave almost any peptide bond and digest proteins to single amino acids. Pretreatment with pronase resulted in a dose-dependent reduction in adhesion ($p=0.028$).

The ability of these enzymes to cleave HMC-1 cell surface adhesion molecules was investigated. Adhesion molecule expression was not altered after trypsin or proteinase k treatment (Figure 3.33). Proteolytic activity of pronase was confirmed, since, after 20 minutes pretreatment (10μg/ml pronase), surface expression of ICAM-1, E-cadherin, CD9 and CD69 were reduced. Interestingly, CD11a (LFA-1) expression was not reduced by pronase treatment. LFA-1 was selectively expressed on HMC-1 (and not HLMC) and may be a marker of immature, undifferentiated mast cells.

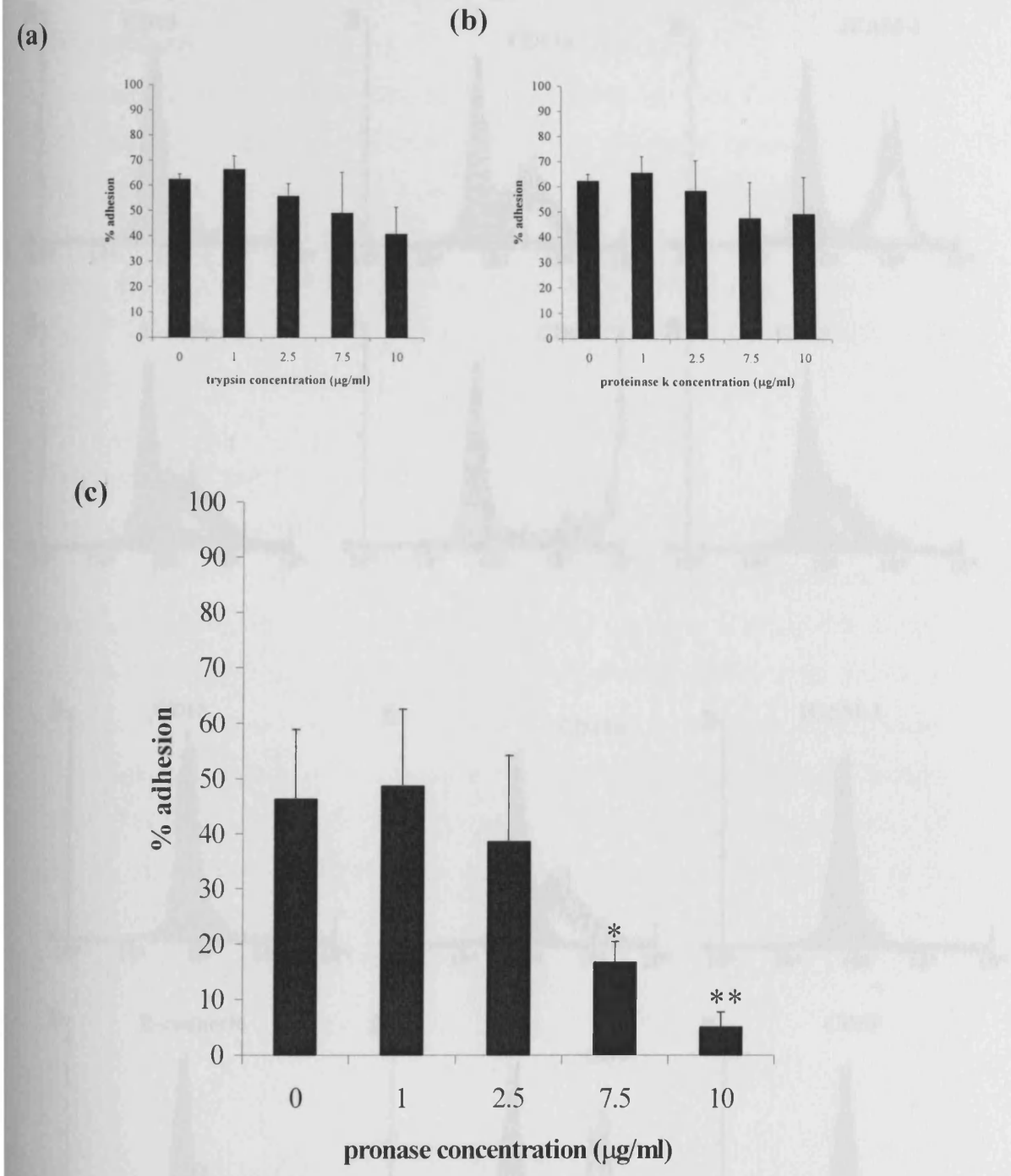


Figure 3.32 **Effect of removal of HLMC surface proteins prior to adhesion to BEAS-2B (n=3). a) trypsin (b) proteinase k (c) pronase**
Proteases were added at the appropriate concentration for 20min at 37^{0}C in serum-free medium, and removed by centrifugation and replacement of medium. Cell viability was assessed by trypan blue. Results expressed as percentage adhesion ± sem

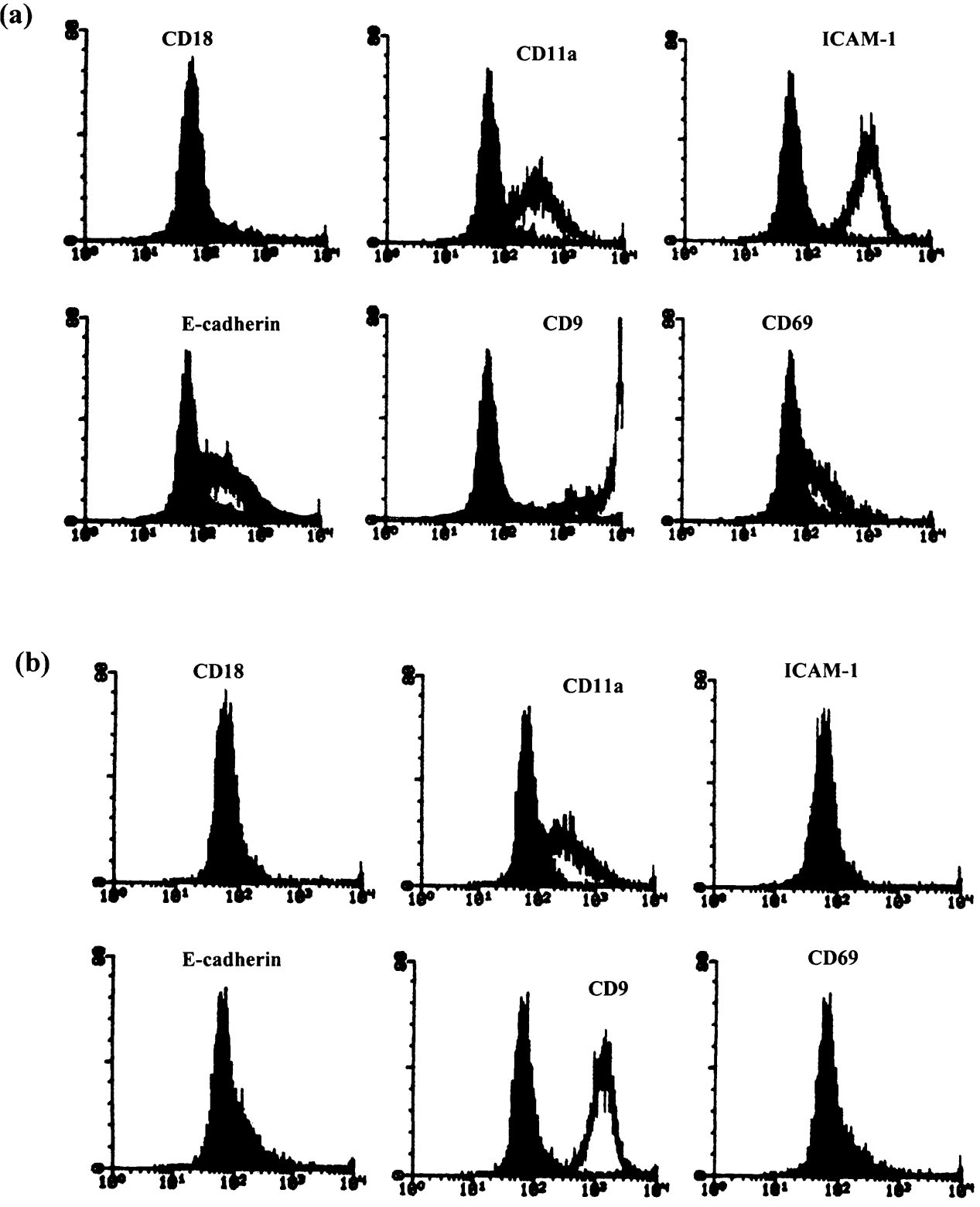


Figure 3.33 Effect on HMC-1 surface adhesion molecules of proteolytic digestion. Cells were treated for 20min at 37°C with 7.5μg/ml protease (a) no enzyme control, trypsin, proteinase k (showed no change in adhesion molecules measured). (b) pronase
Results are representative of two separate experiments.

Mast cell treatment with carbohydrates and glycosidases

Monosaccharide blocking

The presence of surface carbohydrate-mediated adhesive interactions was assessed by the addition of several mono-, di- or polysaccharides (galactose, mannose, mannose-6-phosphate, lactose and fucoidin). They have previously shown competitive blocking ability (Colgan et al, 1995; Yednock et al, 1987). No significant effect on adhesion was seen with these carbohydrates when added to either epithelium (n=2) (Figure 3.34a), or HLMC in the presence of cytomix activated or unactivated epithelium (n=3) (Figure 3.34b).

HLMC glycosidase digestion

Selected cell surface carbohydrates were removed from HLMC prior to adhesion using glycosidases (β-galactosidase, endo-α-N-acetylgalactosaminidase and neuraminidase). β-galactosidase signiicantly reduced adhesion to BEAS-2B of both HLMC (*p=0.031) (Figure 3.35a) and HMC-1 (*p=0.045) (Table 3.3). BEAS-2B pretreatment with β-galactosidase did not alter HLMC adhesion (Figure 3.35b). Endo-α-N-acetylgalactosaminidase was tested only on HLMC and significantly reduced adhesion (*p=0.04) (Figure 3.35a). Neuraminidase did not decrease adhesion of HLMC to BEAS-2B, and did not give an additive decrease in the presence of β-galactosidase (Figure 3.35a).

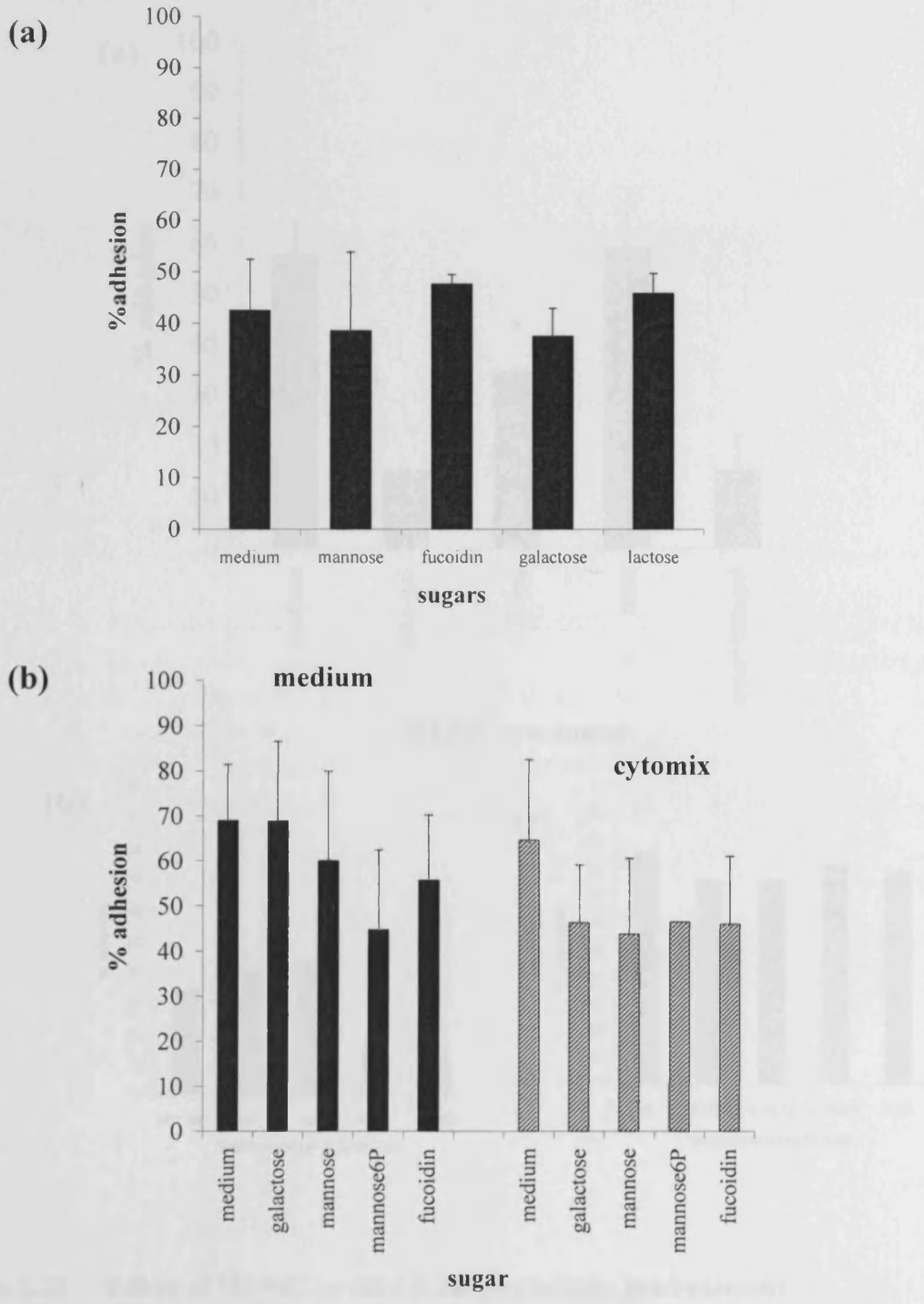


Figure 3.34 Effect of carbohydrate presence on HLMC adhesion to BEAS-2B
The following carbohydrates were added at RT for 30min:
mannose (50mM), mannose-6-phosphate(3.3mM), fucoidin (1mg/ml), galactose (50mM), lactose (50mM). Carbohydrates were added to
(a) BEAS-2B (n=2) (b)HLMC (n=3)
Results are expressed as percentage adhesion ± range (a) or sem (b).

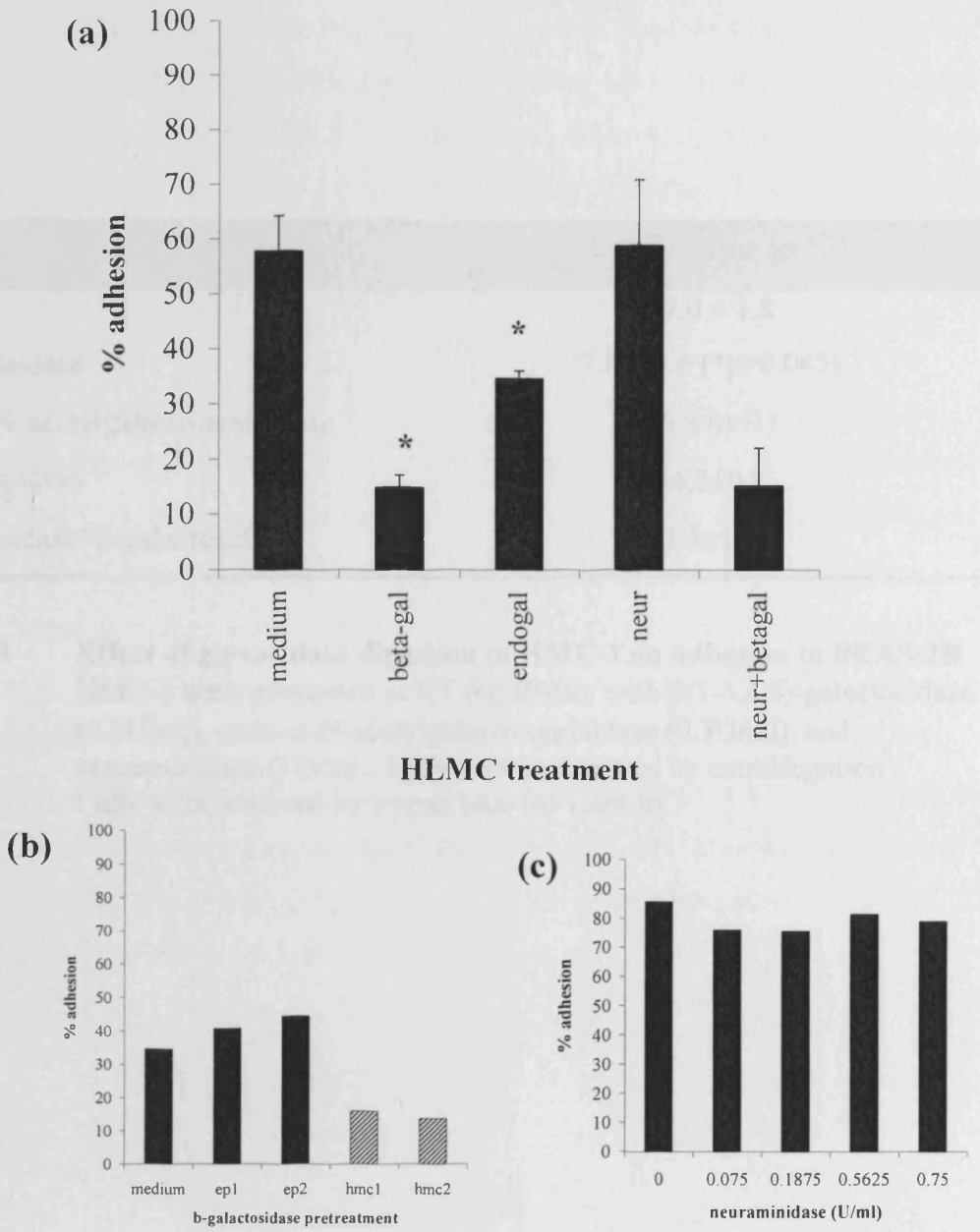


Figure 3.35 Effect of HLMC or BEAS-2B glycosidase pretreatment prior to adhesion to BEAS-2B.

(a) HLMC were treated with β-galactosidase (0.5U/ml), endo-α-N-acetylgalactosaminidase (0.3U/ml), neuraminidase (1U/ml) and both neuraminidase and β-galactosidase (1U/ml;0.5U/ml) (n=3).

(b) BEAS-2B and HMC β-galactosidase pretreatment (0.5U/ml, 1U/ml) (n=1)

(c) HLMC neuraminidase pretreatment (0-0.75U (10μg) /ml)(n=1)

glycosidase	% adhesion (n=3)
Medium	37.0 ± 1.8
β-galactosidase	7.8 ± 7.6 (*p=0.045)
Endo-α-N-acetylgalactosaminidase	36.9 (n=1)
Neuraminidase	34.2±9.8
Neuraminidase+β-galactosidase	3.4±1.3

Table 3.3 **Effect of glycosidase digestion of HMC-1 on adhesion to BEAS-2B**
HMC-1 were pretreated at RT for 30 min with β(1-3,4,6)-galactosidase (0.5U/ml), endo-α-N-acetylgalactosaminidase (0.3U/ml), and neuraminidase (1U/ml). Enzyme was removed by centrifugation. Cells were assessed by trypan blue for viability.

Investigation of lectin involvement in adhesion

Galectin-mediated adhesion may be characterised by competitive inhibition with lactose (D-galactose β1-4 D-glucose) (Baum et al, 1995; Rochon et al, 1994; Inohara and Raz, 1995). To investigate whether galectins were involved in adhesion, lactose and galactose were preincubated with HLMC, BEAS-2B or both, prior to the adhesion assay. No inhibition was seen (n=2) (Figure 3.36a). Lactose was incubated for increased incubation times with HLMC prior to adhesion. There was no inhibition of adhesion observed (n=1) (Figure 3.36b).

Flow cytometry analysis showed no evidence of galectin-1 (Figure 3.37a,b,c) or galectin-3 expression (Figure 3.38, 3.39, 3.40) on unactivated and anti-IgE activated HLMC, HMC-1 or BEAS-2B. Galectin-3 protein was able to bind to HMC-1 and BEAS-2B cells, and was detectable using flow cytometry (Figure 3.39 and 3.40). β-galactosidase treatment of HMC-1 cells did not affect their ability to bind galectin-3 (Figure 3.41), implying adhesion did not involve galectin-3. HLMC did not adhere to either galectin-1 or galectin-3 pre-coated 96-well plates (Figure 3.42a, b). Galectin-1 protein did not block adhesion of HLMC to untreated or cytomix-treated epithelium (Figure 3.43). Galectin-3 function-blocking mAb did not decrease adhesion of unactivated or anti-IgE activated HLMC to BEAS-2B when preincubated with HLMC (Figure 3.44a) or BEAS-2B (Figure 3.44b).

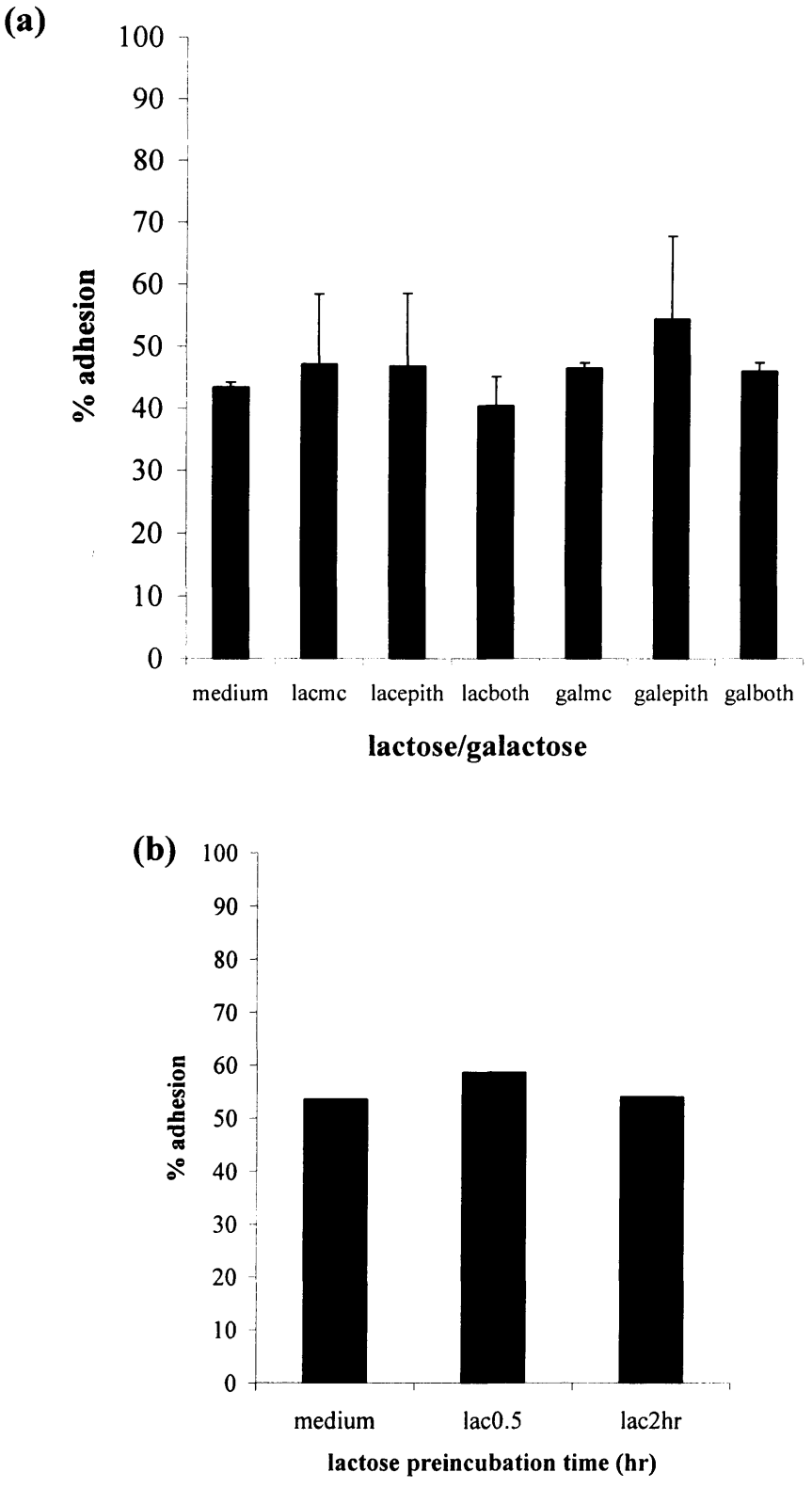


Figure 3.36 Effect of lactose and galactose on adhesion of HLMC to BEAS-2B
HLMC were pretreated at RT for 30minutes with lactose or galactose (50mM) (a) pretreatement of HLMC, BEAS-2B or both (n=2)
(b) extended preincubation time of lactose with HLMC (n=1)
Results expressed as percentage adhesion ± range

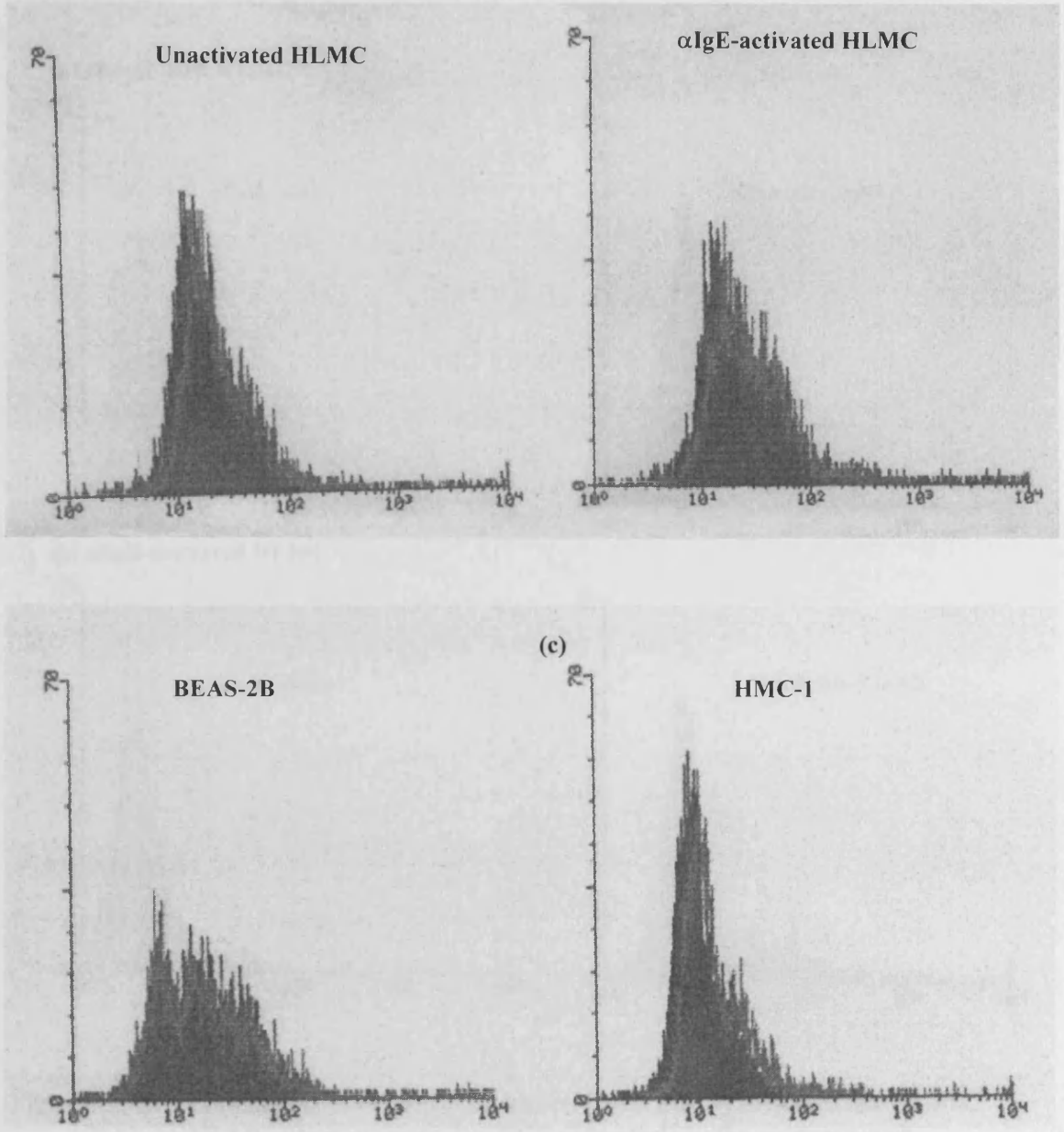


Figure 3.37 Mast cell and epithelial galectin-1 expression
(a) unactivated and anti-IgE activated HLMC
(b) BEAS-2B epithelial cells (removed by EDTA),
(c) HMC-1 mast cell line
Cells were assessed by indirect flow cytometry
Results are representative of two separate experiments

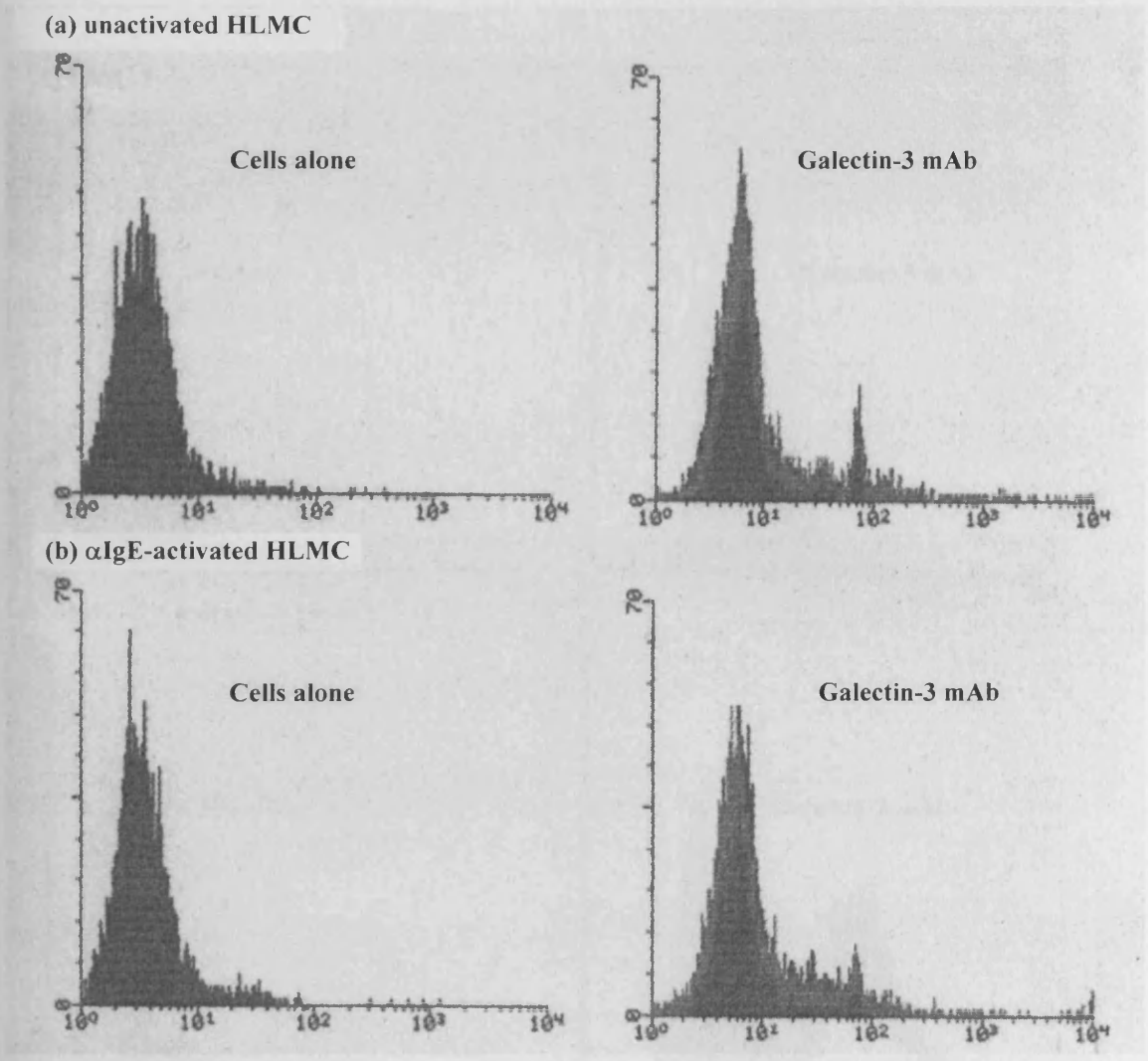


Figure 3.38 Galectin-3 expression of unactivated and IgE-activated HLMC
HLMC were incubated in SCF (10ng/ml) in the presence and absence of anti-IgE (1%, 24hr). Galectin expression was measured by indirect flow cytometry. Results shown are representative of two separate experiments.

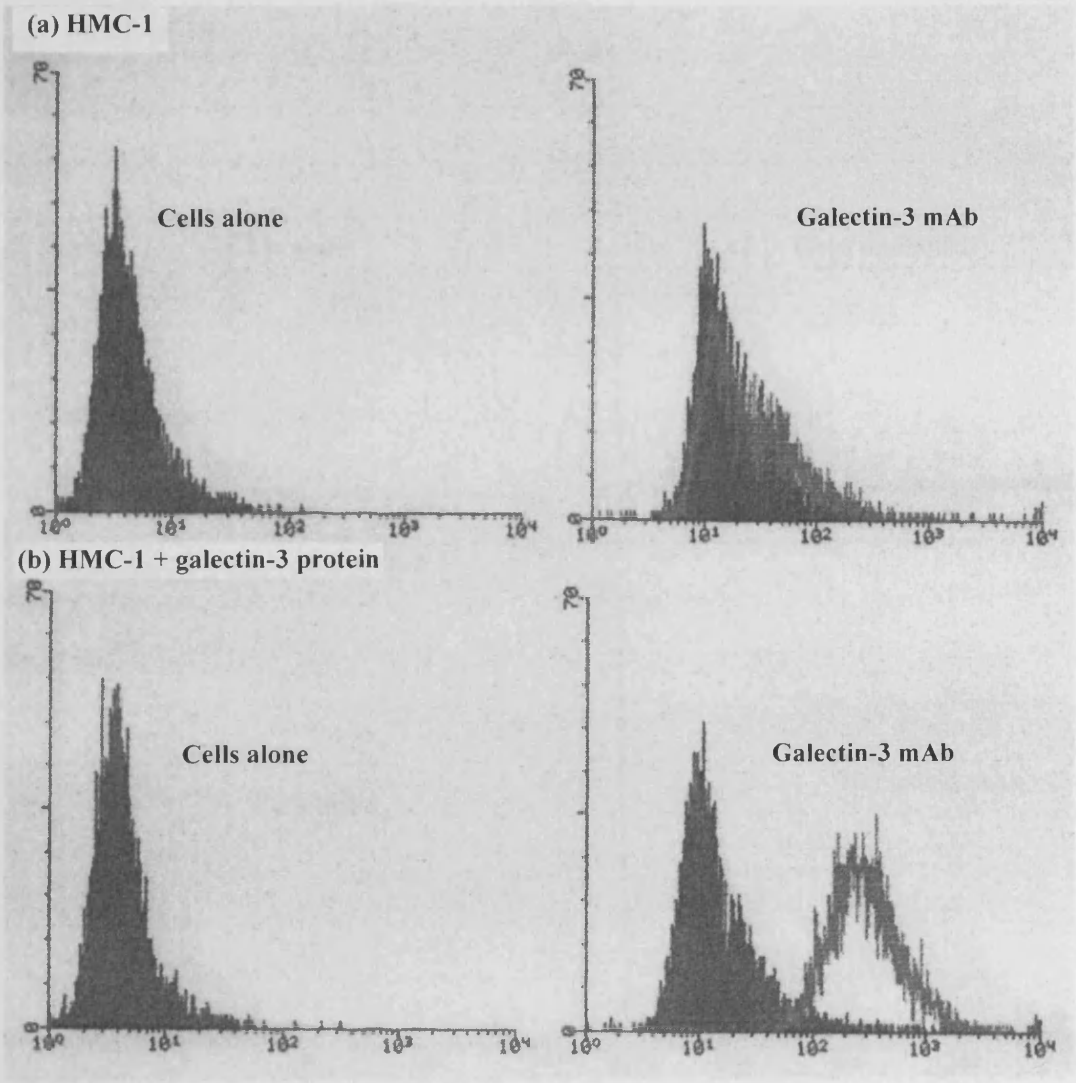


Figure 3.39 HMC-1 galectin-3 expression and ability to adhere to galectin-3
(a) HMC-1 cells alone and (b) HMC-1 preincubated with galectin-3 protein. Galectin-3 expression assessed by indirect flow cytometry with a FITC-labelled secondary antibody.
Results shown are representative of two separate experiments

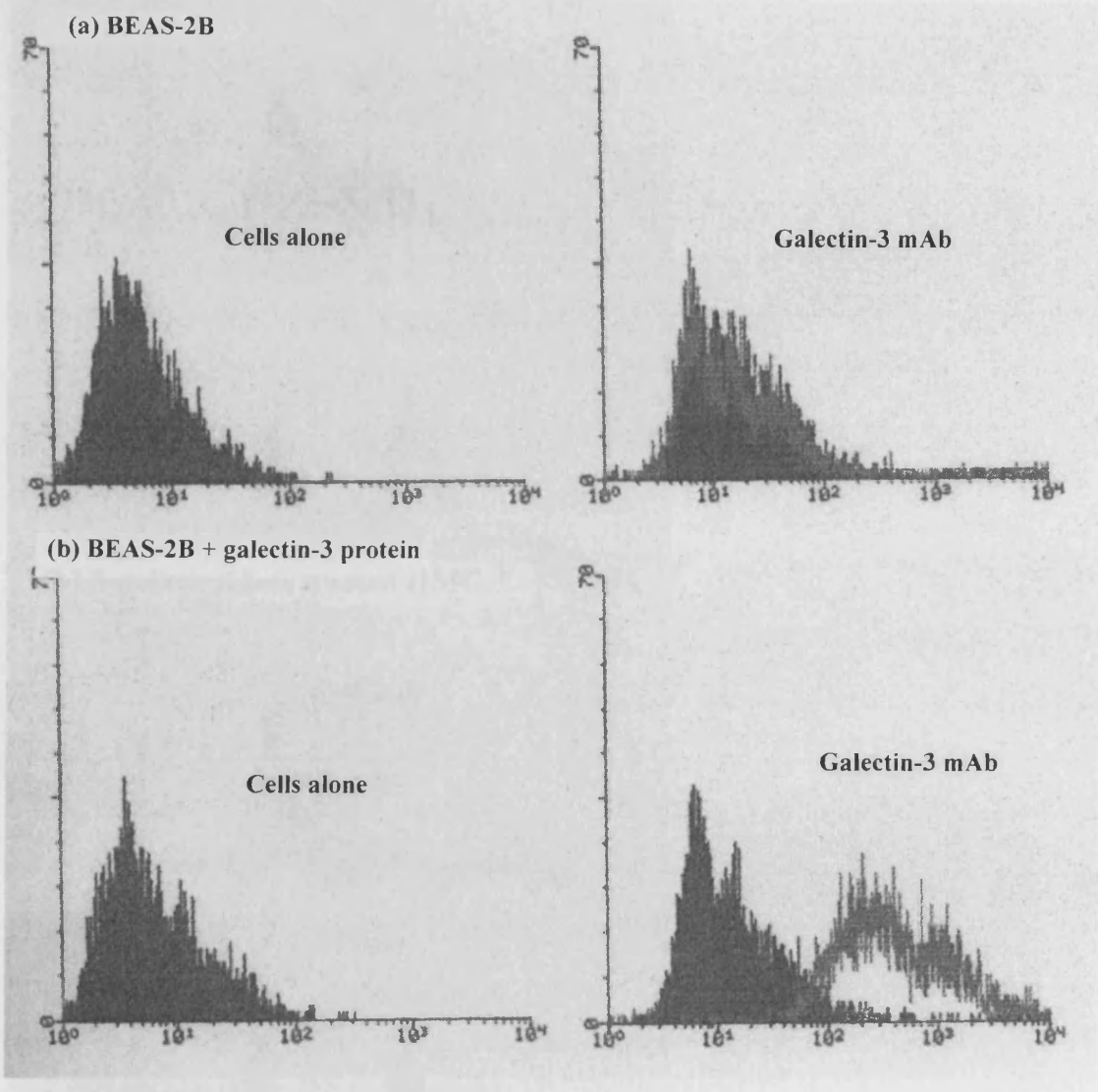


Figure 3.40 BEAS-2B galectin-3 expression and ability to adhere to galectin-3
(a) BEAS-2B cells alone and (b) BEAS-2B preincubated with galectin-3 protein. Galectin-3 expression assessed by indirect flow cytometry with a FITC-labelled secondary antibody. Results shown are representative of two separate experiments.

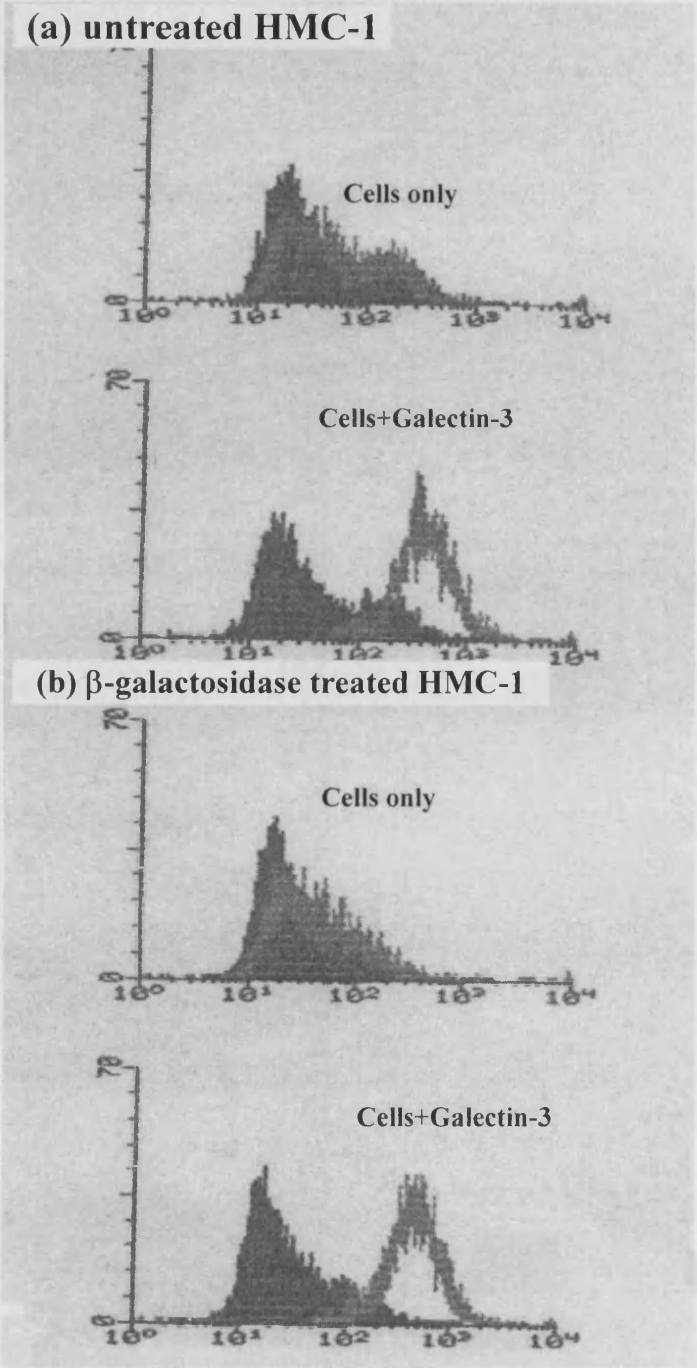


Figure 3.41 Galectin-3 binding of untreated or β-galactosidase-treated HMC-1
(a) untreated or (b) preincubated with β-galactosidase (0.5U/ml) Galectin-3 expression of cells was assessed by indirect flow cytometry with FITC-labelled secondary antibody. Results are representative of two experiments.

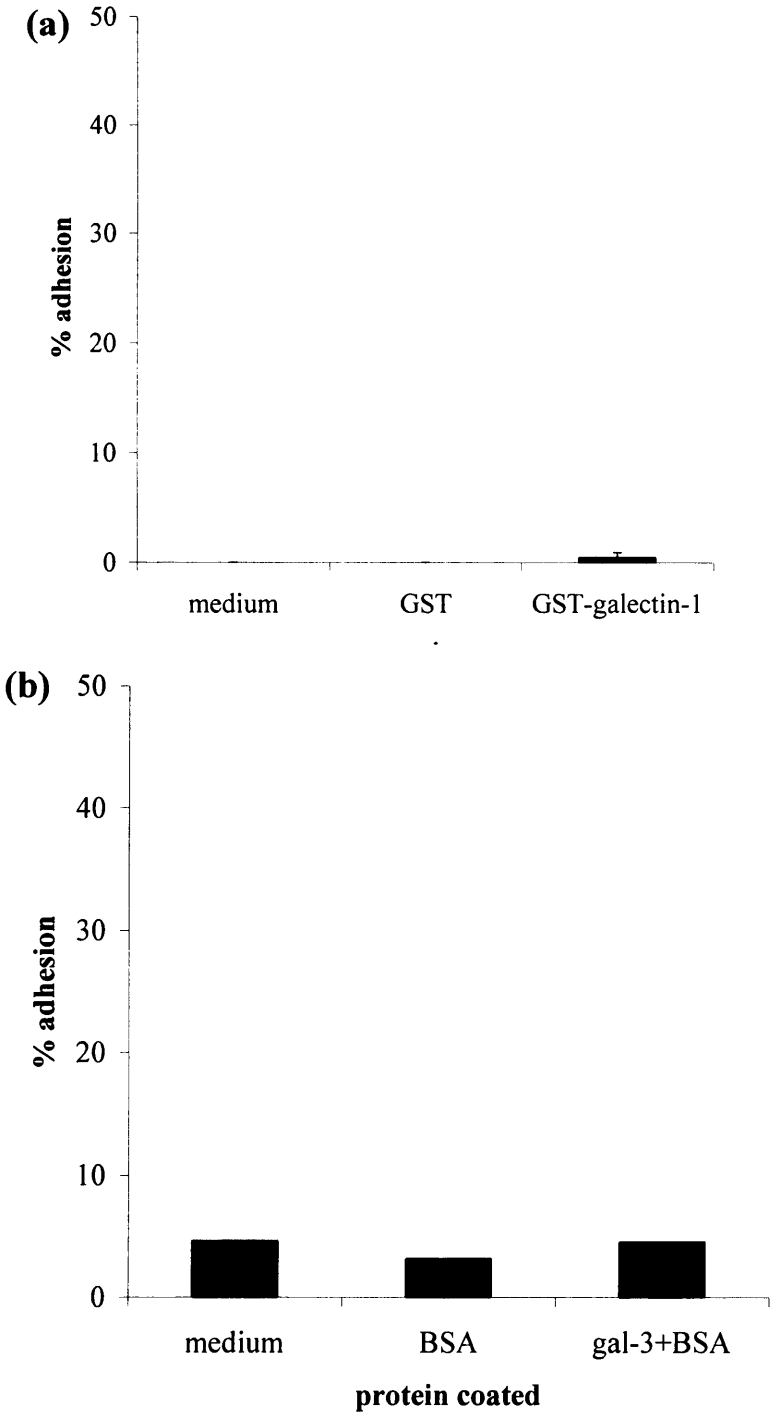


Figure 3.42 HLMC adhesion to galectin-precoated tissue culture plastic (n=3)
(a) galectin-1 (b) galectin-3 were added overnight at 4°C, washed twice with PBS, non-specific binding was blocked with BSA (1hr), and washed twice with PBS prior to use. Data is expressed as mean percentage adhesion ± sem

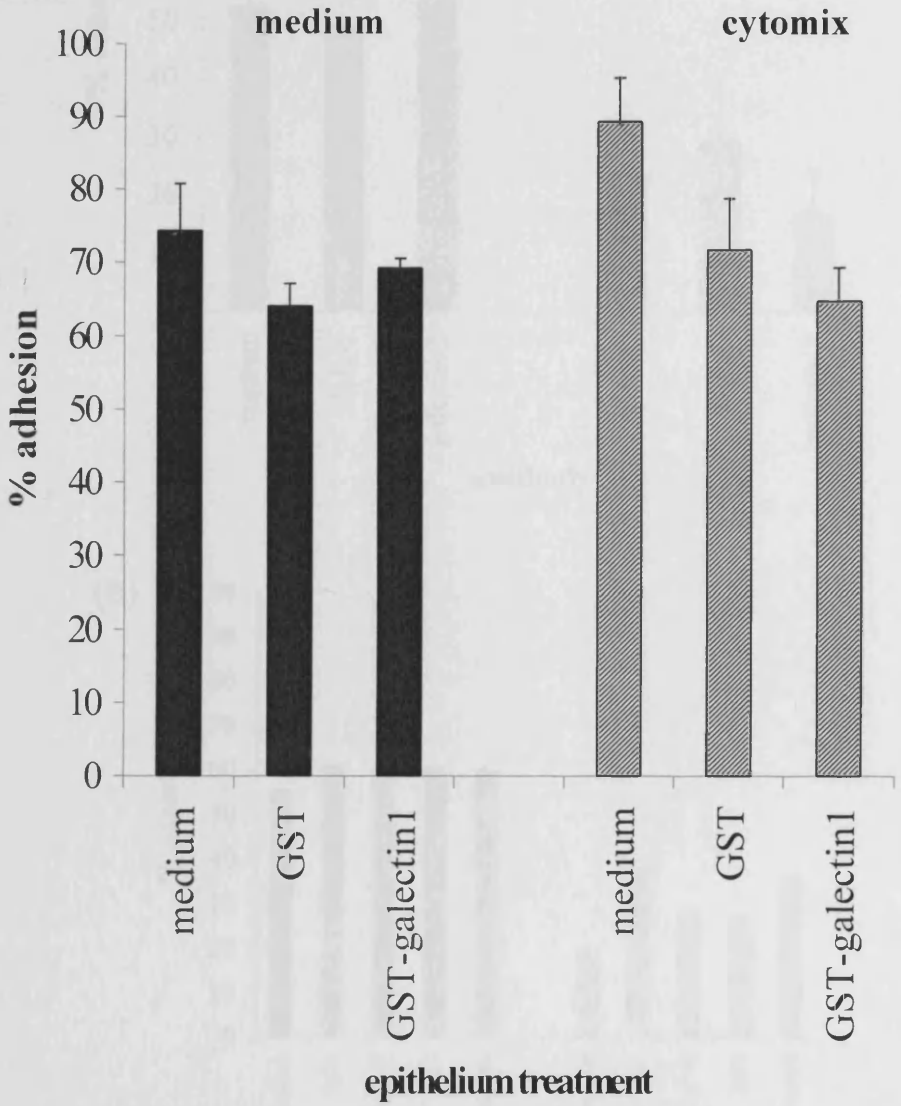


Figure 3.43 HLMC adhesion to unstimulated or cytomix-stimulated BEAS-2B in the presence of galectin-1 protein (n=3).
Galectin-1 (1mg/ml) was preincubated for 30min at RT prior to adhesion assay. Data is expressed as mean % adhesion ± sem

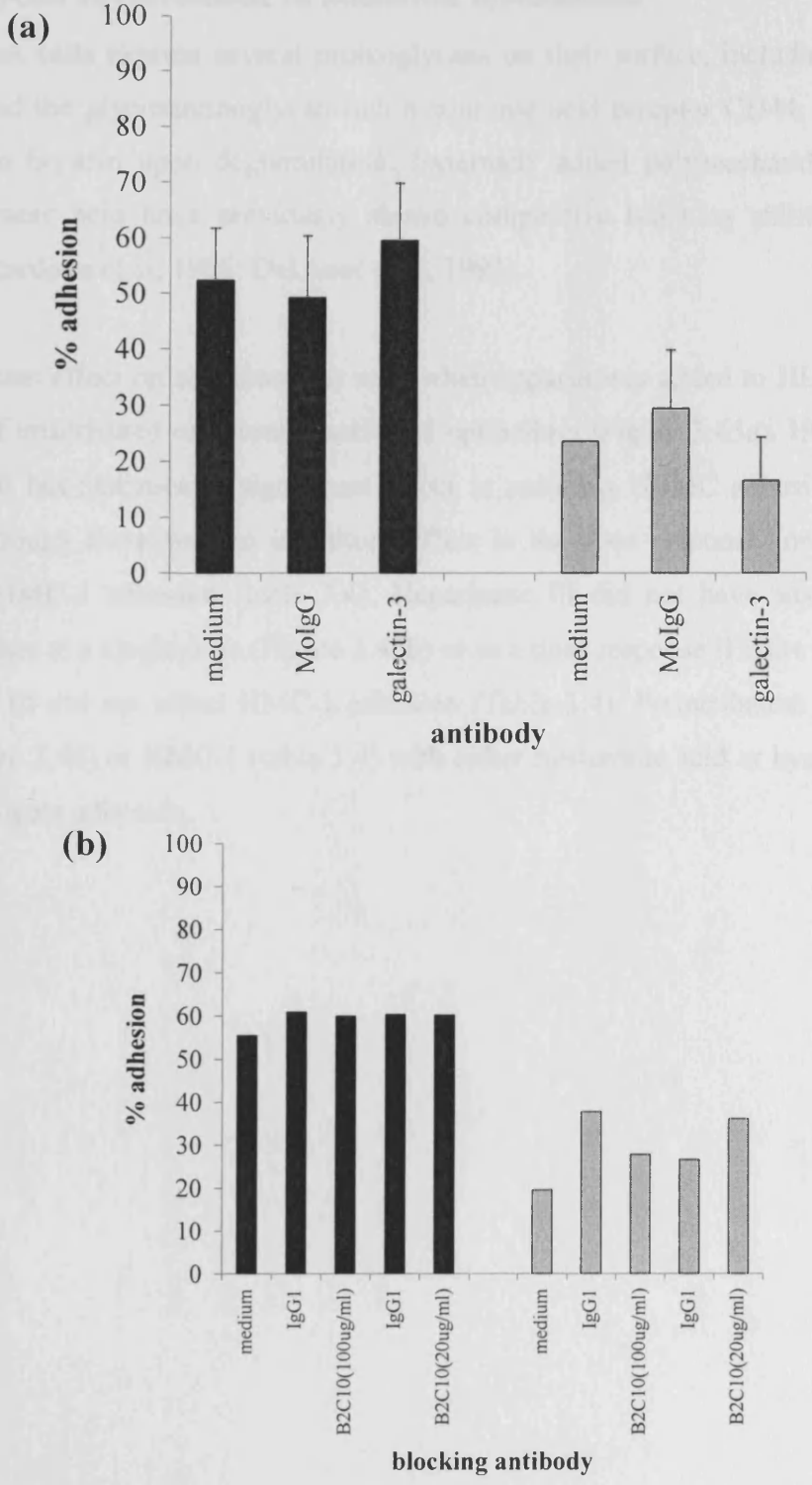


Figure 3.44 The effect of blocking galectin-3 function upon adhesion of unactivated or anti-IgE activated HLMC to BEAS-2B
(a) galectin-3 protein, 1mg/ml, RT, 30min (n=4)
(b) galectin-3 mAb, (B2C10, 20 and 100μg/ml) (n=1).
Results in (a) are expressed as mean percentage adhesion ± sem

Proteoglycan involvement in adhesion mechanism

Human mast cells express several proteoglycans on their surface, including heparan sulphate and the glycosaminoglycan-rich hyaluronic acid receptor CD44. Mast cells also release heparin upon degranulation. Externally added polysaccharides heparin and hyaluronic acid have previously shown competitive blocking ability in other studies (Incardona et al, 1996; DeLisser et al, 1993).

No significant effect on adhesion was seen when heparin was added to HLMC in the presence of unactivated or cytomix activated epithelium (Figure 3.45a). Heparinase I had a small but statistically significant effect in reducing HLMC adhesion (Figure 3.45b), although there was no inhibitory effect in the dose response (n=1) (Figure 3.45c) or HMC-1 adhesion (table 3.4). Heparinase III did not have any effect on HLMC, either at a single dose (Figure 3.45b) or as a dose response (Figure 3.45c) and heparinase III did not affect HMC-1 adhesion (Table 3.4). Preincubation of HLMC (n=3)(Figure 3.46) or HMC-1 (table 3.4) with either hyaluronic acid or hyaluronidase did not abrogate adhesion.

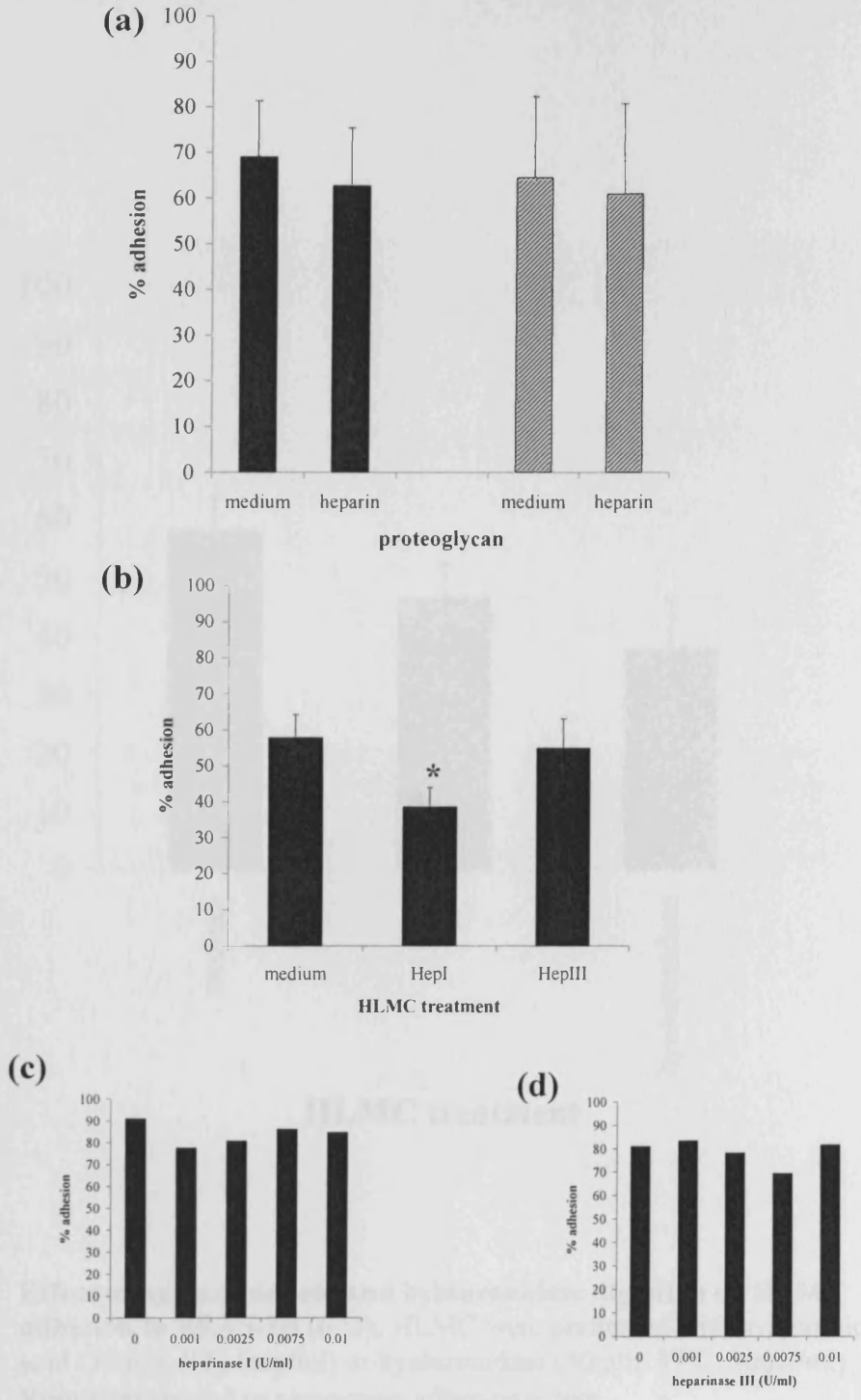


Figure 3.45 Involvement of heparin or heparan sulphate in adhesion
(a) preincubation of HLMC with heparin (1mg/ml, 30min RT) prior to adhesion to unactivated and cytomix-activated BEAS-2B (n=4,3)
(b) effect of heparinase I and III digestion of HLMC (2U/ml, 37°C, 30min) prior to adhesion to BEAS-2B (n=3)
(c) and (d) dose response of heparinase I and III (n=1), (0-0.01U (10μg) /ml). Results expressed as percentage adhesion ± sem

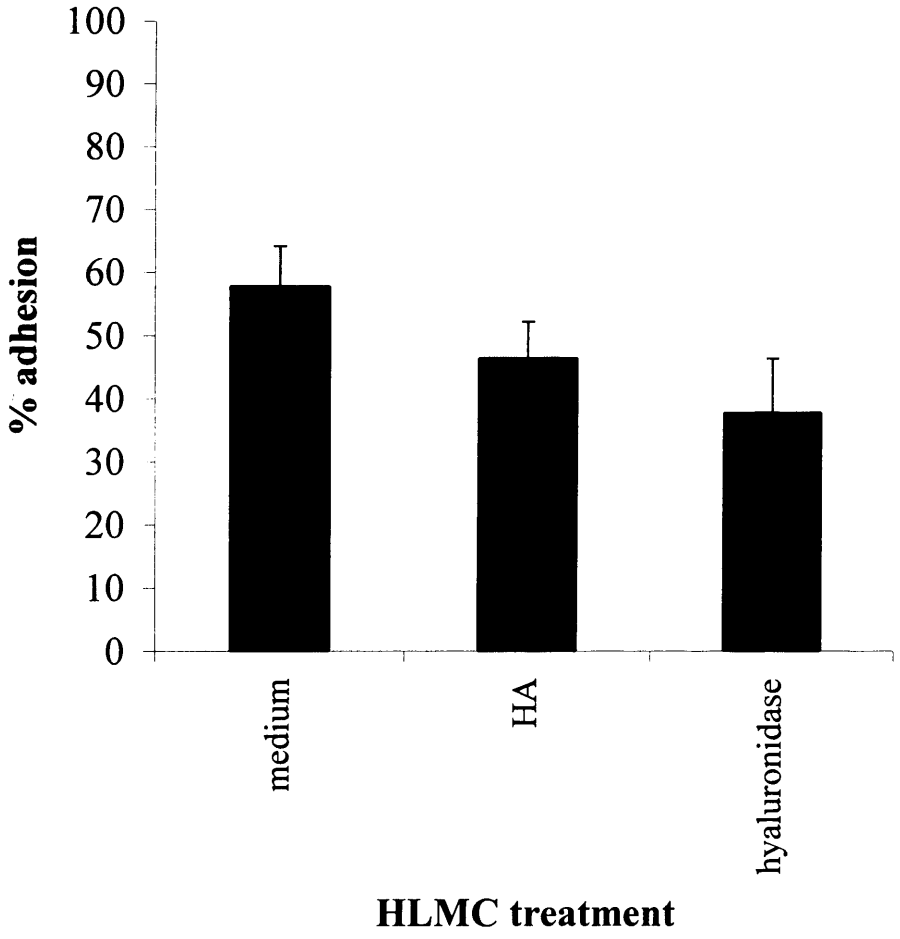


Figure 3.46 Effect of hyaluronic acid and hyaluronidase digestion on HLMC adhesion to BEAS-2B (n=3). HLMC were pretreated with hyaluronic acid (30min, RT, 1mg/ml) or hyaluronidase (30min, 37°C, 300U/ml) Results expressed as percentage adhesion ± sem

Proteoglycan/enzyme	% adhesion (n=3)
Medium	37.0±1.8
heparin	49.6 (n=1)
Heparinase I	34.4±2.3 (p=0.58)
Heparinase III	28.9±4.1
Hyaluronic acid	39.3 (n=1)
Hyaluronidase	27.2±3.9

Table 3.4 **Effect of proteoglycan and proteoglycan-enzyme digestion on HMC-1 adhesion to BEAS-2B.** HMC-1 were pretreated for 30min at RT with heparin (1mg/ml) or hyaluronic acid (1mg/ml). HMC-1 were pretreated for 30min at 37°C with heparinase I (2U/ml), heparinase III (2U/ml) or hyaluronidase (300U/ml).

3.4 Discussion

Asthmatic BAL fluid and bronchial biopsy studies have shown that atopic asthma is characterised by localised bronchial mucosal inflammation, mast cell activation and degranulation (Djukanovic et al, 1990; Tomioka et al, 1984; Flint et al, 1985). Increased mast cell numbers are observed within the asthmatic bronchial epithelium (and correlates with disease severity), but are generally scarce in bronchoalveolar lavage fluid and sputum compared to eosinophils (Gibson et al, 1993; Flint et al, 1985; Wardlaw et al, 1988). Most studies have suggested that intraepithelial migration is responsible for this increase, as variation in local distribution and not total mast cell numbers has been observed (Enerbäck et al, 1986). Controversially, this increase has also been attributed to mast cell proliferation since increased proliferating cell nuclear antigen (PCNA) was observed in mucosal mast cells (Kawabori, 1995). Increased mast cell infiltration of the bronchial epithelium may be important because it would lead to increased exposure to airborne allergens, thereby causing the increased mast cell activation and ongoing degranulation observed in asthma (Laitinen et al, 1993; Pesci et al, 1993; Gibson et al, 1993). In addition, mast cell localisation within the bronchial mucosal microenvironment may expose mast cells to specific epithelial-derived proinflammatory mediators (e.g. GM-CSF, SCF), (Sousa et al, 1993; Cox et al, 1992; Cromwell et al, 1992; Wen et al, 1996). This may result in increased activation and degranulation, or altered mast cell phenotype and interactions with surrounding tissue. The potential regulatory role of mast cell interactions with epithelium would justify further study of this interaction *in vitro*.

The mechanism of mast cell retention within the bronchial epithelium is unknown, although it was thought that specific cell adhesion molecules, similar to those involved in leukocyte-endothelial adhesion/transmigration, or lymphocyte recirculation/homing were involved. Mast cell phenotype and localisation are regulated by local microenvironmental signals including those from surface cell adhesion molecule interactions. Mast cell adhesion may produce signalling-induced changes in cytoskeletal structure, gene expression, cellular differentiation and secretion (Hynes et al, 1992).

In this study, mean adhesion of HLMC to primary (68.4%) and BEAS-2B (60.1%) bronchial epithelial cells was high compared with eosinophil adhesion to BEAS-2B (10.3%). This may explain why, despite similar intraepithelial numbers of both cell types, greater numbers of eosinophils were consistently found in asthmatic BAL fluid and sputum compared to mast cells. The kinetics of cell movement and distribution may involve continuous eosinophil chemotaxis through the epithelium into the bronchial lumen. In contrast, mast cell chemotaxis into the epithelium and intraepithelial retention may occur. Eosinophils are thought to be responsible for most of the observed mucosal tissue damage during inflammation, and therefore low adherence may promote airway inflammation resolution by enhancing epithelium-transmigrated tissue eosinophil removal by the mucociliary escalator. Mast cells sequester specific IgE, prolonging its presence and contributing to immunological 'memory', and therefore it may be advantageous to prevent mast cell loss by retention at the epithelial surface.

Similar proportions of mast cells adhered to primary and BEAS-2B bronchial epithelial cells. In addition, HLMC and HMC-1 adhesion to BEAS-2B were also comparable. This suggested that the mechanism of interaction between cell lines and primary cells was similar and conserved, despite observations that transformed and cancer-derived cells often display altered surface adhesion molecules. The BEAS-2B bronchial epithelial cell line was therefore thought to be most suitable for use in further studies. BEAS-2B cells may possess greater similarity to basal epithelial cell phenotype than ciliated columnar epithelial cells since they adhered to matrix protein, were capable of dividing, did not differentiate upon prolonged culture and displayed a similar profile of mucin expression. Mast cells are commonly found within the bronchial epithelium (Pesci et al, 1993; Djukanovic, 1990; Laitinen et al, 1991). Adherence to basal epithelial cells would be expected to prevent mast cell loss upon epithelial desquamation. The study of mast cell adhesion to the apical surface of BEAS-2B monolayers in this study was therefore thought to be physiologically relevant. In addition, a subgroup of mast cells adherent to the surface of the epithelium in asthma are thought to be the main contributors of mediators such as histamine and tryptase found in asthmatic BAL and sputum during the early asthmatic response (Patterson et al, 1974; Patterson et al, 1977). This interaction may therefore be important *in vivo*.

It should be noted that mast cells were derived from lung resection tissue from older, male smokers with lung cancer. Although lung tissue used appeared to be morphologically normal, cell phenotype may be expected to differ from the normal or atopic asthma phenotype. In addition, *in vivo*, mast cells are continuously exposed to soluble or transmembrane SCF from tissue (bone marrow stromal cells, endothelial cells, fibroblasts, epithelium and smooth muscle cells), or normal human serum (contains ~3ng/ml SCF) (Langley et al, 1993). SCF contributes to mast cell function, mediator release, survival and extracellular matrix receptor expression (Bischoff and Dahinden, 1992), and is optimally active at 1-10ng/ml. Although mast cells were routinely cultured overnight in human recombinant SCF (10ng/ml) prior to use, it may not reflect true physiological conditions.

Cell adhesion receptors expressed by human lung mast cells (HLMC) include integrin, immunoglobulin superfamily and proteoglycan receptors. These include β_1 integrins CD49d/CD29 ($\alpha_4\beta_1$; VLA-4) and CD49e/CD29 ($\alpha_5\beta_1$; VLA-5), β_3 integrin CD51/CD61 ($\alpha_v\beta_3$; vitronectin receptor), CD54 (ICAM-1), CD50 (ICAM-3), CD58 (LFA-3), CD43 (leukosialin), CD44 (Pgp-1) and CD9 (p24) (Agis et al, 1996; Füreder et al, 1997). HMC-1 cells also express a similar profile of adhesion receptors, possibly reflected in the similar proportion of adherent cells observed in this study (Agis et al, 1996). HMC-1 cells express *c-kit* and contain tryptase, but do not express FcεRI (Nilsson et al, 1994a). HMC-1 also express several integrins ($\alpha_3\beta_1$, $\alpha_L\beta_2$ and to a lesser extent $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$), IGSF (ICAM-1, ICAM-3, LFA-3), E-cadherin and the cell surface proteoglycan CD44. Selectins, VCAM-1, or $\alpha_E\beta_7$ were not expressed by HMC-1 cells (Trautmann et al, 1997; Valent et al, 1990).

Conflicting results exist of leukocyte β_2 integrin CD18 expression by mast cells. Previous studies have shown that HMC-1 or human lung, skin or gut mast cells did not express CD18 (Agis et al, 1996; Valent et al, 1989). In the present study, however, flow cytometry showed ~20% purified HLMC expressed CD18 in three separate patients. HLMC appeared to express very small amounts of CD11b and CD11c. The percentage of CD11b and CD11c positive cells together accounted for the CD18 present. Mast cell types which have also been shown to express CD18 include human stem cell-derived cultured mast cells (CD11a/CD18, CD11b/CD18), HMC-1 subclone

5C6 (resting cells CD11a/CD18 positive; CD11b, CD11c positive upon 24h PMA stimulation), human uterine mast cells (CD11c/CD18) and upto 50% inflamed skin mast cells (increased CD18) (Weber et al, 1995; Guo et al, 1992). Differences in CD18 expression and CD11 isotype may be due to separation protocol, mAbs used, mast cell phenotype, degree of mast cell activation, inflammatory status of the source tissue or equipment sensitivity.

The intraepithelial T cell integrin $\alpha_E\beta_7$ (CD103) is a ligand for intestine and breast epithelial E-cadherin, and is suggested as the T cell-epithelial cell mucosal homing mechanism (Cerf-Bensussan, 1987; Shimoyama et al, 1989; Cepek et al, 1993; Cepek et al, 1994; Higgins et al, 1998). Because intraepithelial T cells and mucosal mast cells have identical tissue localisation, their homing/retention mechanisms were thought to be identical. This theory was strengthened by the observation that induction of $\alpha_E\beta_7$ was observed on activated IL-3 cultured murine mast cells (12hr pretreatment with TGF-β (5ng/ml) or anti-IgE) (Smith et al, 1994). Mast cell $\alpha_E\beta_7$ adherence to epithelial E-cadherin has been widely accepted as the mechanism involved in mast cell localisation to epithelium, although it has not been directly confirmed (Smith and Weis, 1996). Murine mast cells may posess phenotypic differences to human mast cells. Murine mucosal mast cells express both $\alpha_4\beta_7$ and $\alpha_4\beta_1$ (Smith et al, 1994), in contrast to HLMC in the present study, which only expressed $\alpha_4\beta_1$ (CD49d/CD29). Genistein has previously been shown to inhibit CD29 and CD49d-clustering- and anti-IgE-induced histamine release by human basophils, but was less efficacious on human lung mast cell histamine release (Warner et al, 1995; Lavens et al, 1992). Interestingly, HLMC histamine release was not induced by integrin clustering or tissue fibronectin (Warner et al, 1995; Lavens et al, 1996).

Bronchial epithelial cells constitutively expressed ICAM-1 (the CD11a/CD18 ($\alpha_L\beta_2$; LFA-1) counterligand), but not VCAM-1 (the CD49d/CD29 ($\alpha_4\beta_1$; VLA-4) counterligand) (Atsuta et al, 1997; Bloemen et al, 1993). BEAS-2B cell adhesion molecule expression may be increased by cytokine stimulation: increased ICAM-1 (by IFN-γ) and VCAM-1, (by IL-4) have been shown (Atsuta et al, 1997). In the present study, indirect flow cytometry showed that cytomix increased BEAS-2B ICAM-1 expression and induced VCAM-1 expression. E-cadherin expression

remained unchanged post-cytomix treatment. Human bronchial epithelial cells have also been shown to constitutively express a number of integrin subunits important for maintaining normal epithelial structure ($\alpha_{1\text{-}6}\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$) and as integral components of hemidesmosomes ($\alpha_6\beta_4$) as well as CD44, LFA-3 and HLA-I (Atsuta et al, 1997; Wang et al, 1996b).

Possible candidate cell adhesion molecule pairings involved in HLMC adhesion to bronchial epithelium were therefore β_2 integrin/ICAM-1, $\alpha_4\beta_1$/VCAM-1 or $\alpha_E\beta_7$/E-cadherin. The lack of effect of function-blocking mAb, Ca^{2+}/Mg^{2+} chelation or Mn^{2+} activation showed that these cell adhesion molecule pairings were probably not involved in adhesion. Unlike leukocyte-endothelial or eosinophil-epithelial adhesion, HLMC adhesion did not alter after epithelial activation with cytomix, despite the observed increases in ICAM-1 and VCAM-1. Although increased expression of CD11b, CD11c and ICAM-1 has been observed on HMC-1 cells after PMA pretreatment (Weber et al, 1995), PMA did not affect adhesion, further implying that the β_2 integrin/ICAM-1 adhesion pathway was not involved. Neither untreated or stimulated HLMC (TGF-β or anti-IgE) expressed $\alpha_E\beta_7$, and HLMC pretreatment with TGF-β did not increase adhesion. This implied that the proposed $\alpha_E\beta_7$/E-cadherin interaction was not involved in HLMC to BEAS-2B. In addition to murine mast cell $\alpha_E\beta_7$ induction, TGF-β has been shown to elevate expression of the β_1, β_2 and β_3 families of integrins (Heino et al, 1989; Ignotz et al, 1989). TGF-β may also induce or downregulate/regulate immunoglobulin superfamily (IGSF) cell adhesion molecule function, including ICAM-1, PECAM-1, carcinoembryonic antigen (CEA) and NCAM (Lastres et al, 1994).

Bronchial epithelial cells produce TGF-β and SCF (Magnan et al, 1994; Aubert et al, 1994; Vignola et al, 1997; Wen et al, 1996). Their effect on mast cells have been previously investigated. TGF-β does not directly cause mast cell degranulation (Broide et al, 1989). There is conflicting evidence of mast cell TGF-β receptor expression. Murine and human mast cells (cultured and HMC-1) express TGF-β receptors and treatment with TGF-β produced a polarised morphology and several hundred-fold greater chemotaxis when compared to SCF (Gruber et al, 1994; Meininger et al, 1992; Olsson et al, 2000). Another study of human lung and uterine

mast cells, and HMC-1 showed that the TGF-β receptor (CD105) was not expressed (Agis et al, 1996).

The mast cell SCF receptor *c-kit* is a member of the receptor tyrosine kinase (RTK) family and is produced by endothelial cells, epithelial cells, smooth muscle cells and mast cells themselves. SCF promotes proliferation, differentiation and maturation of mast cells, is a chemoattractant for immature mast cells and enhances extracellular matrix receptor expression (Nilsson et al, 1994a,b). SCF may also alter mast cell mediator content, induce or enhance release in response to FcεRI stimulation, or produce similar signalling responses to those observed upon FcεRI crosslinking (intracellular free Ca^{2+}, MAP kinases, mRNA induction for early response genes) (Bischoff and Dahinden, 1992; Coleman et al, 1993). SCF has been found in nasal lavage fluid after allergen provocation of allergic subjects (Nilsson et al, 1998). The presence of SCF is linked to tissue mast cell numbers (Finotto et al, 1997). SCF is also directly involved in mast cell adhesion to fibroblasts.

Adhesion may directly involve the *c-kit* receptor by allowing attachment to the extracellular domain of fibroblast cell surface transmembrane SCF (Adachi et al, 1992). Cultured murine mast cell adhesion required SCF-*c-kit* interactions and this interaction was necessary for mast cell proliferation, maturation and survival. These findings were confirmed by investigating adhesion of a murine mast cell line that lacked the extracellular domain of membrane *c-kit* and therefore did not adhere to fibroblasts (Mekori et al, 1997). Cultured murine mast cell adherence to fibroblasts through transmembrane SCF-*c-kit* interaction was functional and led to mast cell histamine and eotaxin release (Hogaboam et al, 1998). However, human skin mast cell attachment to fibroblasts was not altered by addition of excess soluble SCF or function-blocking mAbs to the SCF binding site of *c-kit*, possibly reflecting murine/human phenotype differences (Trautmann et al, 1997). HLMC adhesion to BEAS-2B in the present study showed similar characteristics to the findings of Trautmann et al, 1997. SCF or genistein pretreatment of HLMC did not modulate adhesion and polyclonal SCF-receptor mAb did not inhibit adhesion to BEAS-2B. These results confirmed that the SCF/*c-kit* interaction and subsequent signalling were

probably not involved in adhesion. Lack of inhibition by genistein further confirmed this conclusion.

Mast cell products are involved in fibroblast proliferation and the apparent epithelial basement membrane thickening associated with asthma. A detailed study of the interaction between human mast cells and fibroblasts has been carried out recently (Trautmann et al, 1997). The high baseline adhesion of HMC-1 (>90%) to fibroblasts was not mediated by known adhesion receptors (including β_1 and α_v). HMC-1 adhesion to fibronectin and collagen I (both >70%) were β_1-mediated and adhesion to vitronectin was α_v–mediated. Adhesion was not inhibited by mAbs interfering with *c-kit*, in contrast to murine mast cell adhesion to fibroblasts (Adachi et al, 1992). Adhesion was only partially sensitive to trypsin or EDTA-mediated calcium chelation (implying cadherin, integrin or C-type lectins were unlikely to be involved). Adhesion was reduced approximately 50% by pronase pretreatment showing partial involvement of proteins in adhesion. Cell surface carbohydrates and lectins were not involved in adhesion since tunicamycin pretreatment and adhesion in the presence of carbohydrates (including lactose and N-acetylgalactosamine) did not inhibit adhesion.

In the present study, adhesion of HLMC to BEAS-2B was also not inhibited by function blocking β_1 and $\alpha_v\beta_3$ mAbs. This result shows lack of direct involvement of these integrins in adhesion. In addition, adherence to the human plasma fibronectin required for epithelial adherence, or epithelial-produced extracellular matrix molecules was also not occurring. Similar properties existed between mast cell adhesion to fibroblasts and HLMC adhesion to BEAS-2B (both were not mediated by common cell adhesion molecules, not Ca^{2+}-dependent and were mediated by a mast cell protein). However in contrast, glycosidase pretreatment of HLMC showed adhesion to epithelium was carbohydrate-dependent.

Mast cell adhesion to epithelium has previously been studied using dog mastocytoma cells and tracheal epithelium (Varsano et al, 1988). Mastocytoma cells were used because they closely resemble mast cells, were available in large numbers in a relatively pure form and did not require extensive purification procedures. The kinetics of adhesion to epithelium and phenotype of dog mastocytoma cells were

similar to HLMC adhesion to epithelium in this study (constant adhesion independent of mast cell concentration, adhesion plateauing at 30min, epithelium-adherent mast cells were not spread, and mast cells preferentially adhere to epithelium, not matrix-coated areas). Mastocytoma cells adhered preferentially to epithelial cells present in tracheal tissue sections, and adhered poorly to human placenta collagen I, collagen IV, and human serum fibronectin (<7.5%), which may contribute to preferential epithelial retention of mast cells. A high baseline adherence of dog mastocytoma cells to cultured dog tracheal epithelium (35±13%) was observed, adhesion was not Ca^{2+}/Mg^{2+}-dependent and did not require energy. These findings were similar to HLMC adhesion to BEAS-2B and suggested that spontaneous adhesion was mediated by a simple ligand-receptor interaction that did not require energy (in contrast to integrin activation required for leukocyte-endothelium adhesion). The epithelium in this study was fixed with paraformaldehyde prior to mast cell EDTA or EGTA pretreatment. The epithelial surface adhesion molecule was sensitive to fixation (suggesting a conformational change may be required, and that adhesion was not non-specific). Mastocytoma cells did not express CD18, and adhesion was not mediated by mast cell CD18 (Varsano et al, 1988). In contrast to HLMC adhesion in the present study, adhesion was sensitive to proteolytic digestion with both pronase and proteinase k (but not trypsin), suggesting a mast cell surface protein was required for adhesion. HLMC adhesion to BEAS-2B in this study was only pronase-sensitive (which may have been due to selective adhesion receptor cleavage - confirmed by flow cytometry assessment of protease-treated HMC-1 cell adhesion molecules). This showed that a mast cell-expressed peptide(s) was required for mast cell adhesion to bronchial epithelium. The serine protease trypsin (and tryptase) act on PAR-2 and therefore PAR-2 signalling was probably not involved in mediating adhesion.

Mast cell activation with FcεRI aggregation, A23187 or PMA increased adhesion to fibronectin and laminin which was external divalent cation-dependent (Thompson et al, 1989a,b; Dastych et al, 1991). Conflicting results have been obtained when murine and human mast cell adhesion to laminin have been studied. Stimulated murine mast cells (cell lines and cultured) express laminin receptors and adhere to laminin via CD49f/CD29 ($\alpha_6\beta_1$, VLA-6) *in vitro* (Fehlner-Gardiner et al, 1996). Both IgE-and A23187-dependent activation increased murine mast cell adhesion to laminin

(Thompson et al, 1990). Human dermal connective tissue-type mast cells (CTMC) constitutively express laminin receptors, and upon degranulation (A23187 or morphine sulphate) there is loss of CTMC laminin binding and downregulation of laminin receptor expression, enabling mast cells to mobilise away from the site of challenge after release of mediators (Walsh et al, 1991a).

IgE-dependent activation of murine mast cells would be expected to cause an increase in their adhesion to epithelium through $\alpha_E\beta_7$ induction (Smith et al, 1994; Smith and Weis, 1996). However, in the present study, IgE-dependent activation decreased HLMC adhesion to bronchial epithelium in a dose-dependent manner (40% inhibition occurred at 1% anti-IgE). Mast cell de-adhesion upon activation may enhance mast cell migration towards the epithelial surface or migration towards regional lymph nodes following antigen exposure (Wang et al, 1998). The mechanism involved in IgE-induced de-adhesion of mast cells was not clear. Receptor dilution or membrane fluidity changes upon granule/plasma membrane fusion, surface receptor cleaveage by mast cell protease/glycosidase(s), or cell surface receptor down-regulation upon FcεRI signalling were possible mechanisms involved. Downregulation of cell surface receptors may occur by internalisation, proteolytic cleavage from the cell surface (e.g. L-selectin) or functional loss of binding sites by conformational change.

The signalling cascade post-FcεRI aggregation involves activation of the src family protein kinase lyn (constitutively FcεRI β chain-associated), resulting in receptor subunit (β and γ chain) tyrosine phosphorylation of the cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) consensus sequences (Jouvin et al, 1994). β chain ITAM tyrosine phosphorylation results in additional lyn recruitment, and γ chain, syk. Syk recruitment and activation results in phospholipase C-γ tyrosine phosphorylation and rapid hydrolysis of inositol phospholipids. This yields the second messengers inositol triphosphate (IP_3) which induce mobilisation and rise of intracellular Ca^{2+} $[Ca^{2+}]_i$, and diacylglycerol (DAG) which activate protein kinase C (PKC), leading to phosphorylation of other diverse proteins (Ozawa et al, 1993). Granule discharge takes place by compound exocytosis, involving a combination of granule-granule and granule-plasma membrane fusion encompassing most of the

storage granule pool. ATP, $[Ca^{2+}]_i$, the cytoskeleton and G-proteins (Rac and Rho) are involved in regulation of mast cell exocytosis.

Substance P-induced human cutaneous mast cell histamine release involved G-protein and protein kinase C signalling, but not tyrosine kinases (since it was inhibited by pertussis toxin and PMA, but not genistein) (Columbo et al, 1996). The opposite profile was observed for IgE-mediated histamine release, and was not unexpected, since tyrosine phosphorylation is an integral component of IgE-mediated signalling (Columbo et al, 1996). In addition, mediator release requires the presence of extracellular calcium and intact glycolytic and oxidative phosphorylation pathways. Human lung and skin mast cells release and generate PGD_2 and LTC_4 on stimulation either with A23187 or anti-IgE, although only skin mast cells respond to other secretagogues such as substance P, opiates, polyamines and compound 48/80, partially dependent on extracellular calcium and fully dependent on oxidative phosphorylation. The immunosuppressant FK506 inhibits SCF- and IgE-mediated (but not A23187-mediated) histamine release from HLMC (Sperr et al, 1996; De Paulis et al, 1991). FK506 also inhibited IgE-mediated LTC_4 and PGD_2 release (De Paulis et al, 1991). FK506 was also active in a similar manner on human uterine and skin mast cells (Sperr et al, 1996; De Paulis et al, 1992). FK506 inhibited IgE-mediated RBL-2H3 exocytosis and serotonin release, but did not affect early membrane-associated events (FcεRI aggregation, PI hydrolysis, increase in $[Ca^{2+}]_i$ (Hultsch et al, 1991; Hultsch et al, 1990). Therefore the lack of effect, in this study, of A23187, FK506, cytochalasin D, pertussis toxin, EDTA and sodium azide implied that degranulation itself was not involved in regulation of mast cell adhesion.

In this study, IgE-mediated activation led to decreased human lung mast cell adhesion to bronchial epithelium. IgE-mediated serotonin release has been shown to result in increased actin polymerisation and associated membrane ruffling (Pfeiffer et al, 1985). Ionophore activation of mast cells caused a more persistent signal and larger histamine release compared to FcεRI-mediated signals. However, although both methods of activation caused RBL-2H3 cell spreading, actin cytoskeleton rearrangement and membrane ruffle formation, A23187 stimulation caused less pronounced membrane changes (Sahara et al, 1990). This may be due to their

different mechanisms of action, and may explain why IgE-mediated but not A23187-mediated activation of HLMC caused de-adhesion. Crosslinked FcεRI is clustered, internalised and leads to tyrosine kinase signalling, phosphoinositol breakdown, and Ca^{2+} influx. A23187 binds and transports Ca^{2+} directly into the cells.

Either receptor internalisation, or phosphoinositol breakdown may have caused membrane fluidity/surface morphology changes associated with reduced adhesion. In addition, formation of IgE-mediated deep membrane grooves may cause redistribution of surface adhesion molecules. Since A23187 did not affect adhesion, this implied that all components of signalling post-Ca^{2+} influx and including degranulation itself did not directly affect adhesion. Therefore signalling changes specific for FcεRI and prior to increased $[Ca^{2+}]_i$ caused the observed decreased adhesion. Activation of protein kinase C and increased $[Ca^{2+}]_i$ are both important steps in the signalling pathway leading to mast cell degranulation, although activation of PKC with PMA alone does not induce RBL cell degranulation (Cunha-Melo et al, 1989).

Degranulation leads to release of heparin proteoglycan, histamine and tryptase. In this study, the lack of effect on adhesion following pretreatment with HLMC lysate suggested there was no involvement of signalling by histamine and other preformed mediators in this process. Mast cell heparin was not involved in adhesion since neither preincubation with heparin or heparinase pretreatment decreased adhesion.

The histamine level in human mast cells (both MC_{TC} and MC_T types) ranges from 1 to 3 pg/cell (Castells et al, 1987). In resting cells, histamine is stored in secretory granules at acidic pH, is positively charged and bound to carboxyl groups on proteins or proteoglycans. When mast cells are activated, histamine dissociates from the proteoglycan matrix in exchange for extracellular sodium at neutral pH. Confirmation that histamine was not implicated in the interaction of mast cells with epithelium was obtained when HLMC were pretreated with histamine and adhesion was not altered. β-tryptase is the major enzyme in all mast cells and accounts for upto 20% of the protein content. Lung mast cells contain 11pg and skin mast cells 35 pg β-tryptase per cell (Schwartz et al, 1987). Tryptase is a serine endoprotease that has trypsin-like activity. Tryptase is involved in fibrogenesis and is known to stimulate proliferation,

chemotaxis and collagen production by fibroblasts (Ruoss et al, 1991; Gruber et al, 1997; Cairns and Walls, 1997). Tryptase also stimulates epithelial and endothelial proliferation, ICAM-1 expression, IL-8 production and granulocyte recruitment (Cairns and Walls, 1996; Blair et al, 1997). Adhesion was measured in the presence and absence of leupeptin to confirm serine proteases such as tryptase did not cause reduced adhesion. Although baseline adhesion in the presence of leupeptin was reduced, adhesion in the presence of leupeptin was consistently equal or lower than with anti-IgE activation alone, showing tryptase was not involved in causing the observed decreased adhesion.

Newly formed mediators such as arachidonic acid metabolites (LTC_4 and PGD_2), PAF and cytokines/chemokines (including protein or mRNA for TNF-α, GM-CSF, SCF, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, MIP-1α) are synthesised after mast cell activation. Although they would not be present in HLMC lysates, these mediators were probably not involved in decreasing HLMC adhesion to epithelium since HLMC preincubation with supernatants from 24hr IgE-activated HLMC did not cause decreased adhesion compared to control supernatants (61.2±4.3% untreated, 57.8±7.8% treated, n=3). FcεRI-mediated downregulation of a cell-surface receptor (through internalisation, proteolytic cleavage or redistribution) was therefore thought to be the most likely explanation for activation-induced reduced adhesion.

Cell surface carbohydrates are important in cell-cell recognition and adhesion (Sharon and Lis, 1989). Lectins are proteins (other than antibodies or enzymes) that are carbohydrate ligands (Barondes et al, 1994). Carbohydrates ligands may be pure carbohydrates or glycoconjugates (attached to proteins or lipids). The combination of a neuraminidase, β-galactosidase and hexosaminidase will reduce most O-linked oligosaccharides to the core structure. The carbohydrate-digesting enzymes used in this study to test the involvement of human mast cell surface carbohydrates in adhesion included α2-3,6,8-neuraminidase (sialic acid residues), endo-α-N-acetylgalactosaminidase (unsubstituted Galβ(1-3) GalNAcα) and β-galactosidase (terminal β1-3 linked galactose residues).

Terminal sialic acid and fucose residues frequently decorate carbohydrates involved in adhesion (e.g. selectins, mucins). The most common substitution of glycans is mono-, di-, or trisialyation. These residues are easily removed by a suitable sialidase. α2-3,6,8-neuraminidase (*Arthrobacter ureafaciens*) is specific for N- and O- acyl, non-reducing terminal branched and unbranched sialic acids in α2-3, 6 or 8 linkages to various monosaccharides (e.g. galactose, sialic acid (NeuAc/NeuGc), N-acetylglucosamine, N-acetylgalactosamine) in lipid- and protein-linked glycans. It is unique among sialidases because branched sialic acids (linked to internal residues) will also be cleaved. α2-3,6,8-neuraminidase preferentially hydrolyses terminal α2,6-linkages between N-acetylneuraminic acid and N-acetylgalactosamine residues in various mucopolysaccharides. The relative cleavage rate is α2,6 > α2,3 > α2,8. In addition, in conjunction with α-fucosidase and α-N-acetylgalactosaminidase, this neuraminidase will remove virtually all carbohydrate from porcine submaxillary mucin. Epithelial cells produce transmembrane and secreted mucins, and their oligosaccharide sequences bind to microorganisms and parasites, which may be removed by ciliary action. Although mast cells do not express selectins, their membrane glycolipids (e.g. gangliosides) do contain sialylated carbohydrates (Weber et al, 1997). Sialomucin cell adhesion molecules such as CD34, CD43, CD45RA, MAdCAM-1, GlyCAM-1 and PSGL-1 mediate adhesion via sialic acid residues. Since neuraminidase pretreatment did not affect adhesion, sialic acid present on these cell adhesion molecules and on mucopolysaccharides or mucins may be excluded from mediating HLMC adhesion to BEAS-2B.

Another important modification found on O-linked oligosaccharides includes mucin α-linked N-acetylgalactosamine. Endo-α-N-acetylgalactosaminidase (*Streptococcus pneumoniae*) is a very specific enzyme and specifically cleaves only unsubstituted Galβ1-3GalNAc disaccharides attached to the serine/threonine residues of core proteins typically found in O-linked glyans present in mucin-like molecules (Bhavanandan and Codington, 1983). Any core structure modification (sialic acid, galactose, fucose, GlcNAc or GalNAc) will block the action of endo-α-N-acetylgalactosaminidase and all additional residues must be removed by other exoglycosidases first. Endo-α-N-acetylgalactosaminidase pretreatment of HLMC

produced approximately 40% reduction in adhesion, showing that adhesion was partially mediated by unsubstituted Galβ1-3GalNAc disaccharide residue(s).

β1-3,4,6-galactosidase (bovine testes) cleaves all beta-linked, non-reducing terminal galactose residues from complex carbohydrates and glycoproteins. It is specific for Galβ1,3-GlcNAc ≥ Galβ1,4-GlcNAc >>Galβ1,6-GlcNAc. Activity of this enzyme against β1-3 galactose residues distinguishes it from most other β-galactosidases which show a preference for Galβ1-4 and cleave the β1-3 linkage very slowly, if at all. β-galactosidase treatment of both HLMC and HMC-1, but not epithelium produced a marked reduction in mast cell adhesion (approximately 75%), suggesting that mast cell-expressed β-galactosides were responsible for most of the adhesion seen.

Therefore since significant attenuation of adhesion was observed after incubation of HLMC with either endo-α-N-acetylgalactosaminidase or β-galactosidase, HLMC terminal β1,3galactose-bearing carbohydrate(s) were responsible for adhesion to unknown (non-β-galactoside-bearing) epithelial ligand(s). Because the endo-α-N-acetylgalactosidase ligand cleaved was unsubstituted and directly linked to the core protein, this was probably the minimal ligand recognised by the epithelial lectin. β-galactosidase would also be able to cleave the terminal β1-3-linked galactose, thereby also reducing adhesion.

Lectins are carbohydrate-binding peptides that are often associated with cell activation and differentiation. They are classified into families based on the structure of their highly conserved carbohydrate-recognition domain (CRD). C-type (e.g. endothelial and leukocyte selectins), pentraxin, P-type (e.g. mainly intracellular mannose-6-phosphate receptor), I-type (e.g. macrophage sialoadhesin, B cell CD22, myeloid cell CD33), heparin-binding proteins (e.g. plasma/matrix fibronectin, vitronectin), hyaluronan-binding proteins (e.g. CD44) and S-type (e.g. galectins on many cell types) have been described (Ni and Tizard, 1996). Monosaccharides, polysaccharides and proteoglycans are commonly used to competitively inhibit lectin-mediated adhesion. Galactose, lactose, mannose, mannose-6-Phosphate, fucoidin, heparin and hyaluronic acid were all used in this study. The concentrations used to

attempt inhibition of HLMC-BEAS-2B adhesion had been used previously to successfully and specifically inhibit lectin-mediated adhesion in other systems (Incardona et al, 1996; Yednock et al, 1987; Baum et al, 1995; Rochon et al, 1994; Colgan et al, 1995; Inohara and Raz, 1995; Brassart et al, 1992). In this study, none of these reagents were successful in inhibiting adhesion.

C-type lectins are involved in cell adhesion (e.g. selectins), activation and signalling (e.g. CD69). Mast cells express the C-type lectin MAFA (mast cell function-associated antigen) which is involved in inhibiting FcεRI-mediated secretion. RBL-2H3 mast cell MAFA may be directly or indirectly involved in adhesion (Guthmann et al, 1995). In this study, calcium chelation did not affect adhesion implying C-type lectin and pentraxin-dependent adhesion were not involved in mast cell-epithelial cell adhesion. P-type lectins are mainly intracellular and bind mannose-6-phosphate. Mannose-6-phosphate did not block mast cell adhesion to BEAS-2B. The sialoadhesin family of proteins (I type lectins, siglec – sialic acid-binding Ig-like lectins) may be excluded from involvement in adhesion since neuraminidase did not reduce adhesion. Heparin-binding proteins were not involved in adhesion since, although heparinase I caused a small, statistically significant reduction in HLMC adhesion, there was no effect on HMC-1 adhesion, and neither heparinase III nor heparin inhibited adhesion. Hyaluronan-binding proteins (e.g. human mast cell or epithelial CD44) are also not involved in adhesion, since hyaluronic acid and hyaluronidase had no effect on adhesion. The inhibitory effect of β-galactosidase suggested that the main candidate adhesion molecules involved in HLMC adhesion to BEAS-2B were the S-type lectins (Galectins), characterised by their adhesion to β-galactosides (Barondes et al, 1994). Galectin-1 and –3 have been most studied, and therefore reagents were available to test for their involvement.

Galectin-1 is expressed on a number of epithelial cell lines (Baum et al, 1995) and induced activated T cell apoptosis (Perillo et al, 1995). Galectin-3 has been identified on both types of mast cells (MC_T and MC_{TC}, although more intense staining is observed in skin mast cells compared to those from other locations), eosinophils, neutrophils, macrophages and keratinocytes (Craig et al, 1995; Frigeri and Liu, 1992). Galectin-3 is generally thought to promote non-integrin mediated mast cell adhesion to laminin in the

basal lamina (Thompson et al, 1989a; Frigeri and Liu, 1992). Galectin-3 may bind to IgE (and other lactose-containing glycoproteins) and may thereby cause FcεRI crosslinking and mast cell or eosinophil activation and degranulation. Immunoelectron microscopy showed the presence of human mast cell galectin-3 intracellularly (in the nucleus and/or over secretory granules in the cytoplasm), on the cell surface and in the extracellular space. Activated RBL cells secreted increased surface galectin-3, paralleling cellular degranulation, and suggesting it was secretory granule-associated and released upon degranulation (Frigeri and Liu, 1992).

Galectins lack transmembrane domains (are not membrane anchored), but are secreted via the non-classical secretory pathway and bind specifically to cell surface galactose residues (Barondes et al, 1994). Galectin-1 and galectin-3 self-associate into dimers possessing two CRDs and can therefore mediate cell adhesion by bridging carbohydrate-containing counter-receptors on adjacent cells (or on the same cell, affecting adhesion through inside-out signalling) (Inohara and Raz, 1995; Perillo et al, 1998). This mechanism of action may exclude the involvement of galectins in HLMC adhesion to BEAS-2B, since adhesion was heterotypic and the epithelium did not express β-galactosides involved in adhesion. In addition, galectin-mediated interactions are characterised by inhibition with lactose. Since lactose did not inhibit adhesion, galectins are unlikely to be involved in mast cell adhesion to epithelium. However galectins were not conclusively ruled out from involvement, since the affinity for lactose may be lower than for the physiological ligand, the CRD may not be directly involved in adhesion or other residues/adhesion molecules may also be required in concert (galectins may costimulate adhesion through another cell adhesion molecule).

In this study, galectins-1 and –3 were not expressed on HLMC, HMC-1 and BEAS-2B (flow cytometry). HLMC did not adhere to galectin-1 or galectin-3 protein, and adhesion was not blocked in the presence of galectin-1 protein or adhesion-blocking galectin-3 mAb. Although galectin-1 is expressed by inflamed epithelium, HLMC adhesion to unstimulated or cytomix-stimulated BEAS-2B was not inhibited by galectin-1 protein. In addition, although degranulation was thought to result in galectin-3 secretion, galectin-3 mAb did not affect IgE-activated HLMC adhesion to BEAS-2B.

In summary, human mast cells adhere avidly to human bronchial epithelial cells *in vitro*, mediated by a novel, simple O-glycoprotein-mediated interaction. Mast cell β-galactose present on unsubstituted Galβ(1-3) GalNAcα disaccharide-core peptide was the minimal ligand for an as-yet-unidentified non-β-galactoside-containing epithelial lectin ligand. Previously recognised cell adhesion molecule or carbohydrate-receptor interactions were not involved.

Chapter 4: Effect of the epithelium on HLMC function

4.1 Introduction

HLMC interactions with microenvironment are particularly important in regulating mast cell phenotype. Mast cells are not found in blood, and mature within tissues. Therefore mast cell function is probably highly regulated by interactions with other tissue-derived cells and extracellular matrix. Mast cell interaction with the extracellular matrix protein fibronectin has been shown to regulate extent of degranulation (Hamawy et al, 1992b).

HLMC migration into, localisation within and interaction with bronchial epithelium is believed to increase during asthma (Pesci et al, 1993; Tomioka et al, 1984). The normal bronchial epithelium is a source for established HLMC-active mediators (SCF, TGF-β) and may enhance HLMC survival, mediator release and retention at mucosal surfaces (Wen et al, 1996; Bischoff and Dahinden, 1992; Aubert et al, 1994; Smith et al, 1994). Epithelial activation during asthma has been shown to increase their proinflammatory mediator production (Campbell, 1997). The functional consequences of HLMC coculture with epithelium and epithelial products was investigated.

4.2 Materials

Antibodies

Anti-BrdU mAb (clone IIB5, isotype IgG_1) was purchased from Bradsure Biologicals (Loughborough, UK); FITC-conjugated goat anti-mouse IgG was bought from Dako (Ely, Cambridge, UK); polyclonal anti-human IgE was obtained from Serotec (Kidlington, Oxford, UK).

Reagents

Bromodeoxy uridine (BrdU), PBS tablets, BSA, sodium borate ($Na_2B_4O_7$), Tween-20, propidium iodide, human plasma fibronectin and pharmacological compounds were purchased from Sigma (Poole, UK); ethanol, hydrochloric acid, propan-1-ol, Mayers haematoxylin, xylene and XAM mountant were obtained from Fisher Scientific (Loughborough, UK); tyrodes buffer and FCS were purchased from Life technologies (Paisley, Scotland).

Equipment

Details of 8μm transwells, 48 well chemotaxis chamber and filters (nitrocellulose and polycarbonate) were previously mentioned; glass slides and coverslips were obtained from Fisher scientific (Loughborough, UK); 96-well scintillation plates were purchased from Packard.

Methods

4.2.1 Effect on mast cell survival

Mast cell survival in the presence of epithelial supernatants was assessed by trypan blue exclusion. Epithelial supernatants were from confluent epithelial monolayers grown in $25cm^2$ tissue culture flasks, washed gently three times with 5ml RPMI 1640, and incubated for 24 hours with 5ml fresh medium. Control medium was placed in flasks without epithelial cells for the same period of time, under the same conditions. HLMC survival was assessed at 24 hour intervals.

4.2.2 Epithelial coculture

Epithelial cells were grown at the same density as normal culture by adding to 8μm pore size transwells or 25cm^2 tissue culture flasks. When cells were microscopically assessed as confluent (after 48 hours), HLMC were added in the presence and absence of 1% anti-IgE. Cells were removed using cell dissociation fluid and assessed using indirect flow cytometry, as previously described.

4.2.3 Detection of BrdU incorporation into DNA-synthesising cells

The thymidine analogue bromodeoxyuridine (BrdU) is incorporated into the DNA of proliferating S-phase cells. The BrdU mAb recognises BrdU in denatured, single stranded DNA. Epithelial cells were grown for 24 hours in 25cm^2 flasks. Unactivated and IgE-activated HLMC were added for a further 24 hours. BrdU (10μM) was added to each flask for 1 hour, cells were removed and centrifuged for 10 minutes at 400g at room temperature. The supernatant was aspirated and the pellet was tapped gently. While vortexing, ice-cold 70% ethanol was added drop-wise to a final cell concentration of 10^6 cells/100μl, and incubated for 20 minutes at room temperature. Cell suspension (100μl/tube) was washed with 1ml wash buffer (PBS/0.5% BSA), centrifuged (5 minutes at 400g at room temperature), the supernatant was aspirated and the pellet loosened. The pellet was resuspended in fresh denaturing solution (2M HCl/0.5% BSA), mixed well and incubated for 20 minutes at room temperature. Wash buffer (1ml) was added, mixed well and centrifuged (5 minutes, 400g, room temperature). The supernatant was aspirated, the pellet resuspended in 0.5ml 0.1M sodium borate ($Na_2B_4O_7$, pH 8.5), and incubated for 2 minutes at room temperature. The cells were washed in wash buffer as before and 50μl appropriately diluted primary anti-BrdU mAb (in dilution buffer) was added to the cells and incubated in the dark for 20 minutes at room temperature. Dilution buffer consisted of PBS/0.5% Tween-20/0.5% BSA. Cells were washed in wash buffer and 50μl diluted secondary antibody (FITC-conjugated goat anti-mouse IgG) was incubated with the cells for 20 minutes at room temperature. Cells were washed, resuspended in 0.5ml PI (10μg/ml in PBS) and incubated for 30 minutes at room temperature in the dark. Cells were analysed by flow cytometer for BrdU FITC and PI red fluorescence.

4.2.4 HLMC chemotaxis

Mast cell chemotaxis was originally adapted from eosinophil chemotaxis. Mast cells did not migrate through nitrocellulose filters within 3 hours (even upon coating with upto 100μg/ml fibronectin, or increasing cell concentration added). Polycarbonate filters (5μm and 8μm) were coated with human plasma fibronectin (40μg/ml) for 1 hour at 37°C. The chemotaxis chamber was placed on a flat surface. Pre-warmed controls, chemoattractant and/or samples were rapidly added (to prevent evaporation) to the lower wells (30μl/well) so that a slight positive meniscus was formed. Wells were covered with the 5μm and 8μm filters without allowing formation of air bubbles. The plastic gasket and top chamber were placed over the filters and fixed firmly in place. Cell suspension (50μl/well at $2x10^6$ cells/ml) was added to the upper wells and the whole chamber was placed within a humidified container in the 37°C incubator for 2.5 hours. After incubation, the chamber was disassembled and the upper 8μm filter was discarded. The chamber was soaked and washed in deionised water, blotted onto tissue and allowed to air dry. After each assay, chambers were sterilised by soaking for 30 minutes with 1M sodium hydroxide at 60°C and thorough rinsing with sterile deionised water.

Staining of chemotaxis filter

The lower 5μm filter was carefully removed and stained as follows. Filters were fixed in IMS for 25 minutes, stained in Mayers haematoxylin for 5 minutes, washed in tap water until the filter turned blue evenly, dipped in IMS (approximately 40 times), placed in propan-1-ol for 2 minutes, placed in propan-1-ol/xylene for 2 minutes and placed in xylene for 25 minutes. Filters were mounted in XAM, allowed to air-dry overnight, stored at 4°C and counted within 1 day. Migrated cells on the upper surface of the filter were counted (5 high power fields/well of triplicates).

4.2.5 Effect on pharmacological inhibition of HLMC histamine release

Histamine was released using polyclonal anti-human IgE, stored as frozen aliquots and diluted with tyrodes buffer prior to use. Pharmacological compounds and diluent controls were added (50μl 2x final required concentration) to appropriate wells of a 96 well plate. Releaser or diluent controls were added (25μl 4x final required

concentration) and mixed briefly. Experimental plates were placed for 5 minutes in a waterbath pre-warmed to 37°C to equilibrate temperature prior to addition of HLMC. Upon purification, HLMC were washed by resuspending in tyrodes buffer containing 2% FCS and 1mM Ca^{2+}/Mg^{2+}, cells were centrifuged at 300g for 8 minutes at room temperature, and resuspended in pre-warmed (37°C) buffer at $0.4x10^6$ cells/ml (4x final required concentration). Cells (25µl/well) were immediately placed in pre-prepared experimental 96-well plates using a multi-channel pipette and plates were mixed briefly. Mediator release was allowed to continue for 20 minutes at 37°C and stopped by addition of 10µl 0.5M EDTA. Samples were transferred from experiment plates to plates with 'V'-shaped wells, and were centrifuged at 300g for 3 minutes at 4°C, supernatants (75µl) were removed to new polypropylene plates and stored at -20°C for histamine analysis within one week.

Total mast cell histamine and release without treatment were determined in triplicate for each experiment as follows. Stock mast cells (25µl) was added to 75µl tyrodes buffer and incubated with the experiment plate for 20 minutes, 10µl 0.5M EDTA was added and the cells were added to V-shaped wells of the plate to be centrifuged. After centrifugation, the supernatant was removed for assessment of release during the assay, and the cell pellet was resuspended in ice-cold sterile deionised water, freeze-thawed and stored with the experimental plate prior to histamine assay.

Histamine release was assessed using the previously described histamine assay method, but was measured using solid scintillation in a 96-well plate. Modifications of the previously described histamine assay (chapter 3) were as follows: no [^{14}C]histamine spike was used, the enzyme reaction was halted using 50µl 10M NaOH, 400µl toluene:isoamyl alcohol (4:1) was added to extract [^{3}H]methyl histamine and 75µl of the upper organic phase was transferred using a multichannel pipette directly into the 96 well scintillation plates. Plates were air-dried in a fume hood, sealed with plastic and ^{3}H was measured in the β-counter. % inhibition for compounds tested was calculated as follows:

$$\% \text{ inhibition} = 100 - \left[\frac{\text{pmol histamine released with compound}}{\text{pmol histamine released without compound}} \times 100 \right]$$

4.3 Results

Epithelial coculture and supernatant production

HLMC were cocultured for 24 hours with confluent primary or BEAS-2B epithelial monolayers in the presence and absence of anti-IgE. HLMC adhered to areas of epithelial cell growth and not human plasma fibronectin used for cell attachment or glandular cells (Figure 4.1). Supernatants were collected from HLMC alone, HLMC incubated with epithelium, and anti-IgE activated HLMC incubated with epithelium for 24 hours (Figure 4.2). Appropriate control conditions were also performed as required. Mast cell degranulation under these conditions was confirmed by measurement of histamine levels (Figure 4.3). Histamine release from anti-IgE-treated HLMC in the presence and absence of epithelium were significant, compared to when HLMC were not present.

Effect of epithelial supernatants on HLMC survival and chemotaxis

Epithelial supernatants were incubated with HLMC and trypan blue exclusion was used to monitor mast cell viability upto 72 hours (Figure 4.4). Supernatants incubated with epithelium caused increased mast cell viability, although cytomix did not increase this effect. These epithelial supernatants were also monitored for chemotactic activity in comparison with the established mast cell chemoattractants SCF and TGF-β (Figure 4.5). Supernatants from unactivated and cytomix-activated epithelial cells did not show chemotactic activity.

Effect of epithelial coculture on ICAM-1 expression and epithelial proliferation

Flow cytometry was used to assess whether cocultured HLMC and BEAS-2B cells were distinguishable by size, granularity or ICAM-1 expression, but were not (Figure 4.6). Increased epithelial ICAM-1 was observed upon cytomix treatment, but not upon coculture with unactivated or anti-IgE activated HLMC (Figure 4.7). Unactivated HLMC ICAM-1 expression was not modulated by coculture with unactivated or cytomix-activated epithelium (Figure 4.8). When anti-IgE was added at the time of coculture, no increase in ICAM-1 expression was observed. BEAS-2B cell proliferation was assessed by BrdU incorporation. Although epithelial ICAM-1 expression did not alter, coculture of BEAS-2B with anti-IgE and HLMC did increase BrdU incorporation of BEAS-2B (Figure 4.9).

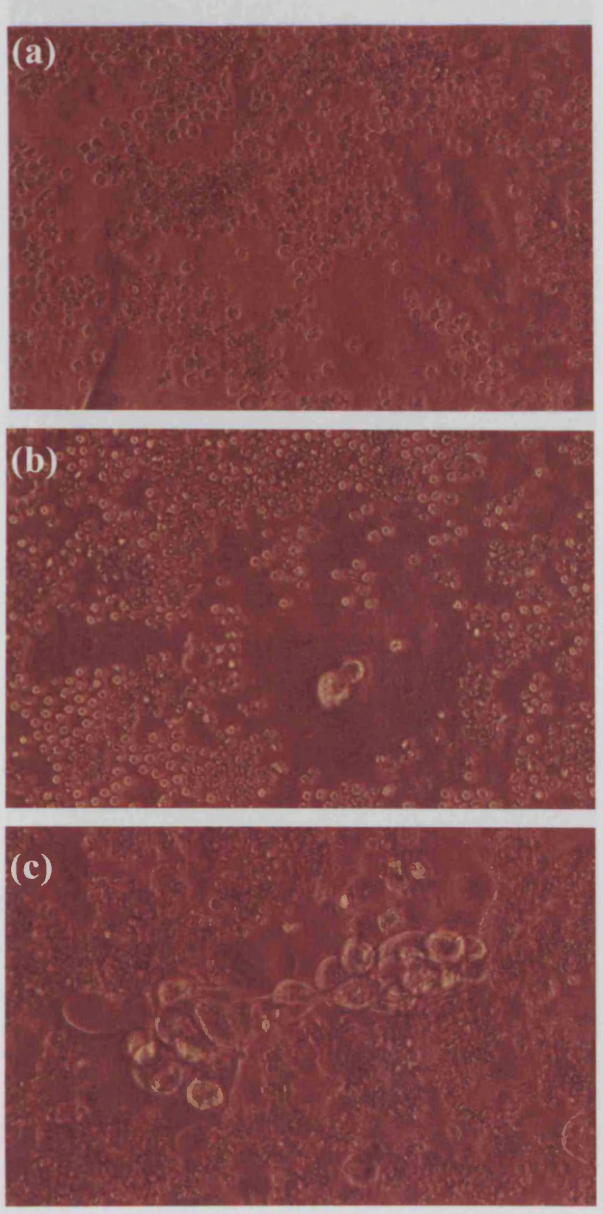


Figure 4.1. HLMC adherence to primary epithelium (x400)
(a) normal epithelium. (b) and (c) show glandular epithelium present from digested or undigested explant cultures respectively. Cultures were checked visually prior to use and if glandular epithelium was present, the culture was not used to generate supernatants from coculture.

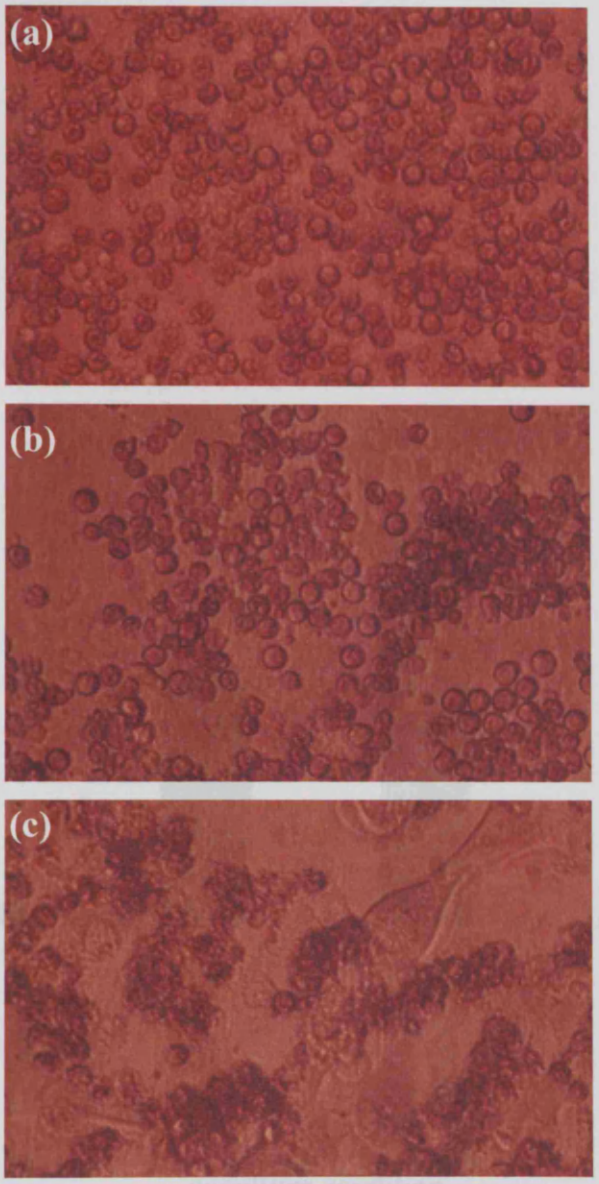


Figure 4.2 **Coculture of HLMC (at 1x10^6/ml, 24hr) with primary epithelial cells (x400).** (a) no epithelial cells present (b) HLMC cocultured with epithelial cells (c) immunologically-activated (αIgE) HLMC cocultured with epithelial cells. HLMC show selective adherence to epithelium, and increased aggregation upon activation. Supernatants were harvested for use in eosinophil chemotaxis and survival assays. Results shown are representative of four separate bronchial epithelial donors.

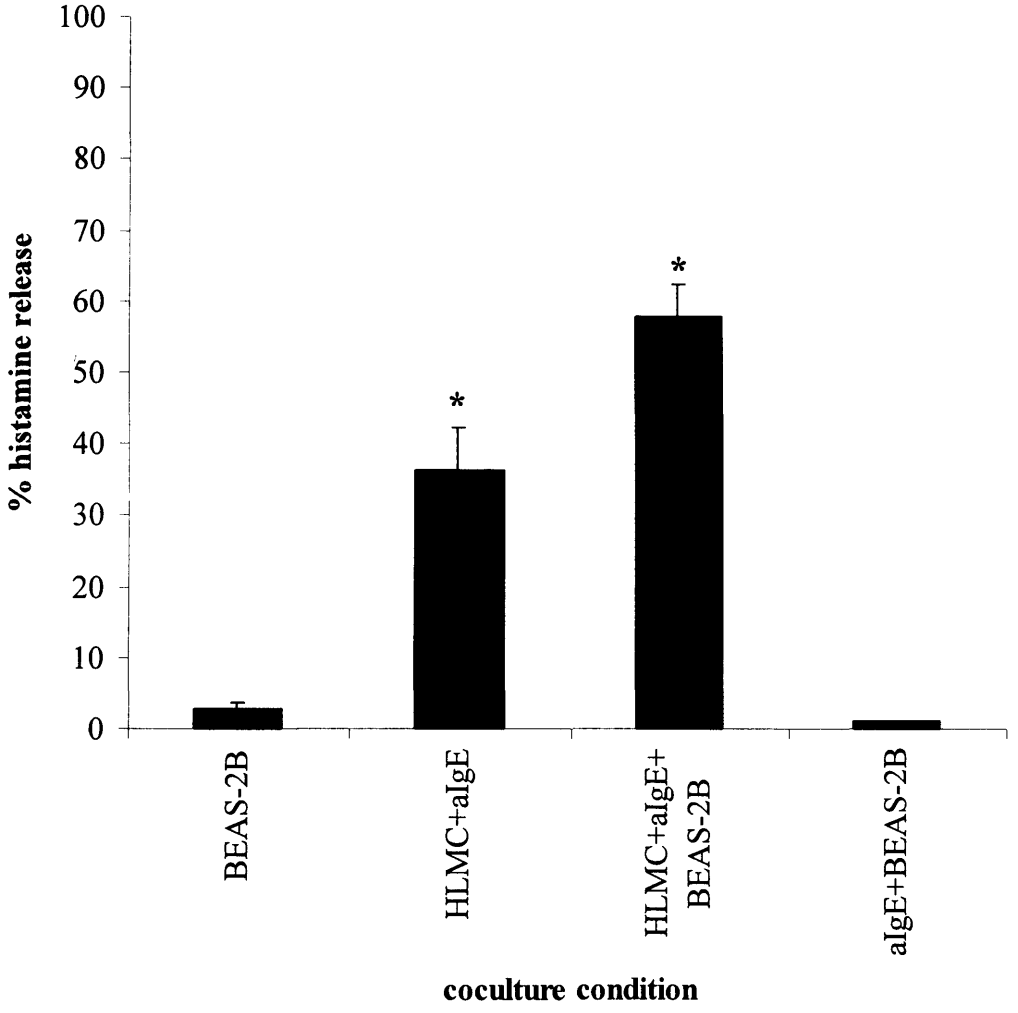


Figure 4.3 **Histamine release of HLMC-epithelium coculture supernatants (n=3).** Anti-IgE treatment of HLMC caused significant (*$p<0.005$) histamine release compared to appropriate controls. The presence of epithelium did not significantly increase HLMC histamine release upon immuniological challenge.

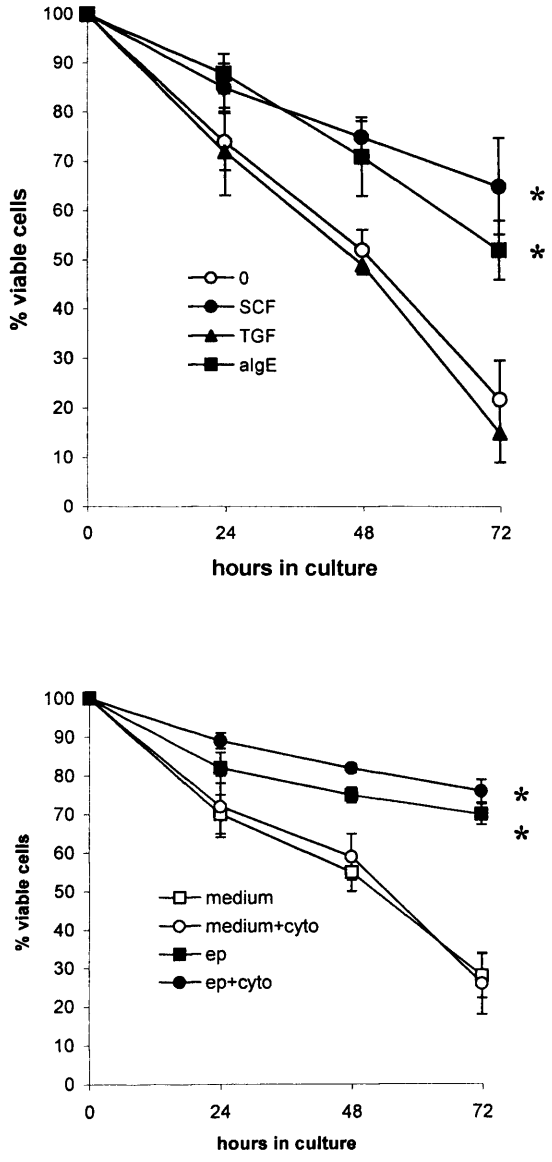
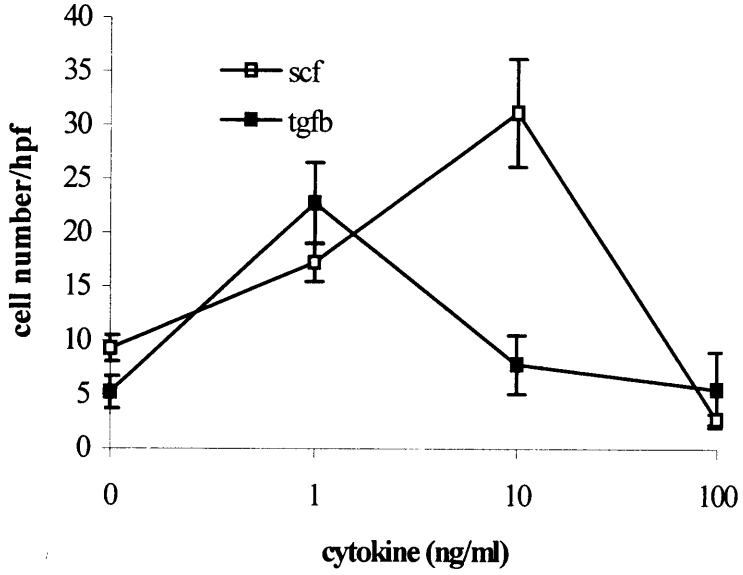


Figure 4.4 **Mast cell viability after incubation with HLMC or epithelial supernatants (n=3).** (a) HLMC supernatant (after 24 hour culture of HLMC with 10ng/ml SCF, SCF+TGF-β (5ng/ml) or SCF+αIgE (1%). (b) BEAS-2B supernatant (after 24 hours with SAGM medium ± cytomix (50ng/ml TNF-α, IL-1β and IFN-γ). Control medium without addition to epithelium, was also assessed. Significant enhancement of viability in the presence of epithelium, compared to controls is shown (*$p<0.05$).



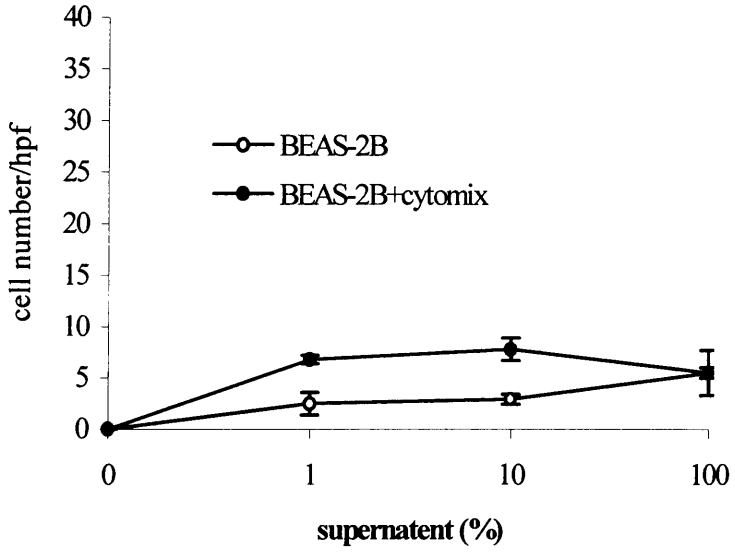


Figure 4.5 **HLMC chemotaxis in response to increasing concentrations of** (a) mast cell chemoattractants SCF and TGF-β and (b) supernatants from unactivated or cytomix-activated BEAS-2B monolayers (n=3). The appropriate controls were also measured, and did not cause chemotaxis. Results are expressed as the average cell number/high power field (x400) from ten measurements of each triplicate.

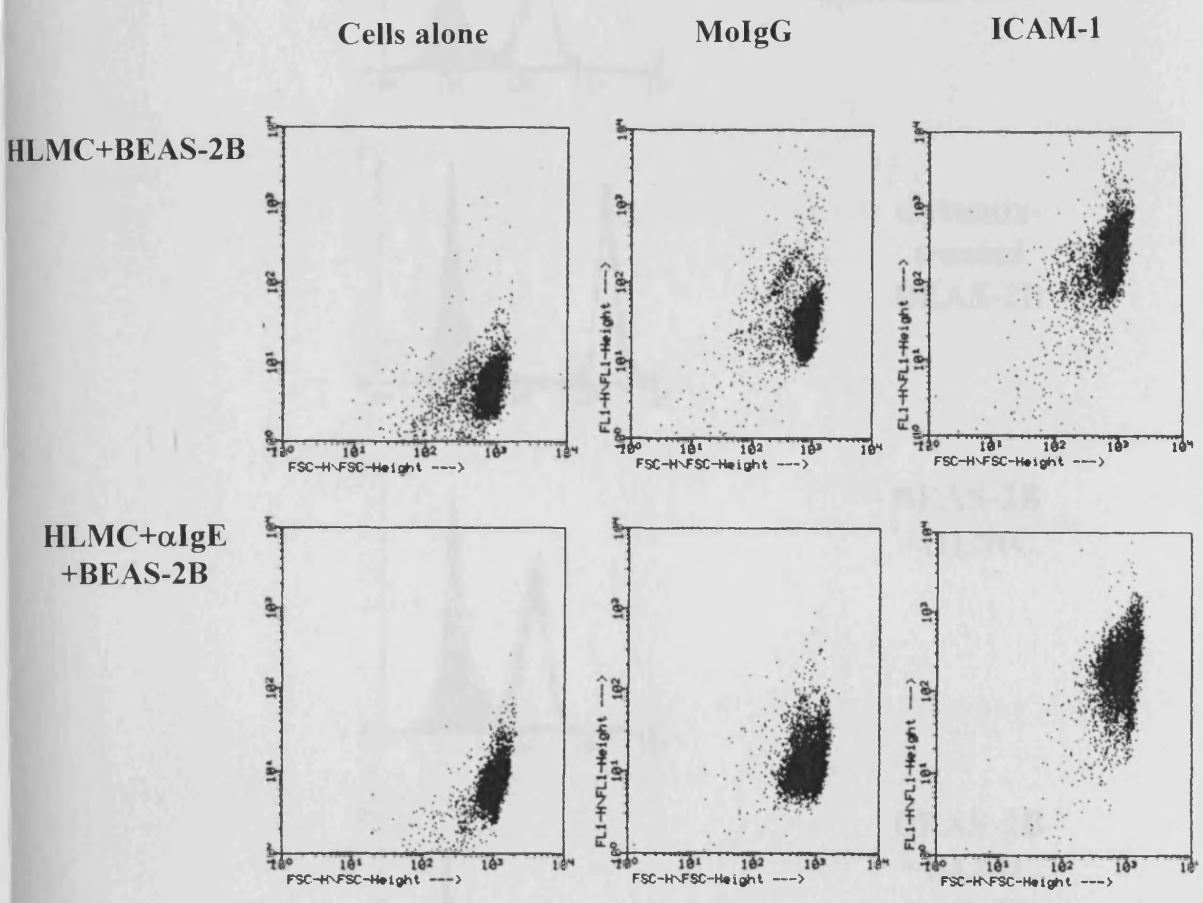


Figure 4.6 **Flow cytometry analysis of ICAM-1 expression of cocultured HLMC and BEAS-2B (representative of three experiments)**
Unlabelled or ICAM-1-labelled HLMC were not distinguishable from BEAS-2B epithelial cells by this method.

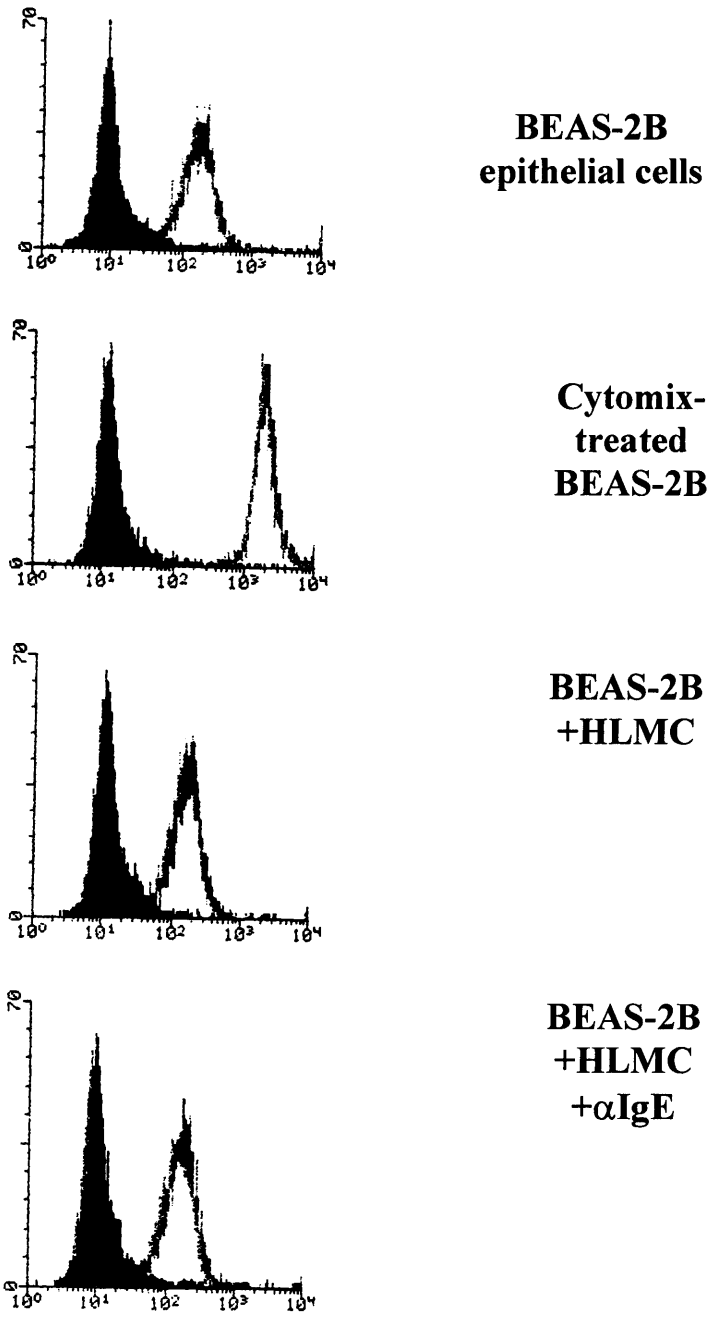


Figure 4.7 **Effect on BEAS-2B epithelial ICAM-1 expression of coculture with unactivated and αIgE-activated HLMC**
Basal and cytomix-treated BEAS-2B cell ICAM-1 were measured as controls. Human lung mast cells were incubated with BEAS-2B monolayers for 24 hours in the presence and absence of αIgE. ICAM-1 expression of BEAS-2B cells were assessed using indirect flow cytometry. Results shown were representative of two experiments.

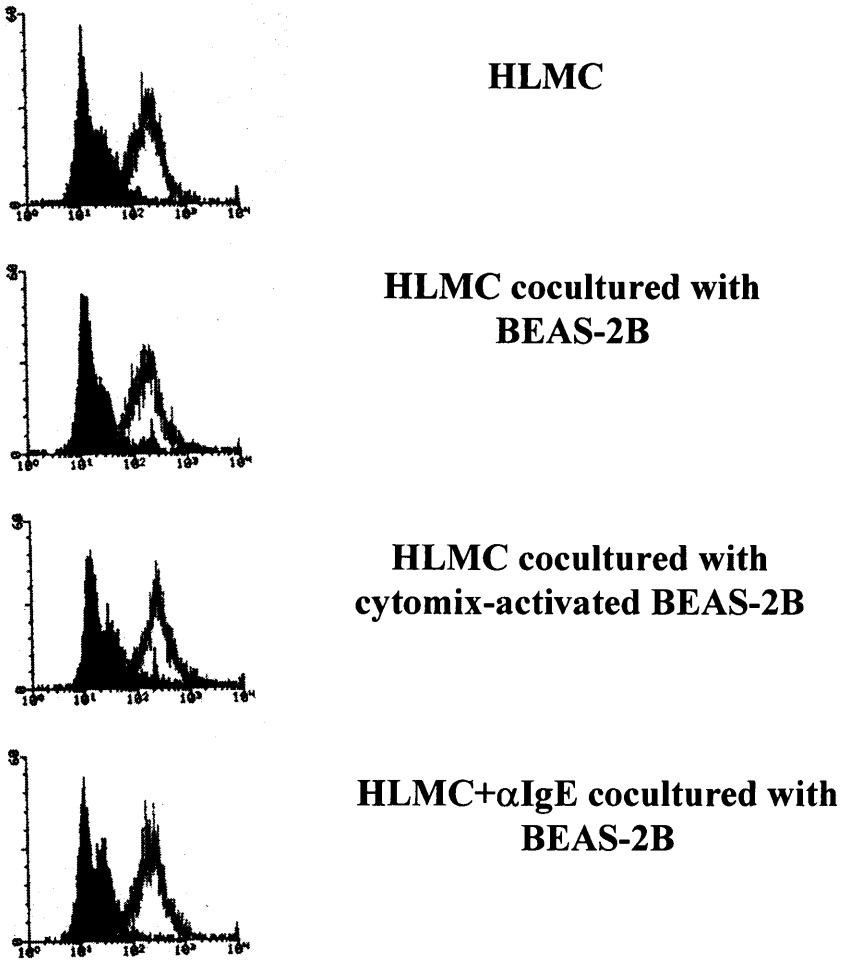


Figure 4.8 **Effect on HLMC ICAM-1 expression upon anti-IgE activation and coculture with BEAS-2B.**
Human lung mast cell ICAM-1 expression in the absence of coculture, upon incubation with unactivated and cytomix-treated BEAS-2B monolayers for 24 hours in the presence and absence of αIgE was measured. ICAM-1 expression of BEAS-2B cells was assessed using indirect flow cytometry. Results shown were representative of two experiments.

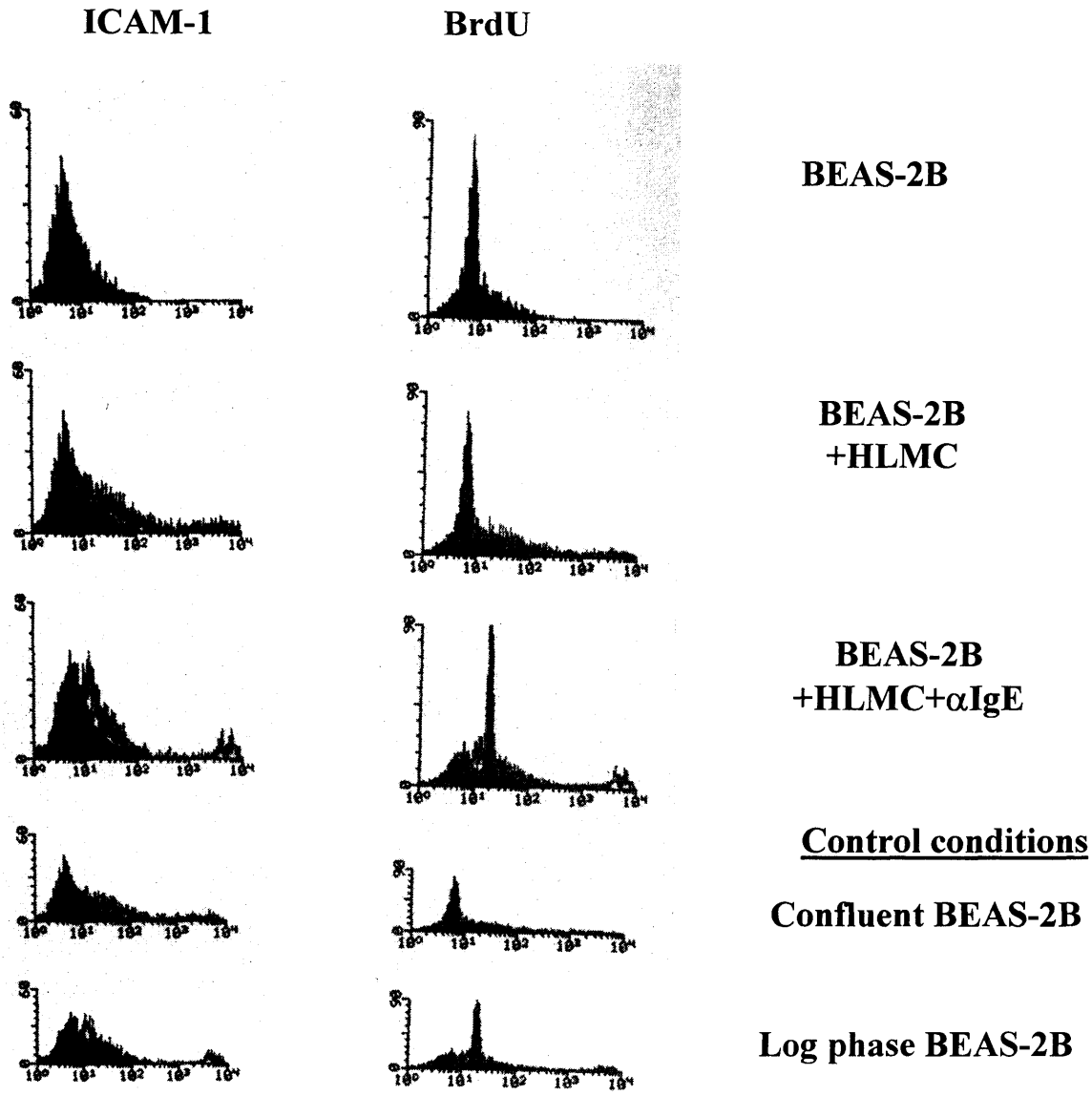


Figure 4.9 **BEAS-2B ICAM-1 expression and BrdU incorporation (proliferation) upon coculture with unactivated and anti-IgE-activated HLMC.** Measurement of proliferation was confirmed using confluent and log-phase BEAS-2B at the same time as the experiment. Results shown are representative of two experiments.

Effect of HLMC adherence to epithelium on degranulation

HLMC degranulation was assessed by histamine release and was measured by solid scintillation counting and comparison with a linear histamine standard curve (Figure 4.10). Initial experiments measured non-adherent HLMC histamine release (polypropylene tissue culture plastic) in comparison with HLMC adherent to BEAS-2B for 1 hour prior to histamine release. There was no significant difference between histamine release of adherent and non-adherent cells in response to increased anti-IgE (Figure 4.11). Maximum release occurred at 0.3% anti-IgE. Histamine release of nonadherent (polypropylene), nonadherent and adherent HLMC (nonad, adep) cocultured with unactivated and cytomix-activated BEAS-2B (ep-, ep+) were assessed. Preliminary data showed that adherence of HLMC resulted in enhanced degranulation compared to non-adherent cells. The activation status (and therefore increased ICAM-1/VCAM-1 expression) did not appear to be involved in regulating this effect (Figure 4.12).

Although HLMC adherence appeared to be important for degree of degranulation, no difference in relative pharmacological inhibition profiles were observed between non-adherent and epithelial-adherent cells for the potent histamine release inhibitors ascomycin and salbutamol (Figure 4.13). The results for theophylline were variable, and this may have been due to variable precipitation from solution. Sodium cromoglycate appeared to be more active on non-adherent mast cells in the absence of epithelium. Since cromoglycate generally requires preincubation at high concentrations to show maximal efficacy, the presence of BEAS-2B epithelial cells may have 'diluted' its available concentration.

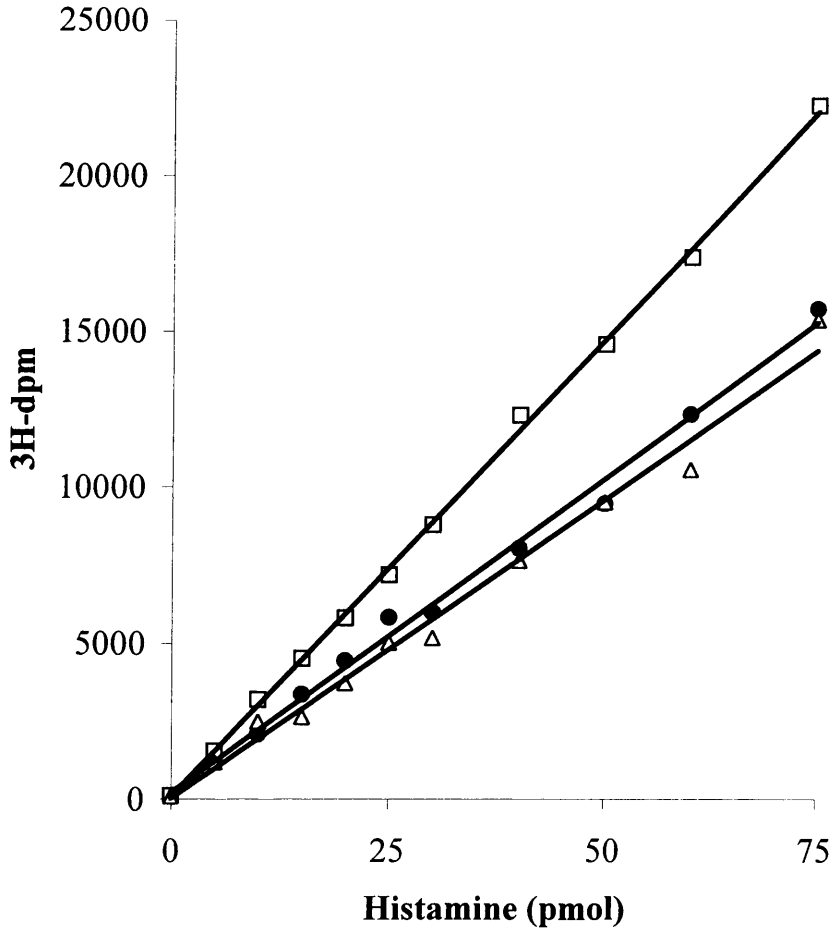


Figure 4.10 Histamine assay standard curves from 96-well plate solid scintillation counting, representative of three separate assays. Triplicate samples and standards were measured at each assay. Variations between different stock enzyme batches may account for the differences in gradient which sometimes occurred (two of the graphs shown above were form the same batch of enzyme).

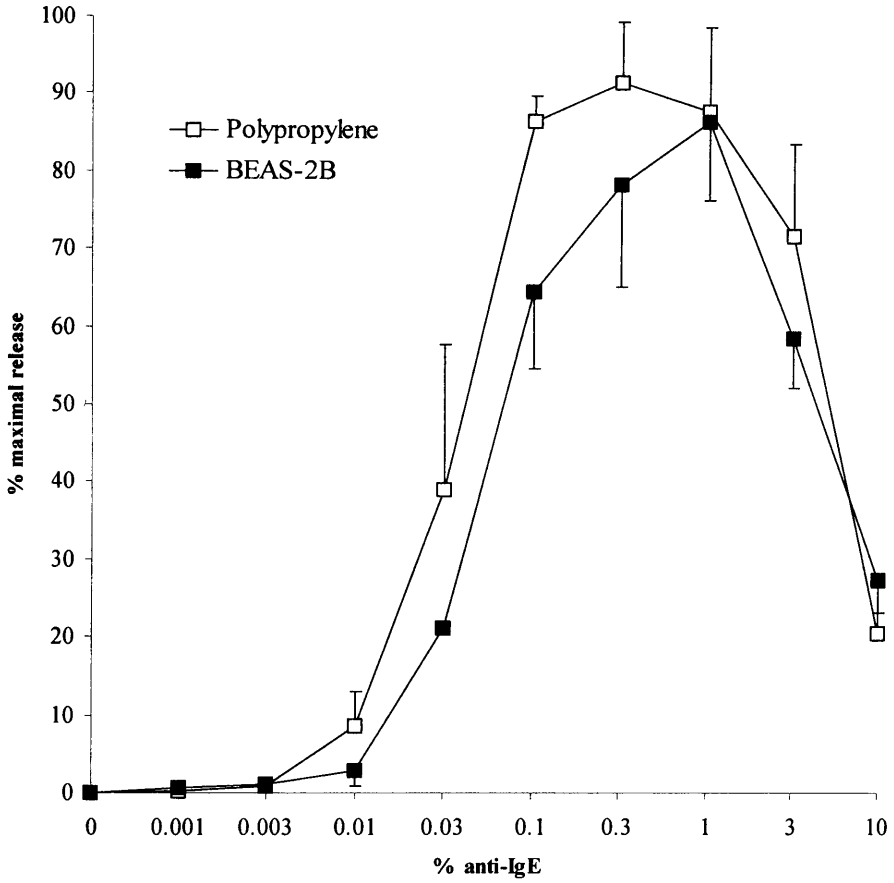
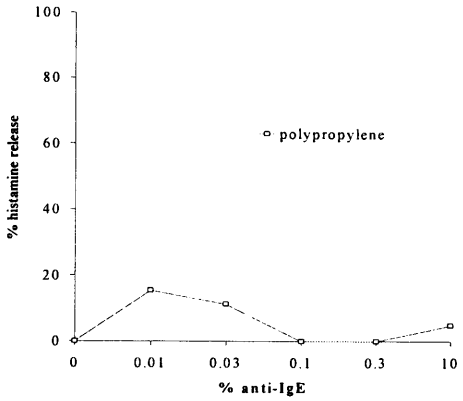
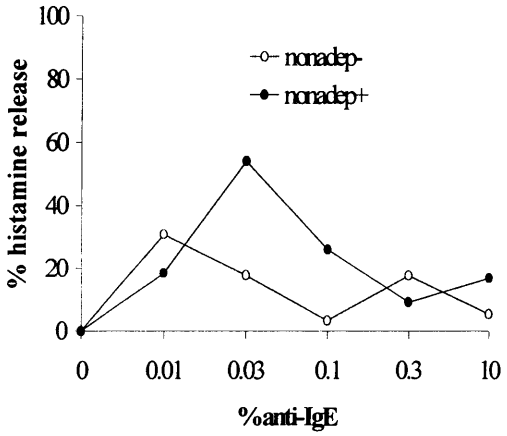


Figure 4.11 **Comparison of histamine release upon anti-IgE activation of non-adherent (polypropylene, n=4) or adherent (BEAS-2B, n=3) .** Maximal (100%) release was assessed in the absence of diluent control. All the above values are in the presence of diluent, and background histamine release has been subtracted.





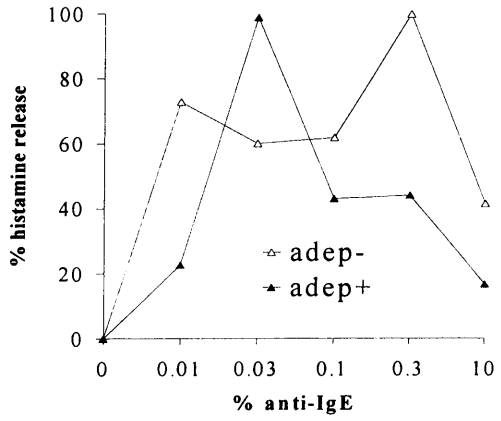


Figure 4.12 Preliminary assessment of histamine release of non-adherent and adherent HLMC to unstimulated and stimulated BEAS-2B (24hr cytomix). (a) control HLMC (polypropylene)
HLMC co-cultured with unstimulated or stimulated BEAS-2B
(b) non-adherent HLMC (c) adherent HLMC
(n=1, representative of three experiments).

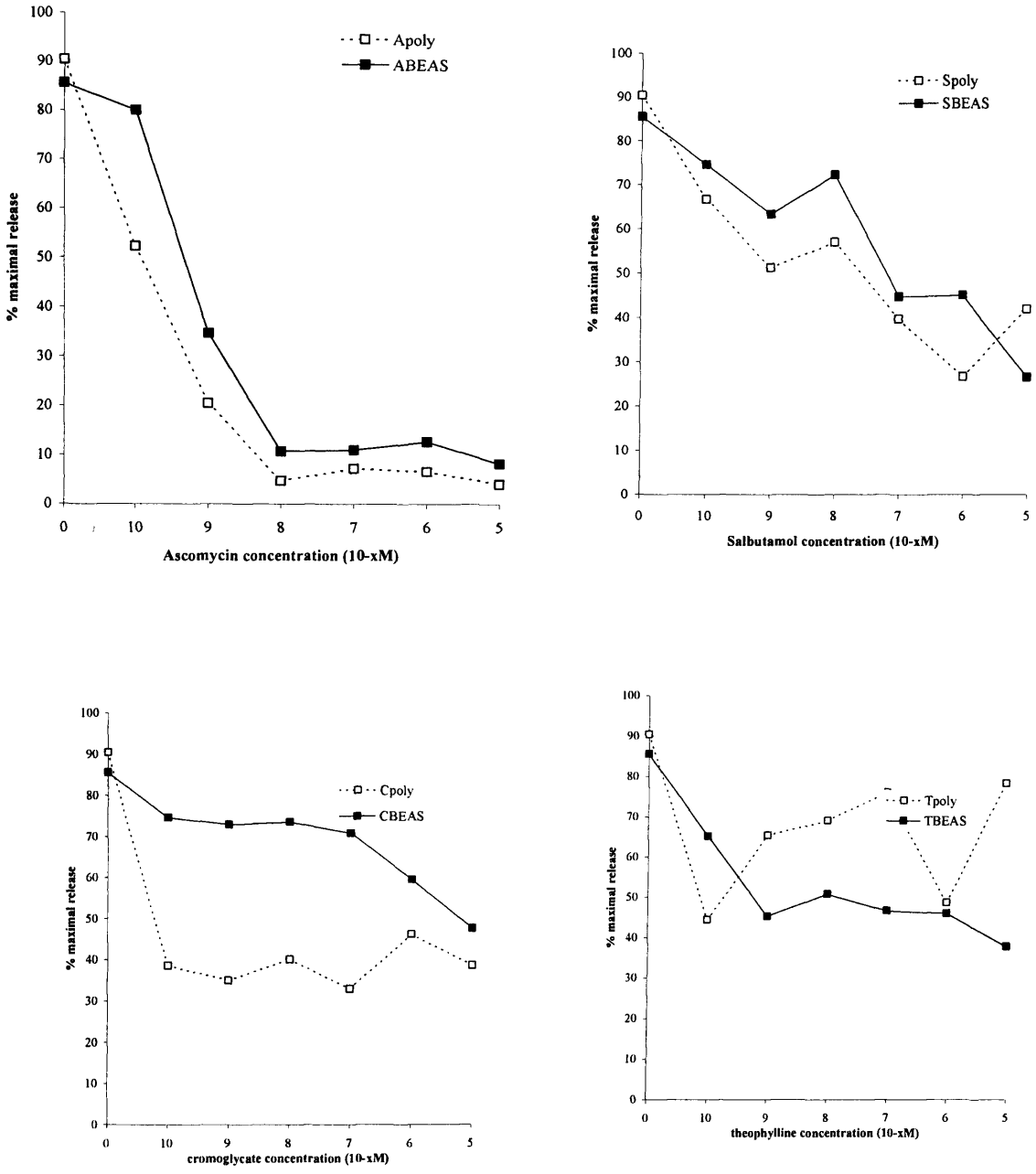


Figure 4.13 Pharmacological inhibition of non-adherent (polypropylene) and adherent (BEAS-2B) HLMC (n=3) Effect of increasing ascomycin, salbutamol, sodium cromoglycate and theophylline on HLMC histamine release. The three donors varied greatly in their histamine release, and therefore error bars were not shown in this preliminary study. Maximal (100%) release was assessed in the absence of diluent control. All the above values are in the presence of diluent, and background release has been subtracted.

4.4 Discussion

It should be noted that the results discussed in this chapter were preliminary studies. Supernatants used in these studies were confirmed to be from mast cells that degranulated in response to anti-IgE activation. Supernatants from HLMC that did not degranulate significantly were discarded. Results shown used BEAS-2B cells for coculture. Where used for comparison, primary explant-derived epithelial cell supernatants produced similar results.

Mast cell supernatants showed significant HLMC survival activity when derived from HLMC cultured in the presence of SCF or SCF+anti-IgE for 24 hours. Both conditions are believed to enhance HLMC mediator release. Supernatants from epithelial coculture also resulted in enhanced HLMC viability compared to control medium not incubated with BEAS-2B cells. Additional cytomix activation of BEAS-2B cells did not increase survival activity further.

This study confirmed the established chemotactic activity of SCF and TGF-β on human mast cells. Although epithelial cells produce both of these mediators (and others), unactivated and cytomix-stimulated BEAS-2B epithelial supernatant did not result in significant chemotactic activity for HLMC at the concentrations produced *in vitro*.

Measurement of the effect of coculture on either cell type (HLMC or BEAS-2B) ICAM-1 expression and BrdU incorporation required the ability to distinguish cell types. Unexpectedly, their size/granularity were not distinguishable by flow cytometry. The ability of BEAS-2B cells to readhere was utilised in separating cells after short-term trypsin treatment.

Although cytomix readily increased epithelial ICAM-1 expression, coculture with anti-IgE-activated HLMC or mast cell supernatants failed to modulate expression. Under identical assay conditions, HLMC ICAM-1 expression was also not modulated, further confirming the lack of involvement of ICAM-1 in adhesive interactions between HLMC and epithelium.

Log phase growth of BEAS-2B caused BrdU incorporation, in contrast to confluent cells. This confirmed the assay was able to detect cell proliferation. Epithelial proliferation was enhanced when cocultured with anti-IgE-activated HLMC.

In general, most studies have investigated pharmacological inhibition of mediator release from non-adherent mast cells. Cell adherence is known to contribute to intracellular signal transduction, and may lead to changes in mast cell phenotype, function and response to activation. Mature mucosal mast cells are tissue-resident and therefore adherent cells may reflect the *in vivo* mast cell phenotype. Investigation of the mechanism of mast cell degranulation and histamine release have been carried out mostly using the rat basophilic cell line RBL-2H3. RBL-2H3 cell adherence resulted in cytoskeleton changes, cell spreading, granule redistribution to the cell periphery and enhanced IgE-mediated secretion (Hamawy et al, 1992b). The signalling pathways resulting from mast cell adhesion and activation may converge, and the overall intracellular signalling resulting from adherence may increase likelihood of degranulation.

Comparison of adherent cells and cells in suspension may be expected to result in differences in cell function or phenotype. PMA abolished the IgE-mediated $[Ca^{2+}]_i$ increase in non-adherent, but not adherent cells (Wolfe et al, 1996). RBL-2H3 cell adhesion to the ECM protein fibronectin was partially integrin-mediated, and focal adhesion complexes were formed. Non-covalently attached proteins (such as talin, vinculin, α-actinin, filamin, $pp125^{FAK}$) co-localised at the integrin cytoplasmic domain (Clark and Brugge, 1995). Adhesion resulted in tyrosine phosphorylation of several proteins, including $pp125^{FAK}$ and the cytoskeletal protein paxillin (Hamawy et al, 1993a; Hamawy et al, 1994b). Although cell adherence did not directly cause histamine release, adherence was essential for activation-induced (FcεRI, A23187 or PMA) $pp125^{FAK}$ tyrosine phosphorylation (Hamawy et al, 1993). Spontaneous adhesion of mouse mast cells to vitronectin, but not entactin, led to FAK phosphorylation, and was regulated by PKC and Ca^{2+} mobilisation. FcεRI aggregation of nonadherent cells also caused FAK phosphorylation, and therefore may represent a point of signal transduction pathway convergence between cell

adhesion and FcεRI cross-linking (Bhattacharyya et al, 1999). The effects of cell adhesion and FcεRI aggregation were additive. Mast cells in tissues may therefore have a basal level of continuously phosphorylated FAK, which may require the further increase upon FcεRI aggregation to produce activation and degranulation. However, FcεRI-mediated increased PECAM-1 tyrosine phosphorylation was independent of cell adhesion, Ca^{2+} influx or PKC activation (and occurred equally in adherent and non-adherent cells) (Sagawa et al, 1997).

In this study, there was no difference between degranulation responses of epithelium-adherent or non-adherent HLMC (Figure 4.11). Mast cell adhesion to epithelium was not mediated by integrin- or other known cell adhesion molecules (chapter 3), which may imply that adhesion to epithelium did not contribute to intracellular signalling required for histamine release. However, when adherent and non-adherent HLMC were separated and their responses assessed separately, adherent HLMC may release a greater proportion of their total histamine content upon degranulation (Figure 4.12). There was no significant difference between adherence to activated or unactivated BEAS-2B. Further work is required to characterise this observation.

β_2-adrenergic receptor agonists inhibit IgE-induced histamine and eicosanoid (LTs and PGD_2) release from human lung tissue fragments, dispersed human lung, skin and tonsil mast cells and cultured human mast cells (Church and Hiroi, 1987; Okayama and Church, 1992; Undem et al, 1988; Shichijo et al, 1998; Butchers et al, 1979; Peters et al, 1982; Church and Young, 1983). The β_2-adrenergic receptor (β_2AR) is a 7-transmembrane G-protein coupled receptor linked to G_s. β-agonists activate adenylate cyclase, and caused elevated intracellular cyclic 3',5'-adenosine monophosphate (cAMP) levels which correlated well with inhibition of IgE-mediated HLMC histamine and LTC_4 release (Peachell et al, 1988; Shichijo et al, 1999). Salbutamol inhibited anti-IgE-induced cytokine release (GM-CSF, IL-5 and MIP-1α) (Shichijo et al, 1999).

Immune suppressive agents have also been used in the treatment of asthma, and although effective on mast cells and eosinophils (Cirillo et al, 1990), their main activity is thought to be reduction of T cell activation by prevention of IL-2 mRNA and protein expression (Granelli-Piperno et al, 1984). CsA and FK506 inhibit the late, but not early response after allergen challenge (Geba et al, 1991). CsA inhibits cytokine production (IL-2, IL-3, IL-4 and GM-CSF) in PMA/A23187-activated cultured mouse mast cells (Hatfield and Roehm, 1992) and also inhibits SCF- and IgE-mediated histamine release from HLMC (Sperr et al, 1996).

Human mast cells from different tissue sites demonstrate a range of responses to the inhibitory action of sodium cromoglycate. Disodium cromoglycate was a weak inhibitor of IgE-mediated histamine and cysteinyl leukotriene release from human lung fragments, although it effectively inhibited human cultured and rat mast cell release (Butchers et al, 1979; Church and Young, 1983; Leung et al, 1986; Shichijo et al, 1998). A high concentration (1000μM) sodium cromoglycate modestly inhibits (~10%) IgE-mediated histamine and PGD_2 release from dispersed human lung mast cells, although human skin mast cells were relatively unresponsive (Okayama et al, 1992a,b). Sodium cromoglycate inhibited IgE-induced histamine release from human lung fragments, BAL and enzymatically dispersed lung parenchymal mast cells, although it was a more potent inhibitor of BAL mast cell histamine release (Church and Young, 1983; Church and Hiroi, 1987; Leung et al, 1988; Moqbel et al, 1988).

At clinically relevant concentrations, theophylline is a non-specific phosphodiesterase inhibitor, non-selective adenosine receptor antagonist and mast cell NF-κB inhibitor (Banner and Page, 1995; Banner and page, 1996). HLMC express $P2Y_1$- and $P2Y_2$-purinoceptor mRNA (but not P2Z), and extracellular ATP potentiated IgE-mediated histamine release (Schulman et al, 1999). Theophylline, at concentrations upto 0.1mmol/L, did not inhibit IgE-mediated histamine release from either human lung fragments or cultured human mast cells (Shichijo et al, 1998), but did inhibit HLMC IgE-mediated histamine release (Weston et al, 1997).

Inhibition of histamine release by ascomycin and salbutamol was not altered by the presence of HLMC adherence to epithelium. Unexpectedly, sodium cromoglycate

was less potent in the presence of epithelium, possibly due to a lower available concentration (by uptake into epithelial cells). The effects of theophylline were very variable, probably due to precipitation from solution.

Although human lung mast cells were used, these results may differ from cells from normal or asthmatic subjects. Mast cells were purified from macroscopically normal regions of diseased surgical specimens, which may have affected responsiveness and function. HLMC were derived from whole lung tissue, and may be less activated than the specific population found within inflamed mucosa or BAL during asthma exacerbations. This may be functionally important since it has been shown that BAL mast cells from asthmatic patients release histamine more readily, and high concentrations of nedocromil sodium were more effective on BAL than HLMC (Leung et al, 1988; Moqbel et al, 1988).

Chapter 5: Eosinophil adhesion to bronchial epithelium

5.1 Introduction

Inflammation in asthma is generally characterised by eosinophil infiltration, activation and degranulation, with epithelial damage and desquamation (Gleich, 1990). Eosinophil products include preformed granule proteins, *de novo* generated lipid mediators, cytokines and oxygen metabolites (Kroegel et al, 1994). Eosinophil granule-derived proteins are deposited at high concentrations in the asthmatic airway and bronchial epithelial cells are generally thought to be the major target of eosinophil granule-derived mediators. MBP and ECP cause cilial dyskinesia, with bronchial epithelial cell damage and loss occurring at higher concentrations (Filley et al, 1982; Gleich et al, 1979).

Eosinophils are triggered for degranulation when adherent to large, opsonised targets such as parasitic schistosomulae. In asthma, eosinophils may be misdirected to degranulate at the epithelial surface. The mechanisms governing triggering of degranulation and resolution of tissue damage remain unclear, although eosinophil adhesion to bronchial epithelium and epithelium-derived proinflammatory mediators may be expected to modulate eosinophil activation. Cell adhesion is a requirement for degranulation of primed eosinophils (Kato et al, 1998).

Eosinophil tissue infiltration is determined by cell adhesion receptor expression, function and activation. Eosinophil migration across the vascular endothelial monolayer has been extensively studied compared to eosinophil interactions with bronchial epithelium. Bronchial epithelial cells from asthmatics expressed increased ICAM-1 (Vignola et al, 1994), although it has not been directly implicated in granulocyte adhesion to epithelium.

This study investigated the mechanism of eosinophil adhesion to bronchial epithelium, the effect of proinflammatory mediator activation on cell adhesion molecule expression and adhesion.

5.2 Materials and methods

Antibodies

The majority of the antibodies used were described in previous chapters. PSGL-1 and L-selectin mAbs were received from the leukocyte typing workshop.

Reagents

Histopaque 1083, phosphate buffered saline (PBS) and manganese (Mn^{2+}) were obtained from Sigma (Poole, UK), anti-CD16 microbeads were purchased from Miltenyi Biotec (Bisley, Surrey, UK), heparin was obtained from CP Pharmaceuticals (Wrexham, UK), dextran was purchased from Fluka Biochemika (Glossop, UK), Hanks' balanced salt solution (HBSS) without calcium and magnesium, foetal calf serum (FCS), 1M Hepes, medium 199 and trypsin/EDTA were obtained from Life technologies (Paisley, Scotland), EDTA, NH_4Cl and $KHCO_3$ were obtained from Fisher scientific (Loughborough, UK), sodium chromate (^{51}Cr) was purchased from Amersham (Aylesbury, UK), IL-1β, TNF-α, IFN-γ, IL-5 were purchased from R&D systems (Abingdon, UK).

Equipment

23G lancet, autolet was purchased from Clinisafe (Oxford, UK), haemocytometer was purchased from Weber Scientific (Teddington, UK), 50ml syringes were purchased from Becton Dickenson (Cowley, UK). Cotton wool tipped sticks were obtained from Sherwood medical (St Louis, USA). Mylar sealing tape was obtained from Sigma (Poole, UK). Routine lab plastic and glassware were obtained from Leicester university bulk store. Gamma counter (Auto-gamma counting system) was obtained from Packard Meriden, UK. Standard 48 well chemotaxis chamber and nitrocellulose filter (8μm pore size) were obtained from Neuro probe (MD, USA).

Methods

5.2.1 Separation of eosinophils from peripheral blood

Eosinophils were purified by negative selection from human peripheral blood using a previously published method (Hansel et al, 1991). Granulocytes were separated from mononuclear cells by virtue of their different density within an isotonic sugar gradient formed using Histopaque 1083. It is well established that neutrophils express

CD16 whereas unstimulated eosinophils do not. Anti-CD16-conjugated magnetic microbeads were used to positively select neutrophils from a mixture of granulocytes within a magnetic field using the magnetic cell separation system (MACS). A virtually pure population of eosinophils passed through the field and were collected. The final eosinophil purity and viability were >98% and >99% respectively.

Blood donation

Blood donors were mildly atopic (assessed by eosinophil count 2-12%), asymptomatic asthmatic and non-medicated at time of donation, and were members of staff from Glenfield hospital. Volunteers were selected on the basis of a blood count (the thumb or index finger of the potential donor was swabbed with alcohol and the skin was punctured with a 23G lancet using an autolet. Collected blood (10μl) was mixed with 90μl Kimura stain in an eppendorf tube. A differential blood count was performed using an improved Neubauer haemocytometer. Donors were informed of their total white cell counts and percentage eosinophils. Blood donation was arranged if eosinophil counts were within the range used for these experiments (2-12%).

Peripheral venous blood was collected from a superficial vein (by a nurse) using a tourniquet and alcohol pre-injection swab. Blood was collected using two heparin-preloaded (0.2ml anticoagulant/syringe) 50ml syringes attached to a 21G sterile butterfly mounted needle.

Eosinophil separation

Within 5 minutes of donation anticoagulated blood (100μl) was removed to an eppendorf for a differential count and 10ml 6% sterile filtered dextran in Hanks' balanced salt solution (HBSS) without calcium and magnesium was added to each 50ml syringe, with gentle mixing. The syringe was fixed at a 45° angle for 45 minutes to allow sedimentation to occur. The plasma layer from each syringe (containing leukocytes and platelets), was transferred to two 50ml centrifuge tubes and sterile wash buffer with 2% heat-inactivated FCS was added. Sterile wash buffer was prepared in advance (15ml 1M Hepes, 5ml 0.5M EDTA in PBS, 50ml 10x HBSS without Ca^{2+} and Mg^{2+}, made up to 500ml and autoclaved. The samples were centrifuged at 200g for

15minutes at room temperature. The leukocyte pellets were resuspended in 20ml wash buffer and 10ml layered carefully over 10ml of a synthetic, sucrose-based polymer, centrifuge gradient solution of density 1.083g/ml (Histopaque 1083) taking care to preserve the interface between the cell suspension and the density gradient. The tubes were then centrifuged at 400g for 25min at room temperature. After centrifugation the mononuclear cell layer (monocytes and lymphocytes) located at the interface was carefully removed. The remaining fluid was removed and the inside walls of the tubes wiped with cotton wool tipped sticks (Q-tips). Red blood cells were removed from the remaining granulocyte pellet by resuspension in 2ml ice-cold sterile-filtered lysis buffer (0.15M NH_4Cl and 0.01M $KHCO_3$ adjusted to a pH between 7.2 and 7.4) and the resultant suspension was transferred to fresh 50ml tubes. The volume of the cell suspension was made up to 50ml with wash buffer and centrifuged for 8min at 250g at 4°C. The pellet was resuspended in 10ml wash buffer, and an aliquot was removed for a differential cell count. The tube was filled with wash buffer and centrifuged as before. The count was used to determine the total numbers of granulocytes present (eosinophils and neutrophils) to calculate the amount of microbeads required.

Anti-CD16 microbeads were added to the cell pellet at 50µl per $5x10^7$ cells and the same volume of wash buffer was added. Cells were incubated on ice for 40min, with occasional gentle mixing. At the same time the separation column was prepared. The three-way tap was connected, a 20ml syringe was connected to the horizontal arm of the valve, and the outflow arm was connected to a 24G needle with its plastic cover cut just below the end of the needle. Another 20ml syringe without a plunger was connected above the column for loading of the cells and buffer. The system was flushed with PBS and then wash buffer, to remove air bubbles from the steel wool of the column and to ensure that buffer filled the whole column. The tap was set to allow fluid out through the column at a slow drop-wise rate. As far as possible, the system was kept cool, the buffers were chilled and the column and its plastic mounting were refrigerated prior to use. The column was positioned in the magnetic field and the bead/cell mixture were loaded onto the column and allowed through slowly, while adding small volumes of wash buffer. A total of 40ml cell suspension was collected and centrifuged for 8min at 250g at 4°C. The pellet was resuspended in a known volume depending on the expected yield, and an aliquot was removed for counting. The total number and percentage purity

of eosinophils was assessed. The separation column was taken out of the magnetic field and washed thoroughly with wash buffer to remove the retained neutrophils. The column was then washed thoroughly with PBS and water, and stored in alcohol.

5.2.2 Assessment of eosinophil cell adhesion molecule expression

The presence of eosinophil surface adhesion molecules was assessed using indirect flow cytometry. The method and reagents were identical to HLMC flow cytometry detailed in chapter 3.

5.2.3 Eosinophil adhesion assay

(i) Radioactive labelling and activation of eosinophils

Purified eosinophils were resuspended in 2ml medium 199 with FCS and labelled by addition of $3.7x10^4$ Bequerels of ^{51}Cr (sodium chromate) per 10^6 cells (Walsh et al, 1990). The cells were incubated at 37°C for 60min with occasional gentle shaking. If necessary the cells were labelled in a number of separate tubes and stimulated as required (e.g. the lipid mediator PAF) for a further 30 minutes. After the total incubation time was complete, the cells were washed twice in medium 199 with FCS by centrifugation at 250g for 5 minutes. The cells were then resuspended at the concentration required.

(ii) Eosinophil adhesion assay

After cells were radiolabelled, stimulated and washed to remove free ^{51}Cr, the granulocytes were resuspended at $1.5x10^6$ cells per ml in medium 199 with FCS. 100µl of cell suspension was added to each well and cells were allowed to adhere for 30min at 37°C. To ensure objectivity and repeatability, non-adherent cells were removed using inverted centrifugation (McClay et al, 1981). Wells were gently filled with maximal amounts of medium 199 containing 2% FCS, ensuring the fluid meniscus formed over the well. The plate was carefully covered with adhesive plastic (Mylar sealing tape), ensuring no trapped bubbles were present. The plate was gently inverted and centrifuged at 18g for 5min at RT. After spinning, the sealing tape was removed and the fluid and non-adherent cells were gently removed. 50µl trypsin/EDTA was added per well and incubated for 10 minutes at 37°C to ensure all cell contents (epithelial cells and adherent granulocytes) were dislodged into solution. The plate was

frozen and thawed to ensure cell lysis (and therefore release of radioactive contents into solution). The contents of each well was carefully transferred to plastic test tubes, each well was thoroughly rinsed with 50μl medium199 and added to the same tube. Adherent eosinophils in each well was assessed in a gamma counter. The percentage adhesion was calculated by comparison of the experimental 96-well count and the average count for total cells added (aliquots of labelled cell suspension were added directly to counting tubes and not placed in the 96-well plate). All experimental conditions were performed in triplicate.

5.2.4 Eosinophil adhesion modulation assays

(i) Eosinophil or epithelial activation

Eosinophil integrins were activated with 5mM Mn^{2+} for 15 minutes at room temperature. 10^{-6} PAF was added to eosinophils for 30 minutes at 37°C prior to adding eosinophils to epithelium. In these experiments, epithelium was stimulated as required for 24 hours with 50ng/ml cytomix (IL-1β, TNF-α and IFN-γ). Wells were washed twice with eosinophil medium (Medium 199/FCS) prior to use.

(ii) Blocking adhesion (mAbs, cation chelation, carbohydrates)

Blocking antibodies were added at optimal saturating concentration for 15 minutes at room temperature to either eosinophils or epithelium, as required.

5.2.5 Eosinophil chemotaxis

Optimal conditions for chemotaxis were originally identified using neutrophils since they were available in greater numbers. The chemotaxis method and staining of filters were carried out as described previously in chapter 3, with the following modifications. Uncoated nitrocellulose filters (8μm pore size) were used, eosinophils were added at 4-5x10^6/ml, and the chamber was incubated for 1.5 hours to allow chemotaxis to proceed.

5.3 Results

Eosinophil cell adhesion molecules have been well characterised. Some common receptors are shown (Figure 5.1, 5.2). Unstimulated eosinophils expressed HLA-1, CD45, CD9 but not FcεRI (29C6) or $\alpha_E\beta_7$ (HML-1). Eosinophil cell adhesion molecules include VLA-4 ($\alpha_4\beta_1$; CD49d/CD29), VLA-6 ($\alpha_6\beta_1$; CD49f/CD29), LFA-1 ($\alpha_L\beta_2$; CD11a/CD18), Mac-1 ($\alpha_M\beta_2$; CD11b/CD18), $\alpha_d\beta_2$, $\alpha_4\beta_7$, L-selectin, PSGL-1 (P-selectin glycoprotein ligand-1), ESL (an E-selectin ligand, bearing sialyl Lewis^X) (Bochner et al, 1991; Bochner and Schleimer, 1994; Wardlaw, 2000). Activated eosinophils express CD69 (Walsh et al, 1996).

A549 alveolar epithelial cells expressed basal levels of ICAM-1 (chapter 2). BEAS-2B cells expressed basal ICAM-1 levels which increased upon cytomix stimulation and VCAM-1 was induced upon cytomix stimulation in agreement with published results showing VCAM-1 induction following cytokine stimulation (Atsuta et al, 1997). NHBE cells express a low basal ICAM-1 level compared to BEAS-2B (Chapter 2). Baseline adhesion of eosinophils to epithelium did not reflect this trend (Figure 5.3), implying ICAM-1 may not be involved.

PAF is a highly potent, non-specific chemoattractant for human eosinophils *in vitro*. (Figure 5.4). A549 or NHBE epithelial cell stimulation with increasing TNF-α (24hr) (Figure 5.5a, b) or A549 stimulation with increasing IFN-γ (24hr) (Figure 5.5c) did not increase adhesion of unactivated or PAF-activated eosinophils. This was in agreement with previous results using NHBE cells (Burke-Gaffney and Hellewell, 1998), although another study showed that 30min preincubation increased eosinophil adhesion to BEAS-2B by 2.5-fold (Sato et al, 1997). Unactivated and PAF-activated eosinophil adhesion to unstimulated A549 was not inhibited by function-blocking mAbs to β_2, $\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, $\alpha_4\beta_1$, $\alpha_4\beta_7$ or β_1 (Figure 5.6), in close agreement with the CD18-, ICAM-1- and VCAM-1-independent basal eosinophil adhesion to NHBE previously described (Burke-Gaffney and Hellewell, 1998).

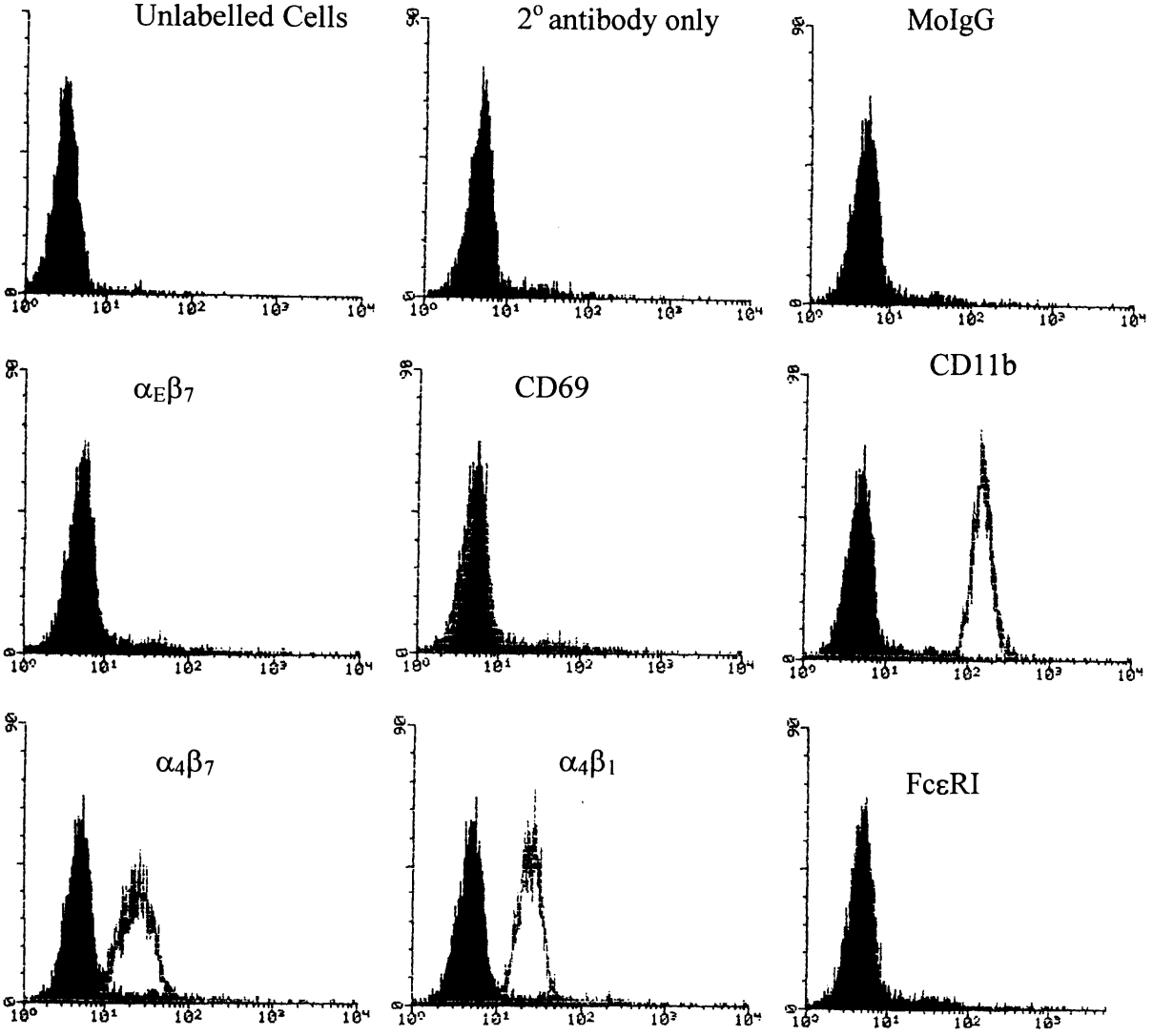


Figure 5.1 **Assessment of peripheral blood eosinophil surface cell adhesion molecules by flow cytometry (I).** Results are representative of four donors. Controls (unlabelled cells, secondary antibody and MoIgG apply to figure 5.2 also). Eosinophils were indirectly labelled with FITC secondary antibody after labelling with the described primary antibodies ($\alpha_E\beta_7$, CD69, CD11b, $\alpha_4\beta_7$, $\alpha_4\beta_1$ and FcεRI).

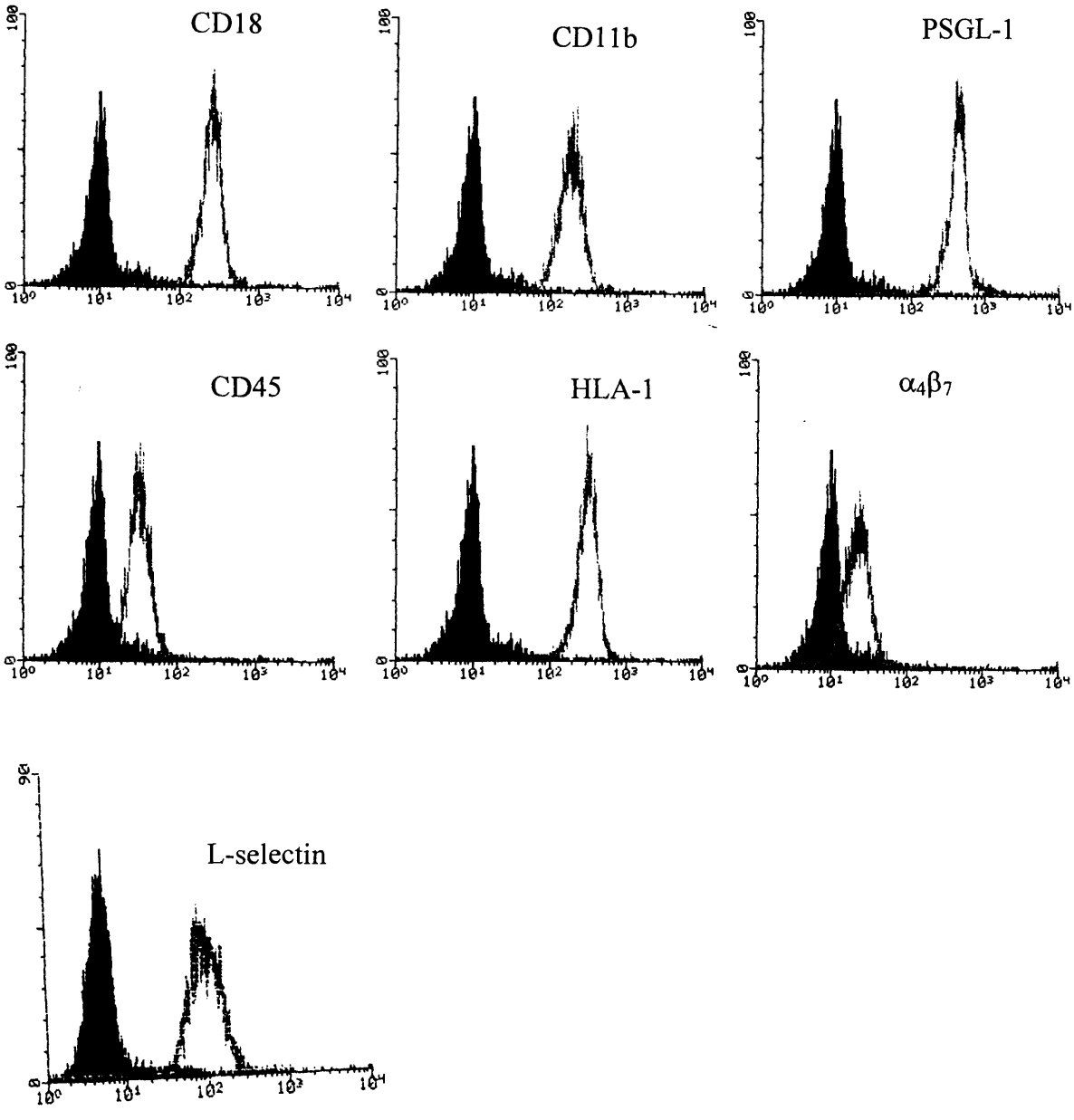


Figure 5.2 **Assessment of peripheral blood eosinophil surface cell adhesion molecules by flow cytometry (II).** Results are representative of four donors. Eosinophils were indirectly labelled with FITC secondary antibody after labelling with the described primary antibodies (CD18, CD11b, PSGL-1, CD45, HLA-1, $\alpha_4\beta_7$ and L-selectin).

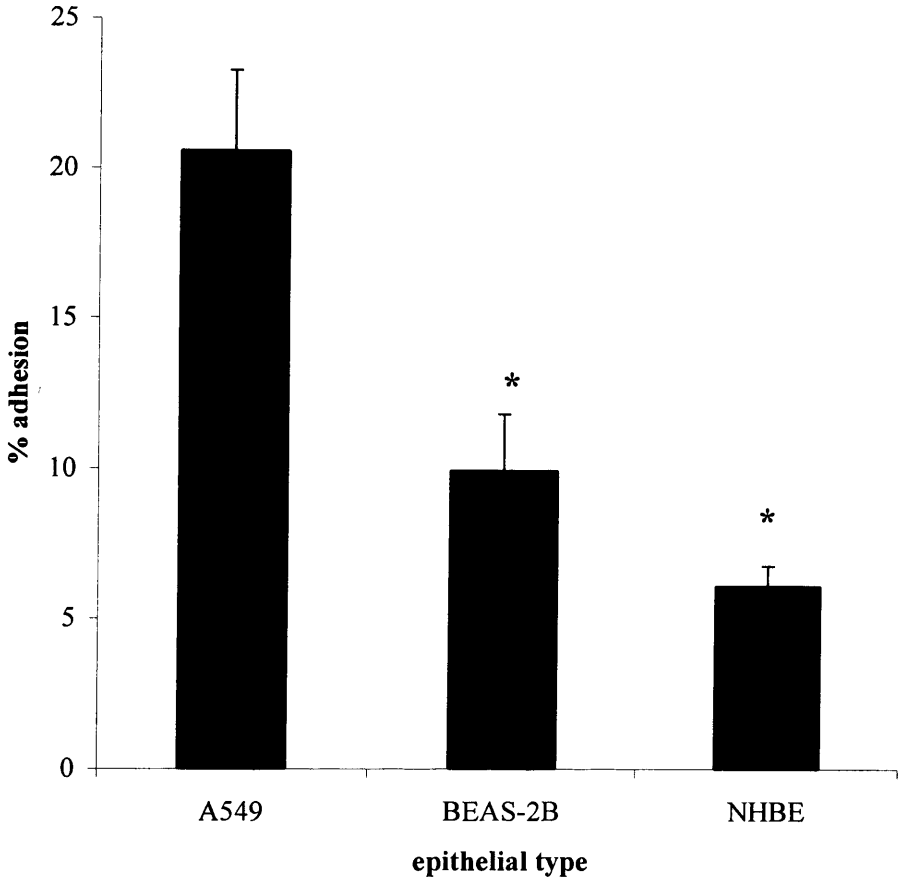


Figure 5.3 **Comparison of eosinophil adhesion to different epithelial cell types (n=3).** Adhesion to A549 (alveolar cell line), BEAS-2B (bronchial cell line) and NHBE (primary epithelial cell) monolayers of peripheral blood eosinophils isolated from the same donors, was carried out at the same time. BEAS-2B and NHBE adhesion was significantly lower than A549 adhesion (*$p<0.005$)

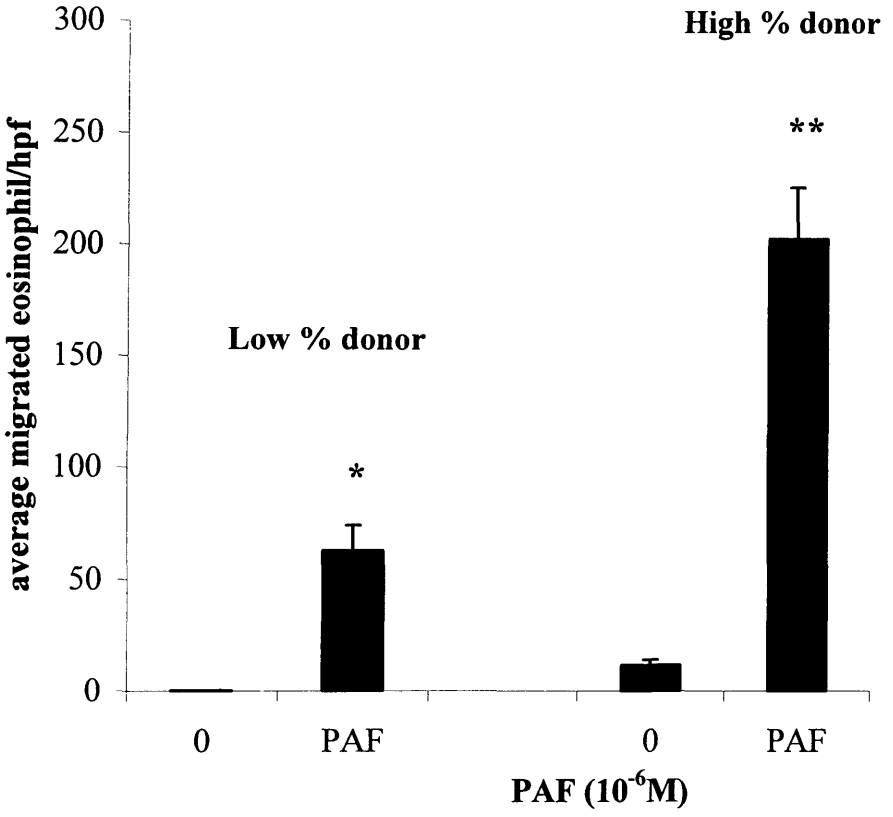


Figure 5.4 **Peripheral blood eosinophil chemotaxis to PAF (10^{-6}M).**
Eosinophil donors were divided into two groups to assess if initial percentage of eosinophils affected subsequent chemotaxis.
Low % donors were defined as <5% (n=3) and high % donors as >5% (n=5). Chemotaxis was assessed by counting 10 high power fields (hpf, x400) of triplicate wells of 8μm nitrocellulose filters (*$p<0.05$, **$p<0.005$, compared to migration in the absence of PAF).

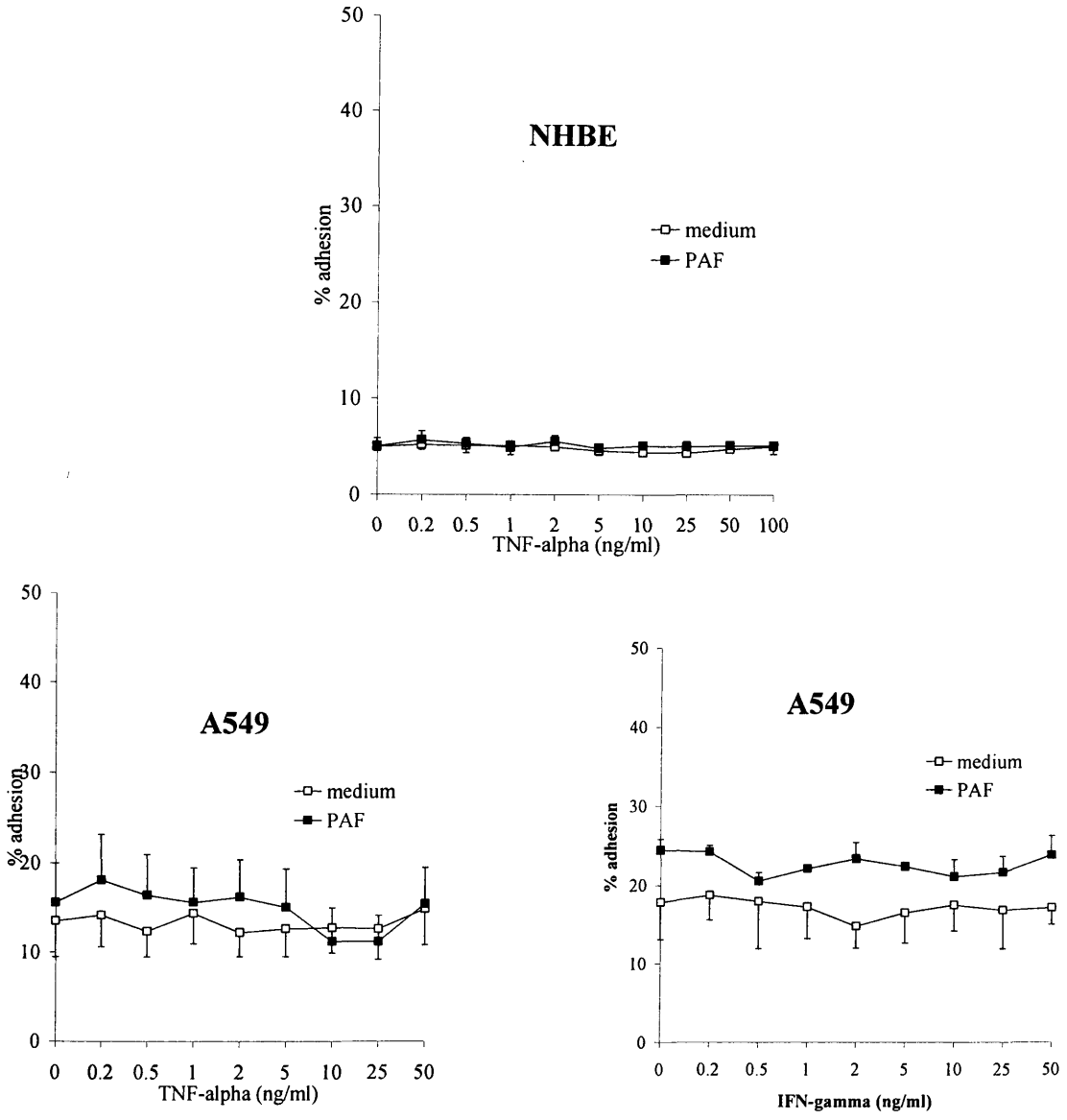


Figure 5.5 **PAF-activated and unactivated peripheral blood eosinophil adhesion** to (a) TNF-α stimulated and unstimulated NHBE primary epithelial monolayers (n=3) (b) TNF-α stimulated and unstimulated A549 alveolar epithelial monolayers (n=5) (c) IFN-γ stimulated and unstimulated A549 alveolar epithelial monolayers (n=3). Results are expressed as percentage adhesion +/- sem

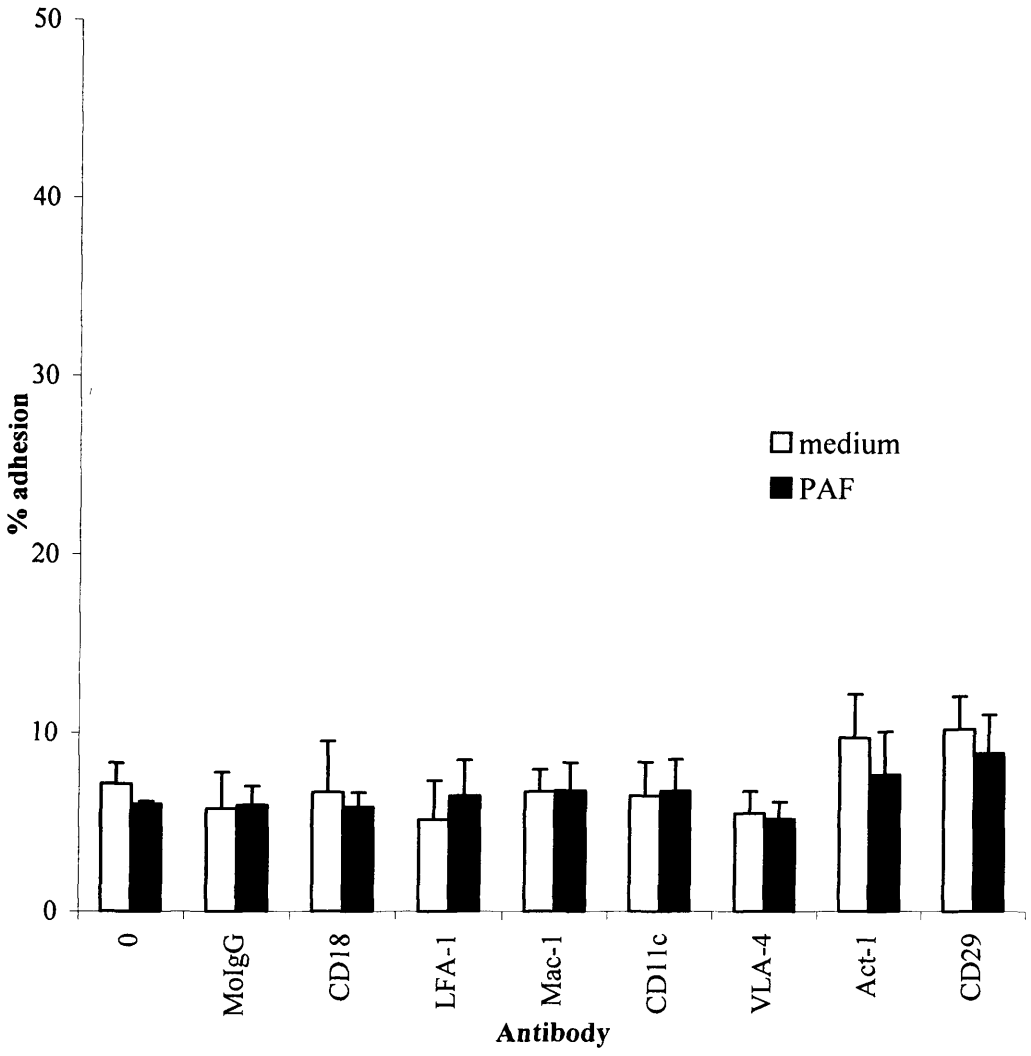


Figure 5.6 **Adhesion of unactivated and PAF-activated eosinophils to A549 monolayers in the presence of function-blocking mAbs (n=3)**
Results are expressed as percentage adhesion ± sem.

Heparin, galactose, mannose, mannose-6-phosphate or fucoidin did not inhibit unactivated eosinophil adhesion to unstimulated or cytomix-stimulated BEAS-2B (Figure 5.7), implying that lectin/carbohydrate and proteoglycan-mediated adhesion were not involved. Unstimulated eosinophil adhesion to 0.4% paraformaldehyde-fixed BEAS-2B did not appear to be cation-mediated and was therefore not thought to be eosinophil integrin-mediated (Figure 5.8). Mn^{2+}-stimulated eosinophil adhesion to cytomix-stimulated BEAS-2B was significantly increased compared to adhesion to unstimulated epithelium. The enhanced adhesion was thought to be integrin-mediated because Mn^{2+} selectively upregulates integrin function. This was confirmed by inhibition of adhesion in the presence of function-blocking CD18 mAb, but not ICAM-1 or E-cadherin mAb (Figure 5.9). In conclusion, this revealed that the enhanced adhesion observed upon adhesion of activated eosinophils to cytomix-treated epithelium involved eosinophil CD18 adhesion to an unknown epithelial ligand (that was not ICAM-1 or E-cadherin).

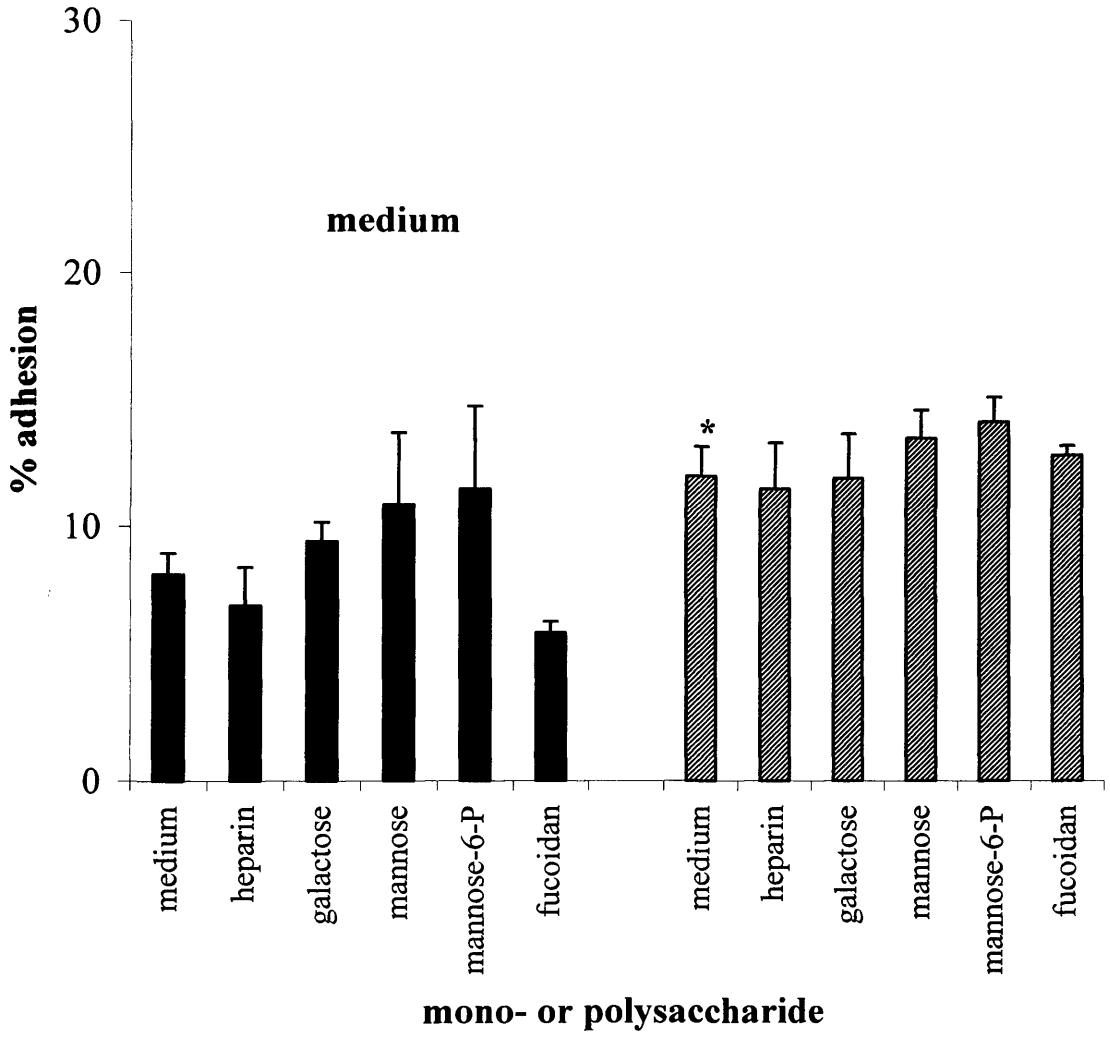


Figure 5.7 **Effect of selected monosaccharides and polysaccharides** on peripheral blood eosinophil adhesion to unstimulated and cytomix-stimulated BEAS-2B (bronchial) epithelial monolayers (n=3). Cytomix treatment significantly increased adhesion ($p<0.05$). Results are expressed as percentage adhesion ± sem.

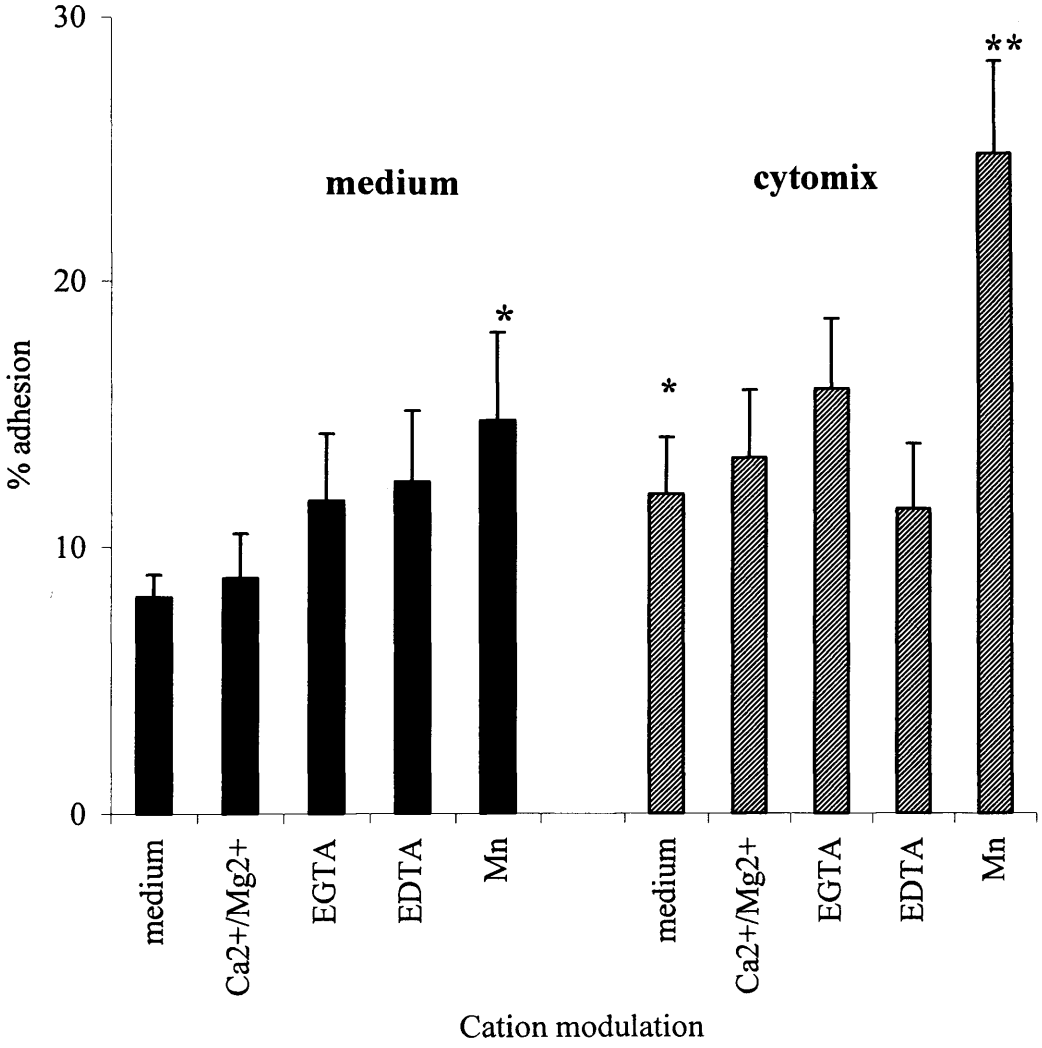


Figure 5.8 **Peripheral blood eosinophil cation modulation prior to adhesion to unactivated and cytomix-activated BEAS-2B (bronchial epithelial) monolayers (n=3)**
Results are expressed as percentage adhesion ± sem. Significance of results was calculated in comparison to medium-treated eosinophil adhesion to unactivated BEAS-2B monolayers (*$p<0.05$, **$p<0.005$)

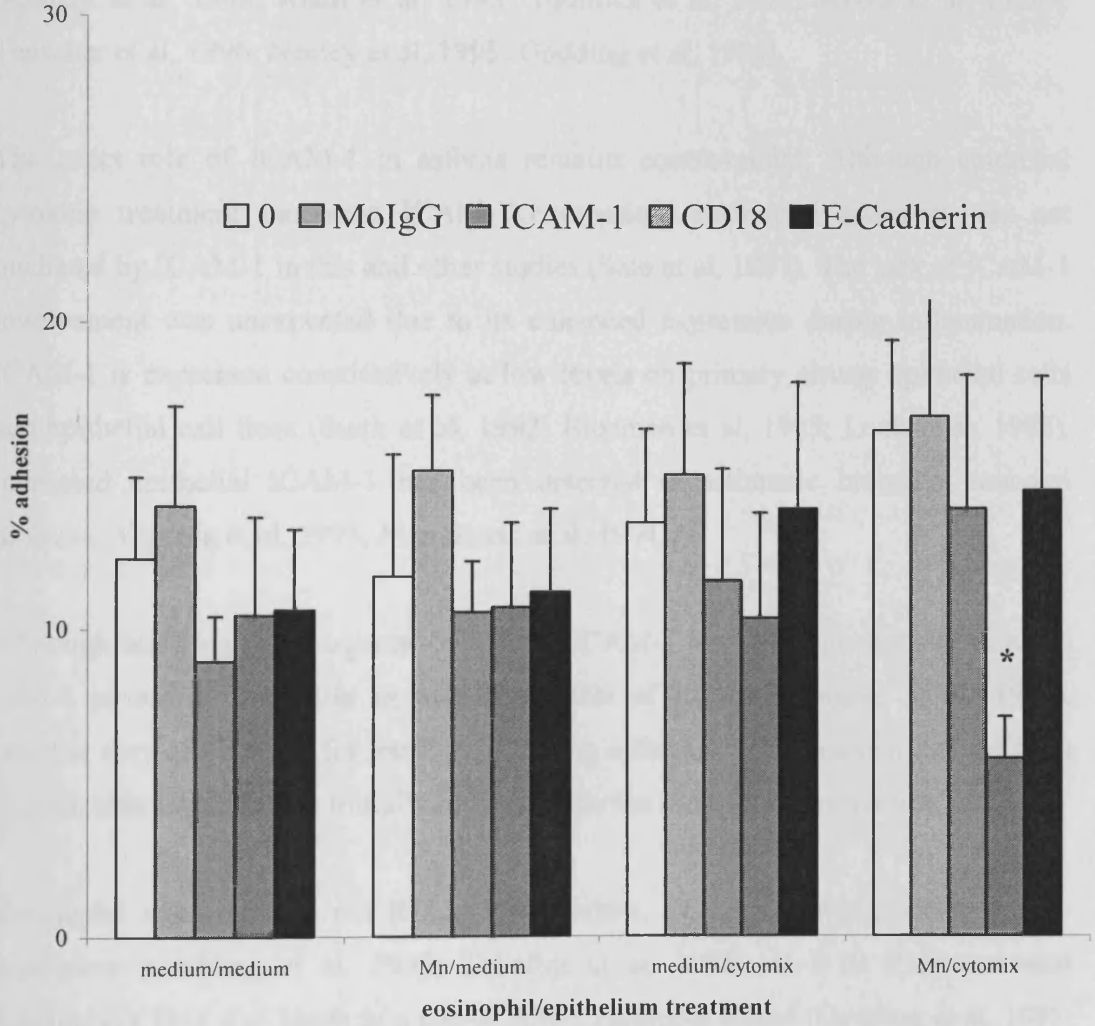


Figure 5.9 **Effect of function blocking mAb upon unactivated and manganese (Mn)-activated eosinophil adhesion to unstimulated and cytomix stimulated BEAS-2B bronchial epithelial monolayers (n=3).** CD18 mAb was preincubated with eosinophils; ICAM-1 and E-cadherin were preincubated with epithelium, 15 minutes prior to adhesion assay. Results are expressed as percentage adhesion ± sem. Significance was compared to MoIgG control for each treatment, *$p<0.005$).

5.4 Discussion

Transmigrated sputum eosinophils expressed increased CD11b compared to blood eosinophils (Hansel and Walker, 1992). Eosinophil activation (PAF, IL-5, eotaxin, RANTES and PMA) resulted in increased CD11b/CD18 expression/activation (Kimani et al, 1988; Alam et al, 1993; Tomioka et al, 1993; Weber et al, 1996b; Tenscher et al, 1996; Neeley et al, 1993; Godding et al, 1995).

The exact role of ICAM-1 in asthma remains controversial. Although epithelial cytokine treatment increased ICAM-1 expression, eosinophil adhesion was not mediated by ICAM-1 in this and other studies (Sato et al, 1997). The lack of ICAM-1 involvement was unexpected due to its enhanced expression during inflammation. ICAM-1 is expressed constitutively at low levels on primary airway epithelial cells and epithelial cell lines (Stark et al, 1992; Bloemen et al, 1993; Look et al, 1992). Increased epithelial ICAM-1 has been detected in asthmatic bronchial mucosal biopsies (Vignola et al, 1993; Manolitsas et al, 1994).

Although blocking mAbs against CD18 and ICAM-1 have been previously shown to inhibit airway eosinophilia in animal models of asthma (Wegner et al, 1990), whether they are ligands for each other during asthmatic inflammation has not been proven, although this was initially thought to be the most likely interaction.

Eosinophil adhesion was not ICAM-1 dependent, although it was partially CD18-dependent (Godding et al, 1995; Takafuji et al, 1996). IL-5 or PMA-activated eosinophil CD18 also binds to a non-ICAM-1 epithelial ligand (Godding et al, 1995; Takafuji et al, 1996). In contrast, PMA-activated eosinophils adhered to RSV-infected A549 cells via CD18/ICAM-1 (Stark et al, 1996), and CD18/ICAM-1 was responsible for PMA or eotaxin-stimulated eosinophil adhesion to activated NHBE cells (Burke-Gaffney and Hallewell, 1998).

This study showed that enhanced eosinophil adhesion to BEAS-2B that occurred upon cytomix stimulation of epithelium and Mn^{2+} activation of eosinophils was partially CD18- but not ICAM-1-dependent. In contrast to eosinophil adhesion to endothelium, adhesion was not enhanced in the presence of PAF.

Chapter 6: Conclusions and future work

Clinical applications

The mortality and morbidity of asthma has risen in recent years, and the reason(s) for this have not been identified. The inflammatory mechanism and mediators involved in asthma are currently being characterised in detail and have led to the understanding that it is a polygenomic syndrome with many causes and triggers which lead to common symptoms. The observation that a single treatment has not cured all asthmatic patients confirmed this hypothesis. Current therapy for asthma includes glucocorticoids, β_2-agonists, theophylline, cromones and anticholinergic agents.

Glucocorticoids are widely used and are the most effective therapy for asthma. Glucocorticoid molecules bound to cytosolic glucocorticoid receptors are translocated to the nucleus and bind as a homodimer to DNA. This results in reduced inducible gene expression (including cytokines and cell adhesion molecules) due to transcription factor inhibition (nuclear factor-κB, NF-κB) (Barnes, 1997; Hancox et al, 1999). Corticosteroids significantly reduce the allergic inflammatory response in the majority of patients, although severe asthma is still poorly controlled even at high doses and severe side effects are observed. Therefore more effective targeted therapy is required for this patient group at highest risk of mortality.

Other recent approaches

Asthma involves a deleterious immune response and includes T_H2 activation, elevated serum IgE levels and airway eosinophilia. T lymphocytes, eosinophils and their associated inflammatory mediator pathways are currently being targeted to generate more specific therapeutic strategies. Recent developments in asthma therapy have included two main approaches:

(i) improvement of existing drugs (increased selectivity, decreased side effects e.g. dissociated glucocorticoids, selective PDE4 antagonists)

(ii) identification of novel targets (T_H2 cell inhibitors, anti-IgE antibodies, antagonists of eosinophil recruitment (VLA-4 receptor antagonists), mediator

antagonists (cysteinyl leukotriene receptor and 5-lipoxygenase, IL-5, CCR3 chemokine receptor, tryptase/Proteinase-activated receptor-2).

Inhibition of T cell cytokine synthesis (corticosteroids, cyclosporin A) and humanised blocking antibodies to cytokines or their receptors have been recently investigated as targets for asthma therapy. Cyclosporin A and FK506 are nonselective immunosuppressants (inhibit both T_H1 and T_H2) and therefore do not restore the balance of these cell subsets. More selective inhibitors of T_H2 cells are being developed and tested (e.g. reduction of circulating $CD4^+$ cells with chimeric anti-CD4 mAb treatment) (Kon et al, 1998). The role of T_H2 cytokines in asthma has remained controversial. Although humanised anti-IL-5 mAb reduced blood eosinophilia for at least 3 months and prevented recruitment after allergen challenge, there was no effect on early or late response to allergen (Leckie et al, 2000). This result implies eosinophils are not as important as previously suggested.

Another novel approach involves reduction of free serum IgE levels by non-anaphylactogenic anti-IgE (e.g. humanised murine mAb E25, rhu-MAb-E25 directed to the FcεRI-binding domain of human IgE). IgE is involved in triggering inflammatory mediator release and antigen presentation. High affinity IgE receptors (FcεRI) are present on mast cells, basophils and dendritic cells, and low affinity IgE receptors (FcεRII, CD23) are expressed by B cells, monocyte/macrophages and eosinophils. In clinical studies, mAb-E25 reduced free serum IgE levels and attenuated both early and late responses to inhaled allergen, inhibited antigen-induced BHR and reduced pulmonary eosinophil infiltration into induced sputum (Fahy et al, 1997; Boulet et al, 1997).

A third important area of current research is the development of cell adhesion antagonists. Cell adhesion molecules play a role in inflammation (e.g. asthma), host defence (virus and bacteria), cancer, (malignancy and metastasis) and heart disease (myocardial infarction and reperfusion injury). Loss of control of adhesion results in unregulated and extensive tissue damage. An early event in inflammation is focal adhesion of leukocytes to vascular endothelium. E-, P- and L-selectin support the

initial attachment of leukocytes to the inflamed vascular endothelium through their recognition of carbohydrate ligands such as sLe^x. Since selective leukocyte recruitment is a key, early step in inflammation, therapies preventing adhesion are attractive for the treatment of pathologic inflammation. Endothelial E- and P-selectin induced by proinflammatory mediators (cytokines, histamine) are concentrated at areas of inflammation. L-selectin is constitutively expressed in leukocytes and shed upon cell activation. Therefore inhibitors targeting adhesion would require local administration and would be active at lower doses than inhibitors aimed at more broadly expressed molecules. Such therapy may also produce fewer side effects than currently available therapies due to their specificity. In addition, airway infiltration by inflammatory cells also includes migration to, and retention at, the bronchial epithelium, and is controlled by leukocyte and epithelial cell adhesion molecule expression and function. These cell adhesion molecules also require upregulation or activation by proinflammatory stimuli. Selective eosinophil interaction with endothelium, tissue infiltration and accumulation in asthma has been studied in detail and is mediated by selectins, and α_4 and β_2 integrins. Increased bronchial epithelial ICAM-1 and *de novo* VCAM-1 may be expressed after inflammatory challenge (Atsuta et al, 1997). Antagonists of these cell adhesion molecules have been intensively investigated.

E-selectin was shown to support the adhesion of neutrophils to cytokine-activated endothelium (Bevilacqua, et al., 1987). Subsequent *in vitro* studies have shown E-selectin is also involved in adhesion of monocytes, memory T lymphocytes, eosinophils and basophils to endothelium. P-selectin also supports eosinophil and neutrophil adhesion to endothelium (Patel, 1998; Woltmann et al, 2000). L-selectin has a role in lymphocyte homing and participates in neutrophil, monocyte and lymphocyte adhesion to activated endothelium (Bevilacqua and Nelson, 1993).
E- and P-selectin recognize oligosaccharide structures including the tetrasaccharide, sialyl $Lewis^x$ (sLe^x) found on leukocytes. L-selectin can also bind similar structures, although there may be a substantial difference in affinities. In addition, L-selectin has been shown to bind sulfatides (sulfated glycolipids) as well as sulfate-and sialic acid-containing mucin-type glycoproteins expressed on high endothelial venules of lymph

nodes. Soluble oligosaccharides related to sLe^x and sLe^a (isomer of sLe^x) are able to inhibit ligand binding and adhesive functions of E- and P-selectin. These compounds have been investigated as anti-inflammatory agents, although their complex structures pose significant obstacles for large-scale synthesis. Previous studies have shown that phosphate- and sulfate-containing carbohydrates unrelated to sLe^x can also interact with P- and L-selectin. L-selectin adhesion can be blocked by PPME (polyphosphomonoester), fucoidan (a sulfated polysaccharide), and a complex core polysaccharide of yeast containing mannose-6-phosphate (Stoolman, et al., 1984).

L-selectin inhibitors (based on sialyl-Lewis^x), inhibit inhaled allergen-induced inflammatory cell influx in sensitised sheep (Abraham et al, 1999). The VLA-4/VCAM-1 interaction has been shown to be important for eosinophil/endothelial adhesion and infiltration, and humanised antibodies to VLA-4 have been developed (Yuan et al, 1996). Small molecule peptide inhibitors of VLA-4 have now been developed (Lin et al, 1999; Jackson et al, 1997). When given by aerosol, there is reduced antigen-induced late responses and airway hyperresponsiveness in allergic sheep (Abraham et al, 1994). A mAb to VLA-4 inhibited eosinophil accumulation (Weg et al, 1993) and mAb to endothelial ICAM-1 prevented eosinophil infiltration into airways and the increase in bronchial reactivity after allergen exposure in sensitised primates (Wegner et al, 1990). The counterreceptor Mac-1 mAb inhibited development of BHR and reduced the levels of ECP in the BAL fluid but did not inhibit airway eosinophilia (Wegner et al, 1990). This suggested that Mac-1 was not essential for eosinophil migration but that it may be involved in eosinophil activation and triggering of mediator release. Eosinophil accumulation has not always been prevented by cell adhesion molecule antagonists in all species studied – e.g. ICAM-1 mAb in brown-Norway rats (Sun et al, 1994).

Cell adhesion molecule antagonists must be carefully investigated *in vivo* for cell adhesion pathway selectivity because many cell types share common pathways, and animal models may vary considerably from humans. It is also not known whether the antibodies studied *in vivo* directly inhibit the specific interaction, or indirectly affect adhesion by inhibiting function of other cell types. In addition, concerns have been

raised that inhibiting immune responses in this manner may lead to more frequent infections or an increased risk of neoplasia.

Future work

Cell-cell interactions and their associated signalling are important in maintaining a cohesive immune system and are involved in tissue structure and immune cell infiltration and function. Adhesive interactions are tightly regulated and may be altered during mucosal inflammation in asthma, which may in turn result in altered inflammatory cell function and activation. Intraepithelial lymphocytes and mucosal mast cells at the bronchial epithelial surface may encounter allergen prior to other inflammatory cell types. Eosinophil recruitment and activation at the bronchial epithelium is increased during the asthmatic late phase. Therefore, cell adhesion molecule-mediated interactions of these cells with bronchial epithelium may be enhanced during airway inflammation and may affect both asthma severity and pharmacological efficacy.

This study has provided evidence of a specific carbohydrate-mediated adhesion mechanism between human lung mast cell surface glycoprotein and bronchial epithelial lectin. In contrast to expectations adhesion was downregulated upon immunological (anti-IgE) but not non-immunological (A23187) activation which was not correlated with reduced surface cell adhesion molecule expression (except ICAM-1, which was not directly involved in the adhesion mechanism). Possible reduced function of as-yet-uncharacterised adhesion molecule or signalling pathways have not been excluded.

Previous mast cell adhesion studies have investigated rodent or human cell lines, or canine mastocytoma cells. These cells are functionally and phenotypically different to primary human lung cells. Therefore it was important to carry out these studies using human cells. In addition, mast cells from different source tissues vary in their products and activation stimuli. Activated human lung mast cells release pre-formed mediators such as tryptase and histamine (in minutes) and newly synthesised cytokines, cysteinyl leukotrienes and prostaglandin D_2 (hours), which may also be

involved in reduction of adhesion, and may be different to human cells from other locations or rodent/canine cells. In this study the epithelial cell line BEAS-2B was used for investigation of adhesion. Biopsy sections with intact areas of epithelium could be used in further experiments to compare proportion and site of adhesion expected *in vivo*. However, it may be difficult to compare asthmatic patients due to characteristic focal denudation and fragility of epithelium.

Although the precise adhesion molecule(s) or family of molecules involved in mast cell interaction with bronchial epithelium was not discovered, it involved heterotypic adhesion to a β-galactoside-containing mast cell glycoprotein. The best-characterised β-galactoside-binding proteins are galectins. However this family of lectins was not involved in adhesion since lactose did not inhibit. Further characterisation is therefore required but may prove to be difficult since no similarities to currently known adhesion molecule classes were observed and both adhesion receptor and ligand are unknown. Since function-blocking mAbs to known families of cell adhesion molecules did not block adhesion there was no molecule (e.g. protein or carbohydrate) that may be linked to a matrix to 'pull out' the involved cell adhesion molecule(s) for further characterisation.

Lectin/carbohydrate immunofluorescent staining may be used to characterise cell surface marker expression. A series of β-galactoside-containing carbohydrates or mucin-containing glycoproteins may be used to attempt inhibition of the adhesive interaction. General or specific inhibitors of carbohydrate or protein synthesis pathways may also be useful for investigating whether the mast cell glycoprotein may be re-established on the cell surface after enzyme-mediated removal. A more comprehensive panel of function-blocking mAb may not be useful, since general features are often shared between all adhesion molecules within a specific family.

A more specific approach may be used to generate novel mAb. Unknown adhesive interactions have previously been characterised using novel mAbs synthesised using mouse hybridomas. These mAbs may be used in adhesion assays or immunocytochemistry. However, this approach may be problematic in this case,

since the epitope directly involved in mediating most of the adhesion appeared to be small and therefore may not be antigenic enough to enable production of a good function blocking mAb. Adhesive interactions in the immune system may involve more than one pair of cell surface molecules for adhesion and/or control of adhesion (e.g. co-receptors). Further investigation of FcεRI-specific signalling may be more useful in characterising the adhesion mechanisms further.

Once the cell adhesion molecule identified in this research has been characterised, it may be possible to produce a cell adhesion molecule-Ig fusion protein (Aruffo et al., 1991; Walz et al., 1990). In addition, recombinant cell adhesion molecules may be transiently expressed by transfecting COS cells with a full-length cell adhesion molecule cDNA-containing expression vector (Seed et al, 1987). COS cell-expressed cell adhesion molecule function may then be tested by competition binding ELISAs or adhesion assays in the presence of the fusion protein (Nelson et al, 1993).

Characterisation of the interaction between HLMC and epithelium may eventually lead to generation of specific, small molecule antagonist(s) to prevent localisation of lung mast cells within the bronchial epithelium, thereby reducing the chance of interactions with aeroallergens. From this study, they would be expected to contain the unsubstituted Galβ(1-3) GalNAcα core peptide, although tertiary interactions/other sites of interaction were not identified, and may also be involved secondarily. Although antagonists may also indirectly prevent subsequent events of the inflammatory cascade (such as eosinophil recruitment, activation and release of epithelium-damaging basic proteins and other proinflammatory mediators), this study indicated that eosinophil interaction with bronchial epithelium would not be directly affected since their adhesion mechanism was different. Until further studies to characterise the physiological role of intraepithelial mast cells are carried out, the functional selectivity and other advantages of this approach cannot be examined. HLMC are involved in physiological control and include epithelial restitution, wound healing, tissue fibrosis, control of adhesion molecule induction (endothelial E-selectin expression upon TNF-α release, Walsh et al, 1991) and innate immunity. It

therefore remains to be seen whether human lung mast cell adhesion molecules are a safe and specific pharmacological target.

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List of publications resulting from this study

Posters were presented at the 54th annual American Academy of Allergy Asthma and Immunology (AAAAI) meeting in Washington D.C. (March 1998).

Poster Abstracts

Sanmugalingam D, Wardlaw AJ, Bradding P (1998). Adherence of human lung mast cells to bronchial epithelium. Abstract 898, *J.Allergy Clin.Immunol.* **101**:S216.

Wardlaw AJ, Gauntlett R, Symon FA, Bradding P, Sanmugalingam D (1998). Eosinophil adhesion to bronchial epithelium. Abstract 914, *J.Allergy Clin.Immunol.* **101**:S220.

Papers

Adhesion of human lung mast cells to bronchial epithelium: evidence for a novel carbohydrate-mediated mechanism. D.Sanmugalingam, A.J.Wardlaw and P.Bradding. *Journal of Leukocyte Biology* (2000) **68**:38-46.
(all experiments presented in this paper were carried out by D.Sanmugalingam)

Interleukin-5 enhances eosinophil adhesion to bronchial epithelial cells. D.Sanmugalingam, E.De Vries, R.Gauntlett, F.A.Symon, P.Bradding and A.J. Wardlaw. *Clinical and Experimental Allergy* (2000) **30**:255-263.
(experiments contributed by D.Sanmugalingam were presented in Figures 2,3 and 4)