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THE PROINFLAMMATORY ACTIONS OF MAST CELL TRYPTASE AND AGONISTS OF PROTEASE ACTIVATED RECEPTOR 2 ON THE HUMAN AIRWAY EPITHELIUM

BY

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ABSTRACT

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THE PROINFLAMMATORY ACTIONS OF MAST CELL TRYPTASE AND AGONISTS OF PROTEASE ACTIVATED RECEPTOR 2 ON THE HUMAN AIRWAY EPITHELIUM

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Mast cell activation and inflammation of the airway epithelium are prominent features of bronchial asthma. The major product of mast cell activation is a unique tetrameric serine protease termed tryptase. This trypsin-like enzyme is emerging as a new mediator of allergic inflammation and it can alter cell function by activating protease activated receptor 2 (PAR-2). Proteolytic cleavage of the amino terminus of this G protein-coupled receptor by tryptase can expose a 'tethered ligand' that binds and activates the receptor. Substantial quantities of tryptase are secreted into the airways of asthmatic subjects, but little is known of the effects on the airway epithelium of exposure to tryptase. The aims of this study have been to investigate the proinflammatory actions of tryptase on airway epithelial cells and to examine the potential role of PAR-2 in airway inflammation.

Tryptase was isolated to high purity and was added to epithelial cells. An increase in ^{3}H-thymidine uptake in 16HBE 14o- cells (maximal at 10 mU/ml) was induced by tryptase without stimulating significant cell proliferation; while at higher concentrations (80 mU/ml), tryptase actually inhibited DNA synthesis and cell growth in an apparently non-cytotoxic manner. Within 3 h of adding tryptase, marked increases in expression of mRNA for IL-6, IL-8 and GM-CSF were detected by reverse transcription-polymerase chain reaction (RT-PCR). This was followed by increased secretion of IL-6 (maximal at 6 h), IL-8 and GM-CSF (both maximal at 24 h and 48 h) into culture supernatants. The actions of tryptase were in all cases inhibited by heat inactivation of the enzyme or by addition of the protease inhibitor leupeptin. The peptide SLIGKV, an agonist for protease activated receptor 2 (PAR-2), induced responses similar to those elicited with tryptase, suggesting that the proinflammatory actions may be mediated through activation of this receptor. PAR-2 was detected in epithelial cells by immunocytochemistry and flow cytometry, and mRNA for this receptor by RT-PCR.

Tryptase induced a progressive decrease in transepithelial resistance (TER) across monolayers of 16HBE 14o- cells at 80 mU/ml though at lower concentrations (10 or 20 mU/ml) could actually increase TER. The peptide agonist of PAR-2 SLIGKV and trypsin were also able to induce a decline in TER. Pretreatment of cells with pertussis toxin, a G_i protein inhibitor, abolished the response to SLIGKV, but not that to tryptase, suggesting that processes other than PAR-2 activation may mediate the decline in TER induced by tryptase. The decreases in TER were reflected in increased permeability of the monolayers to radiolabeled mannitol and inulin tracers but not to albumin. Tryptase and SLIGKV-induced increases in epithelial permeability were accompanied by reduced expression of the tight junction proteins occludin and ZO-1 in immunocytochemistry. With tryptase but not with SLIGKV, there was loss of occludin and ZO-1 from cell extracts as detected by Western blotting, suggesting that proteolytic degradation of occludin and subsequent breakdown of ZO-1 may have occurred.

Supernatants from A549 epithelial cells incubated with tryptase or SLIGKV for 24 h stimulated the migration of neutrophils across monolayers of these epithelial cells, though tryptase itself was without direct chemotactic activity in this system. Neutrophil transmigration was blocked by pretreating the supernatants with a neutralising antibody against IL-8. These cells secreted large amounts of IL-8 in response to treatment with tryptase or the PAR-2 agonist, and it would seem that IL-8 release was largely responsible for the neutrophil chemotactic activity observed.

Tryptase and other agonists of PAR-2 activation could play key roles in epithelial inflammation in asthma and in other allergic conditions, and these must represent attractive targets for therapeutic intervention.

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All the studies presented in this thesis were solely done by the author except for the study of electron microscopy employed in epithelial monolayers which was performed in part with the help of Dr. Godfrey.

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2. Perng, D.W., S.H. Leir, S.J. Compton, P.M. Lackie, S.T. Holgate, and A.F. Walls. 1999. Mast cell tryptase stimulates cytokine synthesis and secretion from bronchial epithelial cells: a role for protease activated receptor 2 (PAR-2). *Am J Respir Crit Care Med. 159:A336.*
3. Berger, P., D.W. Perng, H. Thabrew, J.A. Cairns, S.J. Compton, A.R. McEuen, R. Marthan, J.T. Lara, and A.F. Walls. 2000. Proliferation of human airway smooth muscle cells in response to mast cell tryptase and activation of protease activated receptor 2 (PAR-2). *Am J Respir Crit Care Med. 161:A467.*
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Abbreviations

APRT	adenine phosphoribosyltransferase
Ab	antibody
Ala	alanine
APC 366	N-(1-hydroxy-nathothoyl)-L-arginyl-prolinamide
APS	Amonium persulphate
Arg	arginine
ASA	aspirin sensitive asthma
BAL	bronchoalveolar lavage
BAPNA	N-α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CGRP	calcium gene-related peptide
CPC	cetylpyridinium chloride
CPM	counts per minute
DAB	diaminobenzidine
DMSO	dimethylsulphoxide
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(aminoethyl ether)-N,N,N',N'-tetraacetic acid
EIB	exercise-induced bronchoconstriction
ELISA	enzyme linked-immuno-sorbent assay
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
Gly	glycine
GM-CSF	granulocyte-macrophage colony stimulating factor

GPCR	G-protein-coupled receptor
GRK	G-protein receptor kinase
H_1	histamine receptor type 1
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyepitheylpiperazine-N′2-ethanesulphonic acid
HRP	horseradish peroxidase
HR-SEM	high-resolution scanning electron microscopy
ICAM-1	intercellular adhesion molecules-1
IFN-γ	interferon gamma
Ig	Immunoglobulin
IL	interleukin
kDa	kilo dalton
LDH	lactate dehydrogenase
LT	leukotriene
Lys	lysine
MC_T	mast cell containing tryptase, but not chymase
MC_{TC}	mast cell containing tryptase and chymase
MEM	minimum essential medium
MES	2-[*N*-morpholino]ethanesulphonic acid
MMP	Matrix metalloproteinase
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MTS	(3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium)
NAD	Nicotinamide adenine dinucleotide
PAR	protease-activated receptor
PBS	Phosphate-buffered saline
PG	prostaglandin

PHM	peptide histidine-methionine
PKC	proteine kinase C
PLC	Phospholipase C
RANTES	Regulated on activation, normal T-cell expressed and secreted
RT-PCR	reverse transcription- polymerase chain reaction
SCF	stem cell factor
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	standard error
Ser	serine
TER	transepithelial resistance
TGF	transforming growth factor
Thr	threonine
TJ	tight junction
TNF	tumour necrosis factor
Tris-HCL	tris[hydroxymethyl] aminomethane hydrochloride
Tween-20	polyoxyethylene-sorbitan monolaurate
Val	valine
VIP	vasoactive intestinal peptide

CHAPTER 1

GENERAL INTRODUCTION

1 General Introduction

Mast cell activation and inflammation of the airway epithelium are prominent features in bronchial asthma. The major product of mast cell activation is the tryptic serine protease tryptase, and substantial quantities are released into the airways of asthmatics. This unique mast cell protease is emerging as a major indicator of allergic inflammation, which may be able to alter cell function by activating protease activated receptor 2 (PAR-2). However, little is known of the actions of tryptase on the airway epithelium. The aim of the present study has been to examine the proinflammatory effects of mast cell tryptase and agonists of PAR-2 on epithelial cells, and to examine the potential role of this abundant mast cell product in altering epithelial function and in stimulating the influx of inflammatory cells.

1.1 Mast Cells and Asthma

1.1.1 Pathogenesis of Asthma

In the 1997 Expert Panel Report (National Heart, Lung and Blood Institute, 1997), asthma was defined as a "chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T lymphocytes, macrophages, neutrophils, and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli". This working definition highlights three key aspects of asthma (1) airway inflammation infiltrated with several types of inflammatory cells; (2) airway obstruction that

is reversible either spontaneously or with treatment; and (3) increased airway responsiveness to a variety of stimuli.

Through examination of bronchoscopic lavage fluid and biopsy tissues from asthmatic patients, evidence has emerged to reveal new insights into mechanisms of asthma and features that link altered lung function to a specific type of mucosal inflammation. Mast cells, eosinophils, neutrophils, epithelial cells, macrophages, and activated T cells appear to make key contributions to the inflammatory process of asthma (Beasley et al., 1989; Djukanovic et al., 1990a; Jeffery et al., 1989; Laitinen et al., 1985). The histopathological findings are characterised by injury to airway epithelium, thickening of the subepithelial basement membrane, airway smooth muscle cell hyperplasia/hypertrophy, increased numbers of mucus-secreting cells, and a multicellular infiltration (Dunnill et al., 1969). An initial trigger in asthma may be attributed to the release of inflammatory mediators from mast cells, macrophages, T lymphocytes, and epithelial cells (Busse et al., 1993). These substances may thus induce the migration and activation of other inflammatory cells, such as eosinophils and neutrophils, to the airways where they cause disruption in epithelial integrity, mucus hypersecretion, change in mucociliary function, and increased airway smooth muscle responsiveness.

1.1.2 Roles of Mast Cells in Asthma

Mast cells have been implicated in prticular as participants in early and late phase asthmatic reactions to allergens, exercise-induced bronchoconstriction, nocturnal asthma, aspirin-sensitive asthma, and in airway hyperresponsiveness. Mast cell activation appears to be the dominant component of the immediate response to allergen challenge. Immediate reactions usually occur within minutes, resolve 30 to 60 min later and are characterized by airway obstruction. Late-phase reactions begin approximately 3 to 4 hr after challenge and reach maximal intensity by 4 to 8 hr. The airway obstruction resolves in 12 to 24hr (Lam et al., 1983; Metzger et al., 1986).

Bronchoalveolar lavage fluid obtained from allergic rhinitis patients 12 min after segmental antigen instillation (immediate response) has revealed a significant increase in levels of histamine and tryptase, whereas the response at 48 hr has been characterised by increased levels of interleukin-5 (IL-5), eosinophils, and eosinophil granular proteins (Sedgwick et al., 1991). However, in some asthmatic patients with allergen inhalation challenge, increased numbers of mast cells have been found in late-phase reactions and the number of mast cells may be correlated significantly with their severity (Crimi et al., 1991).

Exercise-induced bronchoconstriction (EIB) is a term to describe the airway narrowing that follows vigorous exercise. EIB usually occurs in persons with clinically documented asthma. Animal studies have demonstrated that breathing dry air at a high flow rate for 5 min can induce acute mucosal injury, associated with mast cell degranulation, leukocyte infiltration, disruption of the ciliated epithelium, and increased vascular leakage (Freed et al., 1995; Omori et al., 1995). Increased numbers of degranulated mast cells and eosinophils has been reported in asthmatics 3 hr after exercise (Crimi et al., 1992), suggesting a role for mast cells in EIB. Nocturnal worsening of bronchoconstriction is not an uncommon event in the asthmatics (Turner-Warwick, 1988). Histamine concentrations in bronchoalveolar lavage fluid at 4:00 a.m. has been reported to be significantly higher in a group of nocturnal asthmatics than in non-nocturnal asthmatics, and the peak plasma histamine levels at 4:00 a.m. can coincide with the nadir in lung function in nocturnal asthmatics, suggesting the links between mast cell activation and the nocturnal asthma attack (Barnes et al., 1980; Jarjour et al., 1992).

Increased serum histamine and tryptase level have been reported in some aspirin-sensitive asthmatic (ASA) patients after aspirin ingestion (Bosso et al., 1991). Immunohistochemical studies of nasal polyps obtained from ASA patients have revealed degranulated mast cells and abundant eosinophils (Yamashita et al., 1989), indicating that the release of mediators of anaphylaxis from mast cells and eosinophils may be a mechanism for aspirin-induced asthma.

Airway hyperresponsiveness is a cardinal feature of asthma. Several studies have shown the presence of inflammatory cells, which were related to airway hyperresponsiveness in human subjects after inhalation of ozone (Seltzer et al., 1986), toluene diisocyanate (Fabbri et al., 1987) and allergen (De Monchy et al., 1985; Woolley et al., 1995). The association between the mast cells and airway hyperresponsiveness has been established with a significant correlation being found between the degree of airway responsiveness and the number of mast cells (Kirby et al., 1987).

Numerous studies have shown a significant increase in the numbers of mast cells in bronchoalveolar lavage fluid collected from subjects with bronchial asthma (Flint et al., 1985; Tomioka et al., 1984; Walls et al., 1990). In asthmatic subjects, a twofold to sixfold increase in mast cell numbers has been reported in atopic and non-atopic asthmatics (Adelroth et al., 1990; Casale et al., 1987; Flint et al., 1985; Kirby et al., 1987; Wardlaw et al., 1988). These luminal mast cells are likely to be the first to be triggered by inhaled allergen. On entering the respiratory tract, allergen will bind readily to specific immunoglobulin E (IgE) on high-affinity IgE receptors of mast cells, triggering the release of potent mediators, which can induce an acute episode of airway obstruction by directly increasing airway smooth muscle tone (Aalbers et al., 1993; Sedgwick et al., 1991; Wenzel et al., 1990; Wenzel et al., 1991). In addition, mast cells express and release a wide variety of proinflammatory mediators, which can initiate, regulate, and maintain immunological and inflammatory reactions, mediating alterations in adhesion molecule expression, cell recruitment, and inflammatory cell survival.

Tryptase, the unique serine protease of human mast cells, has come to be regarded as an important marker of mast cell activation in asthma and other inflammatory diseases. Elevated concentrations of tryptase have been detected in the bronchoalveolar lavage of asthmatics and the levels may be dramatically increased following endobronchial allergen challenge (Wenzel et al., 1988; Broide et al., 1991). Elucidation of the mediator roles of tryptase in the airways must be an important objective.

1.2 Characteristics of Mast Cells

1.2.1 Origin and Maturation

Human mast cells originate from pluripotent CD34+ human progenitor cells in the bone marrow (Bressler et al., 1990; Kirshenbaum et al., 1991). It is believed that circulating committed mast cell progenitors first adhere to endothelial cells and then migrate through tissues to specific locations determined by the resident mix of connective-tissue components and cellular elements. Mast cells reside in these tissues and switch into their mature phenotype. Several growth factors are known to influence the growth and differentiation of mast cells. These include IL-3, IL-4, IL-10, c-*kit* ligand and stem cell factor (SCF) (Huang et al., 1990; Martin et al., 1990; Rennick et al., 1985; Thompson-Snipes et al., 1991). Migration of mast cells can be stimulated by IL-3 or SCF and can be further potentiated by the combination of IL-3 and SCF (Matsuura and Zetter, 1989; Meininger et al., 1992). All human mast cells are categorised by the presence of tryptase.

1.2.2 Functional Heterogeneity

Human mast cells have been categorised on the basis of their content of tryptase and chymase: MC_T containing tryptase only and MC_{TC} containing both tryptase and chymase. MC_T are found predominantly in the mucosal tissues of respiratory and gastrointestinal tracts, whereas the MC_{TC} are preferentially located in connective and submucosal tissues (Craig et al., 1986; Irani et al., 1986; Irani et al., 1989).

In bronchial biopsy tissue, IL-4 is present in both of these mast cell subsets whereas IL-5, IL-6, and TNF-α have been localised exclusively to MC_T cells (Bradding et al., 1993). Mast cell populations can vary greatly in the extent to which they will release histamine in response to anti-IgE. Histamine release from mast cells in nasal polyps, lung and breast skin has been reported to be

consistently higher than that in the colon mucosa and foreskin (Lowman et al., 1988). In contrast, mast cells from human lung, colon, adenoids, tonsils, and nasal polyps release histamine in response to substance P, while those from skin tissue can degranulate with the rapid secretion of histamine with this stimulus. These differences, however, are not related directly to MC_T and MC_{TC} phenotype, but purely tissue location. Human mast cells are heterogeneous in histochemical characteristics, mediator release, and response to stimuli, and may have diverse roles in inflammatory processes.

1.2.3 Mediators

Mast cells degranulate and release a variety of prestored and newly formed mediators after IgE dependent or nonimmunological activation (Fig. 1-1). Nonimmunological stimuli include neuropeptides (Hagermark et al., 1978), complement components (Schulman et al., 1988), muscle relaxants and analgesics (Moss and Rosow, 1983; Stellato et al., 1991), histamine-releasing factors (Guranowski et al., 1981) and certain eosinophil granule proteins (O'Donnell et al., 1983). The major products of mast cell activation will be reviewed.

1.2.3.1 Histamine

Histamine, perhaps the most extensively studied preformed mediator of mast cells, is stored in the secretory granules after passive uptake of histidine and conversion to histamine by histidine decarboxylase (Bauza and Lagunoff, 1981). It is associated with carboxyl groups or proteins by ionic linkage. After degranulation, histamine rapidly dissociates from the proteoglycan-protein complex. It is metabolized within minutes of release. Histamine was the first mediator implicated in the pathogenesis of asthma. There are three types of histamine receptor which mediate multiple effects on the airways (Barnes, 1991). Through H_1-receptor, histamine can induce bronchoconstriction, activation of sensory reflexes, and microvascular leakage. The H_2-receptor can

mediate mucus secretion and vasodilatation and the H_3-receptor can modulate inhibition of cholinergic neurotransmission (Ichinose and Barnes, 1989; Liu et al., 1990). In addition, recent data have suggested that histamine may be involved in the control of late inflammatory reactions associated with allergic disorders through the production of IL-8 and IL-6 by endothelial cells (Delneste et al., 1994; Jeannin et al., 1994). Histamine concentrations in BAL fluid from asthmatics are 3-fold to more than 10-fold higher than those in normal subjects (Broide et al., 1991; Crimi et al., 1991; Zehr et al., 1989). After allergen challenge, levels of histamine may be increased within minutes, and elevations of more than 100-fold have been measured after segmental challenge (Liu et al., 1991; Wenzel et al., 1988).

1.2.3.2 Proteoglycans

Proteoglycans are composed of glycosaminoglycan side chains and a single-chain protein core. The presence of heparin and chondroitin sulfate E has been noted in human mast cells (Stevens et al., 1988; Thompson et al., 1988). Tryptase can be stabilised as an enzymatically active tetramer by association with heparin and in its absence, it dissociates rapidly into inactive monomers (Schwartz and Bradford, 1986). The biological functions of mast cell proteoglycans may be related to the binding of histamine, neutral proteases and acid hydrolase, and they may be important in regulating the activity and stability of endogenous enzymes.

1.2.3.3 Neutral proteases

Three major proteases, tryptase (Schwartz et al., 1981; Smith et al., 1984), chymase (Schechter et al., 1983; Wintroub et al., 1986), and carboxypeptidase (Goldstein et al., 1989; Goldstein et al., 1987), have been purified from human mast cells to date. These enzymes represent the dominant protein components of secretory granules in mast cells and perform optimally near neutral pH. Tryptase is the most important marker of mast cell activation. The biochemical

characteristics and potential roles of tryptase will be described in more detail later.

Chymase, a monomer of 30 kDa, is stored fully active in mast cell secretory granules. Its enzymatic activity may be assessed by the cleavage of synthetic *p*-nitroanilide peptide conjugates or of angiotensin I (Wintroub et al., 1984). Unlike tryptase, the stability of chymase is not affected by heparin and enzymatic activity can be inhibited by classical biological inhibitors of serine protease like α_1-antichymotrypsin, α_1-protease inhibitor and α_2-macroglobulin (Sayama et al., 1987; Schechter et al., 1989). Several biological activities of chymase have been observed in vitro. Chymase converts angiotensin I to angiotensin II approximately 4-fold more efficiently than angiotensin-converting enzyme (Reilly et al., 1982; Urata et al., 1990; Wintroub et al., 1984). Chymase can act as a potent stimulus of mucus production from glandular cells (Sommerhoff et al., 1989).

Carboxypeptidase has been less extensively studied than tryptase and chymase. Its molecular weight is about 34.7 kDa. It appears to be present selectively in the MC_{TC} subpopulation (Irani et al., 1991). Carboxypeptidase in human mast cells does not cleave des-Arg bradykinin or substance P, unlike bovine pancreatic carboxypeptidase, but it does cleave Leu-enkephalin, neurotensin, and kinetensin (Goldstein et al., 1991). Carboxypeptidase is released from mast cells in the same complex with proteoglycans as that to which chymase is bound, and it is likely to act in concert with the other mast cell proteases (Goldstein et al., 1992).

1.2.3.4 Lipid mediators

Lipid mediators, including leukotriene B_4 (LTB_4), leukotriene C_4 (LTC_4) and prostaglandin D_2 (PGD_2), are released from mast cells on mast cell activation. Metabolism of arachidonic acid proceeds either through the cyclooxygenase pathway to prostaglandins and thromboxanes, or through the lipoxygenase pathway to leukotrienes. Human mast cells generate PGD_2 after activation through the IgE receptor or stimulation with calcium ionophore (Levi-Schaffer

and Shalit, 1989). Purified mast cells derived from human lung tissue produce LTC_4 in excess of LTB_4 and also generate substantial amounts of PGD_2 (MacGlashan et al., 1982; Peters et al., 1984). LTC_4 is a potent bronchoconstrictor and can increase microvascular permeability, whereas the major biological effects of LTB_4 are on leukocyte adhesion and migration (Dahlen et al., 1981; Dahlen et al., 1980; Drazen et al., 1980; Ford-Hutchinson et al., 1980). The bronchoconstrictor response of asthmatic airways has been observed to be particularly sensitive to the inhalation of PGD_2 (Hardy et al., 1984).

1.2.3.5 Cytokines

Mast cells are a source of numerous cytokines. By activating the IgE receptor, expression of mRNA for a variety of cytokines has been detected in rodent mast cells, including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IFN-γ, and TGF-β (Burd et al., 1989; Plaut et al., 1989; Seder et al., 1991). Mast cells also represent a potential source of TNF-α, for which levels of mRNA can be upregulated after antigen challenge in mice within 60-90 min (Gordon and Galli, 1991). Moreover, mast cell-derived TNF-α and TGF-β have been suggested to contribute to the increased deposition of extracellular matrix proteins which is observed in certain chronic allergic disorders, such as asthma (Brewster et al., 1990). The release of TNF-α and other cytokines can influence the recruitment and function of other effector cells, which can then induce further progression of the inflammatory response by providing additional sources of cytokines (Galli and Costa, 1995).

Human mast cells can express IL-4, IL-5, IL-6 (Bradding et al., 1992; Okayama et al., 1995), IL-13 (Burd et al., 1995; Jaffe et al., 1996) and the CD40 ligand (Gauchat et al., 1993), which may contribute to the regulation of IgE and T cell differentiation toward a T helper 2 phenotype. The cytokines released from mast cells are likely to participate in allergic and inflammatory responses and to have a role in host defense.

1.3 Mast Cell Tryptase

The value of tryptase as a marker of mast cell activation has until recently attracted more attention than the mediator actions of this abundant mast cell product. Gomori in 1953, first observed the presence of an enzyme capable of hydrolysing 3-chloroacetoxy-2-naphthoic anilide in mast cells in tissue sections. A few years later, Benditt and Arase (Benditt and Arase, 1959) described an enzyme in the mast cells of numerous species resembling bovine α-chymotrypsin, and it was termed *mast cell chymase* (Lagunoff and Benditt, 1963). Shortly thereafter, Glenner and Cohen (1960) found another enzyme in mast cells of human skin, which cleaved a substrate of trypsin (N_α-benzoyl-D,L-arginine-β-naphthylamide hydrochloride) and this was termed *mast cell tryptase* (Lagunoff and Benditt, 1963). In the early 1980's, tryptase was purified for the first time (Schwartz et al., 1981). Only in the last decade have studies indicated the potential importance of this abundant mast cell protease as a mediator of inflammation, and as a new target for therapeutic intervention in asthma and other inflammatory diseases.

1.3.1 Biochemical Characteristics

Tryptase isolated from human tissues, is a tetrameric serine protease with a molecular weight of 110 to 130 kDa, and subunits of 31 to 38 kDa each with a catalytic site (Schwartz et al., 1981; Smith et al., 1984). Determination of the crystal structure has revealed that the four active sites of the tetramer are directed towards an oval central pore, restricting access for macromolecular substrates and enzyme inhibitors (Pereira et al., 1998). Two tryptase cDNA molecules have been cloned from a human lung mast cell library and sequenced (Miller et al., 1990; Miller et al., 1989), and termed α and β tryptase. Three cloned independently from a human skin mast cell library (Vanderslice et al., 1990), have been named I, II and III. The β-tryptase cDNA encodes a 245-amino acid protein which is 90% identical in sequence to α-tryptase and 98% to 100% identical to tryptase I, II and III.

The application of assays for tryptase with specific antibodies with different sensitivities for α and β tryptase has suggested that α-tryptase may be released constitutively in an inactive form, while β or β-like tryptase may be released on anaphylactic degranulation (Sakai et al., 1996; Schwartz et al., 1995). The α tryptase has a more restricted substrate specificity than β tryptase, on account of variation at a single amino acid in the substrate binding cleft (Huang et al., 1999). High levels of tryptase have been measured in human lung tissue (11 $\mu g/10^6$ mast cells) and skin tissue (35 $\mu g/10^6$ mast cells) (Schwartz et al., 1987). No tryptase has been detected in other cell types except for negligible quantities in peripheral blood basophils (Sedgwick et al., 1991).

Tryptase is highly unstable unless bound to heparin or other proteoglycans (Schwartz and Bradford, 1986). Tryptase rapidly dissociates into inactive monomers in the absence of heparin and this is associated with conformational changes which render the tryptase inactive. The interaction with heparin could be crucial in regulating the proteolytic activity as naturally occurring inhibitors have not been found. Heparin-free tryptase, which can convert to an inactive loose tetrameric intermediate, can be reactivated with heparin. However the conversion to inactive monomers appears to be an irreversible reaction (Addington and Johnson, 1996). In addition, low pH and high salt concentrations can stabilize tryptase in an active form (Addington and Johnson, 1996; Schwartz, 1994; Schwartz and Bradford, 1986). Recent evidence indicates that spontaneous inactivation involves reversible changes which convert the active site to a nonfunctional state (Selwood et al., 1998). The association of activity loss with an intrinsic fluorescence decrease and loss of the major substrate-binding site is consistent with the disruption of a critical ionic bond at the active site. This can be reversed by the addition of dextran sulfate. The affinity for glycosaminoglycans appears to be the primary factor controlling the proteolytic activities of this enzyme.

1.3.2 Substrates and Inhibitors

Tryptase, like pancreatic trypsin, cleaves substrates, preferentially at peptide and ester bonds, on the carboxyl side of basic amino acids at lysine and arginine residues (Tanaka et al., 1983). Tripeptide substrates with basic amino acid residues in the S1 and S2 positions are preferred by tryptase (Cromlish et al., 1987). Unlike trypsin, tryptase seems to cleave relatively few protein substrates. It has been shown that tryptase is capable of degrading certain neuropeptides, including vasoactive intestinal peptide (VIP), peptide histidine methonine (PHM) and calcitonin gene-related peptide (CGRP) (Tam and Caughey, 1990; Walls et al., 1992). Tryptase can rapidly cleave and inactivate fibrinogen as a coagulable substrate for thrombin (Schwartz et al., 1985). It can also activate matrix metalloproteinase III (MMP-3) (Gruber et al., 1989) and urokinase plasminogen activator (Stack and Johnson, 1994), as well as cleaving gelatinase and, to a certain extent, fibronectin (Lohi et al., 1992).

Unusually for a serine protease, no endogenous inhibitor has been found for tryptase in plasma, lung, and urine. Soybean trypsin inhibitor, an inhibitor of trypsin and many other tryptic proteases, fails to inhibit tryptase activity (Alter et al., 1990). Tryptase is, however, inhibited by small molecular weight inhibitors such as leupeptin, diisopropyl fluorophosphate, and phenylmethylsulphonyl fluoride. Divalent cations such as calcium, and benzamidine and its derivatives, can act as competitive inhibitors of tryptase (Alter and Schwartz, 1989). Neutrophil lactoferrin (Elrod et al., 1997), neutrophil myeloperoxidase (Cregar et al., 1999) and antithrombin-III (Alter et al., 1990) can inhibit tryptase activity by antagonising the glycosaminoglycans which stabilise the tetrameric structure of tryptase, thereby accelerating the rate of dissociation into monomers.

1.3.3 Pathophysiological Roles of Tryptase

Tryptase can efficiently degrade VIP, PHM and CGRP (Tam and Caughey, 1990). As VIP and PHM can relax airway smooth muscle, their degradation by tryptase could contribute to the increase in bronchial responsiveness and the decrease in immunoreactive VIP in airway nerves associated with asthma. Relatively few protein substrates have been found for tryptase. This protease can, however, inactivate fibrinogen as a clottable substrate for thrombin and could thus have anticoagulant properties (Schwartz et al., 1985; Thomas et al., 1998). Tryptase is also able to promote matrix degradation by activating latent collagenase (Gruber et al., 1988), which is dependent on the activation of MMP-3 (Gruber et al., 1989). This suggests that tryptase may play a role in catabolism of collagen deposition. Moreover, tryptase has been shown to degrade gelatinase and fibronectin, the components of pericellular matrices and basement membrane, indicating a role for mast cell tryptase in connective tissue degradation.

Certain features of bronchial asthma may be reproduced by the transfer of purified tryptase into animal models. Bronchial hyperresponsiveness is a cardinal feature in the pathogenesis of asthma. Mast cell tryptase can greatly increase the sensitivity and the magnitude of the contractile response of isolated dog bronchial smooth muscle to histamine (Sekizawa et al., 1989). Tryptase can also potentiate contraction in human sensitized bronchi via a calcium-related mechanism, providing a link between mast cell tryptase and human airway hyperresponsiveness in vitro (Johnson et al., 1997). In addition, inhalation of tryptase can induce bronchoconstriction and airway hyperresponsiveness in allergic sheep (Molinari et al., 1996). Treatment with APC 366, a synthetic tryptase inhibitor, has been reported to reduce the peak early bronchoconstriction response as well as the late response to allergen (Clark et al., 1995). In addition, allergen-induced airway hyperresponsiveness, increased albumin concentration and tissue eosinophilia were also inhibited. Administration of AMG-126737, a more potent tryptase inhibitor, into the trachea of sensitised guinea pigs has also been found to inhibit early and late phase bronchoconstriction and the development of airway hyperresponsiveness (Wright et al., 1999).

Increased microvascular leakage has been observed following the injection of tryptase into the skin of guinea pigs (He and Walls, 1997). This was blocked by antihistamines and appeared to be mediated by tryptase-induced mast cell activation. It has been suggested that tryptase-induced vascular permeability can be mediated through the release of bradykinin, primarily through prekallikrein activation, but also through a direct release from kininogens (Imamura et al., 1996). Within 6 h of tryptase injection into guinea pig skin or into the peritoneum of mice, the accumulation of large numbers of neutrophils and eosinophils may be provoked (He et al., 1997a; Molinari et al., 1995). Tryptase may thus act as an important stimulus for granulocyte recruitment in allergic disease.

Tryptase can induce profound alterations in the behaviour of several cell types (Fig. 1-3). In addition to contributing to acute inflammatory reactions, tryptase could have a role in processes of chronic disease. Tryptase can induce proliferation of the fibroblasts (Cairns and Walls, 1997; Hartmann et al., 1992; Rouss et al., 1991) and increase the synthesis and secretion of collagen type I from fibroblasts (Cairns and Walls, 1997). This protease can also stimulate the upregulation of mRNA for collagen and fibroblast chemotaxis (Gruber et al., 1997), implicating tryptase in airway remodeling. Moreover, tryptase can act as a potent growth factor for airway smooth muscle cells (Brown et al., 1995) and epithelial cells (Cairns and Walls, 1996). The release of tryptase into the airways may contribute, at least in part, to hyperplasia of myofibroblasts and smooth muscle cells, and the reepithelisation that is observed in the bronchial tissues of asthmatics. Tryptase has been found to act as a chemoattractant for purified preparations of human blood neutrophils and eosinophils in vitro and to stimulate the release of the eosinophilic cationic protein from eosinophils (Walls et al., 1995). Tryptase could also contribute indirectly to granulocyte recruitment and stimulate the release of IL-8 and upregulate the expression of intercellular adhesion molecule 1 (ICAM-1) on epithelial cells (Cairns and Walls, 1996). There is a need for the actions of tryptase as a stimulus for granulocyte accumulation to be studied in more complex models, and to determine in more detail the processes involved.

1.4 Protease Activated Receptor-2 (PAR-2)

The mechanisms whereby tryptase can alter cell function are unclear. However, the discovery of a series of protease-activated receptors (PARs) may provide clues. These are novel family of G-protein-coupled receptors (GPCRs) which undergo a unique process of activation by proteolytic cleavage of the amino terminus, exposing a tethered ligand. This can then bind and activate the cleaved receptor molecule. To date, four structurally related PARs have been identified and cloned: PAR-1, PAR-3 and PAR-4, which are activated by thrombin, and PAR-2, which may be activated by trypsin and mast cell tryptase (Dery et al., 1998).

1.4.1 Mechanism of PAR-2 Activation

Most GPCRs are activated by a soluble ligand with reversible binding to extracellular and transmembrane domains of their receptors at the cell surface. In contrast, serine proteases activate PARs via a unique mechanism that involves recognition of the receptor by the protease, cleavage of the receptor at a specific enzymatic site within the extracellular amino terminus, and finally exposure of a new amino terminus which binds and activates an extracellular loop (Dery et al., 1998) (Fig. 1-4). GPCR activation is associated with a change in the conformation of the receptors with G protein subunits in the plasma membrane, initiating signal transduction.

Analysis of cleavage of peptides, corresponding to potential proteolytic sites of PAR-2, indicates that tryptase and trypsin can hydrolyse the Arg-Ser bond, followed by exposure of the tethered ligand domain (Molino et al., 1997a). This new N-terminus (NH_2-SLIGKVDGT....), the first 5 or 6 residues of which serves as a tethered ligand, activates the receptor by interacting at a site in the extracellular loop of the receptor (Gerszten et al., 1994; Lerner et al., 1996). Synthetic peptides corresponding to the tethered ligand domains of PAR-1 and PAR-2 (eg. SFLLRN for human PAR-1, SLIGKV for human

PAR-2) are able to act as full agonists on their respective receptors (Nystedt et al., 1994; Vu et al., 1991).

PARs are coupled to phospholipase $C\beta_1$, which hydrolyses phosphatidylinositol 4,5-biphosphate and generates inositol 1,4,5-triphosphate and diacylglycerol. Inositol 1,4,5-triphosphate stimulates the release of calcium from intracellular stores, which induces the influx of extracellular calcium (Hung et al., 1992). Diacylglycerol could activate protein kinase C which mediates desensitization of PAR-2 by receptor phosphorylation and uncoupling from G-proteins or by negative feedback control of signaling (Berridge, 1993; Bohm et al., 1996a; Nystedt et al., 1994). Trypsin, tryptase and activating peptides for PAR-2 can stimulate the generation of inositol triphosphate and the mobilisation of intracellular Ca^{2+} in a variety of cell types (Corvera et al., 1997; Kong et al., 1997; Nystedt et al., 1995; Nystedt et al., 1994; Nystedt et al., 1995). In enterocytes and transfected cells, PAR-2 agonists can stimulate the release of arachidonic acid and generation of PGE_2 and $PGF_{1\alpha}$, suggesting the activation of phospholipase A2 and cyclo-oxygenase (Kong et al., 1997). Agonists for PAR-2 could activate the mitogen-activated protein kinases, extracellular signal-related kinase 1 and 2 and p38 in smooth muscle cells and enterocytes (Belham et al., 1996; Yu et al., 1997). The signalling pathways activated by PARs may be associated with growth and inflammation.

1.4.2 Characteristics of PAR-2

The DNA sequence encoding PAR-2 was first isolated from a mouse genomic library (Nystedt et al., 1994). The predicted protein is similar in structure to the thrombin receptor and has a similar mechanism of activation. This receptor was activated by low concentrations of trypsin and by a peptide (SLIGRL) derived from the receptor sequence, but was not activated by thrombin. Human PAR-2 was subsequently cloned and revealed to have a sequence of 397 amino acids which was 83% identical to the mouse receptor sequence. The human protease-activated receptor 2 gene, by in situ

hybridization, was mapped to chromosomal region 5q13, where the gene for the related PAR-1 has been located (Nystedt et al., 1995).

Little is known of the mechanisms leading to the attenuation of signal transduction by PARs. Through investigation of the GPCRs for hormones and neurotransmitters, signal attenuation have been found to be at the level of the agonists, the receptor, the G proteins, and steps downstream of the cascade. Cleavage of PARs could remove the tethered ligand before it is exposed and render cells unresponsive to protease. Cathepsin G, neutrophil elastase, and proteinase 3 have been reported to cleave PAR-1 to remove the tethered ligand domain and to render cells unresponsive to thrombin (Molino et al., 1997a; Renesto et al., 1997). Trypsin and tryptase at low concentrations have been found to cleave a peptide corresponding to human PAR-2 sequence at a site between Arg^{36} and Ser^{37}, which would expose the tethered ligand domain of receptor and thus activate cellular response. At higher concentrations, both proteases showed the potential to hydrolyse the PAR-2 peptide, including the secondary cleavage at Lys^{41}-Val^{42}, which could thereby disrupt the tethered ligand and limit activation (Molino et al., 1997a).

G protein receptor kinases (GRKs) have been known to mediate agonist-dependent phophorylation of GPCRs and initiate desensitisation, which depends on arrestins. Beta-arrestins could redistribute to the plasma membrane to interact with the GRK-phosphorylated receptor, uncouple the receptor from G-proteins and terminate the signal cascade (Dery et al., 1998). PAR-1 and PAR-2 have several potential sites for GRK phosphorylation in the C terminal tail. Replacement of all the Ser and Thr residues in the C terminal tail with Ala will cause PAR-1 to be insensitive to regulation by GRK-3. Second-messenger kinase, protein kinase C (PKC), could also mediate the desentisitation of GRCPs. Multiple PKC consensus sites haven been found in the C terminal tail and the third intracellular loop of PAR-1 and PAR-2, suggesting that PKC may have a role in the phosphorylation of GRCPs and mediate desensitisation (Ishii et al., 1994; Vouret-Craviari et al., 1995; Vouret-Craviari et al., 1995).

Rapid internalisation of PAR-1 and PAR-2 has been observed after activation by proteolysis or by activating peptides. Trypsin and the peptide agonist can trigger rapid endocytosis of PAR-2 in transfected epithelial cells (Bohm et al., 1996a). Trypsin exposure can result in internalisation of PAR-2 into early endosomes and then lysosomes. Endocytosis and lysosomal degradation of PARs may contribute to desensitisation through depleting receptors from the cell surface and degrading activated receptors in lysosomes. Resensitisation of cells to proteases on the other hand requires the synthesis of new receptors and/or the mobilisation of intracellular pools of receptors. It has been reported that resensitisation of PAR-2 transfected epithelial cells and enterocytes following trypsin-induced desensitisation may be inhibited markedly by disrupting the Golgi store of PAR-2. However, this was little affected by inhibiting new protein synthesis, suggesting that resensitization required mobilisation of pools of the receptor (Bohm et al., 1996a). .

1.4.3 Pathophysiological Roles of PAR-2

PAR-2 mRNA has been detected in cells of the kidney, stomach, pancreas, liver, colon, and small intestine by Northern blot analysis (Nystedt et al., 1995; Nystedt et al., 1994; Nystedt et al., 1995). Immunocytochemically PAR-2 has been localised to human keratinocytes (Santulli et al., 1995), vascular endothelial cells (Mirza et al., 1996), epithelial cells of the gastrointestinal tract (Bohm et al., 1996), gastric smooth muscle cells (Hollenberg et al., 1996), pancreatic acinar cells, lung alveolar cells (Bohm et al., 1996) and hippocampal neurons (Smith-Swintosky et al., 1997). The presence of PAR-2 in bronchial epithelial cells and airway smooth muscle cells (D'Andrea et al., 1998) calls attention to the roles of this receptor in the pathogenesis of airway diseases.

Little is known of the pathophysiological role of PAR-2. However, it has been reported that trypsin and agonists of PAR-2 can induce the relaxation of rings of rat aorta tissues through nitric oxide production by endothelium (al-

Ani et al., 1995; Saifeddine et al., 1996). Intravenous injection of SLIGRL, the activating peptide of mouse PAR-2, has been found to produce a marked fall in blood pressure in the rat, which was also consistent with release of nitric oxide from endothelial cells (Hwa et al., 1996). However, the role of PAR-2 in human blood vessels is less certain. The peptide agonists for PAR-2, SLIGKV and SLIGRL, appeared to have no vasodilator effect in human large and small coronary artery and pulmonary arteries (Hamilton et al., 1998).

PAR-2, like PAR-1, may have several proinflammatory actions. Trypsin and thrombin activate PAR-2 and PAR-1 respectively on keratinocytes, to induce the synthesis of GM-CSF (Wakita et al., 1997). Expression of mRNA for PAR-2 in human endothelial cells may be upregulated by TNF-α, IL-1α as well as bacterial lipopolysaccharide in a dose-dependent manner (Nystedt et al., 1996). The induction of PAR-2 by inflammatory mediators supports the concept that PAR-2 may be involved in inflammatory processes. Intraplantar administration of PAR-2 agonists and trypsin can elicit an increase in vascular permeability in rat hindpaws (Kawabata et al., 1998). This reaction was abolished by pretreatment with compound 48/80 to deplete bioactive amines in mast cells, suggesting that the activation of PAR-2 may induce acute inflammation via mast cell deganulation. Moreover, intraperitoneal injection of agonists of PAR-2 can stimulate leukocyte accumulation in the peritoneal cavity of rat and the effect can be completely abolished by platelet-activating receptor antagonists. This suggests that PAR-2 activation may have a role in several early events in the inflammatory reaction, including leukocyte rolling, adherence, and recruitment through a mechanism which could involve the release of platelet-activating factor. (Vergnolle, 1999). A novel process of protease-induced neurogenic inflammation has recently been suggested by the finding that trypsin and tryptase can stimulate neurones to release the neuropeptides, calcitonin gene-related peptide and substance P, through the activation of PAR-2 (Steinhoff et al., 2000).

Apart from its proinflammatory roles, PAR-2 has been reported to have a bronchoprotective effect through paracrine actions. PAR-2 activation can

result in a marked and prolonged inhibition of bronchoconstriction in rats associated with the release of prostaglandin E_2 (Cocks et al., 1999; Lan et al., 2000). In contrast, administration of SLIGRL to guinea pigs has been reported to cause bronchoconstriction (Ricciardolo et al., 2000). This was inhibited by a combination of tachykinin NK_1 and NK_2 receptor antagonists and was potentiated by inhibition of nitric oxide synthase, suggesting a neural mechanism may be involved. In allergic sheep airways, inhalation of PAR-2 agonists has been found to induce bronchoconstriction and this response was blocked by treatment with histamine antagonists, suggesting a role for PAR-2 activation in mast cell degranulation (Abraham et al., 2000). There is a pressing need for further studies investigating PAR-2 activation in cells of the human airways.

PAR-2 can modulate cell growth and the proliferation of certain cell types. Agonists of PAR-2 can stimulate the proliferation of human endothelial cells (Mirza et al., 1996) and vascular smooth muscle cells (Bono et al., 1997). While PAR-1 agonists stimulate the growth of keratinocytes, PAR-2 agonists can inhibit their cell growth (Derian et al., 1997). In addition, PAR-2 agonists can inhibit DNA synthesis in human pancreatic cancer cells, though the mechanism is not clear (Kaufmann et al., 1998). Peptide agonists of PAR-2 and also tryptase can induce the proliferation of lung, but not of dermal, fibroblasts, implicating PAR-2 in the fibroproliferative response (Akers et al., 2000).

In the intestine and pancreas, trypsin probably represents the major endogenous protease for PAR-2. PAR-2 has been localised to the apical and basolateral membranes in enterocytes (Kong et al., 1997) The application of PAR-2 agonists to the apical membrane of enterocytes in culture and the mucosal surface of intact tissue can stimulate prostanoid secretion, providing functional evidence for the expression of PAR-2 at the apical membrane. Trypsin from the intestinal lumen is unlikely to cleave PAR-2 at basolateral locations, and at these sites another protease could activate these receptors. Tryptase is one candidate.

Tryptase as well as peptide agonists can stimulate mobilisation of intracellular Ca^{2+} in neuronal cells (Steinhoff et al., 2000), colonic myocytes (Covera et al., 1997), and endothelial cells (Molino et al., 1997a). No antagonist of PAR-2 is available, but it seems likely that some of the actions of tryptase may be mediated through PAR-2. The extent to which tryptase may activate PAR-2 and alter epithelial function deserves investigation.

1.5 The Airway Epithelium and Asthma

The airway epithelium has long been recognized as a barrier which can protect underlying tissues from external stimuli. Increasingly there is evidence indicating that the epithelium can contribute in an important way to airway function. It can regulate fluid, ion and mediator transport across the interface between lumen and interstitial tissues, modulate smooth muscle tone, induce the influx and aggregation of inflammatory cells during inflammation and injury, and secrete a variety of mediators. Epithelial cells can thus act as central modulators of the inflammatory response. The modulatory role comprises both target and effector type responses (Martin et al., 1997; Polito and Proud, 1998). Epithelial cells influenced by external stimuli can alter mucus secretion, ciliary clearance, and ion transport. As effector cells, epithelial cells can respond to external and internal stimuli by generating a broad range of biologically active mediators. Recently, it has been proposed that the airway epithelium is fundamentally disordered in chronic asthma, as is manifested by increased fragility. Moreover, epithelial cells may be of an altered phenotype and secrete to a much greater degree mucus, cytokines, growth factors and various mediators with the potential to promote inflammation, fibroblast and smooth cell proliferation, and matrix deposition (Holgate et al., 1999). There is a need for greater understanding of the potential role of the epithelium in asthma, and for the effects of mediators from epithelial cells, mast cells and other cells to be defined.

1.5.1 Structure of the Airway Epithelium

The normal lining of the central airways is a pseudostratified epithelium, consisting of upper ciliated columnar epithelial cells and lower basal cells, attached to a basement membrane. The epithelium is progressively converted to nonstratified, simple, ciliated and secretory cells in terminal bronchioles. Terminal processes of sensory fibers and numerous mucous glands traverse the epithelial layer throughout the airways. Several types of secretory cells are present within epithelial layers. Goblet cells which produce airway mucus are abundant in the central airways, but less so in the more peripheral airways (Lumsden et al., 1984; Mercer et al., 1994). Clara cells which provide secretory material for the airway lining are found in the bronchioles (Widdicombe and Pack, 1982). With the help of motile cilia on epithelial cells, airway secretion can be transported toward the larynx. The epithelial integrity and cell-cell binding are maintained by several structures. Tight junctions located in the apical border of the epithelial layer maintain the integrity and permeability of the barrier (Godfrey et al., 1992; Walker et al., 1984). Intermediate junctions, which contain the adhesion molecule E-cadherin, bind suprabasal cells below the level of tight junctions and desmosomes bind columnar cells to basal cells (Montefort et al., 1993). Basal cells are attached to the basement membrane by hemidesmosomes, which contain integrins (Stepp et al., 1990).

1.5.2 Mast Cell-Epithelial Cell Interactions

Mast cells are capable of regulating both epithelial transport and barrier functions. Following mast cell activation, acute stimulation of epithelial chloride secretion with accompanying fluid movement occurs, which is mediated, at least in part, by direct effects of histamine, adenosine and eicosanoid metabolites on secretory epithelial cells (McKay and Perdue, 1993). Mast cell activation is also capable of altering epithelial barrier function, an effect which might be associated with release of proteases.

Perfusion of the rat mesenteric artery and jejunal lumen with mast cell protease-II, can develope macromolecular leakage without epithelial damage. This suggests that mast cell granule chymase may increase epithelial permeability via the paracellular route. Histological evaluation of the mucosa did not reveal any significant morphological change in any of the experiments (Scudamore et al., 1995; Scudamore et al., 1995). This is in contrast to the cytotoxic effect provoked by eosinophil granules. The extent to which human mast cell tryptase may alter epithelial permeability has not been investigated.

1.5.3 Epithelial Injury in Asthma

Epithelial damage is a common feature in the central airways of asthmatics (Beasley et al., 1989; Jeffery et al., 1989; Laitinen et al., 1985). Various provocants of asthma attacks, including allergen (Behrens et al., 1987), virus (Empey et al., 1976), bacterial infection (Michel et al., 1992), toluene diisocyanate (Gordon et al., 1985), cigarette smoke (Gerrard et al., 1980), and ozone (Golden et al., 1978) have all been implicated in the induction of epithelial damage and airway inflammation. Typically, the ciliated columnar epithelium is shed, resulting in exposure of basal cells, which remain attached to the basement membrane. Clusters of shed epithelial cells have been found in asthmatic airways, and these form Creola bodies and Curschmann's spirals (Naylor, 1962). Thickening of the basement membrane, deposition of collagen beneath the basement membrane and increased fibroblast activity have been observed in asthmatics (Beasley et al., 1989). Epithelial damage and desquamation are followed by re-epithelisation with simple stratified nonciliated epithelium or goblet cells (Keenan et al., 1982). The damaged area shows an increased mitotic figure and metaplasia, suggesting an undergoing process of regeneration (Lane and Gordon, 1974).

The cause of epithelial damage in asthma is still not clear. The eosinophil-derived proteins, major basic protein (MBP) and eosinophil cationic protein (ECP), have been implicated as major stimuli of epithelial cell exfoliation.

Both proteins are toxic to epithelial cells in vitro, resulting in bleb formation and detachment (Frigas et al., 1980; Motojima et al., 1989; Yukawa et al., 1990). Epithelial cell loss in asthmatics can lead to exposure of nerve endings to irritant factors, enhanced penetration of allergen particles to the inflammatory cells, decreased production of epithelial-derived relaxing factor, loss of the neutral endopeptidase which normally degrades substance P and similar peptides, and reduced capacity of mucociliary clearance. Another consequence of damage to the airway epithelium will be increased access of bronchoconstrictor agonists to airway smooth muscle, thereby leading to airway hyperresponsiveness (Hulsmann and De Jongste, 1996a). All these factors may contribute to the severity of airway obstruction and bronchial hyperresponsiveness in asthmatics (Joos et al., 1994).

1.5.4 Mediators Produced by Epithelial Cells

The airway epithelium can respond to injury, infection, or inflammation by producing various cytokines and mediators to modulate airway inflammation. These include PGE_2, $PGF_{2\alpha}$, TNF-α, IL-1, IL-6, IL-8, IL-10, IL11, RANTES, GM-CSF, G-CSF, MCP-1, endothelin, fibronectin, nitric oxide, oxygen radicals and so on (Knobil and Jacoby, 1998). It is believed that in airway diseases, activation or dysfunction of epithelial cells themselves will result in attraction, maintenance and activation of various inflammatory cells in the epithelium. Environmental stimuli like air pollutants, bacteria and ozone directly affect epithelial cells to increase the generation of GM-CSF, IL-8 or TNF-α (Devalia et al., 1993; Khair et al., 1994), which can activate specific inflammatory cells such as macrophages, eosinophils and neutrophils. Epithelial cells may thus have a role in stimulating the migration of several different inflammatory cell types into the airway lumen.

The epithelium has been reported to produce mediators of airway smooth muscle relaxation, prominent among which is PGE_2. This eicosanoid inhibits the release of acetylcholine from airway parasympathetic nerves, which may

direct the airway smooth muscle contraction (Walters et al., 1984). Airway epithelial cells can also produce agents of bronchoconstriction such as endothelin (Black et al., 1989; MacCumber et al., 1989) and $PGF_{2\alpha}$ (Churchill et al., 1989; Widdicombe et al., 1989). Those epithelial cell derived cytokines which were investigated in the present studies are reviewed below.

1.5.4.1 IL-8

IL-8, also known as neutrophil-activating peptide, is a chemoattractant protein for neutrophils. It is a member of the the CXC chemokine family, which has the first two N-terminal cysteine (C) residues separated by one nonconserved residue (X). This proinflammatory mediator is secreted by a variety of cell types other than epithelial cells, including monocytes, neutrophils, endothelial cells, fibroblasts and mitogen-stimulated lymphocytes (Alam, 1997). IL-8 is considered to be the most potent chemotatic factor for neutrophils in the lung. Antibodies against IL-8 can block neutrophilic inflammation in various models (Alam, 1997; Kunkel et al., 1991). In addition to stimulating neutrophil chemotaxis, there is evidence that IL-8 can function as a chemotactic factor for cytokine-primed eosinophils (Shute, 1994; Erger and Casale, 1995). IL-8 levels have been found to increase in patients with severe asthma (Shute et al., 1997), cystic fibrosis (Koller et al., 1997) and in most patients with sepsis (Hack et al., 1992) and to correlate with some important clinical, biochemical, and inflammatory parameters.

1.5.4.2 IL-6

Il-6 is produced by various cell types in addition to epithelial cells, including T- and B-cells, monocytes, fibroblasts, kerainocytes, endothelial cells, mesangial cells, and several tumour cells. IL-6 can regulate the growth and differentiation of various cells with major activities on the immune system, haematopoiesis, and inflammation (Kelley, 1990; Kishimoto, 1989). IL-6 induces final maturation of B-cell into antibody producing cells and is a potent growth factor for myeloma and plasmacytoma cells. It stimulates T-cell growth and cytotoxic T-cell differentiaion, promotes megakaryocyte

development and synergises with other cytokines to stimulate multipotent hematopoietic progenitors (Ishibashi et al., 1989), and can provoke acute phase reactions in response to inflammation (Heinrich et al., 1990) or tissue injury (Hack et al., 1992). These multiple actions are integrated within a complex cytokine network, where several cytokines induce or are induced by IL-6 and the final effects result from either synergistic or antagonistic activities between IL-6 and other cytokines such as IL-1, IL-2, IL-3, IL-4, IL5, GM-CSF and IFN-γ.

1.5.4.3 GM-CSF

Human granulocyte-macrophage colony stimulating factor (GM-CSF) is produced by macrophages, endothelial cells, and activated T cells as well as epithelial cells. GM-CSF can enhance the expression of adhesion molecules on epithelial cells and endothelial cells, prime granulocytes to release substantial amounts of mediators on stimulation, and prolong survival of inflammatory cells (Lopez et al., 1986; Soloperto et al., 1991), thereby promoting the infiltration with inflammatory cells and their local activation. Human tracheal epithelial cells constitutively release GM-CSF, which is inhibited by glucocorticoid (Churchill et al., 1992). Increased levels of GM-CSF have been found in the asthmatic airway (Mattoli et al., 1991; Sousa et al., 1993), which can prolong the survival of eosinophils and promote the release of mediators from those cells (Soloperto et al., 1991). Recombinant GM-CSF is used clinically to accelerate haematopoietic recovery following chemotherapy or radiotherapy and bone marrow transplantation (Costello, 1993).

1.5.5 Adhesion Molecules Expressed by Epithelial Cells

The airway epithelium can express adhesion molecules that bind inflammatory cells migrating from capillaries. Such expression may influence the nature of the inflammatory process in the airways. Intercellular adhesion molecule 1 (ICAM-1), a member of the immunoglobulin (Ig) superfamily, is

expressed constitutively on the human airway epithelium and its expression can be induced by cytokines such as INF-γ and TNF-α (Bloemen et al., 1993; Look et al., 1992; Tosi et al., 1992), by infection with parainfluenza virus (Tosi et al., 1992), and to a small extent by human mast cell tryptase (Cairns and Walls, 1996). Upregulation of ICAM-1 is associated with increased influx and binding of both neutrophils (Tosi et al., 1992) and eosinophils (Wegner et al., 1990). Increased ICAM-1 expression has been shown in epithelial cells obtained from asthmatics, but not from normal subjects or subjects with chronic bronchitis (Vignola et al., 1993). In a primate model of asthma, a monoclonal antibody to ICAM-1 as been found to attenuate airway eosinophilia and hyperresponsiveness (Wegner et al., 1990). Blocking the actions of this adhesion receptor may attenuate neutrophil adhesion to the endothelium and inhibit neutrophil transendothelial migration (Barton et al., 1989; Smith et al., 1988).

1.6 Permeability of the Airway Epithelium

The airway epithelium forms the interface between the external environment and airway tissues and is a site for deposition of potentially noxious molecules on inhalation. It consists of ciliated and non-ciliated cells tightly attached to each other and anchored to the basement membrane. The permeability of the airway will determine the extent to which foreign molecules will penetrate into underlying tissues, as well as the flux of water and solutes into the airways.

1.6.1 The Epithelium as a Diffusion Barrier

At the apico-lateral borders of epithelial cells are narrow belt-like stuctures known as tight junctions. Tight junctions are considered to have a major role in regulating the diffusion by the paracellular pathway of ions, water, hydrophilic molecules, and inflammatory cells. The transepithelial movement

of solutes can occur through one of two parallel pathways. They can cross the paracellular tight junctions from the airway lumen, diffuse along the intercellular space and traverse the basement membrane, connective tissue and endothelial cells of capillaries to enter the bloodstream or lymphatics. Alternatively, they can be transported across the apical membrane of the epithelium, and once they have entered the cell, they can be transported across the basolateral membrane where it traverses the lateral intercellular space, basement membrane, connective tissue, and capillary endothelial cells (Lewis et al., 1995). The ultrastructure and morphology of human airway tight junctions have been investigated by freeze electron microscopy and tight junctions have been characterised by measuring the strand number, junctional depth, and indexes of junctional complexity (Carson et al., 1990; Elia et al., 1988; Godfrey et al., 1992). Considerable variation exists in the morphological features of individual tight junctions.

1.6.1.1 Molecular Structure of Tight Junctions

The tight junction creates a regulated barrier in the paracellular pathway and forms a functional unit called the apical junction complex. The tight junction complex contains several protein components. There are three known transmembrane proteins of the tight junctions, occludin, claudins, and junctional adhesion molecule. Extending into the paracellular space, they are canditates for creating the seals and channels. Intracytoplasmic proteins include the membrane-associated guanylate kinase (MAGUK) proteins ZO-1 and ZO-2 (zonula occludens 1 and 2) (Beatch et al., 1996; Gumbiner et al., 1991; Stevenson et al., 1986), which couple occludin to cytoplasmic plaque. Other proteins including ZO-3, occludin 1B, cingulin, 7H6, symplekin, and ZA-1TJ, which may be signaling proteins, have been localized to the tight junction.

Occludin, a protein of approximately 65 kDa (composed of four transmembrane domains, two extracellular loops and a large C-terminal cytosolic domain) is present in the tight junctions of both epithelial and endothelial cells (Furuse et al., 1993). Occludin has been found to localise at

the tight junction fibrils by immuno-freeze fracture electron microscopy. When occludin expression is upregulated in cultured monolayers of MDCK cells by transfection, an increase in the number of fibrils and an elevation of transepithelial resistance has been observed (Fujimoto, 1995). Induction of chicken occludin in MDCK cells can cause an increase in transepithelial resistance (TER) and an increase in the number of tight junction strands (McCarthy et al., 1996). Cells treated with a peptide which binds to occludin extracellular loop 2, but not loop 1, have reduced TER and increased paracellular flux of molecules up to 40 kDa (Wong and Gumbiner, 1997). Moreover, when transfected to fibroblasts, which lack endogenous occludin, it confers adhesiveness in proportion to the level of occludin expressed and the adhesiveness is blocked by peptides corresponding to either of the two extracellular loops (Van Itallie and Anderson, 1997). The presence of occludin in tight junction is required to maintain TER and the size-selective barrier. However, embryonic stem cells, being deficient in occludin by gene depletion through homologus recombination, have been reported to establish tight junctions, which contain fibrils and form a paracellular barrier to low molecular weight tracers (Saitou et al., 1998). This suggests that there may be a second barrier protein.

Claudin-1 and claudin-2, which copurify with occludin, are integral membrane proteins of approximately 23-kDa which possess four transmembrane domains without sequence similarity to occludin (Furuse et al., 1998). When introduced into cultured epithelial cells, claudin-1 and claudin-2 are cable of assembling into long branch fibrils in the tight junctions of these cells. These claudin-induced strands are long and have a morphology that resembles endogenous tight junctions very closely, suggesting that claudin-1 and claudin-2 may function as major structural components of TJ strands. To date, 15 members of claudin family have been cloned and designated as claudin-1 to claudin-15 (Tsukita and Furuse, 1999).

A novel member of the Ig superfamily, localised within the tight junctions of both epithelial cells and endothelial cells, has been identified recently and termed junctional adhesion molecule (JAM) (Martin-Padura et al., 1998). It

contains two extracellular Ig domains with a molecular weight of approximately 32 kDa. When introduced into COS cells, which normally lack tight junctions and the expression of this protein, JAM accumulates at sites of cell-cell contact and induces cell-cell adhesion. There is evidence that JAM can mediate adhesion and influence monocyte transmigration. Spontaneous and chemokine-induced transmigration of monocytes can be abrogated by employing a monoclonal antibody against JAM, suggesting that JAM might function in transmigration across epithelial monolayers by providing an direct contact required for monocytes to enter into the paracellular pathway (Martin-Padura et al., 1998). JAM is the first molecular component of TJs that has been reported to modulate transmigration of immune cells across the paracellular seals.

Of several cytoplasmic proteins associated with the tight junction, ZO-1 and ZO-2 are the best characterised. ZO-1 was found by using specific monoclonal antibodies raised to a mouse liver tight junctions (Stevenson et al., 1986). ZO-1 and ZO-2 interact with each other (Gumbiner et al., 1991) and ZO-1 binds to the C-terminal tail of occludin (Furuse et al., 1994). ZO-1 and ZO-2 belong to the membrane-associated guanylate kinase (lMAGUK) family (Anderson et al., 1995), which are often found at the sites of cell-cell contact and may function to couple the extracellular signalling pathway with the cytoskeleton. It is likely that the tight junction associated protein ZO-1 may act as an organizational scaffold, whereas lateral occludin-occludin interactions may stabilize and promote assembly of occludin into a lineal fibril (Mitic and Anderson, 1998). Thus, ZO-1 may anchor occludin at the membrane and instruct its polymerization. It has been demonstrated that both occludin and ZO-2 interact with specific domains within the N-terminal half of ZO-1, whereas the C-terminal half co-sediments with F-actin, suggesting that one function of ZO-1 may be to organize components of the tight junction and link them to the cortical actin cytoskeleton (Fanning et al., 1998).

1.6.1.2 Mechanism of Tight Junction Assembly

Molecules involved in various intracellular signaling pathways have been reported to affect permeability, including tyrosine kinases, Ca^{2+}, protein kinase C (PKC), heterotrimeric G proteins, calmodulin, cAMP, lipid second messengers, and phospholipase C (Balda et al., 1991; Madara et al., 1992). The effects often correlate with changes in actin organization. Reestablishing the architecture of the actin cytoskeleton appears to be critical for the biogenesis of tight junctions. Drugs that perturb the actin cytoskeleton are likely to disrupt the tight junction through effects on actin originating at the perijunctional ring that projects onto the cytoplasmic surface of the tight junction (Hirokawa and Tilney, 1982). Intracellular Ca^{2+} is also essential for tight junction biogenesis. Selective depletion of Ca^{2+} stores prior to initiation of cell-cell contact disrupts the biogenesis of tight junctions (Stuart et al., 1996). Investigations with activators and inhibitors, have shown that TJ formation may be controlled by a network of reactions where G-proteins, phospholipase C, adenylate cyclase, protein kinase C and calmodulin are involved (Balda et al., 1991). Tight junction assembly is illustrated diagramatically (Fig. 1-2).

1.6.1.3 Assessment of Epithelial Integrity

It is widely accepted that transepithelial electrical resistance (TER), generated across an epithelium, is determined by the ion transport properties of its component cells and the selective and restrictive barrier contributed by the tight junctions (Claude, 1978). In vitro, TER can be measured directly in cell culture inserts with specially designed electrodes. The functional integrity of tight junctions can also be evaluated using radiolabeled hydrophilic molecules (eg. ^{14}C bovine serum albumin, ^{14}C mannitol) which can diffuse passively across epithelial monolayers (Godfrey, 1997). Since most hydrophilic molecules do not penetrate the cell membrane, their transport across the epithelium is largely limited to the paracellular pathway. The integrity of tight junctions and changes in barrier properties can be visualised directly using confocal laser scanning microscopy and fluorescent markers.

1.6.2 Alterations in Airway Epithelial Permeability

The airway epithelium normally acts as a tight barrier to protect underlying tissues. Damage to the epithelium, as seen in asthma, may breach this barrier and permit entry of noxious substance into the mucosa and connective tissue, thus worsening the inflammatory state. Epithelial permeability can be modulated by trypsin, cytokines, eosinophil-derived and neutrophil-derived proteins, and bacterial toxins (Lewis et al., 1995). Luminal exposure of isolated human airways to oxidants (H_2O_2) or cationic proteins (poly-L-arginine) can increase both airway responsiveness and permeability (Hulsmann et al., 1996). Rhinovirus infection can impair the functional integrity and increase permeability in the cultured human tracheal epithelium (Ohrui et al., 1998). The aeroallergen Der p1 is able to increase airway permeability and augment transmucosal movement of macromolecules (Herbert et al., 1990; Herbert et al., 1995).

It has been reported that the rate of transepithelial clearance of ^{99m}Tc-DTPA is significantly higher in asthmatics than that in normal subjects, suggesting that the permeability of asthmatic airways may be increased (Ilowite et al., 1989). Bronchial biopsies from asthmatic patients have been revealed by electron microscopy to have opened tight junctions and the widened intercellular spaces in the airway epithelium under electron microscopy (Ohashi et al., 1992).

Mediators released on mast cell activation may affect the permeability of the airway epithelium. Histamine has been reported to increase airway epithelial permeability in vivo both in animal and human studies (Boucher et al., 1978; Braude et al., 1984; Chan et al., 1987). However, it has been reported that histamine has no direct effect on the permeability of epithelial monolayers in vitro (Devalia et al., 1994). Histamine-induced alterations in permeability may thus be attributed to the increase in submucosal hydrostatic pressure, which causes the opening of the tight junctions. TNF-α, another product of mast cells, has been shown to affect the tight junctions in kidney epithelial cells by

lowering the transepithelial resistance and increasing the flow of solute across the monolayers (Mullin and Snock, 1990). The potential of tryptase to alter epithelial permeability remains unclear.

1.7 Aims

The aims of these studies have been to investigate the abilities of human mast cell tryptase and agonists of PAR-2 to interact with airway epithelial cells to:

I) induce mitogenesis,

II) stimulate cytokine release,

III) increase the permeability of monolayers,

IV) recruit neutrophils.

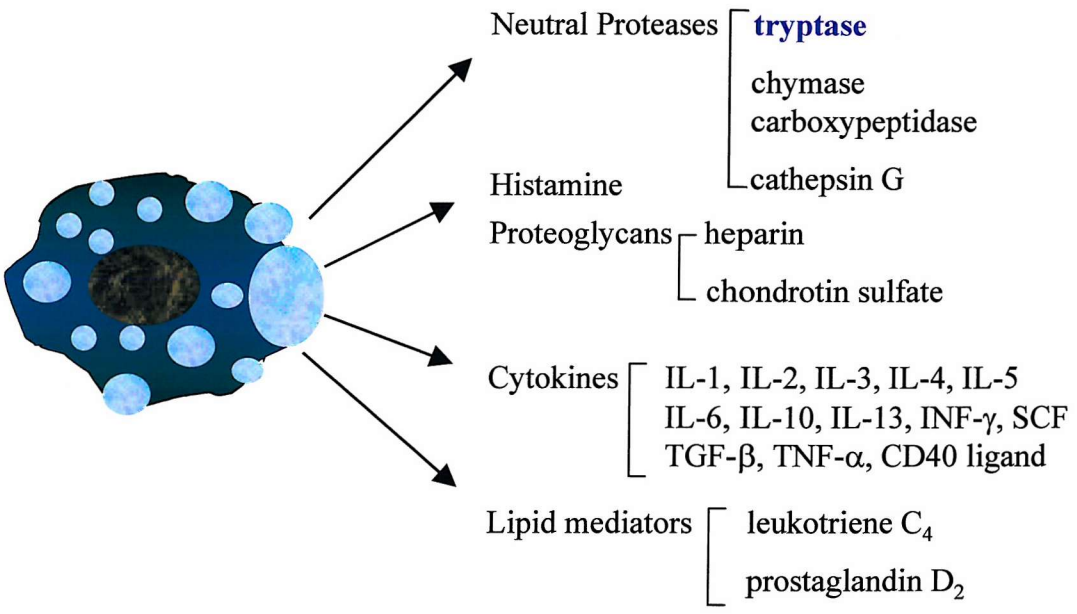


Figure 1-1 Mediators released by mast cells.

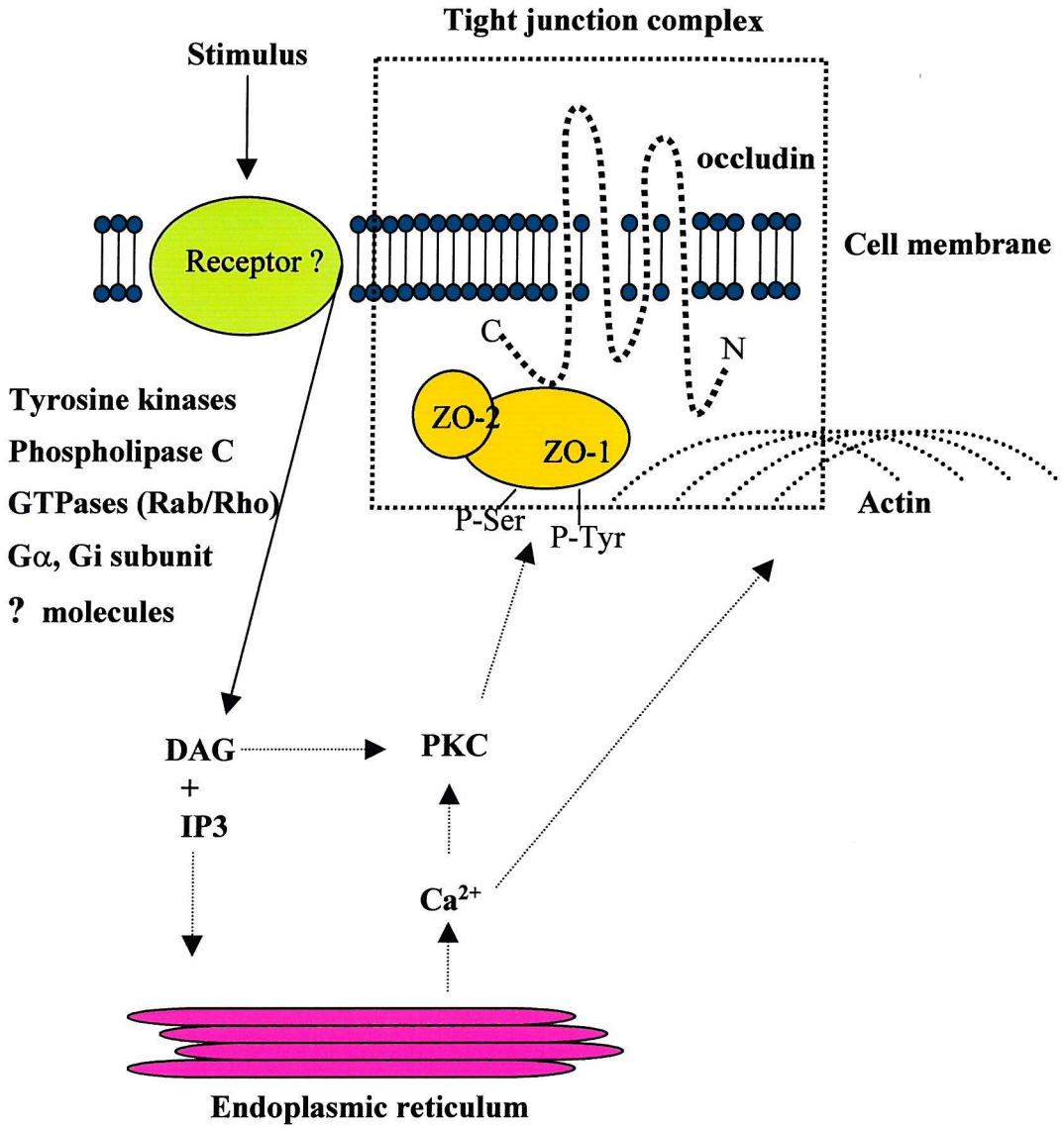


Figure 1-2 Tight junction assembly and intracellular pathways.

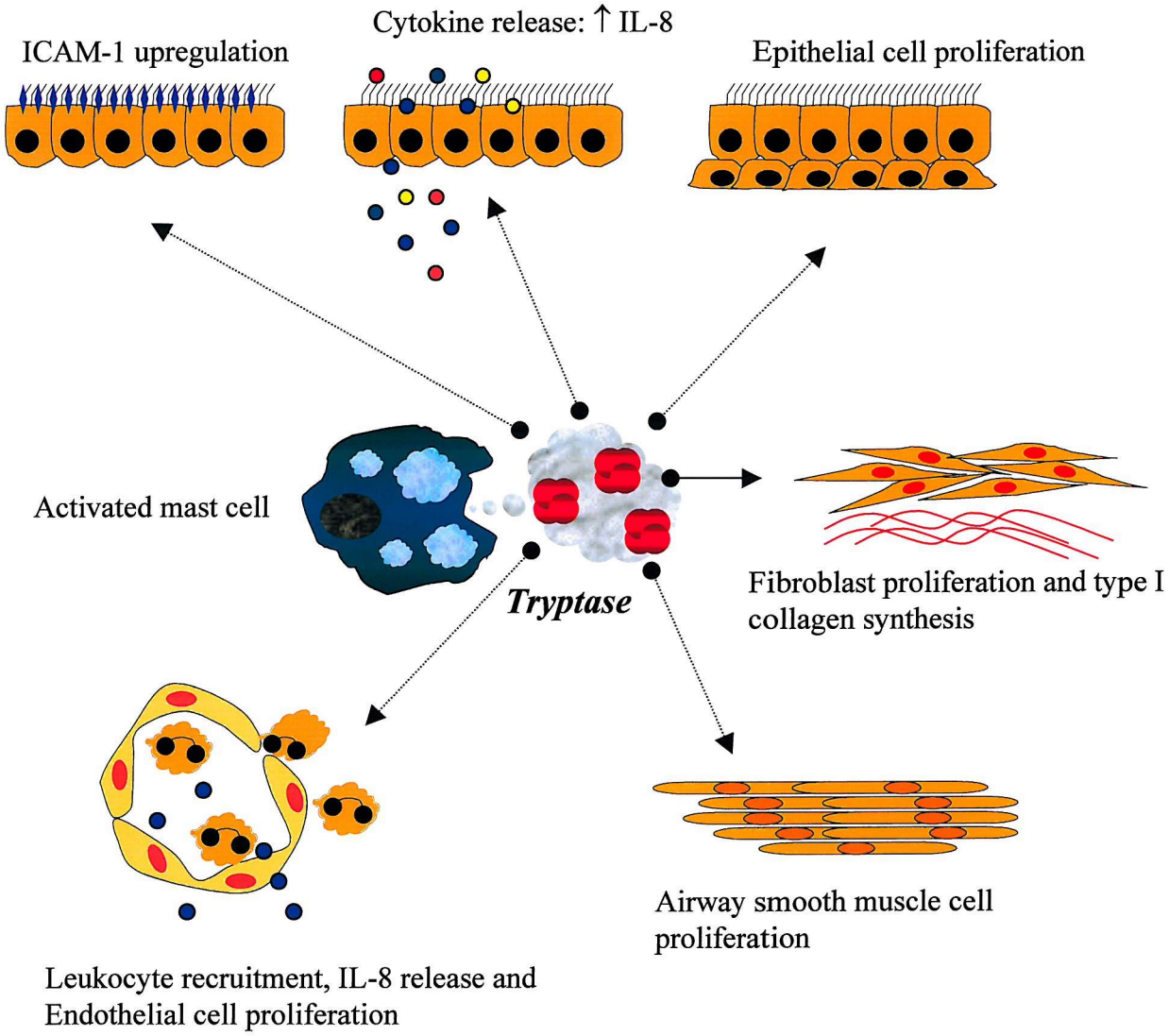


Figure 1-3 Cellular and tissue targets of human mast cell tryptase. ICAM-1, intercellular adhesion molecule 1; IL, interleukin.

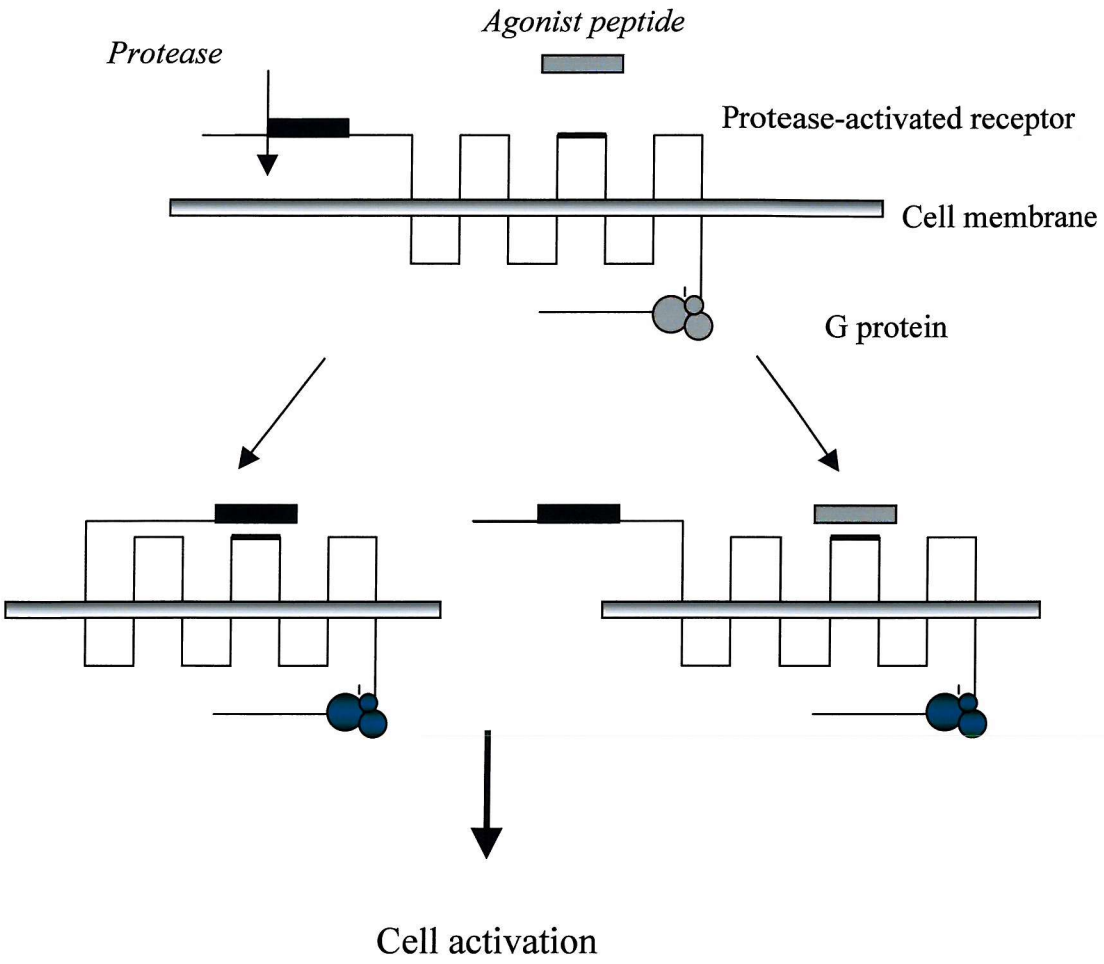


Figure 1-4 Mechanism of activation of protease activated receptor-2 (PAR-2) by protease or activating peptide. Protease cleaves the receptor to expose a tethered ligand, which subsequently interacts with an extracellular domains of the cleaved receptor, resulting in activation of G proteins. The intact receptor can also be activated by synthetic peptide corresponding to the sequence of tethered ligand.

CHAPTER 2

MATERIALS AND METHODS

2 Materials and Methods

2.1 Materials and Reagents

Minimum essential medium (MEM), 10% foetal calf serum (FCS), growth supplement Ultroser G, penicillin, streptomycin, glutamine and Trizol were obtained from Gibco BRL Life Technologies (Paisley, UK); heparin agarose, Sephacryl S-200, N-α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride, leupeptin, porcine lung heparin glycosaminoglycan, BSA (bovine serum albumin), DMSO (dimethylsulphoxide), 3,3' diaminobenzidine tetrahydrochloride, insulin-selenium-transferrin cell culture supplement, the E-toxate assay for endotoxin, collagen type IV, protease type XIV, monoclonal anti-pancytokeratin antibody, monoclonal anti-myosin antibody, monoclonal anti-vimentin antibody, Percoll, propidium iodide, and anti-mouse IgG antibody conjugated to FITC from Sigma Chemical Co. (Poole, Dorset, UK); Coomassie Blue protein assay from Pierce (Chester, UK); molecular weight markers (prestained SDS-PAGE standards), 7.5 % polyacrylamide gel, silver staining reagent and electrophoresis grade agarose from BioRad Laboratories (Hemel Hempstead, Herts, UK); methyl-[^{3}H] thymidine, ^{14}C-mannitol, ^{14}C-inulin and ^{14}C-bovine serum albumin (^{14}C-BSA), and ^{51}Cr sodium chromate in sodium chloride solution from Amersham International plc (Little Chalfont, Bucks, UK); recombinant TNF-α from PharMingen (San Diego, CA); recombinant human epidermal growth factor (EGF), anti-IL-8 antibody, eotaxin ELISA kit, from R&D Systems (Abingdon, UK); ultrafilters from Amicon (Stonehouse, UK).

Cell proliferation assay involving the reduction of the tetrazolium salt (3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTT), and the reverse transcription system including AMV reverse transcriptase, recombinant RNasin ribonuclease inhibitor and oligo(dT) primer were from Promega Ltd. (Southampton, UK); ultrapure dNTP set and 100 base-pair ladder from Pharmacia Biosystems Ltd. (Milton Keynes, UK). IL-8 ELISA kit was from Eurogenetics Ltd. (Teddington, UK);

IL-6 and GM-CSF ELISA kits from BioSource International (Camarillo, CA); PAR-2 agonist peptide SLIGKV-NH_2 and reverse peptide VKGILS-NH_2 were synthesized by Genosys Biotechnologies Ltd (Cambs, UK) and the oligonucleotide primer pair to human PAR-2 by Gibco BRL Life Technologies (Paisley, UK.)

Falcon cell culture inserts were obtained from Becton Dickinson Ltd (Oxford, UK); EVOM micro volt-ohm-meter from World Precision Instruments, (Owslebury, UK); Corning culture inserts for transmigration study from Corning Costar (Bucks, UK); monoclonal mouse anti-occludin and anti-ZO-1 from Zymed Laboratories (San Fransico, CA); biotinylated sheep anti-mouse IgG, avidin-biotin-horseradish peroxidase, goat anti-mouse IgG conjugated to FITC, and rabbit anti-mouse IgG HRP conjugate from DAKO (Copenhagen, Denmark); chemiluminescent substrate SuperSignal West Femto from Pierce (Rockford, IL, USA).

2.2 Cell Culture

2.2.1 Cells

The 16HBE 14o- cell line, kindly provided by Dr. D.C. Gruenert (Cardiovascular Research Institute, University of California, San Francisco, CA) was employed, as it is a normal human airway epithelial cell line derived from the surface epithelium of mainstream, second-generation bronchi (Cozens et al., 1994). The transformed cell line contains the large T antigen of the SV40 virus, a DNA virus of the Papovavirus family. 16HBE 14o- can form tight junctions, generate polar monolayers and express other differentiated features characteristic of native epithelium such as ion transport, secretion, metabolic enzymes and adhesion molecules, and it can demonstrate cilia under defined growth conditions (Gruenert et al., 1995).

The A549 tumour cell line, originated from a human alveolar cell carcinoma, possesses a human karyotype and appears to be derived from a single parent cell. All A549 cells examined by electron microscopy at both early and late passage levels contain multilamellar cytoplasmic inclusion bodies typical of those found in type II alveolar epithelial cells of the lung (Lieber et al., 1976).

Primary human bronchial epithelial cells, isolated by explant culture, enzymatic dissociation, or modified air-liquid interface culture, were employed in some experiments and findings compared to those with the immortalised airway epithelial cells. Working with primary bronchial epithelial cell culture was a challenge, as it proved difficult to obtain clinical materials from surgical patients with any regularity. In most studies, immortalised epithelial cells were used on account of their unlimited growth potential and because they allowed the use of standardised models.

2.2.2 Cell Culture

2.2.2.1 Culture

16HBE 14o- cells: Cells were grown in MEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml gentamycin, and 2mM glutamine at a temperature of 37°C in a humidified atmosphere of 95% air and 5% CO_2 and were routinely employed at passages 20 to 40. Culture media were replaced every two to three days.

A549 cells: Cells were grown in MEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml gentamycin, and 2mM glutamine at a temperature of 37°C in a humidified atmosphere of 95% air and 5% CO_2. Culture media were replaced every three to four days. When cells became confluent, cells were transferred to the other culture flask for subculturing or to culture inserts or 24-well plates for experiments.

Primary bronchial epithelial cells:

Explant Culture Human bronchus, obtained from lung lobectomy, was rinsed several times with Leibovitz's L-15 medium containing penicillin 100 U/ml, streptomycin 100μg/ml, and amphotericin B 0.25 μg/ml. The tissue was cut into 1-2 mm^2 pieces and explanted epithelium side down onto a 6-well culture plate, previously coated with type IV collagen (50 $\mu g/cm^2$). Two ml of LHC-9 medium was added to each well and the explants were incubated at 37 °C in a humidified 5 % CO_2 incubator. The medium was refreshed every 3 to 4 days. After 3 to 4 weeks incubation the epithelial cell outgrowths from the explant appeared approximately 60 % confluence and, usually, the fibroblastic cell contamination was rare as determined by the morphology observed under inverted microscopy. The tissue fragments were then transferred to new culture plate for new outgrowths of epithelial cells.

Enzyme Dissociation Method The bronchus, obtained at surgical lobectomy, was rinsed several times with L-15 medium, trimmed of

connective tissue, and then incubated in Hank's balanced salt solution containing 0.25 % (wt/vol) type XIV protease at 4 °C overnight. The epithelial cells were detached by flushing the lumen and foetal calf serum was added at a final concentration of 10% (vol/vol). Collected cells were centrifuged and resuspended in 5 ml of a dissociating solution containg 0.02% trypsin, 0.02% EGTA (ethyleneglycol-bis-(β-aminoehtyl ether) N,N,N′,N′-tetraacetic acid), 1 % polyvinylpyrrolidone, and 0.002% EDTA in Puck's saline A. The cell suspension was incubated at 37 °C for 5 min, pipetted to dissociate the cells, and then centrifuged in a medium containing Dulbecco's MEM/Ham's F12 (1:1) and 10 % FCS. Cells were washed with serum-free medium by centrifugation and resuspended in a culture medium containing LHC-9 and RPMI-1640 (1:1), Ultroser G 2 %, EGF 1 ng/ml, 2mM L-glutamine, penicillin 100 U/ml, streptomycin 100μg/ml, and amphotericin B 0.25 μg/ml. Cells were seeded in 6-well culture plate, coated with type IV collagen (50 $\mu g/cm^2$), at a density of 5 x 10^4 $cells/cm^2$. Cells had grown to confluence after 5 to 6 days incubation. The purity of epithelial cells appeared to be more than 95 % determined by morphology and by immunocytochemistry with antibodies against cytokeratin for epithelial cells, vimentin for fibroblasts, and myosin for smooth muscle cells.

Modified Air-Liquid Interface Culture The procedure was adopted from methods described previously (Whitcutt et al., 1988; Yamaya et al., 1992). Bronchus tissue was rinsed with L-15 medium contining penicillin 100 U/ml, streptomycin 100μg/ml, and amphotericin B 0.25 μg/ml. The tissue was cut into 1-2 mm^2 pieces and 4 to 5 pieces of tissue were planted epithelium side down onto 6-well culture inserts (growth area of membrane 4.2 cm^2, pore size 0.4 μm) coated with type IV collagen as before. Culture medium, (600 μl) was added into the basal chamber and 50 μl into insert. Culture medium in basal chamber was changed every 48 h and no medium was added to inserts. The bronchial epithelial cells were grown on a porous membrane, on which they formed a continuous epithelial sheet with the basal aspect exposed to medium and the apical surface exposed to air. Cells, grown on the inserts, were

confluent after 7-10 days incubation. The tissue fragments were then transferred to fresh inserts for new growths of epithelial cells.

2.2.2.2 Transfer of Cells

For 16HBE 14o- cells and A549 cells, when cells became confluent, cells were transferred to the other culture flask for subculturing or to 24-well or 96-well culture plates for experiments. To dissociate cells from culture vessels, spent cell culture media was discarded followed by two washes with Hanks' balanced salt solution without calcium and magnesium. A 2ml aliquot of 0.05% trypsin-EDTA was added, swirled briefly and incubated for 10-15 min after discarding the wash solution. When cells were observed by light microscopy to have rounded up, the flask was gently tapped to dislodge the cells. Some 10-15 ml culture medium was added to inactivate the enzymatic actions of trypsin and the cell suspension was centrifuged at 150 g for 10 min. The supernatant was discarded and cells were resuspended in 5 ml of culture medium. The cell density was determined and an appropriate volume of medium was added to the culture flask. The caps of culture flasks were maintained loose to allow gas exchange and placed at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Primary bronchial epithelial cells grown to confluence, were dissociated using trypsin/EGTA/EDTA solution. The cultures were rinsed with HBSS and then incubated with dissociating solution at 37 °C, 5 % CO_2 incubator. The cells generally began to detach from the surface of the plate (monitored using an inverted microscope) within 8 to 10 min. The cell suspension were centrifuged in medium containing 10% serum, washed with HBSS, and resuspended in a serum-free culture medium. Cells were then inoculated into 24-well culture plate at a density of 5×10^4 cells/cm^2 for further experiments.

2.2.3. Enumeration of Cells and Determination of Cell Viability

2.2.3.1. MTT Cell Assay

This non-radioactive cell proliferation assay, purchased from Promega, is a colorimetric method for determining the number of viable cells in proliferation. MTT, a novel tetrazolium compound (3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium), is bioreduced by cells into a formazen. The conversion of MTT into the formazen is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of fromazen product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture. An aliquot of 20 µl of MTT and PMS (phenazine methosulphate, an electron coupling reagent) solution was added to 100 µl cell suspension in each well of a 96-well plate. The plate was incubated for 4 hrs at 37° C in a 5% CO_2 atmosphere and then the absorbance was recorded at 490nm using an ELISA plate reader. The cell numbers were determined using a standard curve constructed with known numbers of cells.

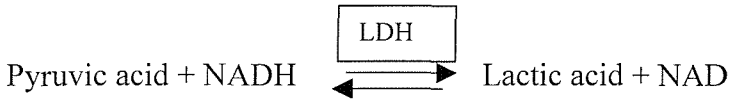
2.2.3.2. Trypan Blue Exclusion

Trypan blue, being a negatively charged vital dye does not interact with the cell unless the membrane is damaged. It may therefore be assumed that cells which exclude dye are viable. A 0.1 ml aliquot of 0.4% trypan blue was mixed thoroughly with 0.1 ml cell suspension and allowed to stand 5 min at room temperature. Stained non-viable and viable cells were counted in a haemocytometer.

2.2.3.3. Lactate Dehydrogenase Assay

In addition to measurements of trypan blue exclusion, cell viability was determined using colorimetric method to measure lactate dehydrogenase (LDH) activity. LDH is a large molecule stored within the cell, which is unable to cross the cell membrane except in cases of cytolytic injury. The

activity of LDH was quantified by a method involving the NADH-dependent reduction of pyruvic acid to lactic acid.



Unreacted pyruvic acid was employed with 2,4-dinitrophenylhydrazine to form an intensely colored "hydrozone", with the absorbance of the coloured product formed being inversely proportional to LDH activity. A 1 ml aliquot of pyruvate substrate was pipetted into an incubation tube containing NADH and placed in a water bath at 37° C for 5 min. Supernatant (0.1 ml) from cultured cells was added, mixed gently, and placed in a water bath at 37° C for exactly 30 min. Colour reagent (1 ml) was then added and mixed. After 20 min, 0.4N sodium hydroxide 10 ml was added and the absorbance value was measured using plate reader at 450 nm wavelength. The LDH value was determined by comparing to a standard curve constructed by plotting the known LDH activity.

2.2.4. Cryopreservation

Cryopreservation involves the storing of cells in a state of suspended animation at the very low temperature (-180° C) of liquid nitrogen . This can prevent possible problems of using cells that have lost their original functions after so many passages. The freezing mixture employed in these studies was made up of 40 % culture medium, 40% FCS, and 20% of the cryopreservative dimethylsulphoxide (DMSO), which acts to reduce the water content of the cells and to prevent ice crystal formation. The cells, at a density of 2 x 10^6/ml were resuspended in the growth medium, free of antibiotics and containing 10% FCS, and placed on ice. An equal volume of freezing mixture was added and mixed thoroughly. Aliquots (1ml) of cell suspension were placed into cold prepared cryotube at a final cell concentration of 1 x 10^6/ml. Cells were frozen slowly at a cooling rate of approximately 1° C/min. This was achieved by

placing vials in an insulated box in -80° C freezer for 24 h, then transferring to liquid nitrogen storage.

To recover cryopreserved cells, they were transferred rapidly into a 37° C water bath, and placed directly into complete growth medium. The cell suspension was centrifuged at 100 g for 5 min to remove cryopreservative. The supernatant was discarded and cells were resuspended in culture medium. Cells were counted and seeded in culture vessels with at least 3 x 10^5 viable cells/ml.

2.3 Tryptase Preparation

2.3.1 Purification

Human mast cell tryptase was purified from lung tissue postmortem using a method based on a modification of that originally described by Smith et al (Smith et al., 1984). The lung tissue (400 to 500 g) was chopped finely, washed with ice cold distilled water, and blended with an equal volume of low salt buffer [10mM morpholinethane sulphonic acid (MES), 0.2 M NaCl, pH 6.1] for 30 sec at low speed. The homogenate was centrifuged at 6000 g for 30 min at 4^O C and the precipitated pellet resuspended in low salt buffer, briefly blended and centrifuged as before. The blending and centrifugation cycle was repeated another three times. The homogenate was then blended with a high salt buffer (10 mM MES, 2 M NaCl, pH 6.1, 400 ml) at high speed for 30 sec and then centrifuged (6000 g, 45 min, at 4^O C). The extraction procedure was performed three times and supernatant was collected. The supernatant fractions of these extractions were pooled and were precipitated with equal volume of 10% cetyl pyridinium chloride to extract glycosaminoglycans, since tryptase has been shown to exist in a complex with heparin proteoglycan. Precipitated material was removed by centrifugation at

6000 g for 45 min at 4^{O} C. The supernatant was then filtered by using a 0.7 and then a 0.4 um microfibre membrane.

The filtrates were dialyzed against distilled water (24 hr, 4^{O} C) and applied to a heparin-agarose column (20 x 1.6 cm) equilibrated with a low salt buffer, at a rate of 1 ml/min. Tryptase-containing fractions were eluted using a gradient between 0.4 M NaCl 10mM MES and 1.5 M NaCl 10mM MES. Fractions of 5 ml were collected at a flow rate of 0.5 ml/min. Tryptase-rich fractions, assayed by BAPNA cleavage were pooled together and concentrated using an Amicon concentrator with a YM-30 membrane. Filtrates were applied to a Sephacryl S-200 gel filtration column equilibrated with high salt buffer (2 M NaCl, 50mM MES) at a rate of 0.4 ml/min. Fractions containing high tryptase activity were again concentrated, passed through a 0.22 μm membrane filter for sterilization and stored at -80° C for experimental use.

2.3.2 Characterisation

2.3.2.1 SDS-PAGE

The purified protein was analysed by electrophoretic separation on SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). Tryptase-containing samples were dissolved in buffer with 10 mM Tris-hydrochloric acid (pH 6.8), 2% SDS (lauryl sulphate, sodium), 5% mercaptoethanol, 10% glycerol and 0.001% bromophenol blue. An equal volume of sample and buffer were mixed and heated to 100° C for 5 min. A 20-40 μl sample was layered on to a 3% polyacrylamide SDS stacking gel and electrophoresis was performed in 10% acrylamide separating gel at 80 mA for 1 hr. Standard markers employed in these studies included bovine milk α-lactoalbumin (14,200), soybean trypsin inhibitor (20,000), bovine trypsinogen (24,000), bovine carbonic anhydrase (29,000), Glyceraldehyde-3-p-dehydrogenase (36,000), egg albumin (45,000) and bovine albumin (66,000).

2.3.2.2 Western Blotting

The unstained gel was incubated for 1 hr in a 10 mM Tris-hydrochloric acid buffer (pH 7.0) with 50mM NaCl and 4M urea. The gel was then incubated for 30 min in a 25 mM Tris transfer buffer (pH 8.3) with 0.2 M glycine and 20% methanol, and nitrocellulose was sandwiched between Scotch-Brite pads in a hinged cassette, and inserted into a Trans-Blot apparatus. Electrophoretic blotting was carried out at 100 V for 1 ½ hr at 8-10° C. Nitrocellulose strips were blocked with 3% BSA in PBS with 0.05% Tween (three 10 min washes at 37° C) and incubated with monoclonal antibody AA5, specific for tryptase (Walls et al., 1990a), in PBS-Tween 20 diluted in 1/200 for 1 hr at room temperature. The washing step was repeated and strips were incubated with biotinylated sheep anti-mouse IgG at a dilution of 1/200 in PBS-Tween 20 for 1 hr at room temperature. After a further washing step, strips were incubated with avidin-biotin-horseradish peroxidase complex in PBS-Tween 20 for 1 hr at room temperature. Following another washing step, the immunoperoxidase staining on transferred protein bands was developed with 3,3' diaminobenzidine tetrahydrochloride (0.5 mg/ml in 40 mM Tris-hydrochloric acid buffer with 0.01% hydrogen peroxide). Strips were washed with distilled water, dried and photographed.

2.3.2.3 Silver Staining

The silver staining protocol employed was based on the instructions provided by the manufacturer. Gels were immersed in fixative solution consisting 40 % methanol and 10 % acetic acid (vol/vol) in deionised water for 30 min followed by immersion in a second fixative solution consisting 10 % methanol and 5 % acetic acid for 30 min. The gel was incubated with a 1 in 10 of a stock oxidiser with gentle agitation for 5 min, followed by washing for 5 min three times in double deionised water. Silver reagent was added at a dilution of 1 in 10 for 20 min, followed by washing for 1 min in deionised water. The gel was then incubated in a 3.2 % developing solution (w/v) until clear and dense bands appeared. The reaction was stopped after addition of 5 % acetic acid.

2.3.3 Assays of Enzyme Activity

The enzymatic activity of purified tryptase was determined using the substrate *N*-α-benzoyl-D,L-arginine-*p*-nitroanilide hydrochloride (BAPNA). After adding 10 µl of enzyme to 90 µl of 20 mM Tris buffer (pH 8.0) containing 1 M glycerol, 1 mg/ml BSA and 20 mM BAPNA in DMSO, absorbance was measured at 410 nm in an ELISA plate reader. Protein concentration was measured spectrophotometrically by the Coomassie brilliant blue dye binding procedure with BSA as the standard. The optical density of each solution was measured in a spectrophotometer at a wavelength of 595 nm. The specific activity of the tryptase preparation was expressed in U/mg where 1 U was taken to represent that amount of tryptase required to hydrolyse 1 µmol substrate per minute at 25° C.

2.3.4 Assays for Endotoxin

E-toxate (Limulus amoebocyte lysate; Sigma) was employed for detection and quantification of endotoxin contamination in the tryptase preparation. According to the manufacturer's instructions, samples and endotoxin standard were diluted to different concentrations with endotoxin-free water and transferred to glass tubes. E-toxate working solution was added to each tube and tubes were mixed gently. After incubation for 1 hr undisturbed at 37° C, a positive reaction was indicated by the formation of a hard gel, which permits complete inversion without disruption of the gel. The presence of a soft gel, turbidity, an increase in viscosity, or a clear liquid was considered negative. The final endotoxin level (EU/ml) was determined by multiplying the inverse of the highest dilution of sample found positive by the lowest concentration of endotoxin standard found positive.

2.3.5 Recombinant Tryptase

This recombinant human mast cell tryptase, supplied by Promega, was expressed in Pichia pastoris via homologous recombination of the cDNA (human skin tryptase I gene) for the mature active tryptase (Niles et al., 1998). Recombinant human tryptase has been reported to cleave specific substrates with kinetics similar to that of human lung mast cell tryptase, but with a K_m slightly higher than that of the native lung tryptase and a K_{cat} 2.4-fold higher. The recombinant tryptase has been described as being approximately twice as active as lung tryptase with the substrate of α-*N*-benzyloxycarbonyl-lysine thiobenzyl ester (Niles et al., 1998). The recombinant tryptase was employed only in the experiments on the alterations of epithelial permeability.

2.4 Enzyme Linked-Immunosorbant Assay (ELISA)

Cytokines (IL-β, IL-5, IL-6, IL-8, GM-CSF, RANTES, and eotaxin) were measured by commercially available sandwich-type ELISA, with monoclonal anti-cytokine antibody bound to polystyrene microtiter wells. The method involved capture of the cytokine present in a defined volume of sample or standard by the antibody on the plate, and removal of non-bound material by washing. Subsequently, a biotinylated antibody to specific human cytokine was added, and excess biotinylated antibody was removed by washing, followed by addition of horseradish peroxidase (HRP) conjugated streptavidin, which binds onto the biotinylated side of the human cytokine sandwich. After removal of non-bound HRP conjugate by washing, a substrate solution was added to the wells. A coloured product was formed in a quantity proportional to the amount of cytokine present in the sample or standard. After the reaction was terminated by addition of a stop solution, absorbance was measured by plate reader. From the absorbance of the samples and those of a standard curve, the concentration of cytokine was determined by interpolation from the standard curves.

2.5 Immunocytochemistry

Immunocytochemistry was employed to localise cellular components in situ with specific antibodies. In the three-step indirect method, the primary antibody, which reacts with antigen in tissue or cells, may be polyclonal, generally raised in rabbits, or monoclonal, mainly raised in a mouse. The second stage comprises of an antibody labeled with the biotin, which has been raised against the animal species of the primary antibody. Biotin may be used as the label as it has a specific chemical affinity for either avidin or streptavidin. For the third stage, a complex of either avidin or streptavidin labeled with an enzyme like horseradish peroxidase or alkaline phosphatase is allowed to bind to the biotin on the secondary antibody. These sites are then visualised by reacting with a chromogenic substrate.

In most of the immunocytohemical studies reported here, the two-step indirect method was used in preference to the three-step indirect method as this procedure is less time-consuming. In this method, an unconjugated primary antibody binds to antigen. A FITC-labeled secondary antibody is then allowed to bind to the primary antibody, and fluorescent staining is detected under a fluorescence microscope.

2.5.1 Immunofluorescent Staining of Primary Epithelial Cells

To confirm the purity of primary cultures of human bronchial epithelial cells, the cells were dissociated and seeded into an eight-chamber slide, which was coated with type IV collagen. When cells were nearly confluent, cells were washed with PBS twice and fixed for 15 min in cold methanol. The samples were blocked with PBS containing 1 % BSA for 30 min. Cells in each chamber were incubated with control IgG, monoclonal anti-pancytokeratin (epithelial cells) (Fig. 2-1A), monoclonal anti-vimentin (fibroblast) (Fig. 2-1B), or monoclonal anti-myosin (smooth muscle cells) (Fig. 2-1C) antibody for 1 h in a appropriate dilution according to the manufacturers' instructions. After appropriate washes, a secondary antibody conjugated to FITC was added

and incubated for an additional 45 min. Propidium iodide (2 μg/ml) was added for 1 min to counterstain the nuclei after washing out the secondary antibody. The cells were washed, mounted, and examined under a fluorescence microscope.

2.5.2 Immunofluorescent Staining of PAR-2

For immunolocalisation of PAR-2 on different cell types, 16HBE 14o- cells, A549 cells, primary bronchial epithelial cells, and human lung fibroblasts (Fig. 2-2) were cultured on chamber slides and fixed in ethanol for 15 min at 4 °C, followed by an additional 3 min incubation with ice-cold acetone. Cells were blocked in PBS-3% BSA with 3% goat serum and then incubated with antiserum (B5), raised in rabbits against a peptide sequence corresponding to a portion of rat PAR-2, at 4 °C overnight (Kong et al, 1997). B5 antibody preabsorbed with the receptor fragment (^{29}TNRSSKGR^{37}SLIGKVC) (10 μM) was applied as a negative control. After further washing, cells were incubated with goat anti-rabbit IgG FITC for 1 h and then mounted in aqueous mountment, before viewing under a fluorescence microscope.

2.5.3 Immunofluorescent Staining of Occludin and ZO-1

After monolayers were exposed to tryptase or the peptide agonist SLIGKV for 6 h or 24 h, cultured insert membranes were removed, washed, and fixed immediately in cold ethanol at 4 °C for 30 min followed by an additional incubation with cold acetone for 3 min at room temperature. Following a blocking stage with PBS containing 3 % goat serum and 3 % BSA, cells were incubated with mouse monoclonal anti-occludin (2 μg/ml) or anti-ZO-1 (15 μg/ml) overnight at 4 °C. After washing, goat anti-mouse IgG FITC conjugate was employed and incubated for 1 h. The insert membranes were mounted in Citifluor and the localisation of occludin and ZO-1 was visualised and

photographed using a Leica DMRBE fluorescence microscope with a MPS 60 photographic system.

2.5.4 Flow Cytometry

Flow cytometry, using fluorescent probes which bind to specific cell associated molecules, is able to determine various phenotypic, biochemical and molecular characteristics of individual cells suspended in a fluid stream. This technology provides a reliable and accurate quantitative analysis of selected physical properties of cells of interest.

To quantify the expression of PAR-2 localised on the cell membrane of 16HBE 14o- cells, A549 cells, primary bronchial epithelial cells, and human lung fibroblasts, cells were grown on the culture dishes and dissociated with non-enzymatic cell dissociation solution after washes with Ca^{2+}/Mg^{2+}-free HBSS twice. Cells were collected, centrifuged with serum-containing buffer, and resuspended with 5% FCS in HBSS at a concentration of 1×10^6 cells/ml. An aliquot of 100 μl cell suspension was immediately placed on ice incubated with 30 μl diluted B5 antiserum in 2% FCS-HBSS containing 0.1% NaN_3 for 1 h. Cells were incubated with goat anti-rabbit IgG FITC conjugate in a 1:50 dilution for 1 h after washing with buffer. Cells were then washed, resuspended and analysed using a FACScan (Becton Dickinson, CA) (Fig. 2-3).

2.6 Statistics

Analysis of variance (ANOVA) was employed to indicate significant differences between groups and results were analysed for statistical significance by two-sample Student's t test or the paired Student's t test. $P < 0.05$ was taken as significant. Calculations were performed principally using the computer software programes, SPSS 9.0 version for Windows.

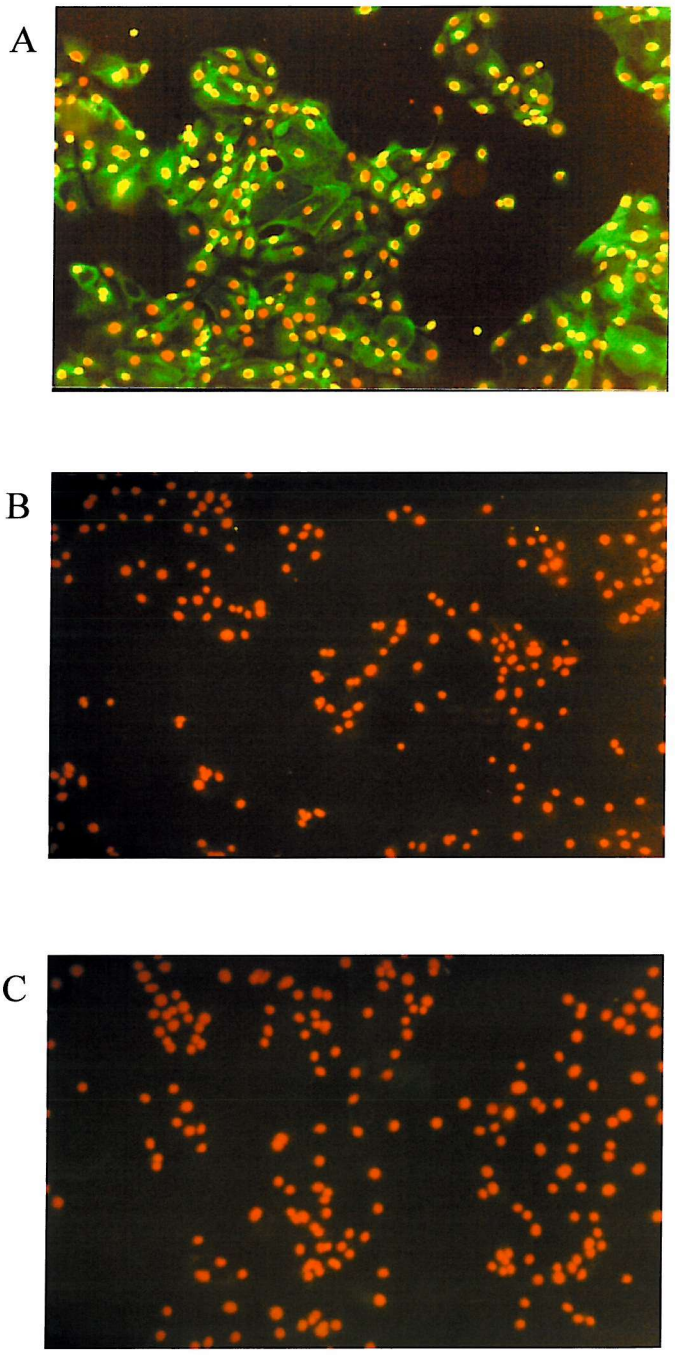


Figure 2-1 Immunofluorescent staining of primary bronchial epithelial cells for (A) cytokeratin, (B) vimentin, and (C) myosin.

Fig. 2-2

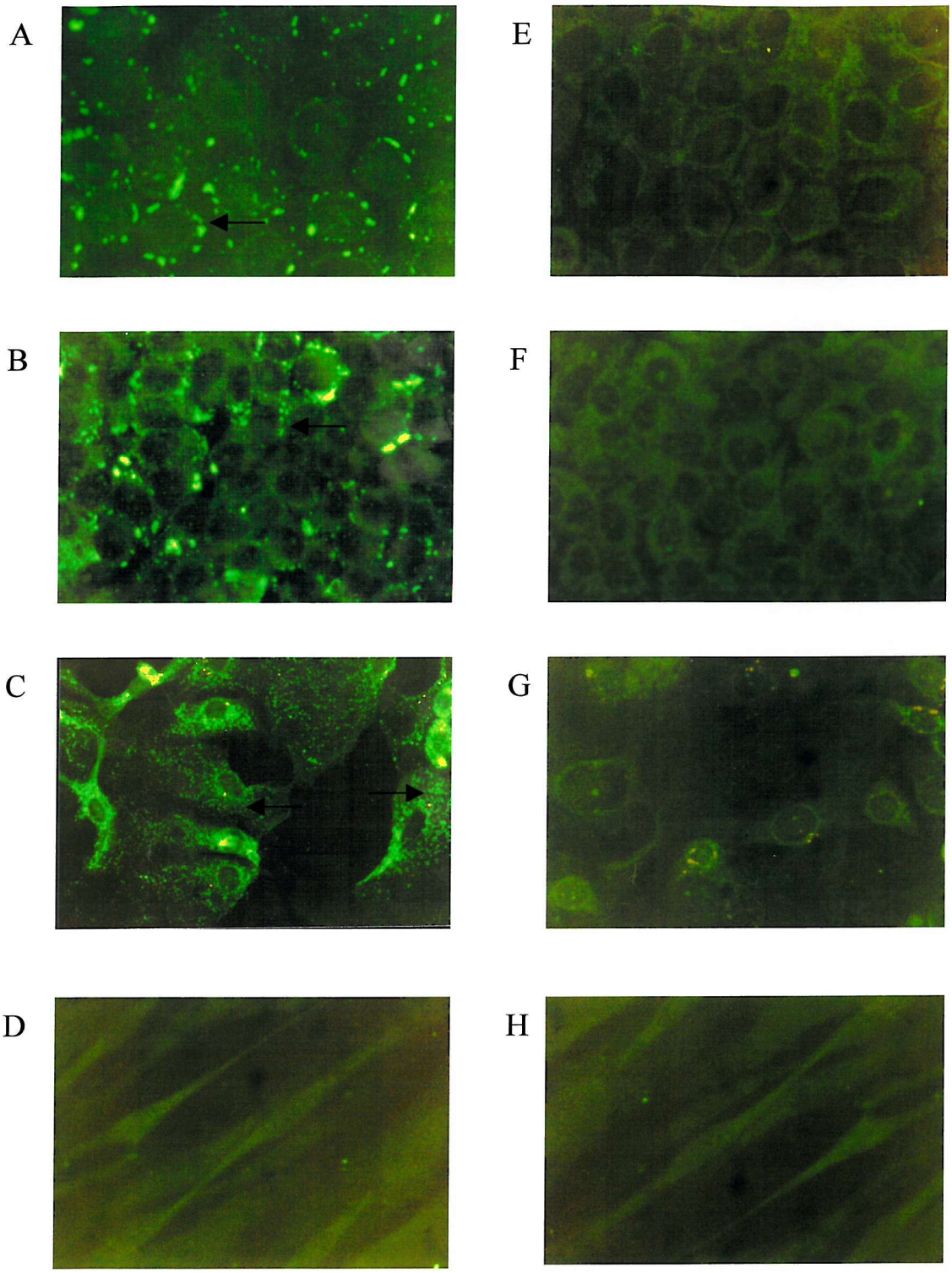


Figure 2-2 Immunofluorescent staining of PAR-2 (arrowed) on (A) 16HBE 14o- cells, (B) A549 cells, (C) primary bronchial epithelial cells, and (D) human lung fibroblasts. PAR-2 was localised using antiserum B5 at a dilution of 1:1000. B5 antibody preabsorbed with the receptor fragment (^{29}TNRSSKGR^{37}SLIGKVC) 10 μM was applied as a negative control (E,F,G, and H corresponding to the cells depicted in A, B, C and D respectively).

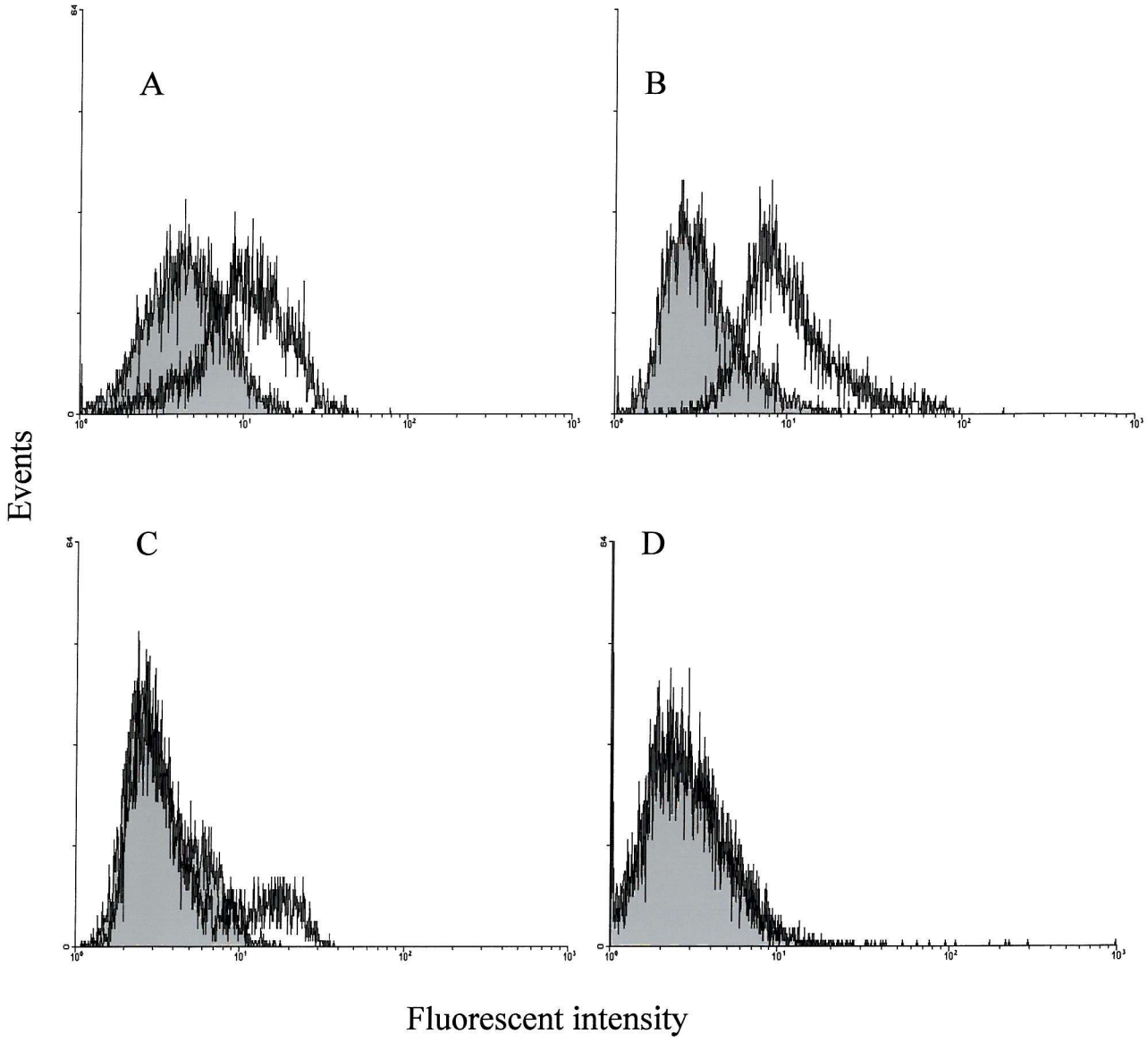


Figure 2-3 PAR-2 expression on the surface of (A) 16HBE 14o- cells, (B) A549 cells, (C) primary bronchial epithelial cells, and (D) human lung fibroblasts (D) detected by flow cytometry. Nonimmune rabbit serum was employed as a negative control (solid area).

CHAPTER 3

TRYPTASE AS A MITOGEN FOR EPITHELIAL CELLS AND STIMULUS FOR CYTOKINE RELEASE

3 Tryptase as A Mitogen for Epithelial Cells and Stimulus for Cytokine Release

3.1 Introduction

The airway epithelium is likely to be exposed to high levels of tryptase in asthma and other inflammatory conditions. The previous observation that tryptase can stimulate cell proliferation and the secretion of IL-8 from the H292 epithelial cell line (Cairns and Walls, 1996) has called attention to the potential for this major mast cell product to modulate epithelial cell function.

In the series of experiments described in this section, the potential of tryptase to induce mitogenesis and the synthesis and release of a range of inflammatory cytokines from 16HBE 14o- airway epithelial cells has been investigated. In parallel experiments a synthetic agonist for PAR-2 has been employed to characterise and compare the cellular responses mediated by PAR-2 with those elicited with tryptase. We report that tryptase may be an important proinflammatory mediator, which can induce increased expression of mRNA for several key cytokines, as well as their release from epithelial cells. Evidence is presented that the mechanism may involve the activation of PAR-2.

3.2 Materials and Methods

Details of methods for the studies in this section are provided below. Procedures used also for the work of other sections are described in Chapter 2 (purification of tryptase, culture of 16HBE 14o- cells, mitogenesis, proliferation assay and ELISA).

3.2.1 Mitogenesis and Cell Proliferation Assay

Cells were seeded in 96-well microtiter plates at a density of 10^5 cells/ml. When close to confluence (70 to 80 %), cells were washed twice with HBSS and growth was arrested using serum-free MEM supplemented with 0.2% Ultrosur G. Cells that achieved complete confluence were not utilized because of the concern for inhibition of cell growth and decreasing cell viability. After 48 h of serum deprivation, purified tryptase was added in the presence of heparin in a weight ratio of 1:1 to stabilize enzymatic activity (Schwartz and Bradford, 1986). FCS and EGF acted as positive controls. In studies to investigate dependency on an intact catalytic site, tryptase (with added heparin) was incubated in the presence or absence of leupeptin 50 μg/ml. The effect of heat treatment was investigated, by heating tryptase at 65°C for 30 minutes. The cells were incubated at 37°C in 95% air and 5% CO_2 for 24h and 0.5 μCi methyl-[^{3}H] thymidine was added to each well for a further 8 h before harvesting and counting in scintillant.

In order to determine cell numbers, cells were incubated for 48 h in the presence or absence of stimulus, washed twice with HBSS and then a 15 μl dye solution containing MTT salt was added to each well. Cells were incubated at 37°C for 4 h, before addition of 100 μl of the stop/stabilization solution. The OD value at 550 nm of each well was determined following an overnight incubation, and the cell numbers were determined using a standard curve constructed using known numbers of cells.

3.2.2 Cytokine Release

Cells were seeded in 24-well plates at a density of 2 x 10^5 cells /ml and grown in 10% FCS MEM. At close to confluence, growth was arrested with serum-free medium and cells were incubated for 24 hours before addition of tryptase (5-80 mU/ml), the PAR-2 agonist peptide, SLIGKV (1-100 μM) or TNF-α 100 U/ml for 3h, 6h, 24h and 48h. Supernatant was stored at -80°C until assaying for cytokines. Cell viability was determined by dye exclusion with trypan blue. Levels of immunoreactive IL-8, GM-CSF, and IL-6 were assayed by ELISA according to the instructions of the manufacturers. The lower limits of sensitivity were 1-3 pg/ml for IL-8, 1 pg/ml for GM-CSF and 2 pg/ml for IL-6.

3.2.3 Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

Following removal of supernatants for cytokine detection described as above, the cells in 24-well plates were washed twice with HBSS and lysed by the addition of Trizol (200 μl/well) and an equal volume of chloroform. The RNA was extracted from the aqueous phase after being centrifuged at 12,000 g for 15 min. RNA was precipitated in the presence of isopropanol at -20°C overnight. The pure RNA was recovered by centrifugation at 12,000 g for 15 minutes at 4°C, washing with 80% ethanol, air-drying and resuspending in DEPC-treated water. The concentration of RNA was measured spectrophotometrically by GeneQuant RNA/DNA calculator (Pharmacia). The RNA (1 μg) was reverse transcribed into cDNA using AMV reverse transcriptase and oligo d(T) as a primer at 42 °C for 60 min followed by heating at 94 °C for 3 min, and cDNA was amplified by PCR using *Taq* DNA polymerase in the presence of a master mix containing specific primer pairs, *Taq* buffer, dNTP, $MgCl_2$. PCR was performed in a programmable thermal cycler under the following conditions: denaturation at 94°C for 20 s, ramp over 45 s, annealing at 50 °C for IL-8, IL-6 primers and at 60 °C for GM-CSF

primer for 30 s, ramp over 45 s, and extension at 72 °C for 1 min. This was repeated for a total of 40 cycles, and followed by terminal polymerization at 72 °C for 10 min.

Amplified PCR-products were electrophoresed on a 2% agarose gel containing 0.5 μg/ml ethidium bromide and compared with a DNA reference marker (Pharmacia Biotech). The gel was visualized under an UV illuminator and photographed on a negative film. The film was scanned and measured by densitometry and each band was quantified by calculating the ratio of IL-8, GM-CSF, IL-6, and PAR-2 cDNA signal to the adenine phosphoribosyltransferase (APRT) control, and mRNA expression presented as a percentage of APRT signal. The APRT specific primer pair amplified a 246 bp PCR product composed of the following sequences: 5′ primer, GCT GCG TGC TCA TCC GAA AG; 3′ primer, CCT TAA GCG AGG TCA GCT CC. The IL-8 specific primer pair amplified a 186 bp PCR product composed of: 5′ primer, GCA GCT CTG TGT GAA GGT GCA, and 3′ primer, CAG ACA GAG CTC TCT TCC AT. The GM-CSF specific primer pair amplified a 215 bp PCR product, composed of: 5′ primer, GCA TGT GAA TGC CAT CCA GG, and 3′ primer, GCT TGT AGT GGC TGG CCA TC. The IL-6 specific primer pair amplified a 628 bp PCR product, composed of: 5′ primer, ATG AAC TCC TTC TCC ACA AGC GC, and 3′ primer, GAA GAG CCC TCA GGC TGG ACT G. The summary of sequence of specific primer pairs for RT-PCR are listed in Table 3-1.

3.2.4 PAR-2 mRNA Expression and Identification

Total RNA was extracted and reverse transcribed as described above. An oligonucleotide primer pair to human PAR-2 (5′ primer, CGG GGC AGG TGA GAG, +40 to +54; 3′ primer, TGC CAT AGA AAA AGC CAA TA, +600 to +619), synthesized by Gibco BRL Life Technologies (Paisley, UK.) was designed to amplify a 580 bp fragment. PCR-products were separated on

a 2% agarose gel and sized by comparison with 100 bp DNA ladder. To confirm the specificity of this DNA fragment, the amplified PCR product was purified using a QIAquick PCR Purification Kit according to the manufacturer's protocol. The DNA fragment was analysed using automatic fluorescent base cycle sequencing (Big Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin Elmer, CA). The purified double-strand DNA was mixed with the reaction buffer, PAR-2 primers and deionised water and added to the thermal cycler GeneAmp 9600 with the following steps: thermal ramp to 96 °C, 96 °C for 10 s, ramp over 50 °C, 50 °C for 5 s, ramp over 60 °C, 60 °C for 4 min; repeated for 25 cycles and finally thermal ramp to 4 °C. The sequencing reactants was subjected to microcentrifugation to remove excess dye terminators. The final product was separated on sequencing gel containing 4.25% acrylamide, 6 M urea and 1 X TBE (Anachem, Bedfordshire, UK.) and analyzed in an ABI Prism 377 DNA Sequencer using software program of ABI Prism Sequence Navigator (Perkin Elmer, CA.).

3.3 Results

3.3.1 Tryptase Preparation

Tryptase was successfully purified from high salt extracts of human lung tissue. On employing heparin agarose and Sephacryl S-200 column chromatography, the tryptase-containing fractions were assaying for BAPNA cleavage and the OD 410 nm measured (Fig. 3-1). After extraction, affinity chromatography and gel filtration, the purity of enzyme was assessed using SDS-PAGE. Gels revealed a single band corresponding to a protein of molecular weight 32 kDa by sliver staining (Fig. 3-2A). The identity was confirmed by Western blotting with AA5, a tryptase specific antibody (Fig. 3-2B). The specific activity of the tryptase preparation employed was between 1.6 and 2.2 U/μg throughout the whole study. Endotoxin levels in the tryptase preparations assayed by the E-Toxate assay were less than 0.06 EU / 1 U tryptase

3.3.2 DNA Synthesis and Cell Proliferation

At the lower end of the concentration range employed, tryptase stimulated a small increase in DNA synthesis in quiescent 16HBE 14o- bronchial epithelial cells. Increases of 38 and 26 % over control values were achieved with 10 and 20 mU/ml tryptase respectively (Fig. 3-3A). However, with a tryptase activity of 80 mU/ml, thymidine uptake was inhibited by 43 %. Under the light microscope, cell monolayers appeared intact following incubation with all concentrations of tryptase employed, and they had reached approximately 90% confluence at the end of each experiment. The dye exclusion test with trypan blue indicated that cell viability was greater than 95 % in each experiment. Heparin, which was added to tryptase in order to stabilise the enzyme activity, had no effect on the mitogenic response of epithelial cells, when added alone at the highest concentration which was added to tryptase.

In order to investigate dependency on an intact catalytic site, tryptase was preincubated with leupeptin (50 μg/ml). When tryptase activity was re-assayed with the chromogenic substrate before addition to cultured cells, the inhibition of enzyme activity was in all cases greater than 95%. This treatment appeared to abolish both tryptase-induced enhancement of thymidine incorporation at 10 mU/ml as well as the decrease induced with 80 mU/ml tryptase (Fig. 3-3B). Incubation of tryptase at either 65°C for 30 min or 100°C for 3 min resulted in the complete loss of BAPNA cleaving activity as well as the ability of the preparation to alter thymidine incorporation.

Cell numbers were determined by the MTT assay after tryptase stimulation for 48 hours (Fig. 3-4). Although there was a trend for an increase in total cell number at this time point, this was not significant. However, there appeared to be a small decrease in numbers at a tryptase concentration of 80 mU/ml. Cell numbers were increased marginally with 10% FCS and with EGF (25ng/ml), though the increase did not achieve significance with EGF.

3.3.3 Expression of mRNA for Cytokines

The 3h time point was selected for the RT-PCR experiments because at this point cytokine mRNA had already increased significantly, whereas cytokine protein secretion was yet to occur. In preliminary experiments, it was found that, at the 6h or 24h time points which were associated with increases in cytokine release (see below), the levels of mRNA for these cytokines had already waned (data not shown). Incubation with tryptase for 3 h provoked an increase in expression of mRNA for IL-8, GM-CSF, and IL-6 mRNA (Fig. 3-5). Increases of a similar order were induced with the PAR-2 peptide agonist SLIGKV and with TNF-α.

Messenger RNA for IL-2 and IL-5 was not detected in unstimulated cells, or following incubation either with tryptase, the PAR-2 agonist or with TNF-α; and that for RANTES was expressed with TNF-α as the stimulus but not with tryptase or the PAR-2 agonist (Fig. 3-6). When tryptase activity was inhibited with 50 μg/ml leupeptin, or inactivated by heating, there was no alteration in expression of mRNA for any of the cytokines investigated. Similarly, VKGILS, the reverse peptide of the PAR-2 agonist was without effect. Densitometry readings from PCR gels and semiquantitative analysis of mRNA are shown (Fig. 3-7).

3.3.4 Cytokine Secretion

No increase in cytokine release into supernatants was observed at 3h, but increased levels of IL-8, GM-CSF, and IL-6 were detected at 6h, 24h, and 48h (Fig. 3-8). Tryptase stimulated IL-8 secretion from epithelial cells in a dose-dependent pattern with maximal concentrations of this cytokine detected at 80 mU tryptase. Comparison of data from these time points, suggested that concentrations of IL-8 appeared maximal at 24 h, and levels remained relatively constant at least until 48h. After 24 hours stimulation, an increase in IL-8 release was observed when as little as 10 mU/ml tryptase was added. Preincubation of tryptase with leupeptin (50mg/ml) or heat inactivation (65°C, 30 min) of this protease abolished its ability to increase IL-8 release (data not shown). The PAR-2 agonist peptide, like tryptase, provoked substantial IL-8 release, whereas the reverse agonist peptide had no effect on IL-8 secretion. TNF-α at 100 U/ml also induced the release of IL-8.

Increased GM-CSF secretion was observed at 24 h following stimulation with tryptase concentrations of some 10 to 20 mU/ml. However, unlike the IL-8 response, secretion appeared to decline with a tryptase concentration of 40 mU/ml. The PAR-2 agonist and TNF also enhanced GM-CSF secretion. Surprisingly the PAR-2 agonist appeared to provoke secretion of GM-CSF

earlier than that of tryptase. Tryptase induced the release of IL-6 at concentrations of 10 and 20 mU/ml, but as was the case with GM-CSF levels, there appeared to be high dose inhibition of the response. Again both the PAR-2 agonist and TNF-α stimulated IL-6 release from epithelial cells and the effects of tryptase on IL-6 secretion were abolished by leupeptin or by heat inactivation of the enzyme. The control peptide VKGILS failed to increase either GM-CSF or IL-6 secretion.

3.3.5 PAR-2 mRNA Expression in Human Airway Epithelial Cells

By RT-PCR analysis it was found that mRNA for PAR-2 was expressed constitutively in the airway epithelial cells studied (Fig. 3-9). Total RNA was extracted and reverse transcribed to cDNA and oligonucleotide primers specific to human PAR-2 were used to directly amplify a 580bp fragment. The PCR product was revealed as a single band of the predicted size by 2% agarose gel electrophoresis. To confirm the identity of this PCR product, the double-stranded DNA was purified and sequenced using an ABI Prism 377 DNA Sequencer. The sequence determined for the DNA fragment was identical to the reference (GeneBank with accession number U34038) for the human PAR-2 gene library. This confirms that the PAR-2 specific transcript is expressed in these cells. When epithelial cells were incubated with tryptase (20 mU/ml), TNF-α (100 U/ml) or the PAR-2 agonist (100 μM) for 3, 6 and 24 h, no alteration in levels of expression of PAR-2 mRNA was observed.

mRNA	5′ sense primer	3′ antisense primer	Product size
APRT	GCTGCGTGCTCATCCGAAAG	AGTCAGTGTTGAGATGATGC	246 bp
IL-1β	AACAGGCTGCTCTGGGATTC	TAAGCCTCGTTATCCCATGT	398 bp
IL-2	GCCACAGAACTGAAACATCT	AGTCAGTGTTGAGATGATGC	252 bp
IL-5	CTGAGGATTCCTGTTCCTGT	CAACTTTCTATTATCCACTC	257 bp
IL-6	ATGAACTCCTTCTCCACAAGCGC	GAAGACCCCTCAGGCTGGACTG	628 bp
IL-8	GCAGCTCTGTGTGAAGGTGCA	CAGACAGAGCTCTCTTCCAT	186 bp
GM-CSF	GCATGTGAATGCCATCCAGG	GCTTGTAGTGGCTGGCCATC	215 bp
PANTES	ATGAAGGTCTCCGCGGCACGCCT CGCTGTC	CTAGCTCATCTCCAAAGAGTTGAT	276 bp
PAR-2	CGG GGC AGG TGA GAG	TGC CAT AGA AAA AGC CAA TA	580 bp

Table 3-1: Oligonucleotides for amplification of specific cDNA

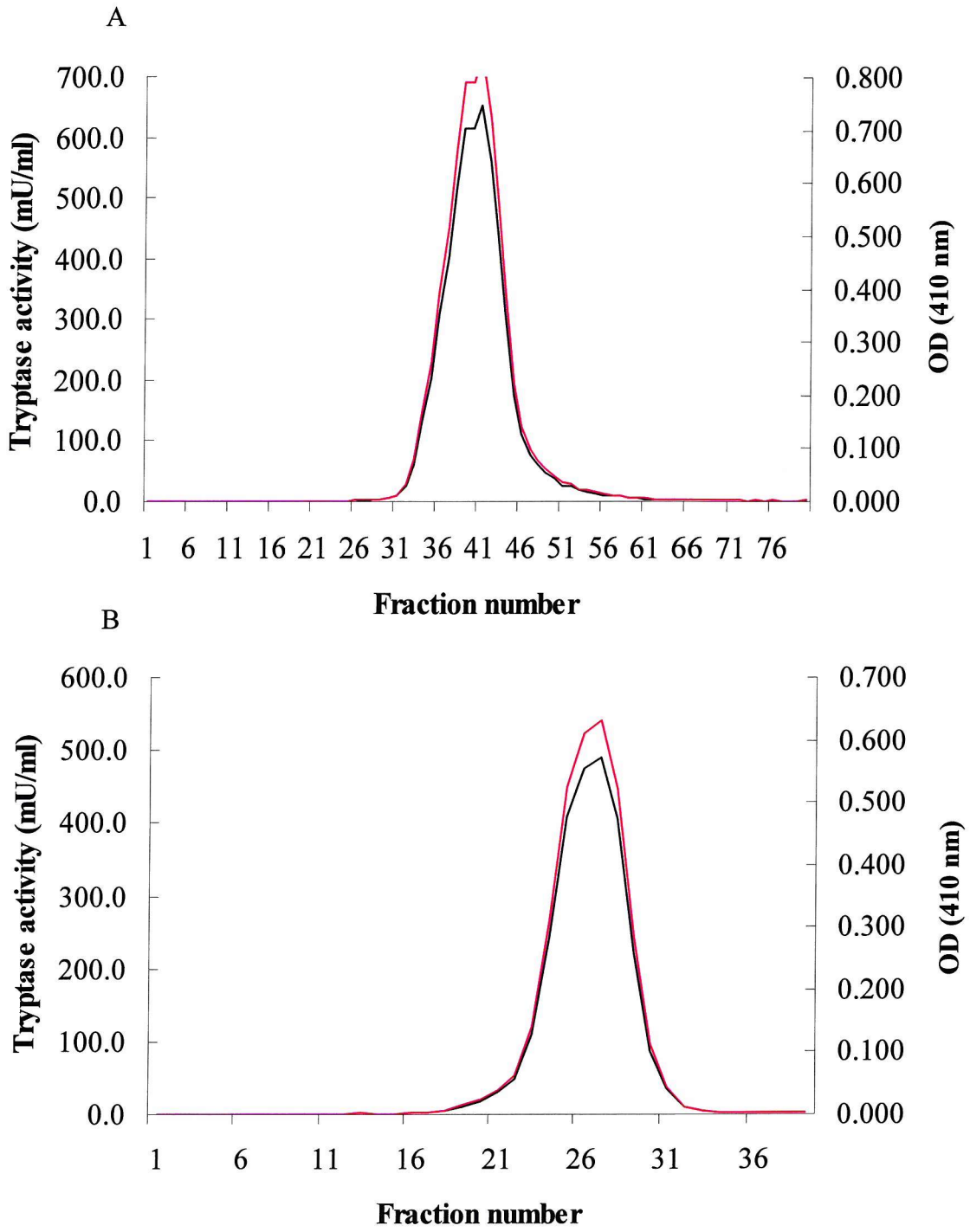


Fig. 3-1 Tryptase activity (blue line) and OD $_{410\ nm}$ (red line) in fractions collected after (A) affinity chromatography and (B) S-200 gel filtration.

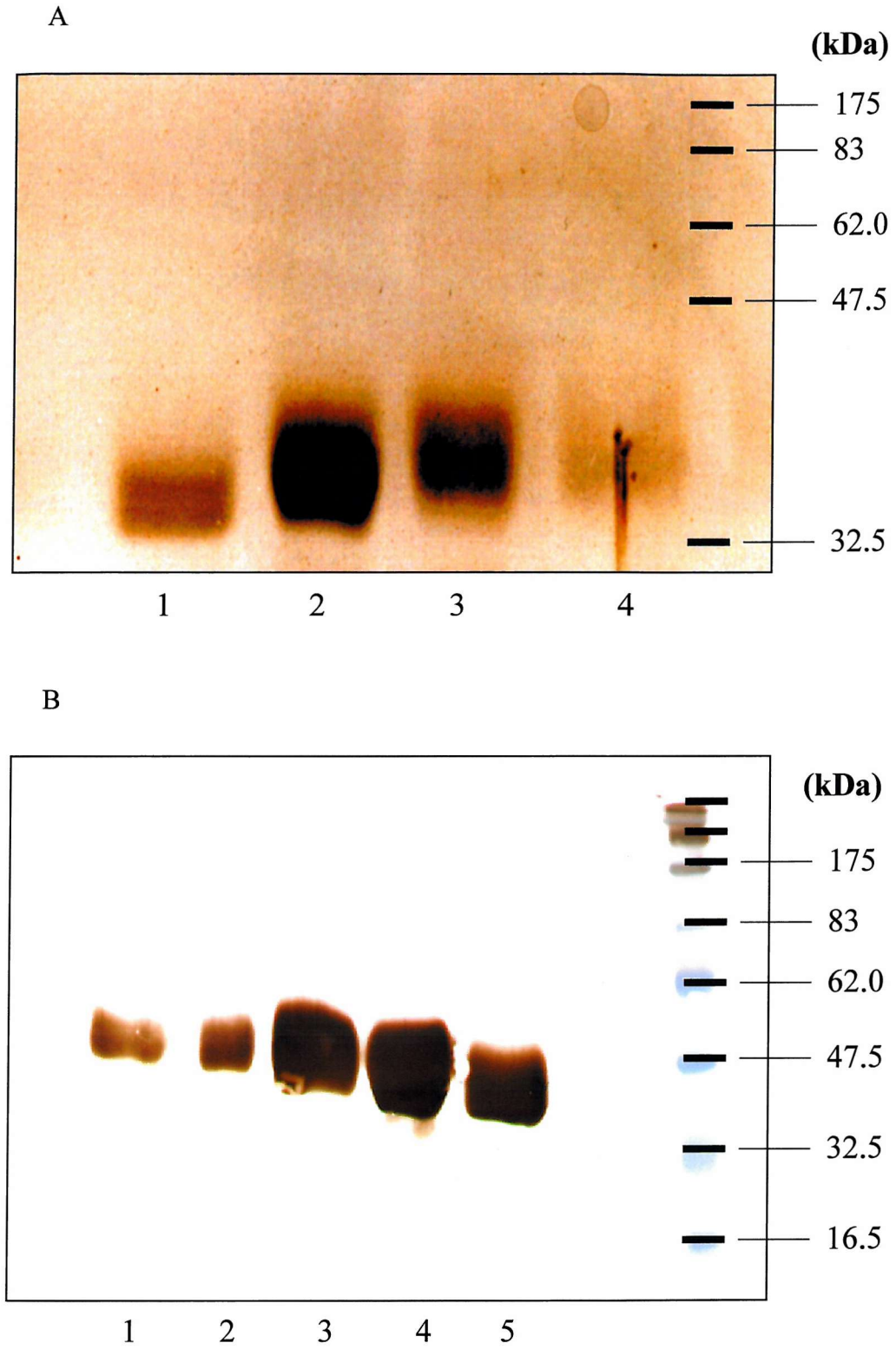


Figure 3-2 Tryptase bands on (A) a silver stained gel (lane 1 to 4 indicated fraction 25 to 28 in S200 gel filtration) and on (B) Western Blots with tryptase specific antibody AA5 (lane 1-5: fraction 24 to 28).

Figure 3-3

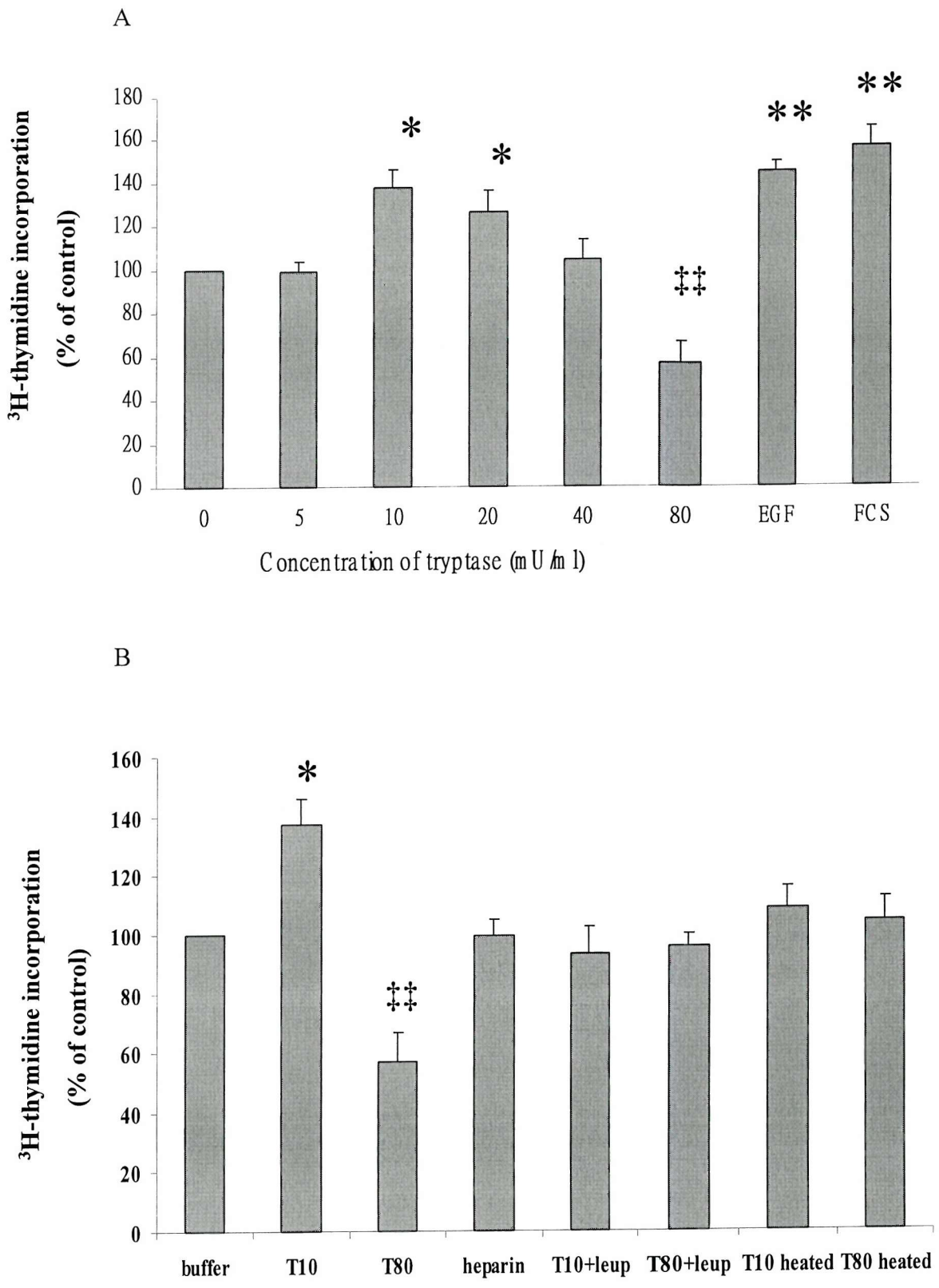

A
^{3}H-thymidine incorporation (% of control)
180
160
140
120
100
80
60
40
20
0
*
*
‡‡
**
**
0
5
10
20
40
80
EGF
FCS
Concentration of tryptase (mU/ml)
B
^{3}H-thymidine incorporation (% of control)
160
140
120
100
80
60
40
20
0
*
‡‡
buffer
T10
T80
heparin
T10+leup
T80+leup
T10 heated
T80 heated

Figure 3-3 ^{3}H-thymidine incorporation in 16HBE 14o- epithelial cells incubated with (A) various concentrations of tryptase, or with EGF 25 ng/ml or 10% FCS, (B) and the effect of preincubating tryptase (T) at 10 mU/ml or 80 mU/ml with buffer or with leupeptin (50 μg/ml; leup), or of heat inactivating the tryptase or of adding heparin alone (10 μg/ml). Mean values (±SEM) are shown for 4 separate experiments each performed with 6 replicates. Significant enhancement of the response (* $P < 0.05$, * * $P < 0.005$) or inhibition (‡‡ $P < 0.005$) compared with the controls is indicated.

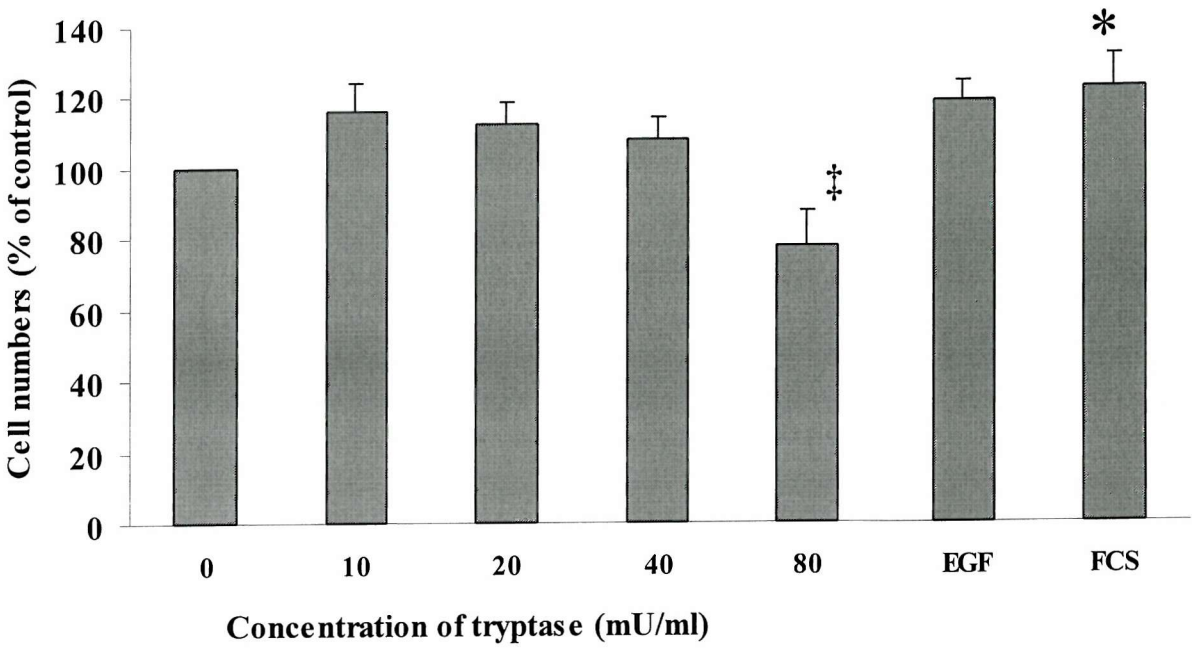


Figure 3-4 Relative numbers of epithelial cells following incubation with various concentrations of tryptase, or with EGF (25 ng/ml) or FCS (10%). Mean values (±SEM) are shown for 3 separate experiments each performed with 6 replicates. A significant enhancement of the response (* $P < 0.05$) or inhibition (‡ $P < 0.05$) compared with the untreated controls is indicated.

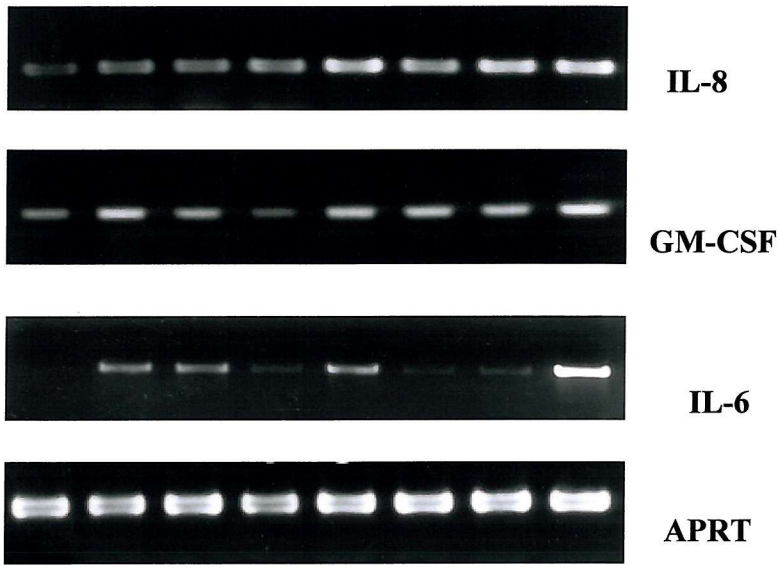


Figure 3-5 Semiquantitative RT-PCR analysis of mRNA for IL-8, GM-CSF, IL-6 and the housekeeping gene APRT following incubation of epithelial cells for 3 h with various concentrations of tryptase, PAR-2 agonist SLIGKV, or with TNF-α. Representative ethidium bromide-stained gels are shown for cells incubated with buffer alone, tryptase (T) at 10 mU/ml, 20 mU/ml, or 40 mU/ml, TNF-α at 100 U/ml, or with the PAR-2 agonist (PA) at 1 μM, 10 μM, or 100 μM.

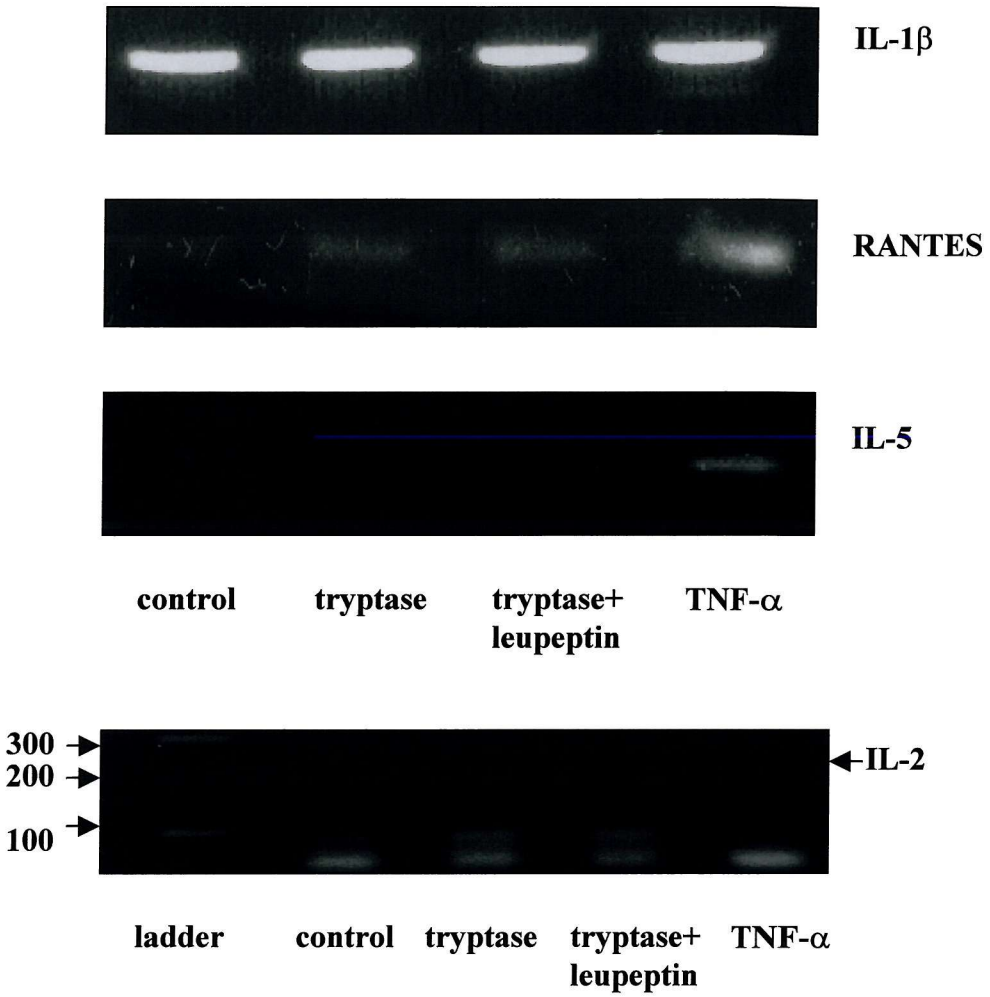


Figure 3-6 Absence of an alteration in mRNA expression for IL-1β, RANTES, IL-5 and IL-2 following addition of tryptase (20 mU/ml) to epithelial cells. Incubation with TNF-α at 100 U/ml slightly enhanced mRNA expression of RANTES and IL-5.

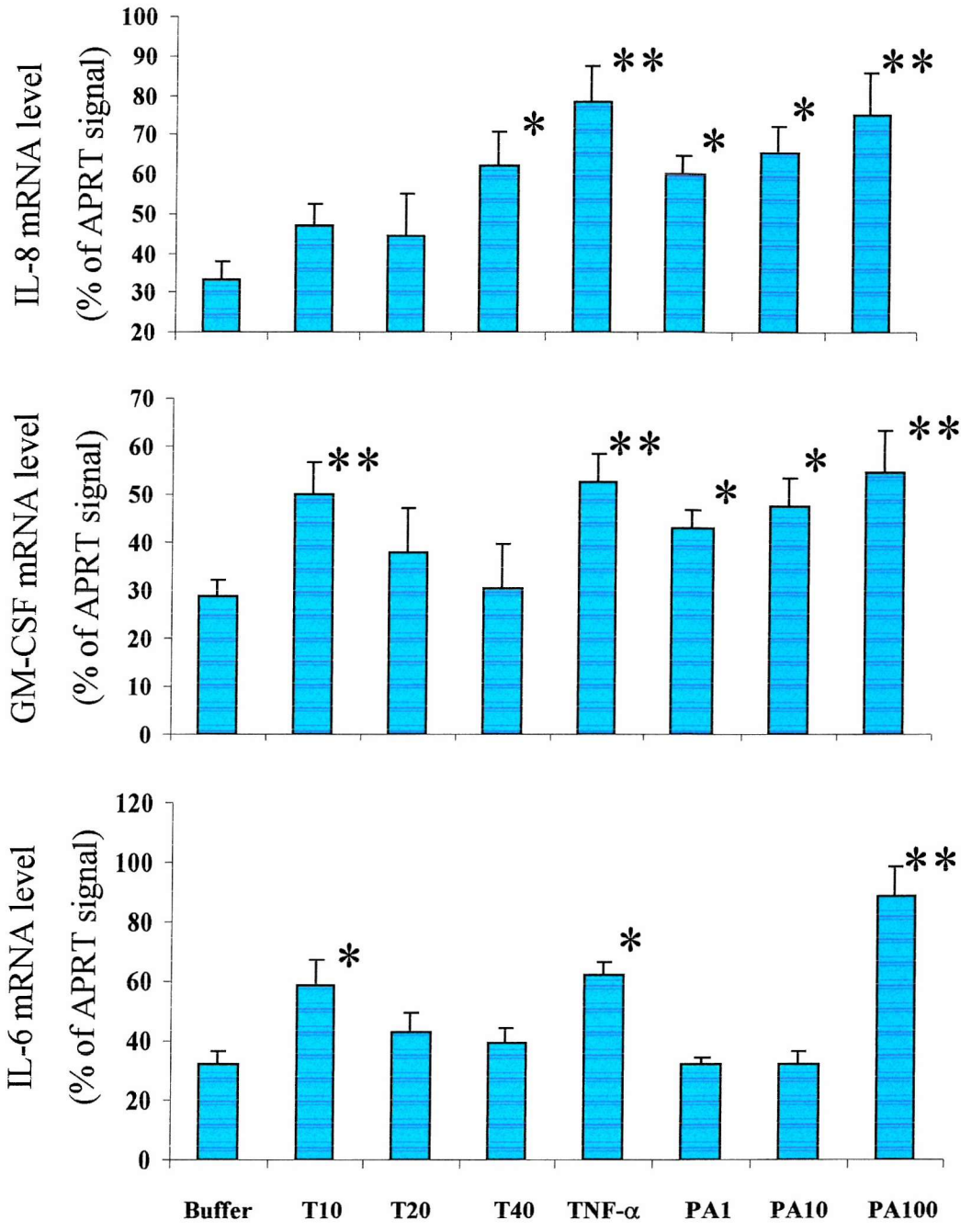


Figure 3-7 Semiquantitative RT-PCR analysis of mRNA for IL-8, GM-CSF, IL-6 and the housekeeping gene APRT following incubation of epithelial cells for 3 h with various concentrations of tryptase, PAR-2 agonist SLIGKV, or with TNF-α. Densitometry readings from PCR gels were determined expressed relative to the signal from APRT. The mean band density (±SEM) is shown for 3 separate experiments. * $P < 0.05$, ** $P < 0.005$ compared with cells incubated with buffer alone.

Figure 3-8

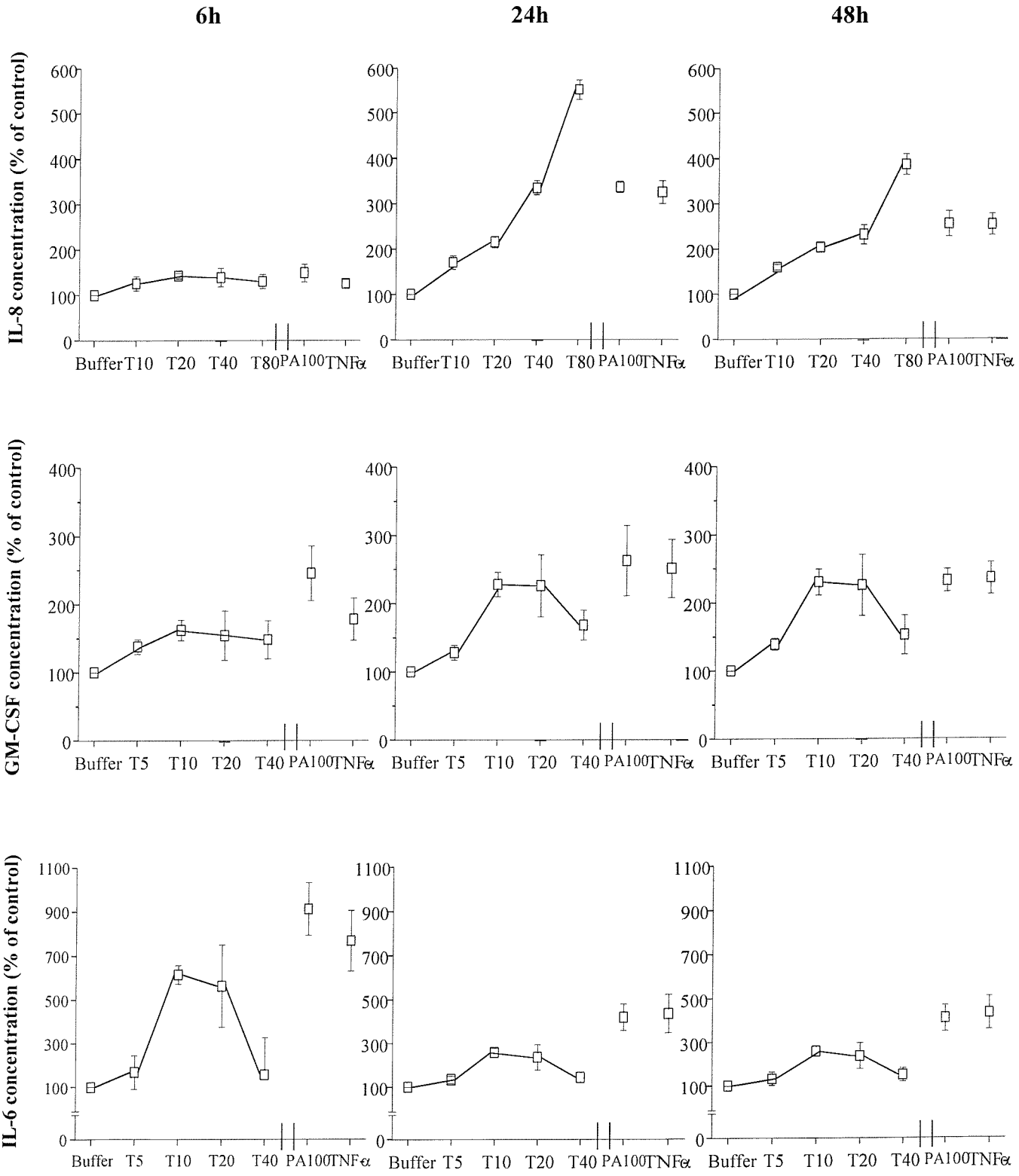

6h
24h
48h
IL-8 concentration (% of control)
GM-CSF concentration (% of control)
IL-6 concentration (% of control)
Buffer
T5
T10
T20
T40
T80
PA100
TNFα

Figure 3-8 IL-8, GM-CSF and IL-6 concentrations in supernatants of epithelial cells incubated for 6 , 24 or 48 h with buffer alone, with various concentrations of tryptase (T) at 20, 40 or 80 mU/ml, or with PAR-2 agonist (PA) at 100 μM, or with TNF-α 100 U/ml (TNF-α). Mean values (±SEM) relative to that of control are shown for four experiments performed in duplicate.

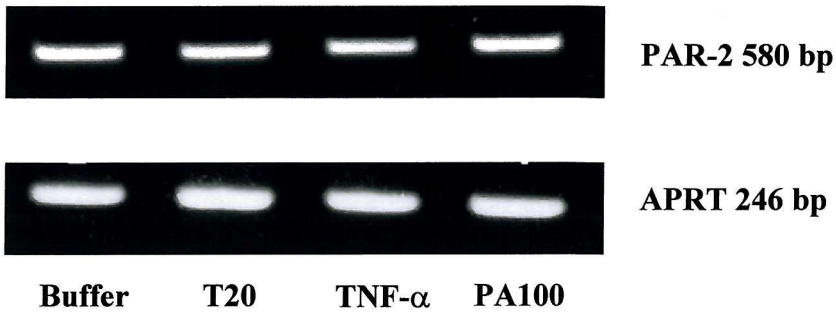


Figure 3-9 RT-PCR analysis of mRNA expression for PAR-2 and APRT in human bronchial epithelial cells incubated for 3 h with buffer alone, 20 mU/ml tryptase (T20), 100 U/ml TNF-α, or 100 μM PAR-2 agonist (PA100). The ethidium bromide stained-gels showed a single band with a 580 bp PCR product which when sequenced was found to be a fragment of the human PAR-2 gene.

CHAPTER 4

MODULATION OF AIRWAY EPITHELIAL PERMEABILITY BY TRYPTASE AND AGONISTS OF PAR-2

4 Modulation of Airway Epithelial Permeability by Tryptase and Agonists of PAR-2

4.1 Introduction

Mast cells may be triggered by inhaled allergen to release a range of potent mediators of inflammation and bronchoconstriction. These products have been implicated in the induction of increased microvascular leakage and airway epithelial permeability in the airways of asthmatics. Allergen challenge of asthmatic subjects can provoke a rapid increase in albumin concentration (a marker of lung permeability) in lavage fluids (Fick et al., 1987); and it has been noted that levels of albumin may be closely correlated with levels of markers of mast cell activation including tryptase and histamine (Salomonsson et al., 1992). However, the mechanisms whereby mast cell products can alter airway epithelial permeability remain unclear.

Tryptase can induce microvascular leakage in animal models (He and Walls, 1997) by a process which appears to involve the activation of mast cells (He et al., 1998; He and Walls, 1997), rather than alterations in endothelial cell permeability (Compton et al., 2000). Certain other proteases including the house dust mite allergen Der p 1 (a cysteine protease) (Wan et al., 1999) and rat chymase (a chymotryptic serine protease) (Scudamore et al., 1998), can increase the permeability of epithelial monolayers in culture, and this has been reported to be associated with the loss of tight junction proteins which normally seal paracellular channels (Wan et al., 1999). It has not been investigated if tryptase or agonists of PAR-2 can alter epithelial permeability or induce alterations in tight junctions.

The aim of the present studies in this section has been to grow monolayers of 16HBE 14o- cells on a porous support and to examine the effects of human mast cell tryptase on permeability. We report that tryptase and agonists of PAR-2 can alter transepithelial resistance, and the paracellular permeability to different sized tracer molecules. We present evidence that tryptase and PAR-2

agonist-induced increases in epithelial permeability are associated with a loss in expression of the tight junction proteins occludin and ZO-1.

4.2 Materials and Methods

Details of methods for the studies in this section are provided below. Procedures used also in the work of other sections are described in Chapter 2 (cell culture and LDH assay).

4.2.1 Cell Preparation

The 16HBE 14o- cells, at a density of 1 x 10^5 cell/insert, were seeded onto Falcon cell culture inserts (0.4 μm pore size) incorporating polyethylene terephthalate track-etched membranes as the growth matrix and the culture inserts were free-standing in 24-well culture plates. Culture medium, 0.3 and 0.8 ml, was added to each culture insert and insert well respectively, and medium was changed every 48 h until the cells had grown to confluence. In preliminary experiments, cells normally became confluent and developed transepithelial resistance (TER) of 300-400 Ω·cm2 by day 5 to 6.

4.2.2 Measurement of Epithelial Cell Permeability

When epithelial monolayers reached a TER of 300-400 Ω·cm2, culture medium was replaced with serum free medium supplemented with 2 % insulin-transferrin-sodium selenite (Sigma, Dorset, UK) and the monolayers were equilibrated for 1 h. Electrical resistance across the confluent monolayers was measured using EVOM micro volt-ohm-meter with a chopstick-styled electrode before and after the addition of agents of interests (Fig. 4-1). Values of TER were obtained by subtracting the contribution of the filter and the bathing solution. To assess the flux of radiolabelled markers, 0.1 μCi ^{14}C-mannitol, 0.5 μCi ^{14}C-inulin or 0.025 μCi ^{14}C-bovine serum albumin (^{14}C-BSA) (Amersham International plc, Amersham, UK) was added to the culture

inserts and the culture plate was agitated gently. The baseline flux of mannitol, inulin or BSA from the apical to basolateral direction was measured from the radioactivity in whole samples removed from wells over a 1 h period. The culture wells were replenished with equal volume of fresh medium after samples were removed for radioactivity counting. At the end of 1 h, the agents of interests were added to inserts and mixed gently. The medium in the culture wells containing radiolabelled markers which had crossed the monolayers was aspirated, added to scintillation cocktail, and counted in a scintillation counter. The cumulative flux in culture wells were determined at each time point and was expressed as counts per minute (CPM).

4.2.3 Inhibition of G Protein

Pertussis toxin was employed to investigate the potential for G protein dependent processes to be involved in tryptase-induced alterations in epithelial permeability. This bacterial toxin can inhibit the actions of G proteins by catalysing the transfer of the ADP-ribose moiety of NAD to the alpha subunit of Gi (Inoue et al., 1990; Murayama and Ui, 1984). Confluent epithelial monolayers were preincubated with pertussis toxin 15 ng/ml for 24 hours, and then medium removed, and replaced with serum-free medium supplemented with 2% insulin-transferrin-sodium selenite. Cells were treated with either tryptase (40 μg/ml), or PAR-2 agonist (500 μM) or pertussis toxin (15 ng/ml) and incubated for 6 h. TER was measured and presented as % change compared with initial value.

4.2.4 Immunostaining of Occludin and ZO-1

After monolayers were exposed to tryptase or agonist peptide for 6 h or 24 h, cultured insert membranes were removed, washed, and fixed in cold ethanol at 4 °C immediately for 30 min followed by additional incubation with cold

acetone for 3 min at room temperature. The samples were blocked with PBS containing 3 % goat serum and 3 % BSA then incubated with mouse monoclonal anti-occludin (2 μg/ml) or anti-ZO-1 (15 μg/ml) overnight at 4 °C. After washes, goat anti-mouse IgG FITC conjugate was employed and incubated for 1 h. The insert membranes were mounted in Citifluor and the localization of occludin and ZO-1 was visualised and photographed using a Leica DMRBE fluorescence microscope with a MPS 60 photographic system. Negative controls were included in which the primary antibodies were replaced with mouse IgG.

4.2.5 Gel Electrophoresis and Immunoblotting

Cells, growing on culture dishes (35mm diameter), were exposed to tryptase or the peptide agonist SLIGKV when confluence was reached. Whole cell extracts were prepared by washing cells with PBS containing aprotinin (1 μg/ml), 4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride (AEBSF) (0.2 mM), E-64[L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane] (20 μM), and EGTA (1 mM). 500 μl of sample buffer (0.125 M Tris-HCl at pH 6.8 with 4 % SDS, 20 % glycerol, 0.1 mg/ml bromophenol blue, and 1.44 M 2-mercaptoethanol) was added to the cells and the lysate was collected and boiled for 10 min. The extracts were electrophoresed on a 7.5 % polyacrylamide gel and transferred to a nitrocellulose membrane. Following the incubation in the blocking buffer (PBS with 5 % skimmed milk and 0.2 % vol/vol Tween-20), the nitrocelluloss membrane was incubated with mouse monoclonal anti-occludin (0.5 μg/ml) or anti-ZO-1 (1 μg/ml) overnight at 4 °C. After appropriate washes, the membrane was incubated with rabbit anti-mouse IgG HRP conjugate for 1 h. The chemiluminescent substrate was added for 5 min after 6 washes with PBS containing 0.2 % Tween. The membrane was then placed in an autoradiographic cassette and the image allowed to develop.

4.2.6 Electron Microscopy

Following exposure of the cell monolayers to agents of interest the culture insert membranes were immediately placed in 3% glutaraldehyde at 4°C in 0.1M sodium cacodylate buffer (pH 7.4). Samples were fixed for at least one hour and transferred to a 0.1M sodium cacodylate (pH7.4) buffer solution. All samples were post-fixed in 1% osmium tetroxide in sodium cacodylate buffer for 90 minutes. The samples were dehydrated through methanols, cleared in propylene oxide and infiltrated overnight and embedded in epoxy resin (Araldite) (Biorad, Herts, UK). Survey 1 μm thick sections were cut and stained with 1% alkaline Toluidine blue for assessment by light microscopy (Axioskop microscope from Carl Zeiss, Oberhochen, Germany). For electron microscopic analysis, ultrathin plastic embedded sections were cut, mounted on uncoated 100-mesh thin-bar grids and stained with uranyl acetate and lead citrate. Micrographs were taken at a magnification of x 98 00 or x 13 000 using a Philips 301 electron microscope.

Certain glutaraldehyde specimens were processed for high resolution scanning electron microscopy (HR-SEM) following storage in 0.1M sodium cacodylate buffer (pH 7.4). Specimens were dehydrated through a graded series of ethanols (70-100%) and washed twice in absolute ethanol prior to critical point drying. Immediately following completion of critical point drying, samples were sputter coated with gold (Polaron E5300) to conduct charge as required for HR-SEM. Samples were subsequently mounted and viewed in a Hitachi 54000 (Nissei Sangyo).

4.2.7 LDH Assay

Cell viability was determined using colorimetric method to measure lactate dehydrogenase (LDH) activity. Supernatants were collected from the culture inserts and wells after incubation with agents of interests for 24 h and the results were expressed as a percentage of total cell LDH determined after homogenization of matched insert membrane of untreated epithelial cells. Morphologically, epithelial monolayers were observed under light microscope and examined using trypan blue exclusion test at the end of each experiment.

4.3 Results

4.3.1 Alterations in TER

At the highest concentration employed (80 mU/ml), tryptase induced a progressive decrease in TER across monolayers of 16HBE 14o- cells (Fig. 4-2). By six hours, TER had declined by approximately 60 % ($P<0.001$), though it appeared to have returned to the initial levels by 24 h. In contrast, with lower concentrations of tryptase (10 or 20 mU/ml), there was an increase in TER of approximately 50 % ($P<0.02$) achieved within 2 h of stimulation, and this increase was still apparent at 24 h. At an intermediate concentration (40 mU/ml), no alteration in TER was observed. The effect of tryptase on TER (both at 80 mU/ml or 10 mU/ml) was abolished by the tryptase inhibitor, leupeptin (50 μg/ml), or by heat inactivation of the protease.

The effect on TER of adding trypsin to epithelial cell monolayers was similar to that with tryptase. Once again at the higher concentration (25 or 50 ng/ml), there was a progressive decline in TER, whereas at a lower concentration (6.25 ng/ml), trypsin increased TER ($P<0.001$) (Fig. 4-3). The peptide agonist for PAR-2, SLIGKV, induced a drop in TER ($P<0.001$) (Fig. 4-4) at a concentration of 250 μM. No apparent increase in TER was observed with a range of lower concentrations of agonist, including 15.6 μM and 7.8 μM. A non-PAR-2 activating peptide with a similar amino acid composition (LSIGKV), was without effect on TER when employed at the same concentration as used with SLIGKV. TNF-α at concentrations of 10 to 200 ng/ml also failed to induce alterations in TER (Fig. 4-5).

Pertussis toxin failed to inhibit the decline in TER induced by tryptase at 6 h, either when cells were treated with this G_i protein inhibitor for 24 h or when it was added at the same time as tryptase (Fig. 4-6). Pretreatment of cells with pertussis toxin did abolish the PAR-2 agonist induced decline in TER, though

this effect was not observed when SLIGKV and pertussis toxin were added simultaneously.

Cytotoxic reactions were not observed in response to tryptase (80 mU/ml) or the PAR-2 agonist SLIGKV (500 μM). Following incubation for 24 h, light microscopic examination revealed that the monolayers of 16HBE 14o- cells remained firmly attached to the underlying porous support. Moreover, the cells were more than 98% viable as indicated by trypan blue exclusion. Cell viability was also assessed by measuring lactate dehydrogenase (LDH) activity in both supernatants and cell lysates. LDH release from cell monolayers exposed to tryptase or SLIGKV for 6 h and 24 h did not differ from that from monolayers incubated with buffer alone (Fig. 4-7).

The effect of adding tryptase or SLIGKV to either the apical or basolateral side of monolayers was investigated. Tryptase decreased TER whether added to chambers on the apical or basal side of monolayers. However, the response to stimulation the basolateral side appeared faster than that of the apical side ($P<0.02$, $n=4$). In contrast, addition of the PAR-2 agonist SLIGKV to the apical side induced a greater decline in TER than when added to the basal side ($P<0.002$, $n=4$) (Fig. 4-8).

4.3.2 Epithelial Permeability

The ability of tryptase and the peptide agonist SLIGKV to alter the permeability of epithelial cell monolayers was investigated by measuring transepithelial flux of ^{14}C-labelled mannitol, inulin and BSA. These hydrophilic molecules diffuse by the paracellular route across epithelial monolayers and the rate of flux will be governed by the leakiness of tight junctions. Concentrations were selected for tryptase (80 mU/ml) or the PAR-2 agonist (500 μM) on the basis of the investigations of TER, choosing those doses which induced maximal responses without apparent damage to the cells. Tryptase induced a significant increase in epithelial permeability to mannitol

within 4 h ($P<0.05$) (Fig. 4-9A) This effect was maintained for the rest of the incubation period, and by 24 h there was an increase in cumulative flux for mannitol of approximately 2.5 fold ($P<0.005$) compared with that with unstimulated monolayers. Tryptase-induced permeability to the larger tracer inulin was less pronounced, and an increase in transepithelial flux of about 1.7 fold was achieved at 24 h ($P<0.005$) (Fig. 4-9B). With BSA as tracer, no alteration in permeability was detected (Fig. 4-9C). Administration of tryptase in the presence of the protease inhibitor leupeptin or heat inactivating the enzyme, abolished the increase in permeability in response to mannitol at 24 h (Fig. 4-10). Findings with the peptide agonist for PAR-2, SLIGKV, mirrored those with tryptase, inducing an increase in flux to mannitol of approximately 2.8 fold and for inulin of 2.3 fold without altering the permeability to BSA (Fig. 4-9). At the same concentration, the peptide LSIGKV was without effect on epithelial permeability to mannitol (Fig. 4-10).

4.3.3 Immunostaining of Occludin and ZO-1

Immunocytochemical studies with fluoresceinated specific antibodies revealed the tight junction-associated proteins, occludin and ZO-1, as continuous bands around unstimulated 16HBE 14o- cells in confluent monolayers. Following incubation of monolayers with tryptase for 6 h, the areas of staining for both occludin (Fig. 4-11) and ZO-1 (Fig. 4-12) became discontinuous and less sharply defined. At 24 h following addition of tryptase, there appeared to be more extensive loss of occludin and ZO-1. The same pattern of findings was observed with the PAR-2 agonist SLIGKV at these time points.

4.3.4 Western Blotting for Occludin and ZO-1

Occludin was detected as a band of 65 kDa in Western blots of whole extracts of 16HBE 14o- cells. In cells incubated with tryptase for 6 h, there was a diminution in the size of the occludin band and this was more pronounced at 24 h (Fig. 4-13A). The protease inhibitor abolished the actions of tryptase. ZO-1 appeared as a band of 225 kDa on Western blots. As with occludin, there was an apparent loss of this protein from cells treated with tryptase (Fig. 4-13B), but not with SLIGKV.

4.3.5 Examination of Epithelial Layers by Light and Electron Microscopy

When sections of 16HBE14o- cells growing on the porous filters were examined under the light microscope, it was found that cells were frequently three to four cell layers deep, rather than strictly "monolayers". The mean depth of the cell 'layers' was approximately 15um. No obvious differences were apparent between the cells treated with tryptase (80 mU/ml), SLIGKV (500 μM), or buffer for 24 h, when three specimens of each were examined.

By electron microscopy, the apical plasma membranes of the 16HBE14o- cells were found to possess numerous microvilli with the usual complement of organelles apparent in the cytoplasm (for example Golgi apparatus, mitochondria, rough endoplasmic reticulum and the presence of well developed nuclei with nucleoli). No cilia were observed within the cultures. At the apico-lateral borders of the cells there was an apparent 'fusion' between the outer leaflets of the apposed plasma membranes, characteristic of epithelial tight junctions. Numerous desmosomes (adhering junctions) were also present between the epithelial cells. Protrusions from the basal surface of the cells into the micropores of the culture inserts were evident (mean depth approximately 1.5 μm) but these protrusions never extended far into the filter and no cellular material was found on the under-surface of the filter. No

obvious differences between the control, tryptase and PAR-2 agonist treated monolayer groups were apparent.

4.3.6 High-Resolution Scanning Electron Microscopy (HR-SEM)

HR-SEM clearly showed the entire surface of the microporous filters, in the control, tryptase and PAR-2 agonist treated monolayer groups to be completely covered with 16HBE14o- cells (Fig. 4-14). In places the 16HBE14o- cells showed a classic "cobblestone" appearance (ie cuboidal/columnar shaped cells) but within the monolayers areas containing more flat "squamous" cells were evident. No obvious differences between the control and treatment groups were evident. The apical membrane of each cell was clearly identified by the presence of numerous well-developed surface microvilli, but well-developed cilia were not found. When the area of the cell borders was examined at higher power, no signs of damage, detachment or loss of cells were found in either of the control, tryptase and PAR-2 agonist treated monolayer groups.

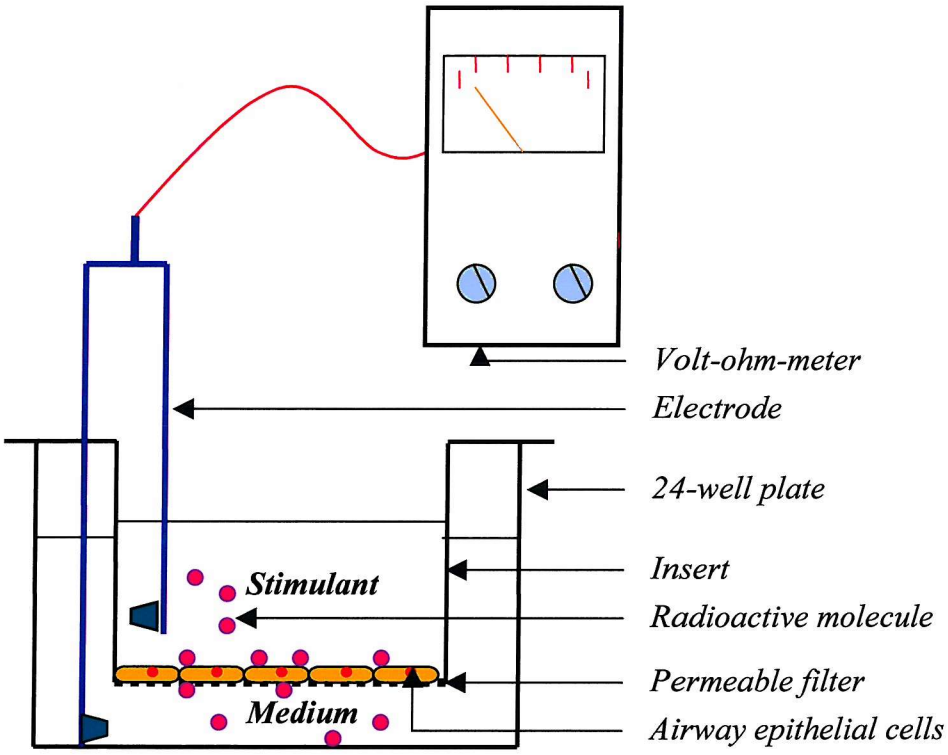


Figure 4-1 Assessment of epithelial permeability by measuring transepithelial resistance and the flux of radiolabeled hydrophilic molecules across monolayers.

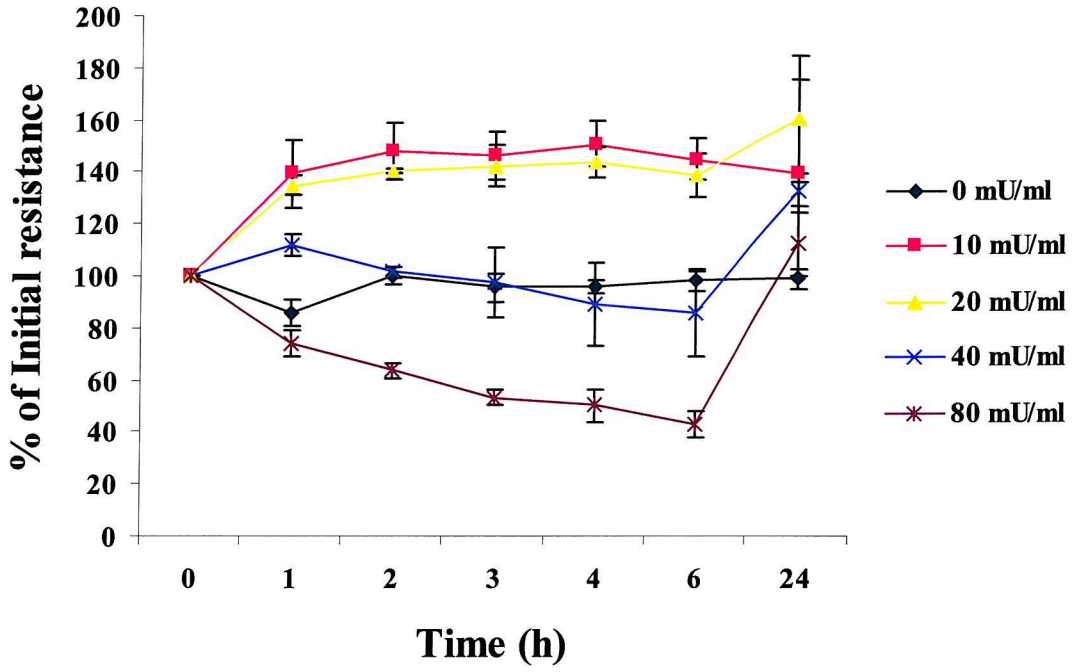


Figure 4-2 Time and dose dependent changes in TER following exposure of bronchial epithelial monolayers to various concentrations of tryptase from apical direction. Results are expressed as a percentage of initial resistance at time zero. Mean ± SEM are shown (n=4).

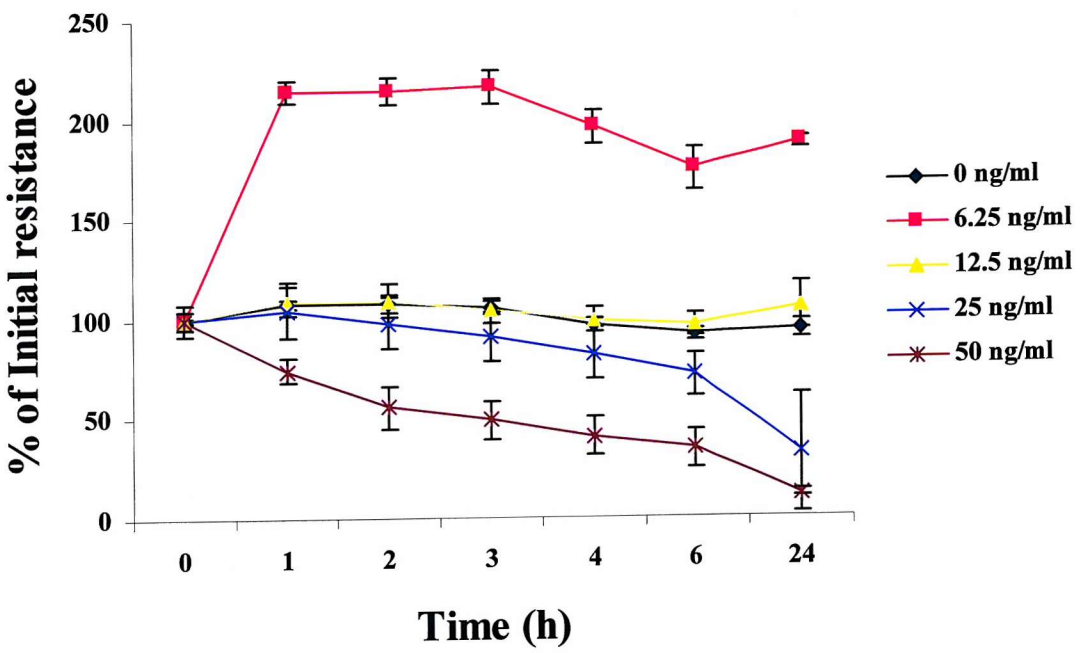


Figure 4-3 Effect of various concentrations of trypsin on apical side on TER across bronchial epithelial monolayers. Mean ± SEM are shown (n=4).

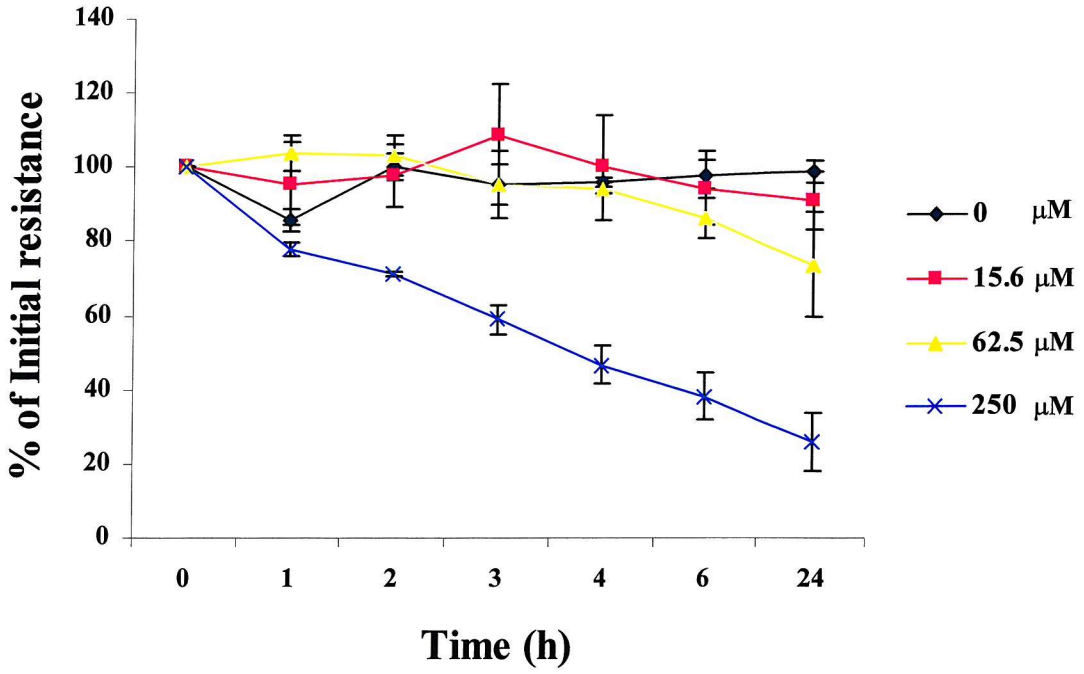


Figure 4-4 Alterations in TER following exposure of bronchial epithelial monolayers to various concentrations of PAR-2 agonists from apical direction. Mean ± SEM are shown (n=4).

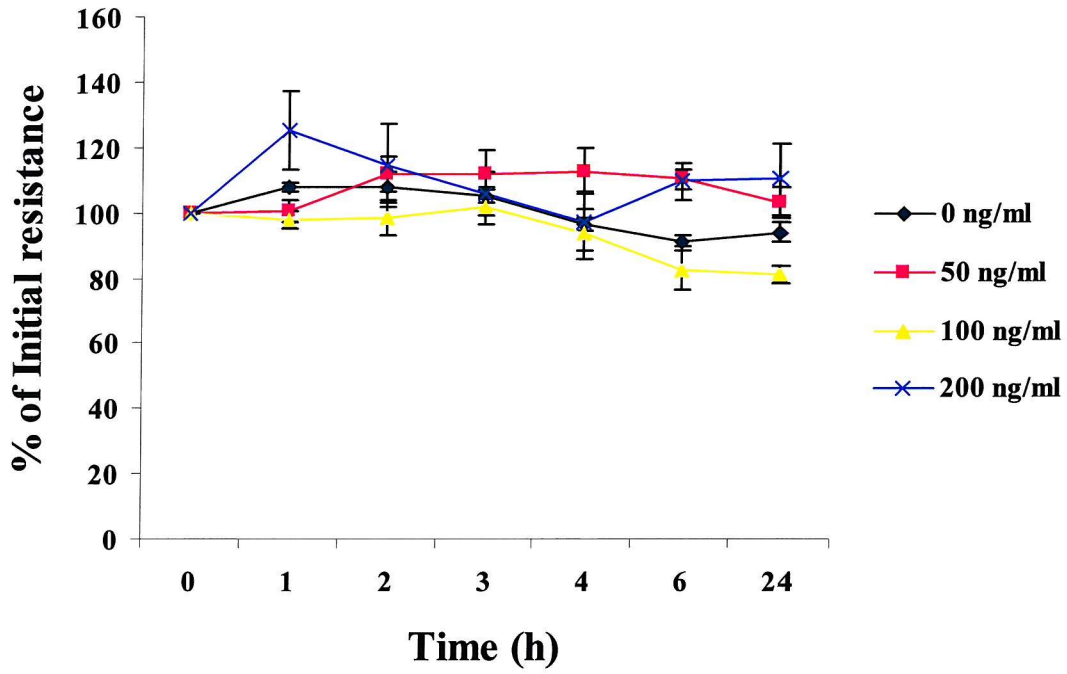


Figure 4-5 Effect of adding various concentrations of TNF-α on apical side for periods up to 24 h on transepithelial resistance (TER) across bronchial epithelial monolayers. Mean ± SEM are shown (n = 4).

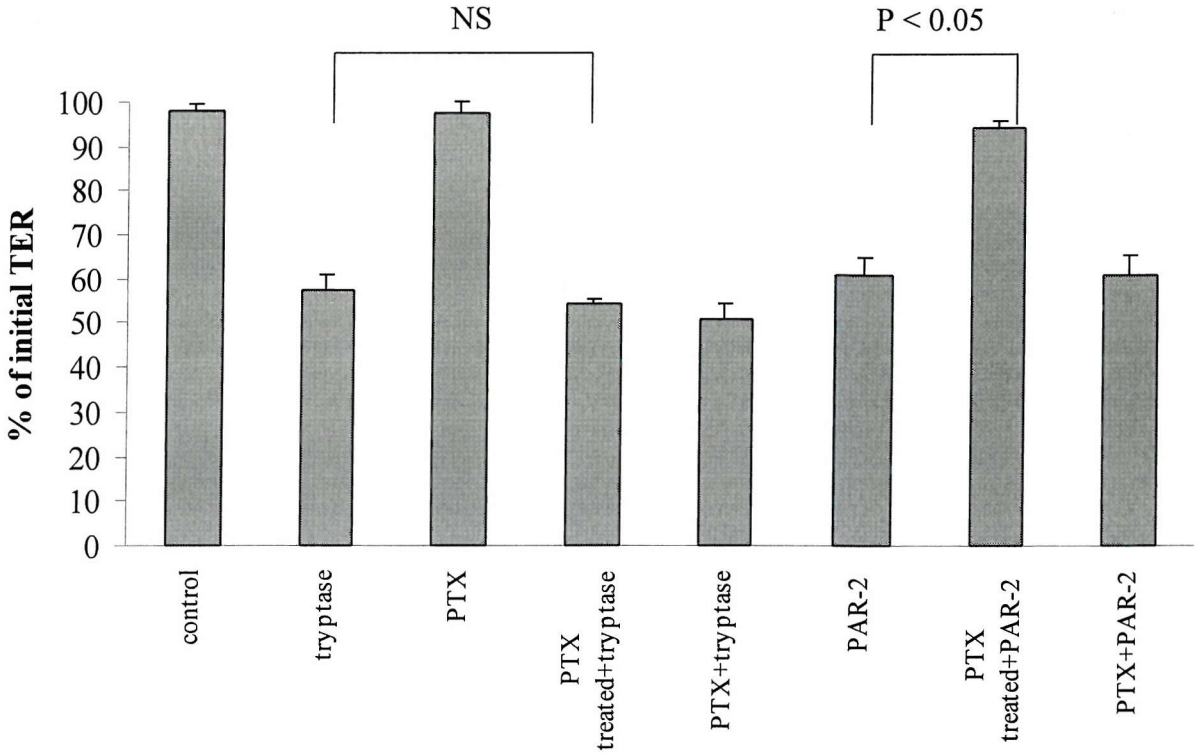


Figure 4-6 Effect of pertussis toxin (PTX) on the reduction in TER induced by tryptase or PAR-2 agonist SLIGKV. Cells were preincubated with pertussis toxin (PTX, 15 ng/ml) for 24 hours then were treated with either tryptase (80 mU/ml), the PAR-2 agonist (500 μM) or with pertussis toxin alone. Pertussis toxin was added to cells at the same time as tryptase (PTX+tryptase) or the PAR-2 agonist (PTX+PAR-2 agonist). TER was measured 6 h after incubation and expressed as a percentage of the initial value. Mean ± SEM (n=4) are shown.

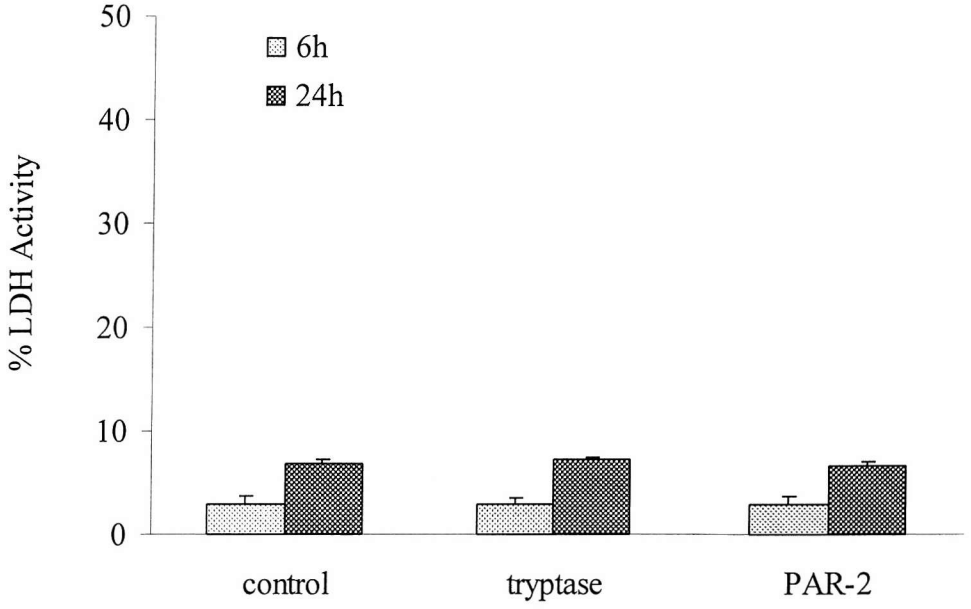


Figure 4-7 LDH activity in supernatants of the epithelial cells treated with buffer alone, tryptase (80 mU/ml) or PAR-2 (500 μM) for 6 h or 24 h. Data are expressed as a percentage of the total levels in cell lysates and supernatants. Mean ± SEM (n=4) are shown.

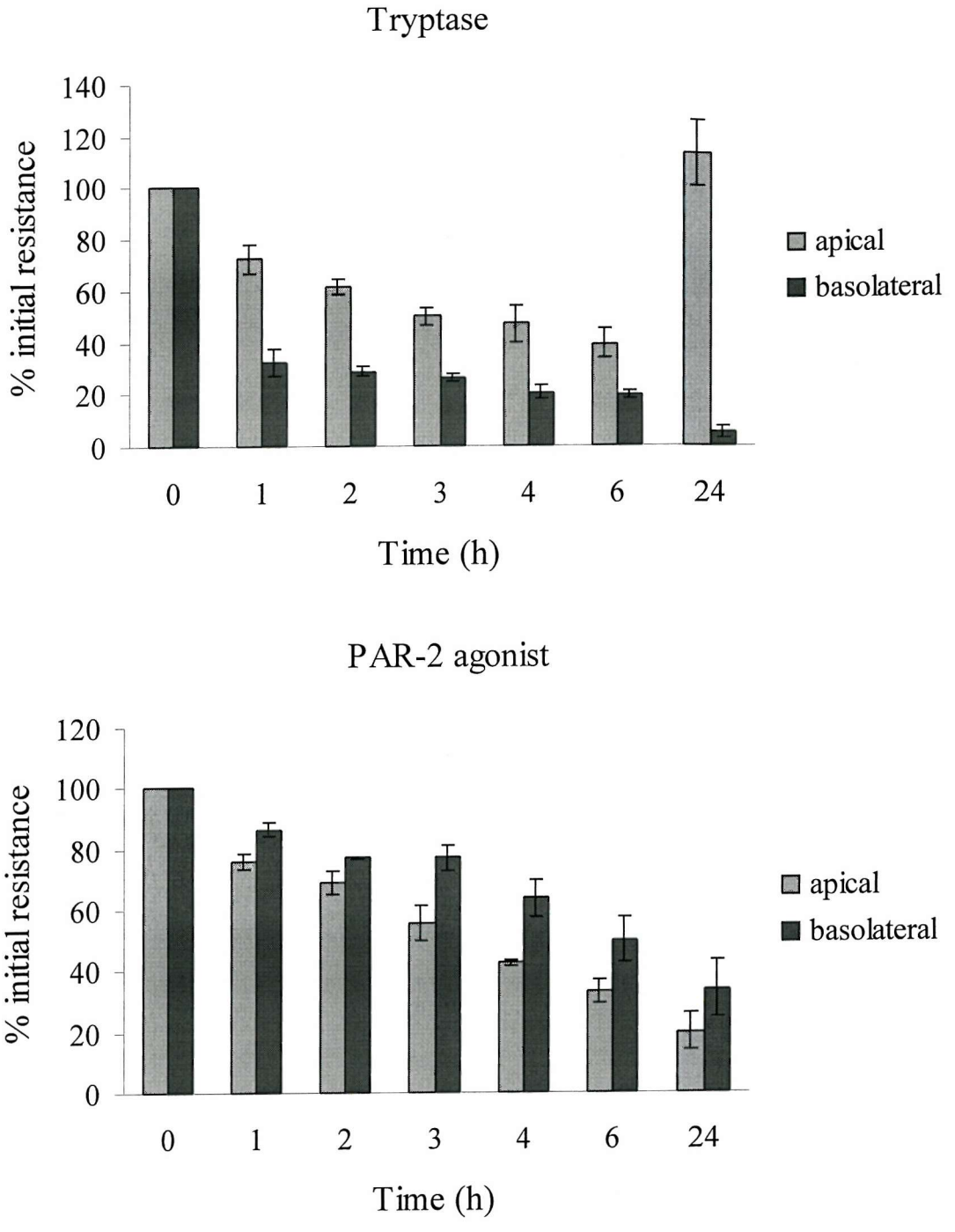


Figure 4-8 The effect of tryptase and PAR-2 agonists on TER with apical and basolateral stimulation. Tryptase (80 mU/ml) or PAR-2 (250 μM) was added to apical or basal chambers and TER was measured at different time points. Data are expressed as a percentage of the initial transepithelial resistance. Mean ± SEM (n=4) are shown.

Figure 4-9

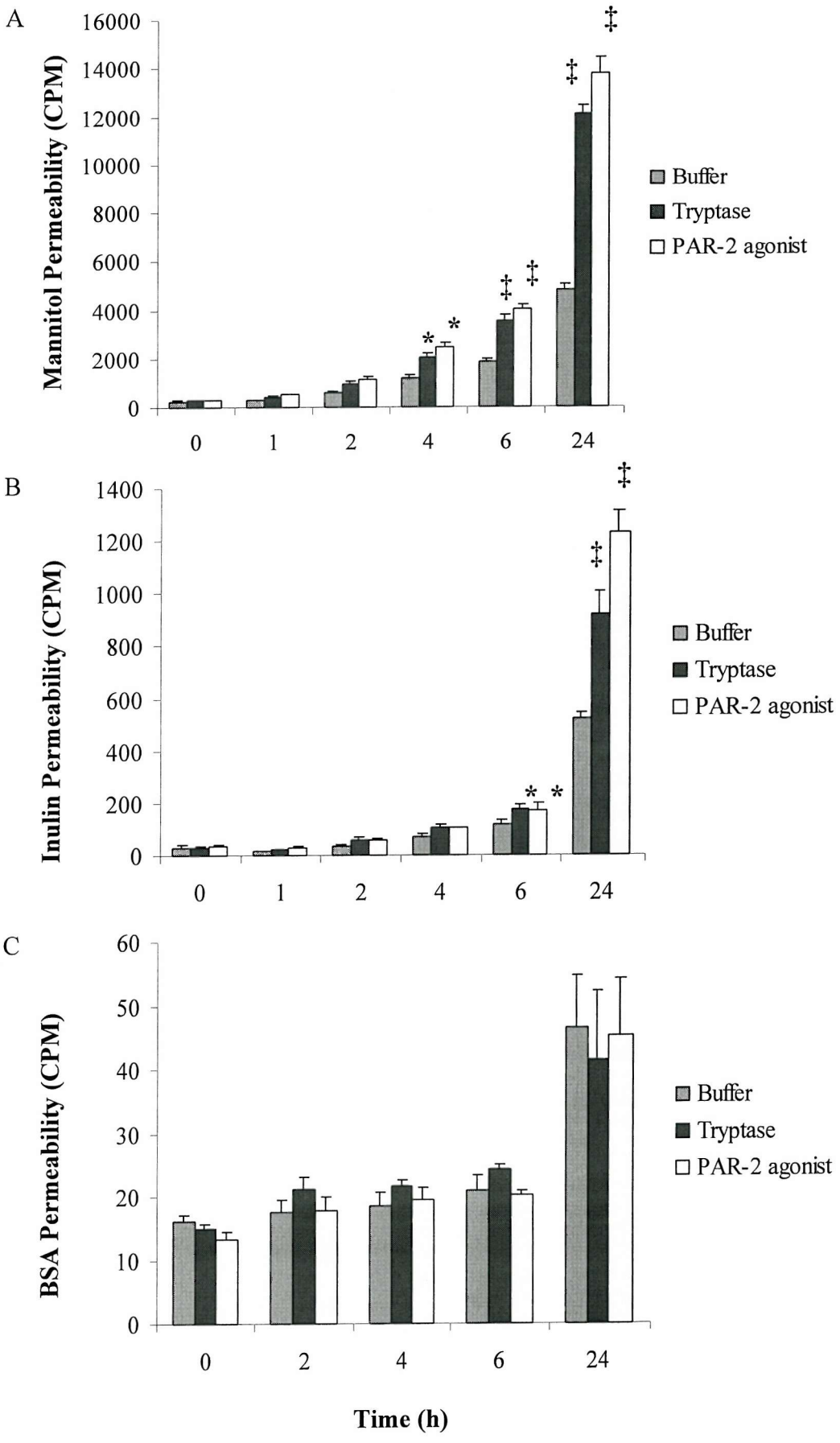

A
Mannitol Permeability (CPM)
16000
14000
12000
10000
8000
6000
4000
2000
0
0
1
2
4
6
24
Buffer
Tryptase
PAR-2 agonist
B
Inulin Permeability (CPM)
1400
1200
1000
800
600
400
200
0
C
BSA Permeability (CPM)
60
50
40
30
20
10
0
Time (h)

Figure 4-9 Effect of adding tryptase (80 mU/ml) or PAR-2 agonist (500 μM) or buffer alone to the apical surface of bronchial epithelial monolayers (grown on inserts) on the permeability to (A) mannitol (MW 184), (B) inulin (MW 5200) and (C) bovine serum albumin (BSA, MW 66,000). * $P < 0.05$ or ‡ indicates $P < 0.005$ compared to controls. All experiments were performed in duplicate with 6 separate experiments for investigation of mannitol and inulin permeability, and 4 for BSA permeability.

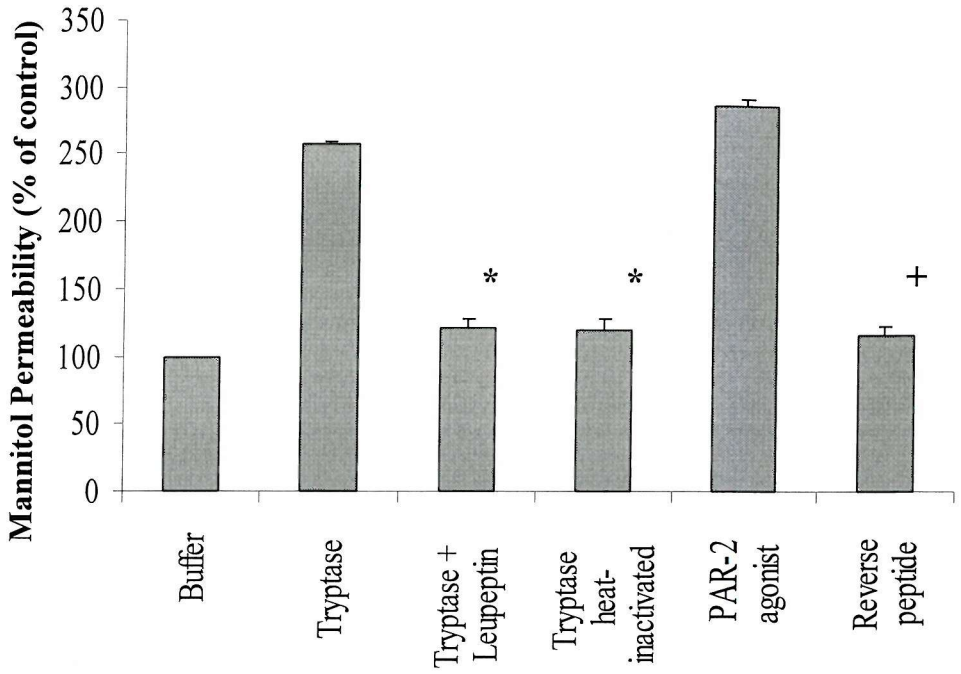


Figure 4-10 Mannitol permeability in epithelial monolayers treated with buffer alone, or with tryptase (80 mU/ml), tryptase preincuabted with leupeptin (50 μg/ml), heat inactivated tryptase (65 °C for 30 min), the PAR-2 agonist SLIGKV (500 μM), or reverse peptide LSIGKV (500μM). Mean values (±SEM) are shown for 4 separate experiments each performed in duplicate. * $P < 0.05$ compared with uninhibited tryptase; $^{+}$ $P < 0.05$ compared with the PAR-2 agonist.

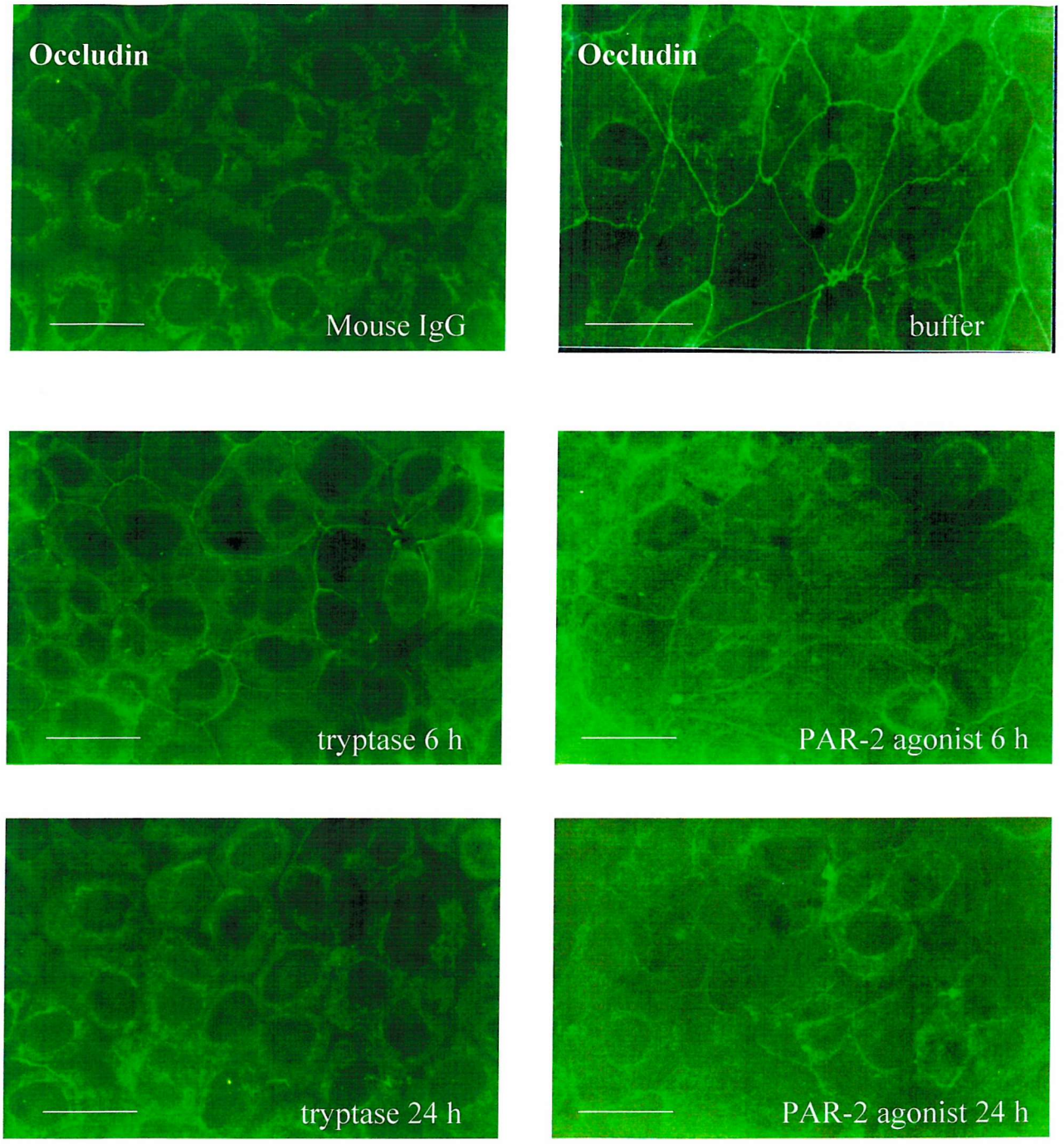


Figure 4-11 Immunostaining of the tight junction protein occludin in epithelial cells incubated with medium alone (buffer) or with tryptase (80 mU/ml), or with the PAR-2 agonist SLIGKV (500 μM), for 6 h or 24 h. Mouse IgG was used as a negative control. Scale bar, 20 μm.

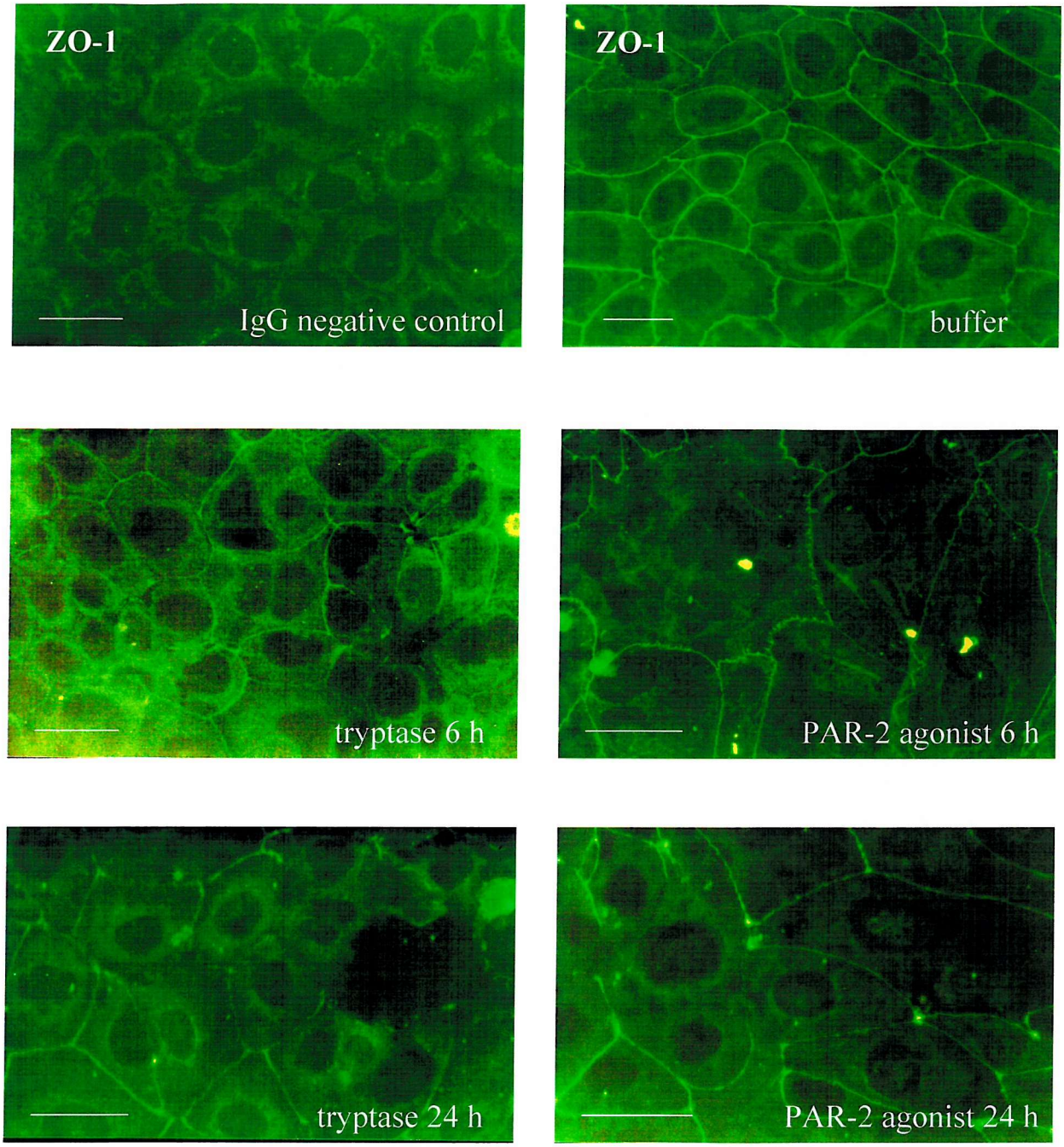
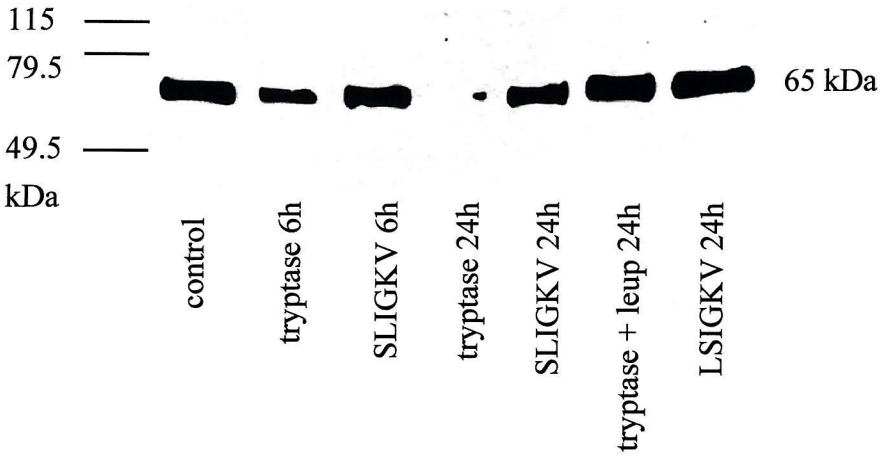


Figure 4-12 Immunostaining of the tight junction protein ZO-1 in epithelial cells incubated with medium alone (buffer) or with tryptase (80 mU/ml), or with the PAR-2 agonist SLIGKV (500 μM), for 6 h or 24 h. Mouse IgG was used as a negative control. Scale bar, 20 μM.

A



B

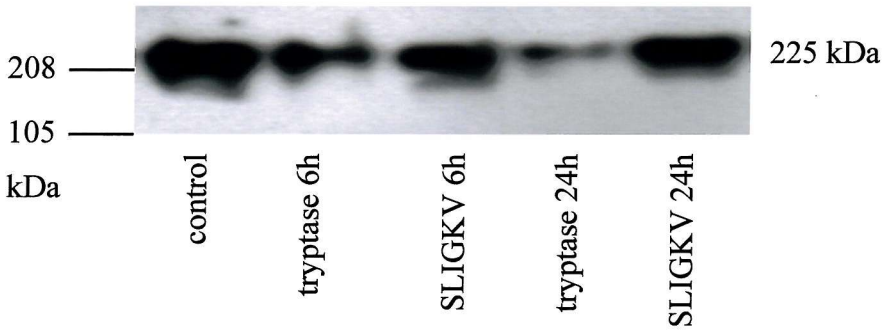


Figure 4-13 Immunoblots for occludin (A) and ZO-1 (B) from 16HBE 14o- cells. Whole cell extracts were prepared from cells incubated with buffer, or with tryptase (80 mU/ml), or with the PAR-2 agonist SLIGKV 500 μM (PA) for 6 h and 24 h respectively. As control, tryptase was preincubated with leupeptin prior to addition and the reverse peptide LSIGKV (500 μM) was employed for 24 h.

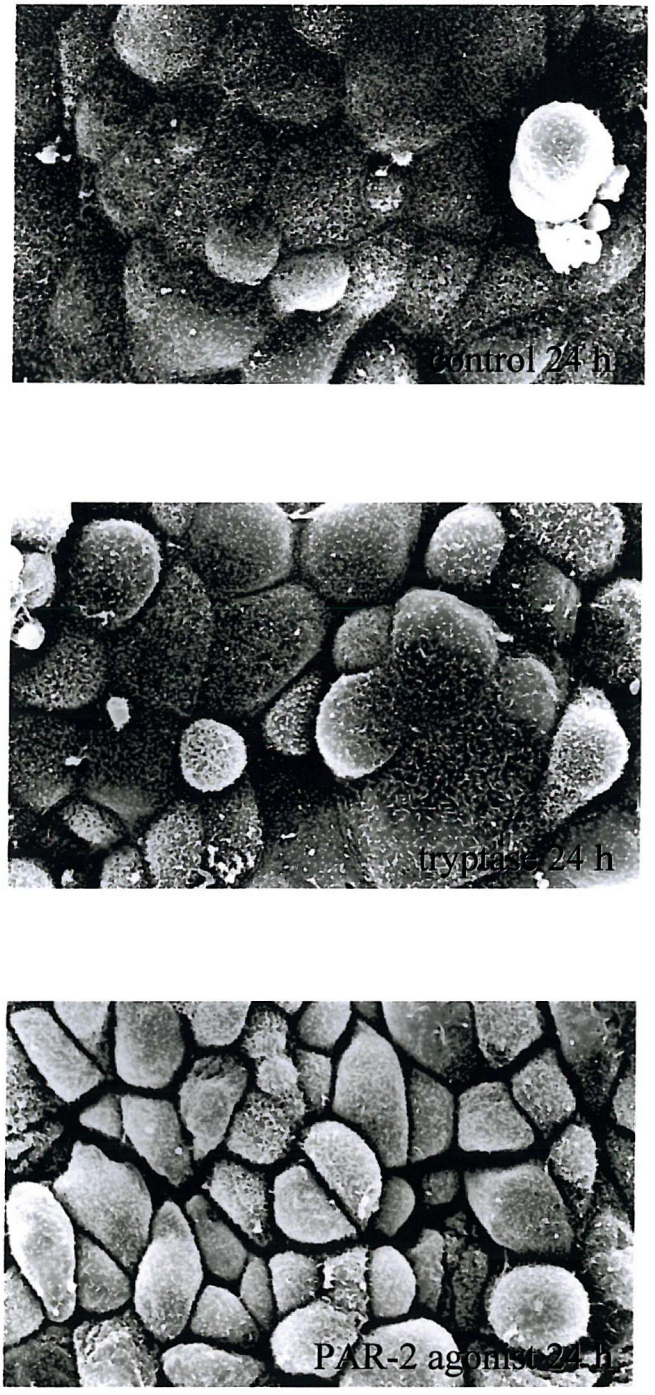


Figure 4-14 Morphology of epithelial cell monolayers. Cells grown on the insert were incubated with buffer, tryptase (80 mU/ml) or the PAR-2 peptide agonist SLIGKV (500 μM) for 24 h. Following measurement of TER and monolayer permeability, insert membranes were removed and processed for high-resolution scanning electron microscopy.

CHAPTER 5

TRYPTASE AND THE ACTIVATION OF PAR-2 AS STIMULI FOR NEUTROPHIL TRANSEPITHELIAL MIGRATION

5 Tryptase and Activation of PAR-2 as Stimuli for Neutrophil Transepithelial Migration

5.1 Introduction

Neutrophils, the predominant inflammatory cells recruited to sites of inflammation, have been shown to be involved in asthmatic airways during spontaneous asthma exacerbations (Fahy et al., 1995), asthmatic airways provoked by allergen or sensitising agents (Nocker et al., 1999; Teran et al., 1996), severe persistent asthma (Jatakanon et al., 1999), and in the airways associated with fatal attack of asthma (Carroll et al., 1996; Sur et al., 1993). Neutrophil numbers in sputum from patients with severe asthma have been found to be higher than those from mild asthma or normal subjects (Jatakanon et al., 1999). Increased neutrophil chemotactic activity has been reported in bronchoalveolar lavage fluid at 4 h after segmental allergen challenge in asthmatic airways (Nocker et al., 1999). However, the mechanisms whereby neutrophils are recruited to asthmatic airways remain unclear.

The accumulation of large numbers of neutrophils and eosinophils has been observed within 3 or 6 h of injecting tryptase into the skin of guinea pigs or the mouse peritoneum (He et al., 1997a). In addition, tryptase has been found to act as a chemoattractant for purified neutrophils and eosinophils in vitro and to stimulate the release of eosinophilic cationic protein from eosinophils (Walls et al., 1995). Moreover, tryptase can provoke IL-8 release from epithelial cells (Cairns and Walls, 1996) and endothelial cells (Compton et al., 1998). The potential of tryptase and agonists of PAR-2 to stimulate granulocyte migration through the epithelium has not been investigated.

The aim of the experiments in this section has been to investigate the ability of tryptase to induce the migration of neutrophils across monolayers of human airway epithelial cells and to examine the potential role of PAR-2 activation.

In parallel studies, we have investigated to what extent neutrophil chemotaxis might be provoked by tryptase in the absence of epithelial cells.

5.2 Materials and Methods

Details of methods for the studies in this section are provided below. Procedures used in the work of other sections are described in Chapter 2 (cell culture and cytokine assay).

5.2.1 Cell Preparation

While confluent, cells were harvested and seeded (100 μl) at a density of 1 × 10^6 cells/ml, onto the lower surface of Corning culture inserts (3.0 μm pore size, 6.5 mm diameter). Cells grown on the lower surface of inserts were first placed on inverted collagen-coated (collagen type IV, 50 μg/cm^2) filters and incubated for 4 h at 37°C. The filters were then turned upward so that the culture inserts were free-standing in 24-well culture plates. Culture medium, in an aliquot of 0.2 or 0.5 ml was added to each culture insert or well respectively, and medium was changed every 48 h until the cells had grown to confluence. At the end of each experiment, the viability of cells growing on the inserts was confirmed by trypan blue exclusion under the light microscope.

5.2.2 Neutrophil Isolation and Labeling

Neutrophils were isolated from whole blood collected by venipuncture from normal donors, employing density gradient centrifugation with isotonic Percoll. Blood (30 ml) was collected into heparinised vacutainers, mixed with 15 ml 6 % dextran normal saline, and sedimented for 45 min to separate the white blood cells from the erythrocytes. The upper leukocyte-rich layer was collected and centrifuged using Percoll density gradient (1.077 and 1.085) to separate neutrophils from mononuclear cells. Contaminant erythrocytes were removed by hypotonic lysis (0.2% NaCl). Isolated neutrophils were labeled with ^{51}Cr (Gallin et al., 1973) before adding them into culture inserts. Neutrophils at a concentration of approximately 4 × 10^7cells/ml in Ca^{2+}/Mg^{2+}

free HBSS were incubated with 0.5 mCi of sodium chromate at 37°C for 1 h with vigorous mixing. After incubation, labeling of neutrophils was terminated by washing three times with Ca^{2+}/Mg^{2+} free HBSS and cells were resuspended in HBSS containing 0.2 % BSA at a density of 1×10^7 cells/ml. Cell numbers were determined and viability was determined by trypan blue exclusion. The purity of neutrophils was examined by staining cytospins by the May Grunwald Giemsa procedure. The isolated neutrophils were at least 97 % pure (Fig. 5-1). Viability was also at least 97%

5.2.3 Chemotaxis and Transmigration

Neutrophil chemotaxis through naked transwell culture inserts, as well as transmigration through layers of A549 cells grown on the culture inserts were investigated (Fig. 5-2). Before adding neutrophils, the culture inserts and well were washed twice with incubation medium. Neutrophils (100 µl) in a suspension of 1×10^7 cells/ml, were placed into inserts (upper compartment) and into the lower compartment was added buffer alone (HBSS-0.2%BSA), TNF-α (1 nM), purified tryptase (80 mU/ml) or peptide agonist SLIGKV (100 µM) or supernatants from cells incubated with TNF-α (1 nM), or with tryptase (80 mU/ml), or with the peptide agonist SLIGKV (100 µM) for 24 h. After 3 h incubation, a 500 µl aliquot of 2% (v/v) Triton X-100 in H_2O was added, and the well contents collected and counted in a gamma counter. The extent of migration was calculated from the radioactivity found in the lower compartment compared with the total radioactivity added to the upper compartment.

5.2.4 Cytokine Assays and Application of Neutralizing Antibody

Confluent A549 cells in 24-well culture plates were serum deprived for 24 h and incubated with TNF-α (1 nM), purified tryptase (80 mU/ml) or the peptide agonist SLIGKV (100 μM) for 24 h. Culture supernatants were collected and their ability to stimulate neutrophil transmigration investigated as described above. A portion was stored for the measurement of cytokines. In certain experiments, supernatants from cells treated with tryptase or peptide agonists were incubated with blocking antibody against IL-8 (10 μg/ml) for 30 min before addition to the lower compartments of the transwell chambers. Levels of immunoreactive IL-8, GM-CSF, and IL-6 were determined by ELISA according to the instructions of the manufacturers.

5.3 Results

5.3.1 Effect of Tryptase and Peptide Agonist SLIGKV on Neutrophil Migration

Cultured supernatants from A549 epithelial cells, treated with tryptase or the peptide agonist SLIGKV for 24 h, induced significant transepithelial migration of neutrophils compared to that of cells treated with buffer alone (Fig. 5-3). Supernatants from cells incubated with TNF-α added at a concentration reported to be effective in this model (Smart et al, 1994) acted as a positive control. The effect of supernatants from tryptase-treated cells was abolished by preincubating tryptase with leupeptin. Supernatants from cells incubated with the reverse peptide VKGILS did not provoke neutrophil migration.

Addition of tryptase or the peptide agonist SLIGKV alone was without effect on neutrophil migration across epithelial cell layers after 3 h incubation (Fig. 5-4). In contrast, TNF-α (1 nM) induced up to 30 % of neutrophils to migrate into the lower chamber. Similarly a direct chemotactic effect was not induced by tryptase across naked filters after a 3-h incubation, a time point optimal for neutrophil migration (Carolan et al, 1996) (Fig. 5-5). There was a trend for TNF-α to induce neutrophil chemotaxis, but this was not significant.

5.3.2 Cytokine Release and Blocking Studies

The failure to demonstrate a direct chemotactic effect for tryptase suggests that chemoattractants may be released from epithelial cells stimulated by either tryptase or the peptide agonist. With the 16HBE 14 o- epithelial cell line, it was observed that tryptase and the peptide agonist can provoke a marked increase in these cells of mRNA for IL-8, GM-CSF and IL-6 as well as the increased secretion of these cytokines (Chapter 3). Investigating levels of these three cytokines with the A549 cells employed in the present studies,

we found that there was the release of substantial quantities of IL-8, as well as the release of smaller amounts of IL-6 from cells incubated with tryptase or the peptide agonist for 24 h (Fig. 5-6). GM-CSF release levels were not affected under the same conditions. Preincubation of tryptase with leupeptin abolished its ability to increase IL-8 and IL-6 release. The PAR-2 agonist peptide, like tryptase, provoked substantial IL-8 and IL-6 release, whereas the reverse peptide had no effect on the secretion of either cytokine.

Preincubation of supernatants from tryptase treated epithelial cells with a blocking antibody against IL-8, before addition to the lower compartment of transwell chambers resulted in complete inhibition of neutrophil transmigration across epithelial cell layers (Fig. 5-7). A similar finding was made when supernatants from SLIGKV-treated epithelial cells were treated with neutralising antibody.

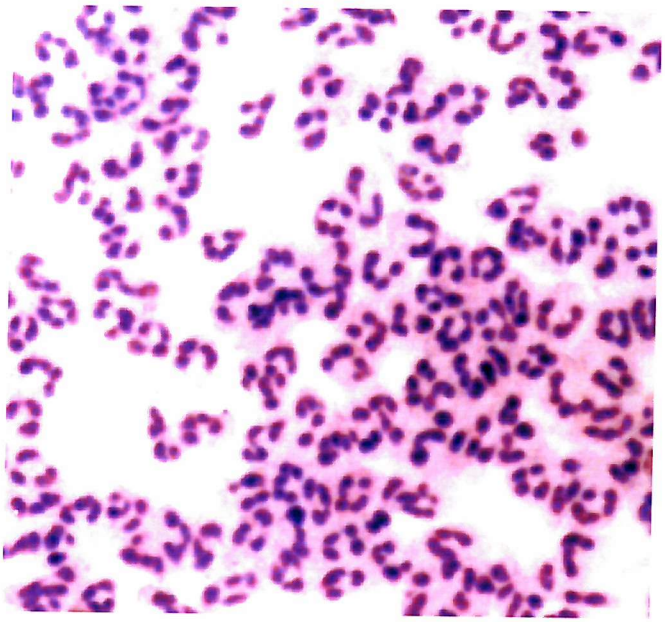


Figure 5-1 Isolated neutrophils.

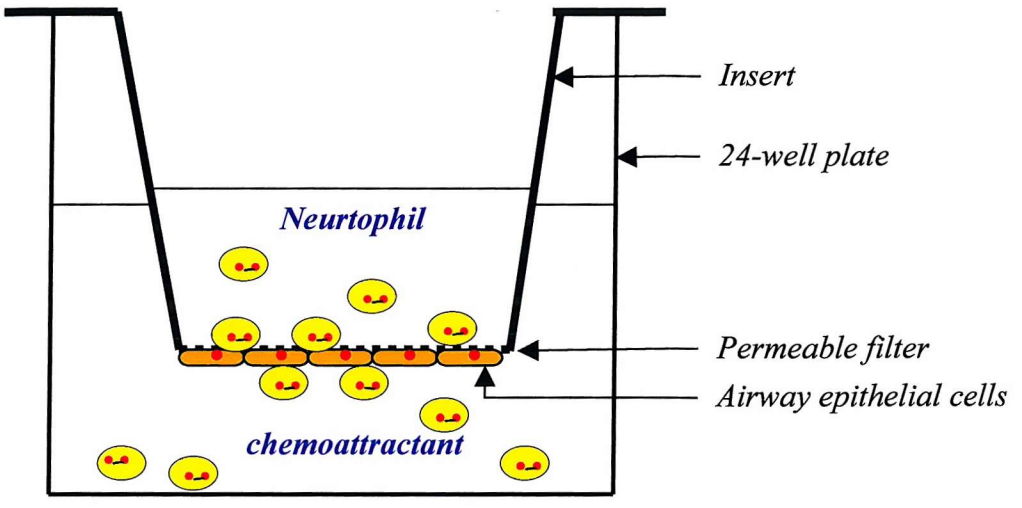


Figure 5-2 Diagramatic representation of the chemotaxis chamber.

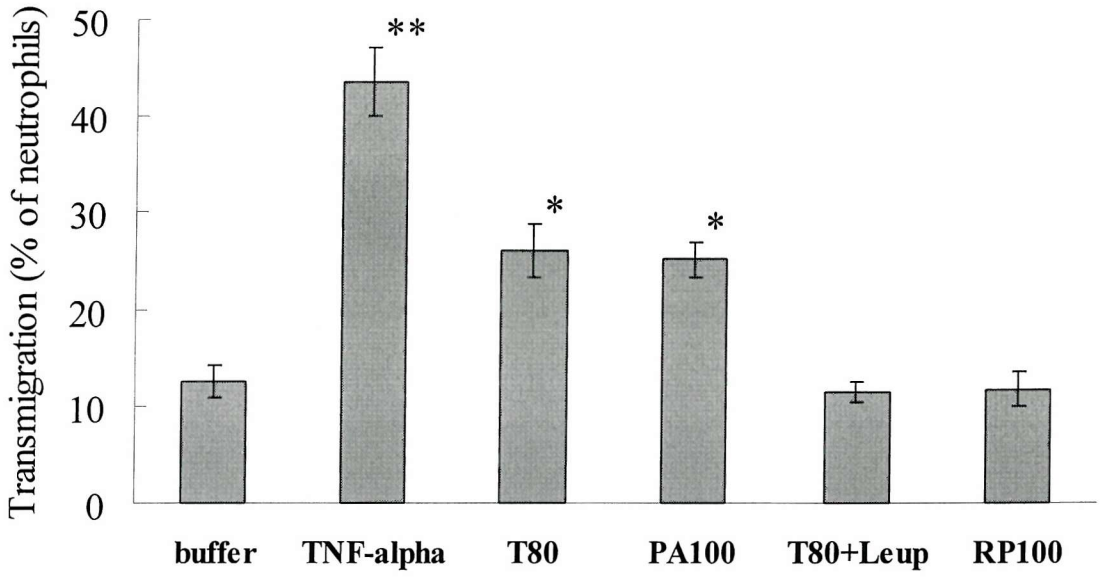


Figure 5-3 Neutrophil migration across epithelial monolayers in response to supernatants, collected from A549 cells after 24 h incubation with buffer, TNF-α (1 nM), tryptase (80 mU/ml; T80), peptide agonist SLIGKV (100 μM; PA), tryptase (80 mU/ml) with leupeptin (50 μg/ml; T80+leup), or reverse peptide VKGILS (100 μM; RP). The extent of transmigration was determined after a 3-h incubation period at 37 °C. Mean values (±SEM) are shown for 4 separate experiments each performed in duplicate. * $P < 0.05$, ** $P < 0.005$ compared with buffer control.

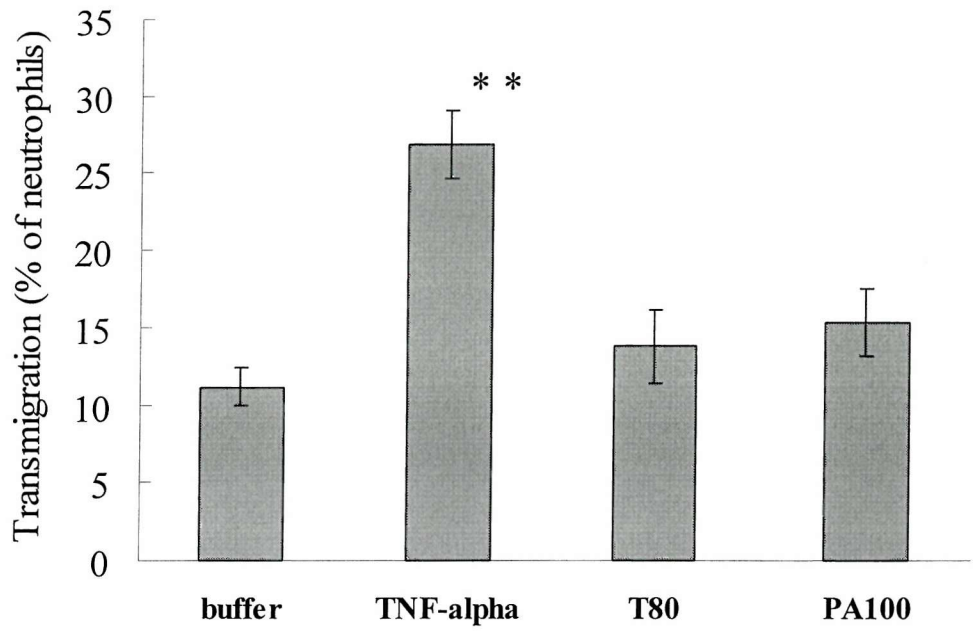


Figure 5-4 Neutrophil migration across epithelial monolayers in response to TNF-α (1 nM), tryptase (80 mU/ml; T80), peptide agonist SLIGKV (100 μM; PA) or buffer with transmigration analysed after incubation for 3 h. Mean values ± SEM are shown for 4 separate experiments each performed in duplicate. ** $P < 0.005$ compared with buffer control.

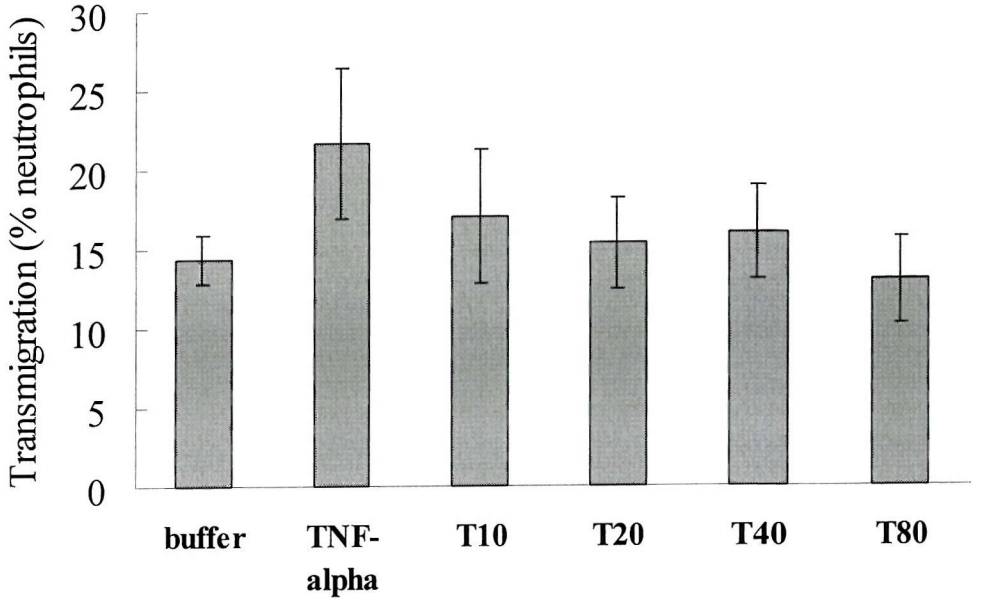


Figure 5-5 Investigation of neutrophil chemotaxis across naked transwell filters following addition of TNF-α (1 nM), tryptase (10, 20, 40 or 80 mU/ml), or buffer. Mean values ± SEM are shown for 4 separate experiments each performed in duplicate.

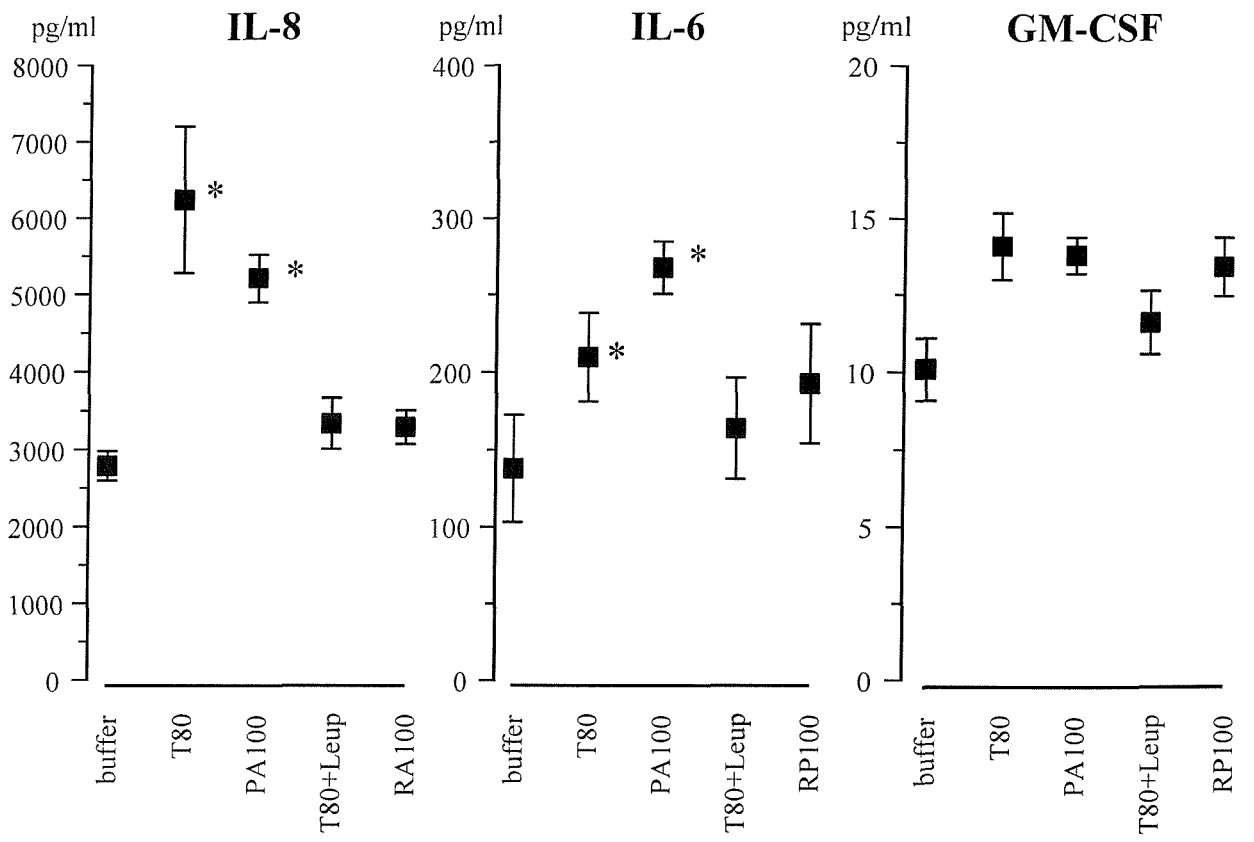


Figure 5-6 Concentrations of IL-8, IL-6 and GM-CSF in supernatants of serum deprived epithelial cells incubated with buffer, TNF-α (1 nM), tryptase (80 mU/ml), peptide agonist SLIGKV (100 μM), or tryptase (80 mU/ml) with leupeptin (50 μg/ml), or reverse peptide VKGILS (100 μM) for 24 h. Mean values ± SEM are shown for 4 separate experiments each performed in duplicate. * $P < 0.05$ compared with buffer control.

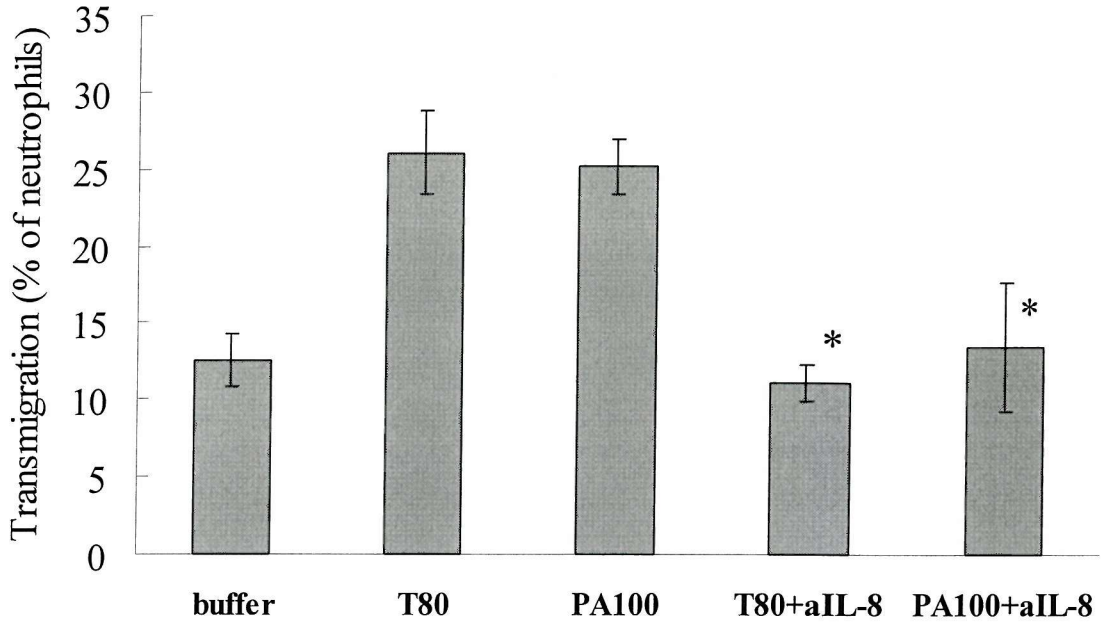


Figure 5-7 Effect of neutralising antibody against IL-8 on the ability of epithelial supernatants to induce neutrophil transepithelial migration. Supernatants, collected from cells after incubation with tryptase (80 mU/ml) or peptide agonist SLIGKV (100 μM) for 24 h, were preincubated with anti-IL-8 antibody (10 μg/ml). Neutrophil migration across epithelial cell monolayers in transwell filters was investigated after 3 h. Mean values ± SEM are shown for 3 separate experiments each performed in duplicate. * $P < 0.05$ compared with corresponding cell supernatant without anti-IL-8 antibody.

CHAPTER 6

GENERAL DISCUSSION

6 General Discussion

These studies provide compelling evidence that mast cell tryptase may be a key mediator of allergic inflammation in the airway epithelium. While not stimulating the proliferation of the epithelial cell line investigated (16HBE 14o-), tryptase was demonstrated to provoke marked increases in expression of mRNA for IL-6, IL-8 and GM-CSF, and to stimulate the release of these cytokines. Tryptase was able to induce alterations in the permeability of the epithelial monolayers and these were related to the breakdown of occludin and the redistribution of tight junction-associated protein ZO-1. Moreover, tryptase provoked neutrophil transmigration across airway epithelial barriers, which appeared to be dependent on the release of IL-8 from epithelial cells. The presence of PAR-2 was demonstrated on the epithelial cells employed by immunocytochemistry and flow cytometry as well as RT-PCR. Peptide agonists of PAR-2 elicited responses which were similar in many respects to those provoked by tryptase. However, some differences did emerge, suggesting that while some of the proinflammatory actions of tryptase on the airway epithelium may be mediated through PAR-2, other processes are likely to be involved.

The observations that tryptase can (1) stimulate cytokine release, (2) alter airway epithelial permeability and (3) induce neutrophil transepithelial migration will be considered separately.

6.1 Tryptase as a Stimulus of Cytokine Release

Tryptase provoked marked increases in the expression of mRNA for IL-6, IL-8 and GM-CSF and stimulated the release of these cytokines from epithelial cells. The finding that the agonist of PAR-2 was able to induce the secretion of these cytokines and with similar patterns of release suggest that activation of this receptor could be of central importance in mediating the proinflammatory actions of tryptase on epithelial cells.

The increases in cytokine expresson and release observed were not related to an increase in epithelial numbers. Tryptase stimulated an increase in DNA synthesis in the 16HBE 14o- cell line, in accord with the previous finding that tryptase is a mitogen for H292 epithelial cells (Cairns and Walls, 1996). However, in contrast with that earlier study, the increase in thymidine incorporation in 16HBE 14o- cells was relatively small and there was not a significant increase in cell numbers following incubation with tryptase. FCS and EGF also provoked comparatively modest increases in DNA synthesis and had little or no effect on cell numbers at concentrations found previously to induce cell proliferation in the H292 epithelial cells. The high baseline rate of proliferation of 16HBE 14 o- cells (and perhaps a greater potential for contact inhibition) is likely to have masked the mitogenic actions of these epithelial cell growth factors, though this cell line does, however, offer major advantages in studies of epithelial cell function, being adherent and able to generate polar monolayers, form tight junctions, and express adhesion molecules (Cozens et al., 1994).

Tryptase at high concentrations actually inhibited DNA synthesis and cell proliferation in 16HBE 14o- epithelial cells. A cytotoxic mechanism exerted by tryptase seems unlikely, as cultured cells were morphologically normal under the light microscope. Moreover, cell viability was greater than 95 % as assessed by trypan blue dye exclusion at each time point. The tryptase was of high purity as confirmed by SDS-PAGE with silver staining and immunoblotting using specific antibody; and endotoxin levels in the tryptase preparation as determined by the E-Toxate assay were less than 0.06 EU / 1 U tryptase. The precise mechanism whereby tryptase at higher concentrations inhibited epithelial cell growth is not clear. However, inhibitory effects of tryptase on cell proliferation have been noted previously when tryptase has been added to primary cultures of human umblical vein endothelial cells (Compton et al., 1998), and a peptide agonist of PAR-2 has been reported to inhibit DNA synthesis in human pancreatic cancer cells at high concentrations (Kaufmann et al., 1998).

In studies employing RT-PCR, we found that tryptase enhanced expression of mRNA for IL-6, IL-8, and GM-CSF. Levels of mRNA for various other cytokines produced by epithelial cells, including IL-1β, IL-2, IL-5, and RANTES, were not altered following incubation with tryptase. Thus, tryptase may be a more selective stimulus for cytokine synthesis than TNF-α, which has been shown to provoke the synthesis of IL-5, and RANTES as well as IL-6, IL-8, and GM-CSF (Adler et al., 1994; Cromwell et al., 1992; Daffern et al., 1999; Salvi et al., 1999; Wang et al., 1996).

The increased expression of mRNA for IL-6, IL-8, and GM-CSF in epithelial cells 3 h after addition of tryptase was followed at later time points by the release of these cytokines into cell supernatants. A substantial increase in the secretion of IL-6 was seen at 6 h; while elevations in concentrations of IL-8 and GM-CSF were more apparent at 24 h and 48 h, at a time when tryptase-induced alterations in IL-6 were less marked. Over the range of tryptase concentrations added, a bell-shaped dose response pattern was observed for the secretion of IL-6 and GM-CSF with greatest release for both cytokines at 10 mU/ml, as was the case with thymidine incorporation. In contrast, maximal IL-8 release did not appear to have been reached even with a tryptase concentration of 80 mU/ml. This would suggest that these responses to tryptase involve different signal transduction mechanisms.

The bell-shaped dose response curves obtained for GM-CSF and IL-6 release, and also for thymidine incorporation could reflect receptor downregulation or inactivation at higher concentrations of tryptase. It has been reported that as well as being able to activate PAR-2, tryptase can disable this receptor by cleaving the N-terminus at one or more sites downstream from the activation site, particularly at high concentrations (Molino et al., 1997a). Alternatively, continuous exposure to the agonist, or repeated activation may lead to receptor downregulation with enhanced degradation and reduced synthesis of receptors, as has been described for PAR-1 in mesangial cells (Zacharias et al., 1995). PAR-2 desensitisation has been reported to be associated with irreversible receptor cleavage, protein kinase C-mediated

termination of signaling, and PAR-2 targeting to lysosomes (Dery et al., 1998).

The actions of tryptase were in all cases dependent on an intact catalytic site. The ability of tryptase both to stimulate or to decrease DNA synthesis was inhibited by the protease inhibitor leupeptin or by heat inactivating the enzyme. The ability of tryptase to enhance mRNA expression for IL-6, IL-8 and GM-CSF and to increase the release of these cytokines was abolished in the same way. In this respect there are parallels with findings with several other proteases which have been reported to induce catalytic-site dependent cytokine release from epithelial cells. Thus epithelial cells have been found to upregulate expression of mRNA for IL-8 in response to neutrophil elastase (Nakamura et al., 1992), to produce IL-6 and IL-8 in response to the serine protease granzyme A (Sower et al., 1996), and IL-6, IL-8, and GM-CSF in response to the proteolytic allergens, Der p 1, a cysteine protease, and Der p 9, a chymotryptic serine protease (King et al., 1998). The potential roles of PARs in mediating similar responses in epithelial cells in response to such diverse proteolytic activities remains to be investigated, though mechanisms could be quite different from those involved in cells stimulated with tryptase.

All of the PARs described to date (PAR-1, PAR-2, PAR-3 and PAR-4) are activated by tryptic serine proteases (Dery et al., 1998), though chymotryptic and elastolytic enzymes may have a role in the inactivation of certain PARs (Schechter et al., 1998). The demonstration that PAR-2 can be activated by tryptase calls particular attention to this receptor in seeking to characterise the actions of tryptase on epithelial cells. A selective antagonist for PAR-2 is not available, but our studies suggest that many of the proinflammatory actions on epithelial cells observed for tryptase could be explained by cleavage and activation of this receptor. The peptide agonist for PAR-2, SLIGKV, was able to reproduce the actions of tryptase on epithelial cells in all cases examined, increasing mRNA synthesis for IL-6, IL-8, and GM-CSF and stimulating the secretion of these cytokines. VKGILS on the other hand, a non-PAR-2 activating peptide with the same amino acid composition, was without any effect on the cells. The synthesis and release of cytokines within hours or days

of PAR-2 activation could complement the more rapid PAR-2 mediated release of prostanoids from the epithelium which has been suggested to represent a bronchoprotective role for this receptor (Cocks et al., 1999; Cocks and Moffatt, 2000).

The presence of immunoreactive PAR-2 was observed in the 16HBE 14o- epithelial cells employed in these studies with the appearance of punctate staining on the cell membrane or within the cytoplasm. This would be in keeping with a study in which PAR-2 has been localised to cells of the bronchial epithelium (D'Andrea et al., 1998). Moreover, we detected mRNA for PAR-2 in the 16HBE cells by RT-PCR and DNA sequencing, though no evidence for increased expression was found following exposure of cells to tryptase or to TNF-α. This result could be a reflection of the high degree of constitutive expression of mRNA for PAR-2 in 16HBE 14o- cells, as TNF-α as well as certain other inflammatory mediators have been found to upregulate mRNA expression of PAR-2 in human umbilical vein endothelial cells (Nystedt et al., 1996).

The cytokines released in response to tryptase and the activation of PAR-2 have a range of potent proinflammatory actions. IL-6 is able to augment immunoglobulin production by B lymphocytes, induce B cell and cytotoxic T cell proliferation and differentiation, and stimulate haemopoietic stem cell growth (Kelley, 1990). IL-8 is a potent granulocyte chemoattractant which has been implicated in neutrophil accumulation in airway inflammation (Adler et al., 1994; Khair et al., 1996; Stockley, 1995). GM-CSF is a key cytokine in modulating the growth and differentiation of haemopoietic cells and can play a central role in mediating cellular responses in inflammation (Spiekermann et al., 1997; Tarr, 1996). Tryptase and other agonists of PAR-2 could provide an important stimulus for the production of these cytokines in the epithelium in allergic inflammation.

6.2 Alterations in Airway Epithelial Permeability

We have found that tryptase can have potent actions on epithelial permeability and can selectively alter the extent to which molecules of different sizes can penetrate. The activation of PAR-2 on epithelial cells induced similar changes, indicating the potential of PAR-2 to mediate increases in epithelial permeability. Addition of both tryptase and the peptide agonist of PAR-2 lead to increased permeability and the loss of tight junctions from epithelial monolayers, though the differences in the responses observed suggest that the effects of tryptase cannot be attributed simply to the activation of PAR-2.

The effect of tryptase on transepithelial resistance was critically dependent on the concentration of tryptase added to cell monolayers with relatively small increments in dose having actions which appeared to be diametrically opposed. Thus, tryptase at 80 mU/ml (40 μg/ml) significantly decreased resistance, but reducing the concentration to 20 or 10 mU/ml (10 or 5 μg/ml) actually stimulated an increase. An apparent tightening of airway epithelial tight junctions was observed also with low concentrations of trypsin, but not with the peptide agonist for PAR-2 over the range of concentrations investigated. A trypsin-induced increase in TER associated with the formation of aberrant strands in tight junctions and a redistribution of ZO-1 has been reported previously (Lynch et al., 1995). It is possible that the secretion of small quantities of tryptase may serve to reduce the permeability of the airways to allergen and other noxious stimuli. The main focus of the present studies was on the ability of tryptase and PAR-2 agonist to increase epithelial permeability, but there would seem to be the potential for tryptase to have protective actions at low doses.

The tryptase-induced decrease in TER was reflected in increased permeability of the monolayers to mannitol and inulin tracers. The concentrations of tryptase capable of increasing epithelial permeability will be achieved in inflammatory conditions of the airways. Tryptase concentrations

of some 50 ng/ml have been reported in bronchoalveolar lavage fluid from patients with symptomatic asthma (Broide et al., 1991), and this is likely to represent a dilution of the extracellular fluid of greater than 100-fold (Rennard et al., 1986). Mast cells in the lung have been estimated to contain about 11 pg/ml tryptase per cell (Schwartz et al., 1987). These cells, as well as being plentiful below the epithelium, may also be free within the lumen of the respiratory tract (Walls et al., 1990) and in asthmatics, may actually be located within the epithelium (Djukanovic et al., 1990). Mast cells in these more superficial locations are the most likely to be activated by inhaled allergen and there is the potential for epithelial cells to be exposed to quite high concentrations of tryptase.

Tryptase is the first product of human mast cells to be demonstrated to alter the permeability of epithelial cell monolayers. Histamine, although implicated as a key mediator of increased permeability in the airway epithelium in both human and animal studies (Boucher et al., 1978; Braude et al., 1984), has been found to have no effect on the permeability of airway epithelial monolayers in vitro (Devalia et al., 1994). Similarly, TNF-α, another product of mast cells, has been shown to reduce TER across monolayers of MDCK cells to increase the permeability to mannitol (Mullin and Snock, 1990). However, TNF-α over a similar range of concentrations failed to alter airway epithelial permeability in the present studies.

Transepithelial flux of albumin was not affected by the concentrations of tryptase employed in these studies, indicating that alterations in permeability were not a consequence of cell detachment. This was supported by examination of cell monolayers by light and electron microscopy. The whole area of the transwell insert was found to be completely covered, generally several cells deep with morphologically healthy epithelial cells, and differences relating to treatment were not observed. Moreover, the actions of tryptase cannot be attributed to cytotoxic processes on the basis of trypan blue exclusion studies, and there were no alterations in LDH levels in cell supernatants.

Tryptase-induced epithelial permeability was dependent on an intact catalytic site, and the effect was abolished by the protease inhibitor or by heat inactivating the enzyme. A selective antagonist of PAR-2 is not available. However, a role for this G protein-coupled receptor is suggested by the observation that both tryptase and the peptide agonist of PAR-2 SLIGKV could induce alterations in TER or epithelial permeability with a similar magnitude and similar time courses. Moreover, SLIGKV, like tryptase was able to alter the distribution of the tight junction associated proteins occludin and ZO-1 as revealed by immunocytochemistry. Treatment of cells with pertussis toxin, which can inhibit the actions of G protein by catalysing the transfer of the ADP-ribose moiety of NAD to the alpha subunit of Gi, was found to reduce the extent of the decrease in the TER stimulated by SLIGKV. Surprisingly, however, pertussis toxin was without effect on the decrease in TER induced by tryptase, suggesting that processes other than PAR-2 activation may be of greater importance in mediating the permeability changes evoked by the tryptase.

The cleavage of occludin on tryptase-treated cells was suggested by the disappearance of this transmembrane tight junction associated molecule from Western blots. The intensity of the band, representing a 65 KDa form of phosphorylated occludin detected by mouse anti-occludin, was reduced in immunoblots from whole cell extracts which had been incubated with tryptase. This would suggest degradation of phosphorylated occludin rather than a shift into the cytoplasmic pool. Several sites in the extracellular loops of occludin have been predicted to be sensitive to trypsin-like enzymes (Fallon et al., 1995). In contrast, activation of PAR-2 in cells had little effect on the levels of occludin detected in immunblots, even though immunostaining for the molecule was reduced. This would suggest that a consequence of PAR-2 activation could be the shift of phosphorylated occludin into the cytoplasmic pool, without apparent degradation.

ZO-1, detected in a 225 kDa form on Western blots, is a cytoplasmic protein and is unlikely to be degraded directly by tryptase. The decrease in levels of

ZO-1, which were observed in these studies following incubation of cells with tryptase, may reflect alterations in cytoplasmic levels of ZO-1. The degradation of occludin could affect the link with ZO-1, leading to its breakdown. As was the case with occludin, the activation of PAR-2 did not alter the total levels of ZO-1 that could be detected on Western blots.

Thus tryptase can alter epithelial monolayer permeability, and this is associated with reduced expression of the tight junction proteins occludin and ZO-1. Other agonists of PAR-2, SLIGKV and trypsin also proved effective in increasing epithelial permeability. However, tryptase-induced responses unlike those of SLIGKV were not inhibited by pertussis toxin, suggesting that processes other than PAR-2 activation may be important in mediating the actions of this major mast cell product. Moreover, treatment of epithelial monolayers with tryptase, but not SLIGKV, resulted in the loss of occludin and ZO-1 from cell extracts. Possibly acting through different mechanisms, tryptase and other stimuli for PAR-2 activation could play key roles in modulating epithelial permeability in allergic airway diseases.

6.3 Neutrophil Chemotaxis

The demonstration that supernatants from epithelial cells treated with tryptase could stimulate neutrophil migration across epithelial monolayers indicates the potential of this protease to stimulate the release of epithelial cell mediators in quantities which are biologically relevant. Activation of PAR-2 on epithelial cells elicited a similar response. As was the case with tryptase as the stimulus, the neutrophil migration provoked could be blocked completely with a neutralising antibody specific for IL-8. Our studies suggest that tryptase may act through PAR-2 to induce IL-8-dependent neutrophil recruitment in asthmatic airways.

It has been reported previously that tryptase can itself act as a chemoattractant for purified neutrophils in modified Boyden chambers at

concentrations of 6 mU/ml and greater (Walls et al., 1995). However, tryptase alone failed to stimulate neutrophil chemotaxis across naked transwell chambers, even with a range of concentrations between 10 and 80 mU/ml. Differences in experimental conditions are likely to account for this discrepancy. In that early study, cells adhering to a 5 μm pore size filter membrane were enumerated after 45 min incubation, while in the present studies, the numbers of cells were determined that had actively migrated across a 3 μm pore size filter after a 3-h incubation.

The chemoattractant activity of tryptase itself may contribute to the ingress of granulocytes immediately following mast cell activation. However, the present studies would suggest that this effect can be dwarfed by the stimulus for neutrophil migration which is released from epithelial cells treated for 24 h with tryptase. Addition of tryptase or SLIGKV to epithelial layers at the same time as neutrophils were added to the apical chambers, failed to stimulate neutrophil transmigration with a 3 h incubation (as was the case using naked filters). Injection of tryptase into the peritoneum of mice has been found to elicit neutrophil accumulation as early as 3 h, though neutrophilia was apparent at 16 h or later post injection (He et al., 1997a). It is possible that the generation of neutrophil chemotactic activity from airway epithelial cells by tryptase will contribute little to the neutrophil accumulation which occurs within the first few hours after allergen challenge, though it could be important in maintaining neutrophil recruitment during later stages of the inflammatory process.

Supernatants from epithelial cells treated for 24 h with tryptase or SLIGKV proved effective in stimulating the migration of neutrophils across epithelial cell monolayers. Once again the actions of tryptase appeared to be dependent on an intact catalytic site, being inhibited by the protease inhibitor leupeptin. The similarity in responses between those stimulated by tryptase and those by SLIGKV strongly suggest that the epithelial cell-dependent migration of neutrophils by tryptase will be mediated largely through the activation of PAR-2. The complete blocking of neutrophil transmigration by pretreating

tryptase or SLIGKV-treated epithelial cell supernatants with a neutralising antibody against IL-8, strongly suggests that IL-8 secretion may have been responsible to a large extent. Moreover it was found that the A549 cells employed, as found for the 16HBE 14o- cell line released substantial quantities of IL-8 in response to tryptase or the peptide agonist of PAR-2.

6.4 Conclusions

The present studies help to establish the idea that tryptase could have an important role in allergic inflammation in the human airway epithelium. Thus, this major mast cell product can stimulate the synthesis and release of potent proinflammatory cytokines including IL-8, IL-6 and GM-CSF. Of these, IL-8 is released in quantities sufficient to induce neutrophil transepithelial migration, and it seems likely that this could provide a mechanism underlying granulocyte recruitment in the human airways in conditions such as asthma.

The ability of tryptase to increase the permeability of airway epithelial cell monolayers suggests that a consequence of tryptase release into the airways could be to increase the penetration of allergens and noxious agents into the tissues. Though the association between mast cell activation and increased epithelial permeability has been established in clinical studies, this is the first report of a defined mast cell product being able to alter the degree of adhesion between airway epithelial cells.

In all studies, the actions of tryptase were dependent on an intact catalytic site, being inhibited by the presence of protease inhibitors or by heat treating the enzyme. More conclusive evidence that PAR-2 activation may account for the effects of tryptase on epithelial cells must await the advent of selective antagonists for this receptor. The presence of PAR-2 on airway epithelial cells and the cell line employed was demonstrated by immunocytochemistry, flow cytometry, and using RT-PCR. mRNA for PAR-2 was detected. Moreover, the peptide agonist for PAR-2, SLIGKV induced changes in epithelial cell

function which were in most cases quite similar to those induced by tryptase. It is possible that the induction of mRNA for cytokines IL-8, IL-6 and GM-CSF and the release of neutrophil chemotactic activity from epithelial cells is dependent largely on the activation of PAR-2. Both tryptase and PAR-2 agonists were able to increase the permeability of epithelial monolayers, but in this case some differences in underlying mechanisms were suggested by the observation of differing responses to pertussis toxin, and magnitude of effects were different when tryptase and SLIGKV were added to the apical or basal side of epithelial cell monolayers. Moreover, tryptase was able to provoke the cleavage as well as the loss of tight junction proteins, a property not shared by the PAR-2 agonist. These findings would suggest that tryptase-induced increases in epithelial permeability may involve processes other than activation of PAR-2.

Tryptase could represent an important mediator of airway inflammation, and this mast cell protease must be considered a promising target for therapeutic intervention in asthma and other allergic conditions. Antagonists of PAR-2 are also likely to have anti-inflammatory properties, but will exhibit a spectrum of actions which will be different from those of tryptase inhibitors.

References

7 References

Aalbers, R., Kauffman, H. F., Vrugt, B., Smith, M., Koeter, G. H., Timens, W., and de Monchy, J. G. (**1993**). Bronchial lavage and bronchoalveolar lavage in allergen-induced single early and dual asthmatic responders. *Am Rev Respir Dis* 147, 76-81.

Abraham, W. M., de Garavilla, L., and Andrade-Gordon, P. (**2000**). Stimulation of protease activated recepotr 2 (PAR-2) in sheep airways causes bronchoconstriction via a histamine-mediated mechanism. *Am J Respir Crit Care Med* 161, A467. (Abstr.).

Addington, A. K., and Johnson, D. A. (**1996**). Inactivation of human lung tryptase: evidence for a re-activatable tetrameric intermediate and active monomers. *Biochemistry* 35, 13511-8.

Adelroth, E., Rosenhall, L., Johansson, S. A., Linden, M., and Venge, P. (**1990**). Inflammatory cells and eosinophilic activity in asthmatics investigated by bronchoalveolar lavage. The effects of antiasthmatic treatment with budesonide or terbutaline. *Am Rev Respir Dis* 142, 91-9.

Adler, K. B., Fischer, B. M., Wright, D. T., Cohn, L. A., and Becker, S. (**1994**). Interactions between respiratory epithelial cells and cytokines: relationships to lung inflammation. *Ann N Y Acad Sci* 725, 128-45.

Adler, K. B., Holden-Stauffer, W. J., and Repine, J. E. (**1990**). Oxygen metabolites stimulate release of high-molecular-weight glycoconjugates by cell and organ cultures of rodent respiratory epithelium via an arachidonic acid-dependent mechanism. *J Clin Invest* 85, 75-85.

Akers, I. A., Parsons, M., Hill, M. R., Hollenberg, M. D., Sanjar, S., Laurent, G. J., and McAnulty, R. J. (**2000**). Mast cell tryptase stimulates human lung

fibroblast proliferation via protease-activated receptor-2. *Am J Physiol Lung Cell Mol Physiol* 278, L193-L201.

Alam, R. (**1997**). Chemokines in allergic inflammation. *J Allergy Clin Immunol* 99, 273-7.

al-Ani, B., Saifeddine, M., and Hollenberg, M. D. (**1995**). Detection of functional receptors for the proteinase-activated-receptor- 2-activating polypeptide, SLIGRL-NH2, in rat vascular and gastric smooth muscle. *Can J Physiol Pharmacol* 73, 1203-1207.

Alter, S. C., Kramps, J. A., Janoff, A., and Schwartz, L. B. (**1990**). Interactions of human mast cell tryptase with biological protease inhibitors. *Arch Biochem Biophys* 276, 26-31.

Alter, S. C., and Schwartz, L. B. (**1989**). Effect of histamine and divalent cations on the activity and stability of tryptase from human mast cells. *Biochim Biophys Acta* 991, 426-30.

Anderson, J. M., Fanning, A. S., Lapierre, L., and Van Itallie, C. M. (**1995**). Zonula occludens (ZO)-1 and ZO-2: membrane-associated guanylate kinase homologues (MAGuKs) of the tight junction. *Biochem Soc Trans* 23, 470-5.

Balda, M. S., Gonzalez-Mariscal, L., Contreras, R. G., Macias-Silva, M., Torres-Marquez, M. E., Garcia-Sainz, J. A., and Cereijido, M. (**1991**). Assembly and sealing of tight junctions: possible participation of G- proteins, phospholipase C, protein kinase C and calmodulin. *J Membr Biol* 122, 193-202.

Barnes, P., FitzGerald, G., Brown, M., and Dollery, C. (**1980**). Nocturnal asthma and changes in circulating epinephrine, histamine, and cortisol. *N Engl J Med* 303, 263-7.

Barnes, P. J. (**1991**). Histamine receptors in the lung. *Agents Actions Suppl* 33, 103-22.

Barton, R. W., Rothlein, R., Ksiazek, J., and Kennedy, C. (**1989**). The effect of anti-intercellular adhesion molecule-1 on phorbol-ester- induced rabbit lung inflammation. *J Immunol* 143, 1278-82.

Bauza, M. T., and Lagunoff, D. (**1981**). Histidine transport by isolated rat peritoneal mast cells. *Biochem Pharmacol* 30, 1271-6.

Beasley, R., Roche, W. R., Roberts, J. A., and Holgate, S. T. (**1989**). Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am Rev Respir Dis* 139, 806-17.

Beatch, M., Jesaitis, L. A., Gallin, W. J., Goodenough, D. A., and Stevenson, B. R. (**1996**). The tight junction protein ZO-2 contains three PDZ (PSD-95/Discs- Large/ZO-1) domains and an alternatively spliced region. *J Biol Chem* 271, 25723-6.

Behrens, B. L., Clark, R. A., Presley, D. M., Graves, J. P., Feldsien, D. C., and Larsen, G. L. (**1987**). Comparison of the evolving histopathology of early and late cutaneous and asthmatic responses in rabbits after a single antigen challenge. *Lab Invest* 56, 101-13.

Belham, C. M., Tate, R. J., Scott, P. H., Pemberton, A. D., Miller, H. R., Wadsworth, R. M., Gould, G. W., and Plevin, R. (**1996**). Trypsin stimulates proteinase-activated receptor-2-dependent and - independent activation of mitogen-activated protein kinases. *Biochem J* 320, 939-46.

Benditt, E. P., and Arase, M. (**1959**). An enzyme in mast cells with properties like chymotrypsin. *J Exp Med* 110, 451-60.

Berridge, M. J. (**1993**). Inositol trisphosphate and calcium signalling. *Nature* 361, 315-25.

Bertog, M., Letz, B., Kong, W., Steinhoff, M., Higgins, M. A., Bielfeld-Ackermann, A., Fromter, E., Bunnett, N. W., and Korbmacher, C. (**1999**). Basolateral proteinase-activated receptor (PAR-2) induces chloride secretion in M-1 mouse renal cortical collecting duct cells. *J Physiol (Lond)* 521 Pt 1, 3-17.

Black, P. N., Ghatei, M. A., Takahashi, K., Bretherton-Watt, D., Krausz, T., Dollery, C. T., and Bloom, S. R. (**1989**). Formation of endothelin by cultured airway epithelial cells. *FEBS Lett* 255, 129-32.

Bloemen, P. G., van den Tweel, M. C., Henricks, P. A., Engels, F., Wagenaar, S. S., Rutten, A. A., and Nijkamp, F. P. (**1993**). Expression and modulation of adhesion molecules on human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 9, 586-93.

Bohm, S. K., Khitin, L. M., Grady, E. F., Aponte, G., Payan, D. G., and Bunnett, N. W. (**1996a**). Mechanisms of desensitization and resensitization of proteinase- activated receptor-2. *J Biol Chem* 271, 22003-16.

Bohm, S. K., Kong, W., Bromme, D., Smeekens, S. P., Anderson, D. C., Connolly, A., Kahn, M., Nelken, N. A., Coughlin, S. R., Payan, D. G., and Bunnett, N. W. (**1996**). Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. *Biochem J* 314, 1009-16.

Bono, F., Lamarche, I., and Herbert, J. M. (**1997**). Induction of vascular smooth muscle cell growth by selective activation of the proteinase activated receptor-2 (PAR-2). *Biochem Biophys Res Commun* 241, 762-4.

Bosso, J. V., Schwartz, L. B., and Stevenson, D. D. (**1991**). Tryptase and histamine release during aspirin-induced respiratory reactions. *J Allergy Clin Immunol* 88, 830-7.

Boucher, R. C., Ranga, V., Pare, P. D., Inoue, S., Moroz, L. A., and Hogg, J. C. (**1978**). Effect of histamine and methacholine on guinea pig tracheal permeability to HRP. *J Appl Physiol* 45, 939-48.

Bradding, P., Feather, I. H., Howarth, P. H., Mueller, R., Roberts, J. A., Britten, K., Bews, J. P., Hunt, T. C., Okayama, Y., Heusser, C. H., and et al. (**1992**). Interleukin 4 is localized to and released by human mast cells. *J Exp Med* 176, 1381-6.

Bradding, P., Feather, I. H., Wilson, S., Bardin, P. G., Heusser, C. H., Holgate, S. T., and Howarth, P. H. (**1993**). Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. The mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation. *J Immunol* 151, 3853-65.

Brass, L. F., Pizarro, S., Ahuja, M., Belmonte, E., Blanchard, N., Stadel, J. M., and Hoxie, J. A. (**1994**). Changes in the structure and function of the human thrombin receptor during receptor activation, internalization, and recycling. *J Biol Chem* 269, 2943-52.

Braude, S., Royston, D., Coe, C., and Barnes, P. J. (**1984**). Histamine increases lung permeability by an H2-receptor mechanism. *Lancet* 2, 372-4.

Bressler, R. B., Friedman, M. M., Kirshenbaum, A. S., Irani, A. M., Schwartz, L. B., and Metcalfe, D. D. (**1990**). Sequential appearance of basophils and mast cells from human bone marrow in long-term suspension culture. *Int Arch Allergy Appl Immunol* 91, 403-10.

Breuer, R., Christensen, T. G., Lucey, E. C., Stone, P. J., and Snider, G. L. (**1987**). An ultrastructural morphometric analysis of elastase-treated hamster bronchi shows discharge followed by progressive accumulation of secretory granules. *Am Rev Respir Dis* 136, 698-703.

Brewster, C. E., Howarth, P. H., Djukanovic, R., Wilson, J., Holgate, S. T., and Roche, W. R. (**1990**). Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 3, 507-11.

Brinkman, G. L. (**1968**). The mast cell in normal human bronchus and lung. *J Ultrastruct Res* 231, 15-23.

Broide, D. H., Gleich, G. J., Cuomo, A. J., Coburn, D. A., Federman, E. C., Schwartz, L. B., and Wasserman, S. I. (**1991**). Evidence of ongoing mast cell and eosinophil degranulation in symptomatic asthma airway. *J Allergy Clin Immunol* 88, 637-48.

Brown, J. K., Tyler, C. L., Jones, C. A., Ruoss, S. J., Hartmann, T., and Caughey, G. H. (**1995**). Tryptase, the dominant secretory granular protein in human mast cells, is a potent mitogen for cultured dog tracheal smooth muscle cells. *Am J Respir Cell Mol Biol* 13, 227-36.

Burd, P. R., Rogers, H. W., Gordon, J. R., Martin, C. A., Jayaraman, S., Wilson, S. D., Dvorak, A. M., Galli, S. J., and Dorf, M. E. (**1989**). Interleukin 3-dependent and -independent mast cells stimulated with IgE and antigen express multiple cytokines. *J Exp Med* 170, 245-57.

Burd, P. R., Thompson, W. C., Max, E. E., and Mills, F. C. (**1995**). Activated mast cells produce interleukin 13. *J Exp Med* 181, 1373-80.

Burgess, L. E., Newhouse, B. J., Ibrahim, P., Rizzi, J., Kashem, M. A., Hartman, A., Brandhuber, B. J., Wright, C. D., Thomson, D. S., Vigers, G. P., and Koch, K. (**1999**). Potent selective nonpeptidic inhibitors of human lung tryptase. *Proc Natl Acad Sci U S A* 96, 8348-52.

Burman, W. J., and Martin, W. J. d. (**1986**). Oxidant-mediated ciliary dysfunction. Possible role in airway disease. *Chest* 89, 410-3.

Busse, W. W., Calhoun, W. F., and Sedgwick, J. D. (**1993**). Mechanism of airway inflammation in asthma. *Am Rev Respir Dis* 147, S20-4.

Cairns, J. A., and Walls, A. F. (**1996**). Mast cell tryptase is a mitogen for epithelial cells. Stimulation of IL- 8 production and intercellular adhesion molecule-1 expression. *J Immunol* 156, 275-83.

Cairns, J. A., and Walls, A. F. (**1997**). Mast cell tryptase stimulates the synthesis of type I collagen in human lung fibroblasts. *J Clin Invest* 99, 1313-21.

Carolan, E. J., and Casale, T. B. (**1996**). Neutrophil transepithelial migration is dependent upon epithelial characteristics. *Am J Respir Cell Mol Biol* 15, 224-31.

Carson, J. L., Collier, A. M., Gambling, T. M., Knowles, M. R., and Boucher, R. C. (**1990**). Ultrastructure of airway epithelial cell membranes among patients with cystic fibrosis. *Hum Pathol* 21, 640-7.

Casale, T. B., Wood, D., Richerson, H. B., Trapp, S., Metzger, W. J., Zavala, D., and Hunninghake, G. W. (**1987**). Elevated bronchoalveolar lavage fluid histamine levels in allergic asthmatics are associated with methacholine bronchial hyperresponsiveness. *J Clin Invest* 79, 1197-203.

Chan, T. B., Eiser, N., Shelton, D., and Rees, P. J. (**1987**). Histamine receptors and pulmonary epithelial permeability. *Br J Dis Chest* 81, 260-7.

Chow, J. M., Moffatt, J. D., and Cocks, T. M. (**2000**). Effect of the PAR-2 activating peptide SLIGRL-NH2 on airway smooth muscle in vitro and in vivo. *Am J Respir Crit Care Med* 161, A438. (Abstr.).

Churchill, L., Chilton, F. H., Resau, J. H., Bascom, R., Hubbard, W. C., and Proud, D. (**1989**). Cyclooxygenase metabolism of endogenous arachidonic

acid by cultured human tracheal epithelial cells. *Am Rev Respir Dis* 140, 449-59.

Churchill, L., Friedman, B., Schleimer, R. P., and Proud, D. (**1992**). Production of granulocyte-macrophage colony-stimulating factor by cultured human tracheal epithelial cells. *Immunology* 75, 189-95.

Clark, J. M., Abraham, W. M., Fishman, C. E., Forteza, R., Ahmed, A., Cortes, A., Warne, R. L., Moore, W. R., and Tanaka, R. D. (**1995**). Tryptase inhibitors block allergen-induced airway and inflammatory responses in allergic sheep. *Am J Respir Crit Care Med* 152, 2076-83.

Claude, P. (**1978**). Morphological factors influencing transepithelial permeability: a model for the resistance of the zonula occludens. *J Membr Biol* 39, 219-32.

Cocks, T. M., Fong, B., Chow, J. M., Anderson, G. P., Frauman, A. G., Goldie, R. G., Henry, P. J., Carr, M. J., Hamilton, J. R., and Moffatt, J. D. (**1999**). A protective role for protease-activated receptors in the airways. *Nature* 398, 156-60.

Cocks, T. M., and Moffatt, J. D. (**2000**). Protease-activated receptors: sentries for inflammation? *Trends Pharmacol Sci* 21, 103-8.

Compton, S. J., Cairns, J. A., Holgate, S. T., and Walls, A. F. (**2000**). Human mast cell tryptase stimulates the release of an IL-8 dependent neutrophil chemotactic activity from human umblical vein endothelial cells. *Clin Exp Immunol (In press)*.

Compton, S. J., Cairns, J. A., Holgate, S. T., and Walls, A. F. (**1998**). The role of mast cell tryptase in regulating endothelial cell proliferation, cytokine release, and adhesion molecule expression: tryptase induces expression of mRNA for IL-1 beta and IL-8 and stimulates the selective release of IL-8 from human umbilical vein endothelial cells. *J Immunol* 161, 1939-46.

Corvera, C. U., Dery, O., McConalogue, K., Bohm, S. K., Khitin, L. M., Caughey, G. H., Payan, D. G., and Bunnett, N. W. (**1997**). Mast cell tryptase regulates rat colonic myocytes through proteinase- activated receptor 2. *J Clin Invest* 100, 1383-93.

Costello, R. T. (**1993**). Therapeutic use of granulocyte-macrophage colony-stimulating factor (GM- CSF). A review of recent experience. *Acta Oncol* 32, 403-8.

Covera, C. U., Dery, O., McConalogue, K., Bohn, S. K., Khitin, L. M., Caughey, G. H., Payan, D. G., and Bunnett, N. W. (**1997**). Mast cell tryptase regulates rat colonic myocyte through proteinase-activated receptor 2. *J Clin Invest* 100, 1383-1393.

Cozens, A. L., Yezzi, M. J., Kunzelmann, K., Ohrui, T., Chin, L., Eng, K., Finkbeiner, W. E., Widdicombe, J. H., and Gruenert, D. C. (**1994**). CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 10, 38-47.

Craig, S. S., DeBlois, G., and Schwartz, L. B. (**1986**). Mast cells in human keloid, small intestine, and lung by an immunoperoxidase technique using a murine monoclonal antibody against tryptase. *Am J Pathol* 124, 427-35.

Cregar, L., Elrod, K. C., Putnam, D., and Moore, W. R. (**1999**). Neutrophil myeloperoxidase is a potent and selective inhibitor of mast cell tryptase. *Arch Biochem Biophys* 366, 125-30.

Crimi, E., Balbo, A., Milanese, M., Miadonna, A., Rossi, G. A., and Brusasco, V. (**1992**). Airway inflammation and occurrence of delayed bronchoconstriction in exercise-induced asthma. *Am Rev Respir Dis* 146, 507-12.

Crimi, E., Chiaramondia, M., Milanese, M., Rossi, G. A., and Brusasco, V. (**1991**). Increased numbers of mast cells in bronchial mucosa after the late-phase asthmatic response to allergen. *Am Rev Respir Dis* 144, 1282-6.

Cromlish, J. A., Seidah, N. G., Marcinkiewicz, M., Hamelin, J., Johnson, D. A., and Chretien, M. (**1987**). Human pituitary tryptase: molecular forms, NH2-terminal sequence, immunocytochemical localization, and specificity with prohormone and fluorogenic substrates. *J Biol Chem* 262, 1363-73.

Cromwell, O., Hamid, Q., Corrigan, C. J., Barkans, J., Meng, Q., Collins, P. D., and Kay, A. B. (**1992**). Expression and generation of interleukin-8, IL-6 and granulocyte- macrophage colony-stimulating factor by bronchial epithelial cells and enhancement by IL-1 beta and tumour necrosis factor-alpha. *Immunology* 77, 330-7.

Daffern, P. J., Jagels, M. A., Saad, J. J., Fischer, W., and Hugli, T. E. (**1999**). Upper airway epithelial cells support eosinophil survival in vitro through production of GM-CSF and prostaglandin E2: regulation by glucocorticoids and TNF-alpha. *Allergy Asthma Proc* 20, 243-53.

Dahlen, S. E., Bjork, J., Hedqvist, P., Arfors, K. E., Hammarstrom, S., Lindgren, J. A., and Samuelsson, B. (**1981**). Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: in vivo effects with relevance to the acute inflammatory response. *Proc Natl Acad Sci U S A* 78, 3887-91.

Dahlen, S. E., Hedqvist, P., Hammarstrom, S., and Samuelsson, B. (**1980**). Leukotrienes are potent constrictors of human bronchi. *Nature* 288, 484-6.

D'Andrea, M. R., Derian, C. K., Leturcq, D., Baker, S. M., Brunmark, A., Ling, P., Darrow, A. L., Santulli, R. J., Brass, L. F., and Andrade-Gordon, P. (**1998**). Characterization of protease-activated receptor-2 immunoreactivity in normal human tissues. *J Histochem Cytochem* 46, 157-64.

De Monchy, J. G., Kauffman, H. F., Venge, P., Koeter, G. H., Jansen, H. M., Sluiter, H. J., and De Vries, K. (**1985**). Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. *Am Rev Respir Dis* 131, 373-6.

Delneste, Y., Lassalle, P., Jeannin, P., Joseph, M., Tonnel, A. B., and Gosset, P. (**1994**). Histamine induces IL-6 production by human endothelial cells. *Clin Exp Immunol* 98, 344-9.

Derian, C. K., Eckardt, A. J., and Andrade-Gordon, P. (**1997**). Differential regulation of human keratinocyte growth and differentiation by a novel family of protease-activated receptors. *Cell Growth Differ* 8, 743-9.

Dery, O., Corvera, C. U., Steinhoff, M., and Bunnett, N. W. (**1998**). Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. *Am J Physiol* 274, C1429-52.

Devalia, J. L., Campbell, A. M., Sapsford, R. J., Rusznak, C., Quint, D., Godard, P., Bousquet, J., and Davies, R. J. (**1993**). Effect of nitrogen dioxide on synthesis of inflammatory cytokines expressed by human bronchial epithelial cells in vitro. *Am J Respir Cell Mol Biol* 9, 271-8.

Devalia, J. L., Godfrey, R. W., Sapsford, R. J., Severs, N. J., Jeffery, P. K., and Davies, R. J. (**1994**). No effect of histamine on human bronchial epithelial cell permeability and tight junctional integrity in vitro. *Eur Respir J* 7, 1958-65.

Djukanovic, R., Roche, W. R., Wilson, J. W., Beasley, C. R., Twentyman, O. P., Howarth, R. H., and Holgate, S. T. (**1990a**). Mucosal inflammation in asthma. *Am Rev Respir Dis* 142, 434-57.

Djukanovic, R., Wilson, J. W., Britten, K. M., Wilson, S. J., Walls, A. F., Roche, W. R., Howarth, P. H., and Holgate, S. T. (**1990**). Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic

asthmatics and healthy control subjects using immunohistochemistry. *Am Rev Respir Dis* 142, 863-71.

Drazen, J. M., Austen, K. F., Lewis, R. A., Clark, D. A., Goto, G., Marfat, A., and Corey, E. J. (**1980**). Comparative airway and vascular activities of leukotrienes C-1 and D in vivo and in vitro. *Proc Natl Acad Sci U S A* 77, 4354-8.

Dunnill, M. S., Massarella, G. R., and Anderson, J. A. (**1969**). A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema. *Thorax* 24, 176-9.

Elia, C., Bucca, C., Rolla, G., Scappaticci, E., and Cantino, D. (**1988**). A freeze-fracture study of human bronchial epithelium in normal, bronchitic and asthmatic subjects. *J Submicrosc Cytol Pathol* 20, 509-17.

Elrod, K. C., Moore, W. R., Abraham, W. M., and Tanaka, R. D. (**1997**). Lactoferrin, a potent tryptase inhibitor, abolishes late-phase airway responses in allergic sheep. *Am J Respir Crit Care Med* 156, 375-81.

Empey, D. W., Laitinen, L. A., Jacobs, L., Gold, W. M., and Nadel, J. A. (**1976**). Mechanisms of bronchial hyperreactivity in normal subjects after upper respiratory tract infection. *Am Rev Respir Dis* 113, 131-9.

Erger, R. A., and Casale, T. B. (**1995**). Interleukin-8 is a potent mediator of eosinophil chemotaxis through endothelium and epithelium. *Am J Physiol* 268, L117-22.

Fabbri, L. M., Boschetto, P., Zocca, E., Milani, G., Pivirotto, F., Plebani, M., Burlina, A., Licata, B., and Mapp, C. E. (**1987**). Bronchoalveolar neutrophilia during late asthmatic reactions induced by toluene diisocyanate. *Am Rev Respir Dis* 136, 36-42.

Fahy, J. V., Kim, K. W., Liu, J., and Boushey, H. A. (**1995**). Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J Allergy Clin Immunol* 95, 843-52.

Fallon, M. B., Brecher, A. R., Balda, M. S., Matter, K., and Anderson, J. M. (**1995**). Altered hepatic localization and expression of occludin after common bile duct ligation. *Am J Physiol* 269, C1057-62.

Fanning, A. S., Jameson, B. J., Jesaitis, L. A., and Anderson, J. M. (**1998**). The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J Biol Chem* 273, 29745-53.

Ferguson, A. C., Whitelaw, M., and Brown, H. (**1992**). Correlation of bronchial eosinophil and mast cell activation with bronchial hyperresponsiveness in children with asthma. *J Allergy Clin Immunol* 90, 609-13.

Fick, R. B., Jr., Metzger, W. J., Richerson, H. B., Zavala, D. C., Moseley, P. L., Schoderbek, W. E., and Hunninghake, G. W. (**1987**). Increased bronchovascular permeability after allergen exposure in sensitive asthmatics. *J Appl Physiol* 63, 1147-55.

Flint, K. C., Leung, K. B., Hudspith, B. N., Brostoff, J., Pearce, F. L., and Johnson, N. M. (**1985**). Bronchoalveolar mast cells in extrinsic asthma: a mechanism for the initiation of antigen specific bronchoconstriction. *Br Med J (Clin Res Ed)* 291, 923-6.

Ford-Hutchinson, A. W., Bray, M. A., Doig, M. V., Shipley, M. E., and Smith, M. J. (**1980**). Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature* 286, 264-5.

Fox, B., Bull, T. B., and Guz, A. (**1981**). Mast cells in the human alveolar wall: an electronmicroscopic study. *J Clin Pathol* 34, 1333-42.

Freed, A. N., Omori, C., and Schofield, B. H. (**1995**). The effect of bronchial blood flow on hyperpnea-induced airway obstruction and injury. *J Clin Invest* 96, 1221-9.

Frigas, E., Loegering, D. A., and Gleich, G. J. (**1980**). Cytotoxic effects of the guinea pig eosinophil major basic protein on tracheal epithelium. *Lab Invest* 42, 35-43.

Fujimoto, K. (**1995**). Freeze-fracture replica electron microscopy combined with SDS digestion for cytochemical labeling of integral membrane proteins. Application to the immunogold labeling of intercellular junctional complexes. *J Cell Sci* 108, 3443-9.

Furuse, M., Fujita, K., Hiiragi, T., Fujimoto, K., and Tsukita, S. (**1998**). Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J Cell Biol* 141, 1539-50.

Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., and Tsukita, S. (**1993**). Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol* 123, 1777-88.

Furuse, M., Itoh, M., Hirase, T., Nagafuchi, A., Yonemura, S., and Tsukita, S. (**1994**). Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. *J Cell Biol* 127, 1617-26.

Galli, S. J., and Costa, J. J. (**1995**). Mast-cell-leukocyte cytokine cascades in allergic inflammation. *Allergy* 50, 851-62.

Gallin JI, Clark RA, Kimball HR (**1973**). Granulocyte chemotaxis: an improved in vitro assay employing 51 Cr-labeled granulocytes. *J Immunol* 110, 233-40.

Gauchat, J. F., Henchoz, S., Mazzei, G., Aubry, J. P., Brunner, T., Blasey, H., Life, P., Talabot, D., Flores-Romo, L., Thompson, J., and et al. (**1993**).

Induction of human IgE synthesis in B cells by mast cells and basophils. *Nature* 365, 340-3.

Gerrard, J. W., Cockcroft, D. W., Mink, J. T., Cotton, D. J., Poonawala, R., and Dosman, J. A. (**1980**). Increased nonspecific bronchial reactivity in cigarette smokers with normal lung function. *Am Rev Respir Dis* 122, 577-81.

Gerszten, R. E., Chen, J., Ishii, M., Ishii, K., Wang, L., Nanevicz, T., Turck, C. W., Vu, T. K., and Coughlin, S. R. (**1994**). Specificity of the thrombin receptor for agonist peptide is defined by its extracellular surface. *Nature* 368, 648-51.

Glenner, G. G., and Cohen, L. A. (**1960**). Histochemical demonstration of a species-specific trypsin-like enzyme in mast cells. *Nature* 185, 846-847.

Godfrey, R. W. (**1997**). Human airway epithelial tight junctions. *Microsc Res Tech* 38, 488-99.

Godfrey, R. W., Severs, N. J., and Jeffery, P. K. (**1992**). Freeze-fracture morphology and quantification of human bronchial epithelial tight junctions. *Am J Respir Cell Mol Biol* 6, 453-8.

Golden, J. A., Nadel, J. A., and Boushey, H. A. (**1978**). Bronchial hyperirritability in healthy subjects after exposure to ozone. *Am Rev Respir Dis* 118, 287-94.

Goldstein, S. M., Kaempfer, C. E., Kealey, J. T., and Wintroub, B. U. (**1989**). Human mast cell carboxypeptidase. Purification and characterization. *J Clin Invest* 83, 1630-6.

Goldstein, S. M., Kaempfer, C. E., Proud, D., Schwartz, L. B., Irani, A. M., and Wintroub, B. U. (**1987**). Detection and partial characterization of a human mast cell carboxypeptidase. *J Immunol* 139, 2724-9.

Goldstein, S. M., Leong, J., and Bunnett, N. W. (**1991**). Human mast cell proteases hydrolyze neurotensin, kinetensin and Leu5- enkephalin. *Peptides* 12, 995-1000.

Goldstein, S. M., Leong, J., Schwartz, L. B., and Cooke, D. (**1992**). Protease composition of exocytosed human skin mast cell protease- proteoglycan complexes. Tryptase resides in a complex distinct from chymase and carboxypeptidase. *J Immunol* 148, 2475-82.

Gordon, J. R., and Galli, S. J. (**1991**). Release of both preformed and newly synthesized tumor necrosis factor alpha (TNF-alpha)/cachectin by mouse mast cells stimulated via the Fc epsilon RI. A mechanism for the sustained action of mast cell-derived TNF-alpha during IgE-dependent biological responses. *J Exp Med* 174, 103-7.

Gordon, T., Sheppard, D., McDonald, D. M., Distefano, S., and Scypinski, L. (**1985**). Airway hyperresponsiveness and inflammation induced by toluene diisocyanate in guinea pigs. *Am Rev Respir Dis* 132, 1106-12.

Gormori, G. (**1953**). Chloroacyl esters as histochemical substrates. *J Histochem Cytochem* 1, 469-470.

Gruber, B. L., Kew, R. R., Jelaska, A., Marchese, M. J., Garlick, J., Ren, S., Schwartz, L. B., and Korn, J. H. (**1997**). Human mast cells activate fibroblasts: tryptase is a fibrogenic factor stimulating collagen messenger ribonucleic acid synthesis and fibroblast chemotaxis. *J Immunol* 158, 2310-7.

Gruber, B. L., Marchese, M. J., Suzuki, K., Schwartz, L. B., Okada, Y., Nagase, H., and Ramamurthy, N. S. (**1989**). Synovial procollagenase activation by human mast cell tryptase dependence upon matrix metalloproteinase 3 activation. *J Clin Invest* 84, 1657-62.

Gruber, B. L., Schwartz, L. B., Ramamurthy, N. S., Irani, A. M., and Marchese, M. J. (**1988**). Activation of latent rheumatoid synovial collagenase by human mast cell tryptase. *J Immunol* 140, 3936-42.

Gruenert, D. C., Finkbeiner, W. E., and Widdicombe, J. H. (**1995**). Culture and transformation of human airway epithelial cells. *American Journal of Physiology* 268, L347-L360.

Gumbiner, B., Lowenkopf, T., and Apatira, D. (**1991**). Identification of a 160-kDa polypeptide that binds to the tight junction protein ZO-1. *Proc Natl Acad Sci U S A* 88, 3460-4.

Guranowski, A., Montgomery, J. A., Cantoni, G. L., and Chiang, P. K. (**1981**). Adenosine analogues as substrates and inhibitors of S- adenosylhomocysteine hydrolase. *Biochemistry* 20, 110-5.

Hack, C. E., Hart, M., van Schijndel, R. J., Eerenberg, A. J., Nuijens, J. H., Thijs, L. G., and Aarden, L. A. (**1992**). Interleukin-8 in sepsis: relation to shock and inflammatory mediators. *Infect Immun* 60, 2835-42.

Hagermark, O., Hokfelt, T., and Pernow, B. (**1978**). Flare and itch induced by substance P in human skin. *J Invest Dermatol* 71, 233-5.

Hamilton, J. R., Nguyen, P. B., and Cocks, T. M. (**1998**). Atypical protease-activated receptor mediates endothelium-dependent relaxation of human coronary arteries. *Circ Res* 82, 1306-11.

Hardy, C. C., Robinson, C., Tattersfield, A. E., and Holgate, S. T. (**1984**). The bronchoconstrictor effect of inhaled prostaglandin D2 in normal and asthmatic men. *N Engl J Med* 311, 209-13.

Hartmann, T., Ruoss, S. J., Raymond, W. W., Seuwen, K., and Caughey, G. H. (**1992**). Human tryptase as a potent, cell-specific mitogen: role of signaling pathways in synergistic responses. *Am J Physiol* 262, L528-34.

He, S., Gaca, M. D., and Walls, A. F. (**1998**). A role for tryptase in the activation of human mast cells: modulation of histamine release by tryptase and inhibitors of tryptase. *J Pharmacol Exp Ther* 286, 289-97.

He, S., Peng, Q., and Walls, A. F. (**1997a**). Potent induction of a neutrophil and eosinophil-rich infiltrate in vivo by human mast cell tryptase: selective enhancement of eosinophil recruitment by histamine. *J Immunol* 159, 6216-25.

He, S., and Walls, A. F. (**1997**). Human mast cell tryptase: a stimulus of microvascular leakage and mast cell activation. *Eur J Pharmacol* 328, 89-97.

Hein, L., Ishii, K., Coughlin, S. R., and Kobilka, B. K. (**1994**). Intracellular targeting and trafficking of thrombin receptors. A novel mechanism for resensitization of a G protein-coupled receptor. *J Biol Chem* 269, 27719-26.

Heinrich, P. C., Castell, J. V., and Andus, T. (**1990**). Interleukin-6 and the acute phase response. *Biochem J* 265, 621-36.

Herbert, C. A., Holgate, S. T., Robinson, C., Thompson, P. J., and Stewart, G. A. (**1990**). Effect of mite allergen on permeability of bronchial mucosa. *Lancet* 336, 1132.

Herbert, C. A., King, C. M., Ring, P. C., Holgate, S. T., Stewart, G. A., Thompson, P. J., and Robinson, C. (**1995**). Augmentation of permeability in the bronchial epithelium by the house dust mite allergen Der p1. *Am J Respir Cell Mol Biol* 12, 369-78.

Hirokawa, N., and Tilney, L. G. (**1982**). Interactions between actin filaments and between actin filaments and membranes in quick-frozen and deeply etched hair cells of the chick ear. *J Cell Biol* 95, 249-61.

Holgate, S. T., Lackie, P. M., Davies, D. E., Roche, W. R., and Walls, A. F. (**1999**). The bronchial epithelium as a key regulator of airway inflammation and remodelling in asthma. *Clin Exp Allergy* 29 Suppl 2, 90-5.

Hollenberg, M. D., Saifeddine, M., and al-Ani, B. (**1996**). Proteinase-activated receptor-2 in rat aorta: structural requirements for agonist activity of receptor-activating peptides. *Mol Pharmacol* 49, 229-33.

Horvat, R., and Palade, G. E. (**1995**). The functional thrombin receptor is associated with the plasmalemma and a large endosomal network in cultured human umbilical vein endothelial cells. *J Cell Sci* 108, 1155-64.

Hoxie, J. A., Ahuja, M., Belmonte, E., Pizarro, S., Parton, R., and Brass, L. F. (**1993**). Internalization and recycling of activated thrombin receptors. *J Biol Chem* 268, 13756-63.

Huang, C., Li, L., Krilis, S. A., Chanasyk, K., Tang, Y., Li, Z., Hunt, J. E., and Stevens, R. L. (**1999**). Human tryptases alpha and beta/II are functionally distinct due, in part, to a single amino acid difference in one of the surface loops that forms the substrate-binding cleft. *J Biol Chem* 274, 19670-6.

Huang, E., Nocka, K., Beier, D. R., Chu, T. Y., Buck, J., Lahm, H. W., Wellner, D., Leder, P., and Besmer, P. (**1990**). The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. *Cell* 63, 225-33.

Hulsmann, A. R., and De Jongste, J. C. (**1996a**). Modulation of airway responsiveness by the airway epithelium in humans: putative mechanisms. *Clin Exp Allergy* 26, 1236-42.

Hulsmann, A. R., Raatgeep, H. R., den Hollander, J. C., Bakker, W. H., Saxena, P. R., and de Jongste, J. C. (**1996**). Permeability of human isolated airways increases after hydrogen peroxide and poly-L-arginine. *Am J Respir Crit Care Med* 153, 841-6.

Hung, D. T., Wong, Y. H., Vu, T. K., and Coughlin, S. R. (**1992**). The cloned platelet thrombin receptor couples to at least two distinct effectors to stimulate phosphoinositide hydrolysis and inhibit adenylyl cyclase. *J Biol Chem* 267, 20831-4.

Hwa, J. J., Ghibaudi, L., Williams, P., Chintala, M., Zhang, R., Chatterjee, M., and Sybertz, E. (**1996**). Evidence for the presence of a proteinase-activated receptor distinct from the thrombin receptor in vascular endothelial cells. *Circ Res* 78, 581-8.

Ichinose, M., and Barnes, P. J. (**1989**). Histamine H3-receptors modulate nonadrenergic noncholinergic neural bronchoconstriction in guinea-pig in vivo. *Eur J Pharmacol* 174, 49-55.

Ilowite, J. S., Bennett, W. D., Sheetz, M. S., Groth, M. L., and Nierman, D. M. (**1989**). Permeability of the bronchial mucosa to 99mTc-DTPA in asthma. *Am Rev Respir Dis* 139, 1139-43.

Imamura, T., Dubin, A., Moore, W., Tanaka, R., and Travis, J. (**1996**). Induction of vascular permeability enhancement by human tryptase: dependence on activation of prekallikrein and direct release of bradykinin from kininogens. *Lab Invest* 74, 861-70.

Inoue, Y., Fishman, P. H., and Rebois, R. V. (**1990**). Differential activation of the stimulatory and inhibitory guanine nucleotide-binding proteins by fluoroaluminate in cells and in membranes. *J Biol Chem* 265, 10645-51.

Irani, A. A., Schechter, N. M., Craig, S. S., DeBlois, G., and Schwartz, L. B. (**1986**). Two types of human mast cells that have distinct neutral protease compositions. *Proc Natl Acad Sci U S A* 83, 4464-8.

Irani, A. M., Bradford, T. R., Kepley, C. L., Schechter, N. M., and Schwartz, L. B. (**1989**). Detection of MCT and MCTC types of human mast cells by

immunohistochemistry using new monoclonal anti-tryptase and anti- chymase antibodies. *J Histochem Cytochem* 37, 1509-15.

Irani, A. M., Goldstein, S. M., Wintroub, B. U., Bradford, T., and Schwartz, L. B. (**1991**). Human mast cell carboxypeptidase. Selective localization to MCTC cells. *J Immunol* 147, 247-53.

Ishibashi, T., Kimura, H., Shikama, Y., Uchida, T., Kariyone, S., Hirano, T., Kishimoto, T., Takatsuki, F., and Akiyama, Y. (**1989**). Interleukin-6 is a potent thrombopoietic factor in vivo in mice. *Blood* 74, 1241-4.

Ishii, K., Chen, J., Ishii, M., Koch, W. J., Freedman, N. J., Lefkowitz, R. J., and Coughlin, S. R. (**1994**). Inhibition of thrombin receptor signaling by a G-protein coupled receptor kinase. Functional specificity among G-protein coupled receptor kinases. *J Biol Chem* 269, 1125-30.

Jaffe, J. S., Raible, D. G., Post, T. J., Wang, Y., Glaum, M. C., Butterfield, J. H., and Schulman, E. S. (**1996**). Human lung mast cell activation leads to IL-13 mRNA expression and protein release. *Am J Respir Cell Mol Biol* 15, 473-81.

Jarjour, N. N., Busse, W. W., and Calhoun, W. J. (**1992**). Enhanced production of oxygen radicals in nocturnal asthma. *Am Rev Respir Dis* 146, 905-11.

Jarjour, N. N., Calhoun, W. J., Schwartz, L. B., and Busse, W. W. (**1991**). Elevated bronchoalveolar lavage fluid histamine levels in allergic asthmatics are associated with increased airway obstruction. *Am Rev Respir Dis* 144, 83-7.

Jatakanon, A., Uasuf, C., Maziak, W., Lim, S., Chung, K. F., and Barnes, P. J. (**1999**). Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med* 160, 1532-9.

Jeannin, P., Delneste, Y., Gosset, P., Molet, S., Lassalle, P., Hamid, Q., Tsicopoulos, A., and Tonnel, A. B. (**1994**). Histamine induces interleukin-8 secretion by endothelial cells. *Blood* 84, 2229-33.

Jeffery, P. K., Wardlaw, A. J., Nelson, F. C., Collins, J. V., and Kay, A. B. (**1989**). Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. *Am Rev Respir Dis* 140, 1745-53.

Johnson, L. G., Dickman, K. G., Moore, K. L., Mandel, L. J., and Boucher, R. C. (**1993**). Enhanced Na+ transport in an air-liquid interface culture system. *Am J Physiol* 264, L560-5.

Johnson, P. R., Ammit, A. J., Carlin, S. M., Armour, C. L., Caughey, G. H., and Black, J. L. (**1997**). Mast cell tryptase potentiates histamine-induced contraction in human sensitized bronchus. *Eur Respir J* 10, 38-43.

Joos, G. F., Germonpre, P. R., Kips, J. C., Peleman, R. A., and Pauwels, R. A. (**1994**). Sensory neuropeptides and the human lower airways: present state and future directions. *Eur Respir J* 7, 1161-71.

Kaufmann, R., Schafberg, H., and Nowak, G. (**1998**). Proteinase-activated receptor-2-mediated signaling and inhibition of DNA synthesis in human pancreatic cancer cells. *Int J Pancreatol* 24, 97-102.

Kawabata, A., Kuroda, R., Minami, T., Kataoka, K., and Taneda, M. (**1998**). Increased vascular permeability by a specific agonist of protease- activated receptor-2 in rat hindpaw. *Br J Pharmacol* 125, 419-22.

Keenan, K. P., Combs, J. W., and McDowell, E. M. (**1982**). Regeneration of hamster tracheal epithelium after mechanical injury. I. Focal lesions: quantitative morphologic study of cell proliferation. *Virchows Arch B Cell Pathol Incl Mol Pathol* 41, 193-214.

Kelley, J. (**1990**). Cytokines of the lung. *Am Rev Respir Dis* 141, 765-88.

Khair, O. A., Davies, R. J., and Devalia, J. L. (**1996**). Bacterial-induced release of inflammatory mediators by bronchial epithelial cells. *Eur Respir J* 9, 1913-22.

Khair, O. A., Devalia, J. L., Abdelaziz, M. M., Sapsford, R. J., Tarraf, H., and Davies, R. J. (**1994**). Effect of Haemophilus influenzae endotoxin on the synthesis of IL-6, IL- 8, TNF-alpha and expression of ICAM-1 in cultured human bronchial epithelial cells. *Eur Respir J* 7, 2109-16.

King, C., Brennan, S., Thompson, P. J., and Stewart, G. A. (**1998**). Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J Immunol* 161, 3645-51.

Kirby, J. G., Hargreave, F. E., Gleich, G. J., and O'Byrne, P. M. (**1987**). Bronchoalveolar cell profiles of asthmatic and nonasthmatic subjects. *Am Rev Respir Dis* 136, 379-83.

Kirshenbaum, A. S., Kessler, S. W., Goff, J. P., and Metcalfe, D. D. (**1991**). Demonstration of the origin of human mast cells from CD34+ bone marrow progenitor cells. *J Immunol* 146, 1410-5.

Kishimoto, T. (**1989**). The biology of interleukin-6. *Blood* 74, 1-10.

Knobil, K., and Jacoby, D. B. (1998). Mediator functions of epithelial cells. In Inflammatory mechanisms in asthma, S. T. Holgate and W. W. Busse, eds. New York: Marcel Dekker Inc, pp. 469-95.

Kobayashi, K., Salathe, M., Pratt, M. M., Cartagena, N. J., Soloni, F., Seybold, Z. V., and Wanner, A. (**1992**). Mechanism of hydrogen peroxide-induced inhibition of sheep airway cilia. *Am J Respir Cell Mol Biol* 6, 667-73.

Koller, D. Y., Nething, I., Otto, J., Urbanek, R., and Eichler, I. (**1997**). Cytokine concentrations in sputum from patients with cystic fibrosis and their relation to eosinophil activity. *Am J Respir Crit Care Med* 155, 1050-4.

Kong, W., McConalogue, K., Khitin, L. M., Hollenberg, M. D., Payan, D. G., Bohm, S. K., and Bunnett, N. W. (**1997**). Luminal trypsin may regulate enterocytes through proteinase-activated receptor 2. *Proc Natl Acad Sci U S A* 94, 8884-9.

Krishna, M. T., Chauhan, A. J., Little, L., Sampson, K., Mant, T. G. K., Hawksworth, R., Djukanovic, R., Lee, T. H., and Holgate, S. T. (**1998**). Effect of inhaled APC 366 on allergen-induced bronchoconstriction and airway hyperresponsiveness to histamine in atopic asthmatics. *Am J Respir Crit Care Med* 157, A456. (Abstr.).

Kunkel, S. L., Standiford, T., Kasahara, K., and Strieter, R. M. (**1991**). Interleukin-8 (IL-8): the major neutrophil chemotactic factor in the lung. *Exp Lung Res* 17, 17-23.

Laan, M., Cui, Z. H., Hoshino, H., Lotvall, J., Sjostrand, M., Gruenert, D. C., Skoogh, B. E., and Linden, A. (**1999**). Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J Immunol* 162, 2347-52.

Lagunoff, D., and Benditt, E. P. (**1963**). Proteolytic enzymes of mast cells. *Ann N Y Acad Sci* 103, 185-98.

Laitinen, L. A., Heino, M., Laitinen, A., Kava, T., and Haahtela, T. (**1985**). Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am Rev Respir Dis* 131, 599-606.

Lam, S., Tan, F., Chan, H., and Chan-Yeung, M. (**1983**). Relationship between types of asthmatic reaction, nonspecific bronchial reactivity, and specific IgE antibodies in patients with red cedar asthma. *J Allergy Clin Immunol* 72, 134-9.

Lan, R. S., Stewart, G. A., and Henry, P. J. (**2000**). Modulation of airway smooth muscle tone by protease activated receptor- 1,-2,-3 and -4 in trachea isolated from influenza A virus-infected mice. *Br J Pharmacol* 129, 63-70.

Lane, B. P., and Gordon, R. (**1974**). Regeneration of rat tracheal epithelium after mechanical injury. I. The relationship between mitotic activity and cellular differentiation. *Proc Soc Exp Biol Med* 145, 1139-44.

Lansing, M. W., Ahmed, A., Cortes, A., Sielczak, M. W., Wanner, A., and Abraham, W. M. (**1993**). Oxygen radicals contribute to antigen-induced airway hyperresponsiveness in conscious sheep. *Am Rev Respir Dis* 147, 321-6.

Lerner, D. J., Chen, M., Tram, T., and Coughlin, S. R. (**1996**). Agonist recognition by proteinase-activated receptor 2 and thrombin receptor. Importance of extracellular loop interactions for receptor function. *J Biol Chem* 271, 13943-7.

Levi-Schaffer, F., and Shalit, M. (**1989**). Differential release of histamine and prostaglandin D2 in rat peritoneal mast cells activated with peptides. *Int Arch Allergy Appl Immunol* 90, 352-7.

Lewis, S. A., Berg, J. R., and Kleine, T. J. (**1995**). Modulation of epithelial permeability by extracellular macromolecules. *Physiological Reviews* 75, 561-589.

Lieber, M., Smith, B., Szakal, A., Nelson-Rees, W., and Todaro, G. (**1976**). A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int J Cancer* 17, 62-70.

Liu, M. C., Hubbard, W. C., Proud, D., Stealey, B. A., Galli, S. J., Kagey-Sobotka, A., Bleecker, E. R., and Lichtenstein, L. M. (**1991**). Immediate and late inflammatory responses to ragweed antigen challenge of the peripheral

airways in allergic asthmatics. Cellular, mediator, and permeability changes. *Am Rev Respir Dis* 144, 51-8.

Liu, S. F., Yacoub, M., and Barnes, P. J. (**1990**). Effect of histamine on human bronchial arteries in vitro. *Naunyn Schmiedebergs Arch Pharmacol* 342, 90-3.

Lohi, J., Harvima, I., and Keski-Oja, J. (**1992**). Pericellular substrates of human mast cell tryptase: 72,000 dalton gelatinase and fibronectin. *J Cell Biochem* 50, 337-49.

Look, D. C., Rapp, S. R., Keller, B. T., and Holtzman, M. J. (**1992**). Selective induction of intercellular adhesion molecule-1 by interferon- gamma in human airway epithelial cells. *Am J Physiol* 263, L79-87.

Lopez, A. F., Williamson, D. J., Gamble, J. R., Begley, C. G., Harlan, J. M., Klebanoff, S. J., Waltersdorph, A., Wong, G., Clark, S. C., and Vadas, M. A. (**1986**). Recombinant human granulocyte-macrophage colony-stimulating factor stimulates in vitro mature human neutrophil and eosinophil function, surface receptor expression, and survival. *J Clin Invest* 78, 1220-8.

Lowman, M. A., Rees, P. H., Benyon, R. C., and Church, M. K. (**1988**). Human mast cell heterogeneity: histamine release from mast cells dispersed from skin, lung, adenoids, tonsils, and colon in response to IgE-dependent and nonimmunologic stimuli. *J Allergy Clin Immunol* 81, 590-7.

Lumsden, A. B., McLean, A., and Lamb, D. (**1984**). Goblet and Clara cells of human distal airways: evidence for smoking induced changes in their numbers. *Thorax* 39, 844-9.

Lynch, R. D., Tkachuk-Ross, L. J., McCormack, J. M., McCarthy, K. M., Rogers, R. A., and Schneeberger, E. E. (**1995**). Basolateral but not apical application of protease results in a rapid rise of transepithelial electrical resistance and formation of aberrant tight junction strands in MDCK cells. *Eur J Cell Biol* 66, 257-67.

MacCumber, M. W., Ross, C. A., Glaser, B. M., and Snyder, S. H. (**1989**). Endothelin: visualization of mRNAs by in situ hybridization provides evidence for local action. *Proc Natl Acad Sci U S A* 86, 7285-9.

MacGlashan, D. W., Jr., Schleimer, R. P., Peters, S. P., Schulman, E. S., Adams, G. K. d., Newball, H. H., and Lichtenstein, L. M. (**1982**). Generation of leukotrienes by purified human lung mast cells. *J Clin Invest* 70, 747-51.

Madara, J. L., Parkos, C., Colgan, S., Nusrat, A., Atisook, K., and Kaoutzani, P. (**1992**). The movement of solutes and cells across tight junctions. *Ann N Y Acad Sci* 664, 47-60.

Martin, F. H., Suggs, S. V., Langley, K. E., Lu, H. S., Ting, J., Okino, K. H., Morris, C. F., McNiece, I. K., Jacobsen, F. W., Mendiaz, E. A., and et al. (**1990**). Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell* 63, 203-11.

Martin, L. D., Rochelle, B. M., Fischer, B. M., Krunchosky, T. M., and Alder, K. B. (**1997**). Airway epithelium as an effector of inflammation: molecular regulation of secondary mediators. *European Respiratory Journal* 10, 2139-2146.

Martin, T. R., Pistorese, B. P., Chi, E. Y., Goodman, R. B., and Matthay, M. A. (**1989**). Effects of leukotriene B4 in the human lung. Recruitment of neutrophils into the alveolar spaces without a change in protein permeability. *J Clin Invest* 84, 1609-19.

Martin-Padura, I., Lostaglio, S., Schneemann, M., Williams, L., Romano, M., Fruscella, P., Panzeri, C., Stoppacciaro, A., Ruco, L., Villa, A., Simmons, D., and Dejana, E. (**1998**). Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J Cell Biol* 142, 117-27.

Matsuura, N., and Zetter, B. R. (**1989**). Stimulation of mast cell chemotaxis by interleukin 3. *J Exp Med* 170, 1421-6.

Mattoli, S., Mattoso, V. L., Soloperto, M., Allegra, L., and Fasoli, A. (**1991**). Cellular and biochemical characteristics of bronchoalveolar lavage fluid in symptomatic nonallergic asthma. *J Allergy Clin Immunol* 87, 794-802.

McCarthy, K. M., Skare, I. B., Stankewich, M. C., Furuse, M., Tsukita, S., Rogers, R. A., Lynch, R. D., and Schneeberger, E. E. (**1996**). Occludin is a functional component of the tight junction. *J Cell Sci* 109, 2287-98.

McKay, D. M., and Perdue, M. H. (**1993**). Intestinal epithelial function: the case for immunophysiological regulation. Cells and mediators (1). *Dig Dis Sci* 38, 1377-87.

Meininger, C. J., Yano, H., Rottapel, R., Bernstein, A., Zsebo, K. M., and Zetter, B. R. (**1992**). The c-kit receptor ligand functions as a mast cell chemoattractant. *Blood* 79, 958-63.

Mercer, R. R., Russell, M. L., Roggli, V. L., and Crapo, J. D. (**1994**). Cell number and distribution in human and rat airways. *Am J Respir Cell Mol Biol* 10, 613-24.

Metzger, W. J., Richerson, H. B., Worden, K., Monick, M., and Hunninghake, G. W. (**1986**). Bronchoalveolar lavage of allergic asthmatic patients following allergen bronchoprovocation. *Chest* 89, 477-83.

Michel, O., Ginanni, R., Le Bon, B., Content, J., Duchateau, J., and Sergysels, R. (**1992**). Inflammatory response to acute inhalation of endotoxin in asthmatic patients. *Am Rev Respir Dis* 146, 352-7.

Miller, J. S., Moxley, G., and Schwartz, L. B. (**1990**). Cloning and characterization of a second complementary DNA for human tryptase. *J Clin Invest* 86, 864-70.

Miller, J. S., Westin, E. H., and Schwartz, L. B. (**1989**). Cloning and characterization of complementary DNA for human tryptase. *J Clin Invest* 84, 1188-95.

Mirza, H., Schmidt, V. A., Derian, C. K., Jesty, J., and Bahou, W. F. (**1997**). Mitogenic responses mediated through the proteinase-activated receptor- 2 are induced by expressed forms of mast cell alpha- or beta-tryptases. *Blood* 90, 3914-22.

Mirza, H., Yatsula, V., and Bahou, W. F. (**1996**). The proteinase activated receptor-2 (PAR-2) mediates mitogenic responses in human vascular endothelial cells. *J Clin Invest* 97, 1705-14.

Mitic, L. L., and Anderson, J. M. (**1998**). Molecular architecture of tight junctions. *Annu Rev Physiol* 60, 121-42.

Molinari, J. F., Moore, W. R., Clark, J., Tanaka, R., Butterfield, J. H., and Abraham, W. M. (**1995**). Role of tryptase in immediate cutaneous responses in allergic sheep. *J Appl Physiol* 79, 1966-70.

Molinari, J. F., Scuri, M., Moore, W. R., Clark, J., Tanaka, R., and Abraham, W. M. (**1996**). Inhaled tryptase causes bronchoconstriction in sheep via histamine release. *American Journal of Respiratory and Critical Care Medicine* 154, 649-653.

Molino, M., Barnathan, E. S., Numerof, R., Clark, J., Dreyer, M., Cumashi, A., Hoxie, J. A., Schechter, N., Woolkalis, M., and Brass, L. F. (**1997a**). Interactions of mast cell tryptase with thrombin receptors and PAR-2. *J Biol Chem* 272, 4043-9.

Molino, M., Woolkalis, M. J., Reavey-Cantwell, J., Pratico, D., Andrade-Gordon, P., Barnathan, E. S., and Brass, L. F. (**1997**). Endothelial cell

thrombin receptors and PAR-2. Two protease-activated receptors located in a single cellular environment. *J Biol Chem* 272, 11133-41.

Montefort, S., Baker, J., Roche, W. R., and Holgate, S. T. (**1993**). The distribution of adhesive mechanisms in the normal bronchial epithelium. *Eur Respir J* 6, 1257-63.

Moss, J., and Rosow, C. E. (**1983**). Histamine release by narcotics and muscle relaxants in humans. *Anesthesiology* 59, 330-9.

Motojima, S., Frigas, E., Loegering, D. A., and Gleich, G. J. (**1989**). Toxicity of eosinophil cationic proteins for guinea pig tracheal epithelium in vitro. *Am Rev Respir Dis* 139, 801-5.

Mullin, J. M., and Snock, K. V. (**1990**). Effect of tumor necrosis factor on epithelial tight junctions and transepithelial permeability. *Cancer Res* 50, 2172-6.

Murayama, T., and Ui, M. (**1984**). [^{3}H]GDP release from rat and hamster adipocyte membranes independently linked to receptors involved in activation or inhibition of adenylate cyclase. Differential susceptibility to two bacterial toxins. *J Biol Chem* 259, 761-9.

Nahori, M. A., Renesto, P., Vargaftig, B. B., and Chignard, M. (**1992**). Activation and damage of cultured airway epithelial cells by human elastase and cathepsin G. *Eur J Pharmacol* 228, 213-8.

Nakamura, H., Yoshimura, K., McElvaney, N. G., and Crystal, R. G. (**1992**). Neutrophil elastase in respiratory epithelial lining fluid of individuals with cystic fibrosis induces interleukin-8 gene expression in a human bronchial epithelial cell line. *J Clin Invest* 89, 1478-84.

National Heart, Lung, and Blood Institute. **(1997)**. Expert Panel Report 2: gidelines for the diagnosis and management of asthma: National Institute of Health, pp. 97-4051.

Naylor, B. (**1962**). The shedding of the mucosa of the bronchial tree in asthma. *Thorax* 45, 79-72.

Niles, A. L., Maffitt, M., Haak-Frendscho, M., Wheeless, C. J., and Johnson, D. A. (**1998**). Recombinant human mast cell tryptase beta: stable expression in Pichia pastoris and purification of fully active enzyme. *Biotechnol Appl Biochem* 28, 125-31.

Nocker, R. E., Out, T. A., Weller, F. R., Mul, E. P., Jansen, H. M., and van der Zee, J. S. (**1999**). Influx of neutrophils into the airway lumen at 4 h after segmental allergen challenge in asthma. *Int Arch Allergy Immunol* 119, 45-53.

Nystedt, S., Emilsson, K., Larsson, A. K., Strombeck, B., and Sundelin, J. (**1995**). Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor 2. *Eur J Biochem* 232, 84-9.

Nystedt, S., Emilsson, K., Wahlestedt, C., and Sundelin, J. (**1994**). Molecular cloning of a potential proteinase activated receptor. *Proc Natl Acad Sci U S A* 91, 9208-12.

Nystedt, S., Larsson, A. K., Aberg, H., and Sundelin, J. (**1995**). The mouse proteinase-activated receptor-2 cDNA and gene. Molecular cloning and functional expression. *J Biol Chem* 270, 5950-55.

Nystedt, S., Ramakrishnan, V., and Sundelin, J. (**1996**). The proteinase-activated receptor 2 is induced by inflammatory mediators in human endothelial cells. Comparison with the thrombin receptor. *J Biol Chem* 271, 14910-5.

O'Donnell, M. C., Ackerman, S. J., Gleich, G. J., and Thomas, L. L. (**1983**). Activation of basophil and mast cell histamine release by eosinophil granule major basic protein. *J Exp Med* 157, 1981-91.

Ohashi, Y., Motojima, S., Fukuda, T., and Makino, S. (**1992**). Airway hyperresponsiveness, increased intracellular spaces of bronchial epithelium, and increased infiltration of eosinophils and lymphocytes in bronchial mucosa in asthma [see comments]. *Am Rev Respir Dis* 145, 1469-76.

Ohrui, T., Yamaya, M., Sekizawa, K., Yamada, N., Suzuki, T., Terajima, M., Okinaga, S., and Sasaki, H. (**1998**). Effects of rhinovirus infection on hydrogen peroxide- induced alterations of barrier function in the cultured human tracheal epithelium. *Am J Respir Crit Care Med* 158, 241-8.

Okayama, Y., Petit-Frere, C., Kassel, O., Semper, A., Quint, D., Tunon-de-Lara, M. J., Bradding, P., Holgate, S. T., and Church, M. K. (**1995**). IgE-dependent expression of mRNA for IL-4 and IL-5 in human lung mast cells. *J Immunol* 155, 1796-808.

Omori, C., Schofield, B. H., Mitzner, W., and Freed, A. N. (**1995**). Hyperpnea with dry air causes time-dependent alterations in mucosal morphology and bronchovascular permeability. *J Appl Physiol* 78, 1043-51.

Pereira, P. J. B., Bergner, A., Macedo-Ribeiro, S., Huber, R., Matschiner, G., Fritz, H., Sommerhoff, C. P., and Bode, W. (**1998**). Human beta-tryptase is a ring-like tetramer with active sites facing a central pore. *Nature* 392, 306-311.

Peters, S. P., MacGlashan, D. W., Jr., Schulman, E. S., Schleimer, R. P., Hayes, E. C., Rokach, J., Adkinson, N. F., Jr., and Lichtenstein, L. M. (**1984**). Arachidonic acid metabolism in purified human lung mast cells. *J Immunol* 132, 1972-9.

Phipps, R. J., Denas, S. M., Sielczak, M. W., and Wanner, A. (**1986**). Effects of 0.5 ppm ozone on glycoprotein secretion, ion and water fluxes in sheep trachea. *J Appl Physiol* 60, 918-27.

Plaut, M., Pierce, J. H., Watson, C. J., Hanley-Hyde, J., Nordan, R. P., and Paul, W. E. (**1989**). Mast cell lines produce lymphokines in response to cross-linkage of Fc epsilon RI or to calcium ionophores. *Nature* 339, 64-7.

Polito, A. J., and Proud, D. (**1998**). Epithelia cells as regulators of airway inflammation. *J Allergy Clin Immunol* 102, 714-8.

Rabe, K. F., Dent, G., and Magnussen, H. (**1995**). Hydrogen peroxide contracts human airways in vitro: role of epithelium. *Am J Physiol* 269, L332-8.

Reilly, C. F., Tewksbury, D. A., Schechter, N. M., and Travis, J. (**1982**). Rapid conversion of angiotensin I to angiotensin II by neutrophil and mast cell proteinases. *J Biol Chem* 257, 8619-22.

Renesto, P., Si-Tahar, M., Moniatte, M., Balloy, V., Van Dorsselaer, A., Pidard, D., and Chignard, M. (**1997**). Specific inhibition of thrombin-induced cell activation by the neutrophil proteinases elastase, cathepsin G, and proteinase 3: evidence for distinct cleavage sites within the aminoterminal domain of the thrombin receptor. *Blood* 89, 1944-53.

Rennard, S. I., Basset, G., Lecossier, D., O'Donnell, K. M., Pinkston, P., Martin, P. G., and Crystal, R. G. (**1986**). Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. *J Appl Physiol* 60, 532-8.

Rennick, D. M., Lee, F. D., Yokota, T., Arai, K. I., Cantor, H., and Nabel, G. J. (**1985**). A cloned MCGF cDNA encodes a multilineage hematopoietic growth factor: multiple activities of interleukin 3. *J Immunol* 134, 910-4.

Ricciardolo, F. L., Steinhoff, M., Amadesi, S., Guerrini, R., Tognetto, M., Trevisani, M., Creminon, C., Bertrand, C., Bunnett, N. W., Fabbri, L. M., Salvadori, S., and Geppetti, P. (**2000**). Presence and Bronchomotor Activity of Protease-Activated Receptor-2 in Guinea Pig Airways. *Am J Respir Crit Care Med* 161, 1672-1680.

Rouss, S. J., Hartmann, T., and Caughey, G. H. (**1991**). Mast cell tryptase is a mitogen for culture fibroblasts. *J Clin Invest* 88, 493-499.

Saifeddine, M., al-Ani, B., Cheng, C. H., Wang, L., and Hollenberg, M. D. (**1996**). Rat proteinase-activated receptor-2 (PAR-2): cDNA sequence and activity of receptor-derived peptides in gastric and vascular tissue. *Br J Pharmacol* 118, 521-30.

Saitou, M., Fujimoto, K., Doi, Y., Itoh, M., Fujimoto, T., Furuse, M., Takano, H., Noda, T., and Tsukita, S. (**1998**). Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. *J Cell Biol* 141, 397-408.

Sakai, K., Ren, S., and Schwartz, L. B. (**1996**). A novel heparin-dependent processing pathway for human tryptase. Autocatalysis followed by activation with dipeptidyl peptidase I [see comments]. *J Clin Invest* 97, 988-95.

Salomonsson, P., Gronneberg, R., Gilljam, H., Andersson, O., Billing, B., Enander, I., Alkner, U., and Persson, C. G. (**1992**). Bronchial exudation of bulk plasma at allergen challenge in allergic asthma. *Am Rev Respir Dis* 146, 1535-42.

Salvi, S., Semper, A., Blomberg, A., Holloway, J., Jaffar, Z., Papi, A., Teran, L., Polosa, R., Kelly, F., Sandstrom, T., Holgate, S., and Frew, A. (**1999**). Interleukin-5 production by human airway epithelial cells. *Am J Respir Cell Mol Biol* 20, 984-91.

Sampson, S. E., Costello, J. F., and Sampson, A. P. (**1997**). The effect of inhaled leukotriene B4 in normal and in asthmatic subjects. *Am J Respir Crit Care Med* 155, 1789-92.

Santulli, R. J., Derian, C. K., Darrow, A. L., Tomko, K. A., Eckardt, A. J., Seiberg, M., Scarborough, R. M., and Andrade-Gordon, P. (**1995**). Evidence for the presence of a protease-activated receptor distinct from the thrombin receptor in human keratinocytes. *Proc Natl Acad Sci U S A* 92, 9151-5.

Sayama, S., Iozzo, R. V., Lazarus, G. S., and Schechter, N. M. (**1987**). Human skin chymotrypsin-like proteinase chymase. Subcellular localization to mast cell granules and interaction with heparin and other glycosaminoglycans. *J Biol Chem* 262, 6808-15.

Schechter, N. M., Brass, L. F., Lavker, R. M., and Jensen, P. J. (**1998**). Reaction of mast cell proteases tryptase and chymase with protease activated receptors (PARs) on keratinocytes and fibroblasts. *J Cell Physiol* 176, 365-73.

Schechter, N. M., Fraki, J. E., Geesin, J. C., and Lazarus, G. S. (**1983**). Human skin chymotryptic proteinase. Isolation and relation to cathepsin g and rat mast cell proteinase I. *J Biol Chem* 258, 2973-8.

Schechter, N. M., Sprows, J. L., Schoenberger, O. L., Lazarus, G. S., Cooperman, B. S., and Rubin, H. (**1989**). Reaction of human skin chymotrypsin-like proteinase chymase with plasma proteinase inhibitors. *J Biol Chem* 264, 21308-15.

Schulman, E. S., Post, T. J., Henson, P. M., and Giclas, P. C. (**1988**). Differential effects of the complement peptides, C5a and C5a des Arg on human basophil and lung mast cell histamine release. *J Clin Invest* 81, 918-23.

Schwartz, L. B. (**1994**). Tryptase: a mast cell serine protease. *Methods in Enzymology* 244, 88-100.

Schwartz, L. B., and Bradford, T. R. (**1986**). Regulation of tryptase from human lung mast cells by heparin. Stabilization of the active tetramer. *J Biol Chem* 261, 7372-9.

Schwartz, L. B., Bradford, T. R., Littman, B. H., and Wintroub, B. U. (**1985**). The fibrinogenolytic activity of purified tryptase from human lung mast cells. *J Immunol* 135, 2762-7.

Schwartz, L. B., Irani, A. M., Roller, K., Castells, M. C., and Schechter, N. M. (**1987**). Quantitation of histamine, tryptase, and chymase in dispersed human T and TC mast cells. *J Immunol* 138, 2611-5.

Schwartz, L. B., Lewis, R. A., and Austen, K. F. (**1981**). Tryptase from human pulmonary mast cells. Purification and characterization. *J Biol Chem* 256, 11939-43.

Schwartz, L. B., Metcalfe, D. D., Miller, J. S., Earl, H., and Sullivan, T. (**1987**). Tryptase levels as an indicator of mast-cell activation in systemic anaphylaxis and mastocytosis. *N Engl J Med* 316, 1622-6.

Schwartz, L. B., Sakai, K., Bradford, T. R., Ren, S., Zweiman, B., Worobec, A. S., and Metcalfe, D. D. (**1995**). The alpha form of human tryptase is the predominant type present in blood at baseline in normal subjects and is elevated in those with systemic mastocytosis. *J Clin Invest* 96, 2702-10.

Scudamore, C. L., Jepson, M. A., Hirst, B. H., and Miller, H. R. P. (**1998**). The rat mucosal mast cell chymase, RMCP-II, alters epithelial cell monolayer permeability in association with altered distribution of the tight junction proteins ZO-1 and occludin. *Eur J Cell Biol* 75, 321-30.

Scudamore, C. L., Pennington, A. M., Thornton, E., McMillan, L., Newlands, G. F., and Miller, H. R. (**1995**). Basal secretion and anaphylactic release of rat mast cell protease-II (RMCP-II) from ex vivo perfused rat jejunum:

translocation of RMCP-II into the gut lumen and its relation to mucosal histology. *Gut* 37, 235-41.

Scudamore, C. L., Thornton, E. M., McMillan, L., Newlands, G. F., and Miller, H. R. (**1995**). Release of the mucosal mast cell granule chymase, rat mast cell protease-II, during anaphylaxis is associated with the rapid development of paracellular permeability to macromolecules in rat jejunum. *J Exp Med* 182, 1871-81.

Seder, R. A., Paul, W. E., Ben-Sasson, S. Z., LeGros, G. S., Kagey-Sobotka, A., Finkelman, F. D., Pierce, J. H., and Plaut, M. (**1991**). Production of interleukin-4 and other cytokines following stimulation of mast cell lines and in vivo mast cells/basophils. *Int Arch Allergy Appl Immunol* 94, 137-40.

Sedgwick, J. B., Calhoun, W. J., Gleich, G. J., Kita, H., Abrams, J. S., Schwartz, L. B., Volovitz, B., Ben-Yaakov, M., and Busse, W. W. (**1991**). Immediate and late airway response of allergic rhinitis patients to segmental antigen challenge. Characterization of eosinophil and mast cell mediators. *Am Rev Respir Dis* 144, 1274-81.

Sekizawa, K., Caughey, G. H., Lazarus, S. C., Gold, W. M., and Nadel, J. A. (**1989**). Mast cell tryptase causes airway smooth muscle hyperresponsiveness in dogs. *J Clin Invest* 83, 175-9.

Seltzer, J., Bigby, B. G., Stulbarg, M., Holtzman, M. J., Nadel, J. A., Ueki, I. F., Leikauf, G. D., Goetzl, E. J., and Boushey, H. A. (**1986**). O3-induced change in bronchial reactivity to methacholine and airway inflammation in humans. *J Appl Physiol* 60, 1321-6.

Selwood, T., McCaslin, D. R., and Schechter, N. M. (**1998**). Spontaneous inactivation of human tryptase involves conformational changes consistent with conversion of the active site to a zymogen-like structure. *Biochemistry* 37, 13174-83.

Shute, J. (**1994**). Interleukin-8 is a potent eosinophil chemo-attractant. *Clin Exp Allergy* 24, 203-6.

Shute, J. K., Vrugt, B., Lindley, I. J., Holgate, S. T., Bron, A., Aalbers, R., and Djukanovic, R. (**1997**). Free and complexed interleukin-8 in blood and bronchial mucosa in asthma. *Am J Respir Crit Care Med* 155, 1877-83.

Sibille, Y., and Marchandise, F. X. (**1993**). Pulmonary immune cells in health and disease: polymorphonuclear neutrophils. *Eur Respir J* 6, 1529-43.

Sibille, Y., and Reynolds, H. Y. (**1990**). Macrophages and polymorphonuclear neutrophils in lung defense and injury. *Am Rev Respir Dis* 141, 471-501.

Simon, R. H., Edwards, J. A., Reza, M. M., and Kunkel, R. G. (**1991**). Injury of rat pulmonary alveolar epithelial cells by H2O2: dependence on phenotype and catalase. *Am J Physiol* 260, L318-25.

Smallman, L. A., Hill, S. L., and Stockley, R. A. (**1984**). Reduction of ciliary beat frequency in vitro by sputum from patients with bronchiectasis: a serine proteinase effect. *Thorax* 39, 663-7.

Smart, S. J., and Casale, T. B. (**1994**). Pulmonary epithelial cells facilitate TNF-alpha-induced neutrophil chemotaxis. A role for cytokine networking. *J Immunol* 152, 4087-94.

Smith, C. W., Rothlein, R., Hughes, B. J., Mariscalco, M. M., Rudloff, H. E., Schmalstieg, F. C., and Anderson, D. C. (**1988**). Recognition of an endothelial determinant for CD 18-dependent human neutrophil adherence and transendothelial migration. *J Clin Invest* 82, 1746-56.

Smith, T. J., Hougland, M. W., and Johnson, D. A. (**1984**). Human lung tryptase: purification and characterization. *J Biol Chem* 259, 11046-51.

Smith-Swintosky, V. L., Cheo-Isaacs, C. T., MR, D. A., Santulli, R. J., Darrow, A. L., and Andrade-Gordon, P. (**1997**). Protease-activated receptor-2 (PAR-2) is present in the rat hippocampus and is associated with neurodegeneration. *J Neurochem* 69, 1890-6.

Soloperto, M., Mattoso, V. L., Fasoli, A., and Mattoli, S. (**1991**). A bronchial epithelial cell-derived factor in asthma that promotes eosinophil activation and survival as GM-CSF. *Am J Physiol* 260, L530-8.

Sommerhoff, C. P., Caughey, G. H., Finkbeiner, W. E., Lazarus, S. C., Basbaum, C. B., and Nadel, J. A. (**1989**). Mast cell chymase. A potent secretagogue for airway gland serous cells. *J Immunol* 142, 2450-6.

Sousa, A. R., Poston, R. N., Lane, S. J., Nakhosteen, J. A., and Lee, T. H. (**1993**). Detection of GM-CSF in asthmatic bronchial epithelium and decrease by inhaled corticosteroids. *Am Rev Respir Dis* 147, 1557-61.

Sower, L. E., Klimpel, G. R., Hanna, W., and Froelich, C. J. (**1996**). Extracellular activities of human granzymes. I. Granzyme A induces IL6 and IL8 production in fibroblast and epithelial cell lines. *Cell Immunol* 171, 159-63.

Spiekermann, K., Roesler, J., Emmendoerffer, A., Elsner, J., and Welte, K. (**1997**). Functional features of neutrophils induced by G-CSF and GM-CSF treatment: differential effects and clinical implications. *Leukemia* 11, 466-78.

Stack, M. S., and Johnson, D. A. (**1994**). Human mast cell tryptase activates single-chain urinary-type plasminogen activator (pro-urokinase). *J Biol Chem* 269, 9416-9.

Steinhoff, M., Vergnolle, N., Young, S. H., Tognetto, M., Amadesi, S., Ennes, H. S., Trevisani, M., Hollenberg, M. D., Wallace, J. L., Caughey, G. H., Mitchell, S. E., Williams, L. M., Geppetti, P., Mayer, E. A., and Bunnett, N.

W. (**2000**). Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism [In Process Citation]. *Nat Med* 6, 151-8.

Stellato, C., de Paulis, A., Cirillo, R., Mastronardi, P., Mazzarella, B., and Marone, G. (**1991**). Heterogeneity of human mast cells and basophils in response to muscle relaxants. *Anesthesiology* 74, 1078-86.

Stepp, M. A., Spurr-Michaud, S., Tisdale, A., Elwell, J., and Gipson, I. K. (**1990**). Alpha 6 beta 4 integrin heterodimer is a component of hemidesmosomes. *Proc Natl Acad Sci U S A* 87, 8970-4.

Stevens, R. L., Fox, C. C., Lichtenstein, L. M., and Austen, K. F. (**1988**). Identification of chondroitin sulfate E proteoglycans and heparin proteoglycans in the secretory granules of human lung mast cells. *Proc Natl Acad Sci U S A* 85, 2284-7.

Stevenson, B. R., Siliciano, J. D., Mooseker, M. S., and Goodenough, D. A. (**1986**). Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J Cell Biol* 103, 755-66.

Stockley, R. A. (**1995**). Role of inflammation in respiratory tract infections. *Am J Med* 99, 8S-13S.

Stuart, R. O., Sun, A., Bush, K. T., and Nigam, S. K. (**1996**). Dependence of epithelial intercellular junction biogenesis on thapsigargin-sensitive intracellular calcium stores. *J Biol Chem* 271, 13636-41.

Suter, S., Schaad, U. B., Roux, L., Nydegger, U. E., and Waldvogel, F. A. (**1984**). Granulocyte neutral proteases and Pseudomonas elastase as possible causes of airway damage in patients with cystic fibrosis. *J Infect Dis* 149, 523-31.

Tam, E. K., and Caughey, G. H. (**1990**). Degradation of airway neuropeptides by human lung tryptase. *Am J Respir Cell Mol Biol* 3, 27-32.

Tanaka, T., McRae, B. J., Cho, K., Cook, R., Fraki, J. E., Johnson, D. A., and Powers, J. C. (**1983**). Mammalian tissue trypsin-like enzymes: comparative reactivities of human skin tryptase, human lung tryptase, and bovine trypsin with peptide 4-nitroanilide and thioester substrates. *J Biol Chem* 258, 13552-7.

Tarr, P. E. (**1996**). Granulocyte-macrophage colony-stimulating factor and the immune system. *Med Oncol* 13, 133-40.

Tegner, H., Ohlsson, K., Toremalm, N. G., and von Mecklenburg, C. (**1979**). Effect of human leukocyte enzymes on tracheal mucosa and its mucociliary activity. *Rhinology* 17, 199-206.

Thomas, V. A., Wheeless, C. J., Stack, M. S., and Johnson, D. A. (**1998**). Human mast cell tryptase fibrinogenolysis: kinectics, anticoagulation mechanism, and cell adhesion disruption. *Biochemistry* 37, 2291-8.

Thompson, H. L., Schulman, E. S., and Metcalfe, D. D. (**1988**). Identification of chondroitin sulfate E in human lung mast cells. *J Immunol* 140, 2708-13.

Thompson-Snipes, L., Dhar, V., Bond, M. W., Mosmann, T. R., Moore, K. W., and Rennick, D. M. (**1991**). Interleukin 10: a novel stimulatory factor for mast cells and their progenitors. *J Exp Med* 173, 507-10.

Tomioka, M., Ida, S., Shindoh, Y., Ishihara, T., and Takishima, T. (**1984**). Mast cells in bronchoalveolar lumen of patients with bronchial asthma. *Am Rev Respir Dis* 129, 1000-5.

Tosi, M. F., Stark, J. M., Hamedani, A., Smith, C. W., Gruenert, D. C., and Huang, Y. T. (**1992**). Intercellular adhesion molecule-1 (ICAM-1)-dependent and ICAM-1- independent adhesive interactions between polymorphonuclear

leukocytes and human airway epithelial cells infected with parainfluenza virus type 2. *J Immunol* 149, 3345-9.

Tosi, M. F., Stark, J. M., Smith, C. W., Hamedani, A., Gruenert, D. C., and Infeld, M. D. (**1992**). Induction of ICAM-1 expression on human airway epithelial cells by inflammatory cytokines: effects on neutrophil-epithelial cell adhesion. *Am J Respir Cell Mol Biol* 7, 214-21.

Tsukita, S., and Furuse, M. (**1999**). Occludin and claudins in tight-junction strands: leading or supporting players? *Trends Cell Biol* 9, 268-73.

Turner-Warwick, M. (**1988**). Epidemiology of nocturnal asthma. *Am J Med* 85, 6-8.

Urata, H., Kinoshita, A., Misono, K. S., Bumpus, F. M., and Husain, A. (**1990**). Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart [published erratum appears in J Biol Chem 1991 Jun 25;266(18):12114]. *J Biol Chem* 265, 22348-57.

Van Itallie, C. M., and Anderson, J. M. (**1997**). Occludin confers adhesiveness when expressed in fibroblasts. *J Cell Sci* 110, 1113-21.

Vanderslice, P., Ballinger, S. M., Tam, E. K., Goldstein, S. M., Craik, C. S., and Caughey, G. H. (**1990**). Human mast cell tryptase: multiple cDNAs and genes reveal a multigene serine protease family. *Proc Natl Acad Sci U S A* 87, 3811-5.

Vergnolle, N. (**1999**). Proteinase-activated receptor-2-activating peptides induce leukocyte rolling, adhesion, and extravasation in vivo. *J Immunol* 163, 5064-9.

Vignola, A. M., Campbell, A. M., Chanez, P., Bousquet, J., Paul-Lacoste, P., Michel, F. B., and Godard, P. (**1993**). HLA-DR and ICAM-1 expression on

bronchial epithelial cells in asthma and chronic bronchitis. *Am Rev Respir Dis* 148, 689-94.

Vouret-Craviari, V., Grall, D., Chambard, J. C., Rasmussen, U. B., Pouyssegur, J., and Van Obberghen-Schilling, E. (**1995**). Post-translational and activation-dependent modifications of the G protein-coupled thrombin receptor. *J Biol Chem* 270, 8367-72.

Vu, T. K., Wheaton, V. I., Hung, D. T., Charo, I., and Coughlin, S. R. (**1991**). Domains specifying thrombin-receptor interaction. *Nature* 353, 674-7.

Wakita, H., Furukawa, F., and Takigawa, M. (**1997**). Thrombin and trypsin induce granulocyte-macrophage colony-stimulating factor and interleukin-6 gene expression in cultured normal human keratinocytes. *Proc Assoc Am Physicians* 109, 190-207.

Walker, D. C., MacKenzie, A., Wiggs, B. R., Hulbert, W. C., and Hogg, J. C. (**1984**). The structure of tight junctions in the tracheal epithelium may not correlate with permeability. *Cell Tissue Res* 235, 607-13.

Walls, A. F. (2000). Structure and function of human mast cell tryptase. In Mast Cells and Basophils, G. Marone, L. M. Lichtensten and S. J. Galli, eds. (New York: Academic Press), pp. 291-309.

Walls, A. F., Bennett, A. R., Godfrey, R. C., Holgate, S. T., and Church, M. K. (**1991**). Mast cell tryptase and histamine concentration in bronchoalveolar larvage fluid from patients with interstitial lung disease. *Clin Sci* 81, 183-188.

Walls, A. F., Bennett, A. R., McBride, H. M., Glennie, M. J., Holgate, S. T., and Church, M. K. (**1990a**). Production and characterization of monoclonal antibodies specific for human mast cell tryptase. *Clin Exp Allergy* 20, 581-589.

Walls, A. F., Brain, S. D., Desai, A., Jose, P. J., Hawkings, E., Church, M. K., and Williams, T. J. (**1992**). Human mast cell tryptase attenuates the vasodilator activity of calcitonin gene-related peptide. *Biochem Pharmacol* 43, 1243-8.

Walls, A. F., He, S., Teran, L., Buckely, M. G., Jung, K. S., Holgate, S. T., Shute, J. K., and Cairns, J. A. (**1995**). Granulocyte recruitment by human mast cell tryptase. *Int Arch Allergy Immunol.*

Walls, A. F., Roberts, J. A., Godfrey, R. C., Church, M. K., and Holgate, S. T. (**1990**). Histochemical heterogeneity of human mast cells: disease-related differences in mast cells subsets recovered by bronchoalverolar larvage. *Int Arch Allergy Appl Immunol* 92, 233-241.

Walters, E. H., O'Byrne, P. M., Fabbri, L. M., Graf, P. D., Holtzman, M. J., and Nadel, J. A. (**1984**). Control of neurotransmission by prostaglandins in canine trachealis smooth muscle. *J Appl Physiol* 57, 129-34.

Wan, H., Winton, H. L., Soeller, C., Tovey, E. R., Gruenert, D. C., Thompson, P. J., Stewart, G. A., Taylor, G. W., Garrod, D. R., Cannell, M. B., and Robinson, C. (**1999**). Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest* 104, 123-33.

Wang, J. H., Devalia, J. L., Xia, C., Sapsford, R. J., and Davies, R. J. (**1996**). Expression of RANTES by human bronchial epithelial cells in vitro and in vivo and the effect of corticosteroids. *Am J Respir Cell Mol Biol* 14, 27-35.

Wardlaw, A. J., Dunnette, S., Gleich, G. J., Collins, J. V., and Kay, A. B. (**1988**). Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. *Am Rev Respir Dis* 137, 62-9.

Wegner, C. D., Gundel, R. H., Reilly, P., Haynes, N., Letts, L. G., and Rothlein, R. (**1990**). Intercellular adhesion molecule-1 (ICAM-1) in the pathogenesis of asthma. *Science* 247, 456-9.

Wenzel, S. E., Fowler, A. A. d., and Schwartz, L. B. (**1988**). Activation of pulmonary mast cells by bronchoalveolar allergen challenge. In vivo release of histamine and tryptase in atopic subjects with and without asthma. *Am Rev Respir Dis* 137, 1002-8.

Wenzel, S. E., Larsen, G. L., Johnston, K., Voelkel, N. F., and Westcott, J. Y. (**1990**). Elevated levels of leukotriene C4 in bronchoalveolar lavage fluid from atopic asthmatics after endobronchial allergen challenge. *Am Rev Respir Dis* 142, 112-9.

Wenzel, S. E., Westcott, J. Y., and Larsen, G. L. (**1991**). Bronchoalveolar lavage fluid mediator levels 5 minutes after allergen challenge in atopic subjects with asthma: relationship to the development of late asthmatic responses. *J Allergy Clin Immunol* 87, 540-8.

Whitcutt, M. J., Adler, K. B., and Wu, R. (**1988**). A biphasic chamber system for maintaining polarity of differentiation of cultured respiratory tract epithelial cells. *In Vitro Cell Dev Biol* 24, 420-8.

Widdicombe, J. G., and Pack, R. J. (**1982**). The Clara cell. *Eur J Respir Dis* 63, 202-20.

Widdicombe, J. H., Ueki, I. F., Emery, D., Margolskee, D., Yergey, J., and Nadel, J. A. (**1989**). Release of cyclooxygenase products from primary cultures of tracheal epithelia of dog and human. *Am J Physiol* 257, L361-5.

Wintroub, B. U., Kaempfer, C. E., Schechter, N. M., and Proud, D. (**1986**). A human lung mast cell chymotrypsin-like enzyme. Identification and partial characterization. *J Clin Invest* 77, 196-201.

Wintroub, B. U., Schechter, N. B., Lazarus, G. S., Kaempfer, C. E., and Schwartz, L. B. (**1984**). Angiotensin I conversion by human and rat chymotryptic proteinases. *J Invest Dermatol* 83, 336-9.

Wong, V., and Gumbiner, B. M. (**1997**). A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. *J Cell Biol* 136, 399-409.

Woolley, K. L., Adelroth, E., Woolley, M. J., Ellis, R., Jordana, M., and O'Byrne, P. M. (**1995**). Effects of allergen challenge on eosinophils, eosinophil cationic protein, and granulocyte-macrophage colony-stimulating factor in mild asthma. *Am J Respir Crit Care Med* 151, 1915-24.

Wright, C. D., Havill, A. M., Middleton, S. C., Kashem, M. A., Dripps, D. J., Abraham, W. M., Thomson, D. S., and Burgess, L. E. (**1999**). Inhibition of allergen-induced pulmonary responses by the selective tryptase inhibitor 1,5-bis-[4-[(3-carbamimidoyl-benzenesulfonylamino)- methyl]-phenoxy]-pen tane (AMG-126737). *Biochem Pharmacol* 58, 1989-96.

Wu, R., Yankaskas, J., Cheng, E., Knowles, M. R., and Boucher, R. (**1985**). Growth and differentiation of human nasal epithelial cells in culture. Serum-free, hormone-supplemented medium and proteoglycan synthesis. *Am Rev Respir Dis* 132, 311-20.

Yamashita, T., Tsuji, H., Maeda, N., Tomoda, K., and Kumazawa, T. (**1989**). Etiology of nasal polyps associated with aspirin-sensitive asthma. *Rhinol Suppl* 8, 15-24.

Yamaya, M., Finkbeiner, W. E., Chun, S. Y., and Widdicombe, J. H. (**1992**). Differentiated structure and function of cultures from human tracheal epithelium. *Am J Physiol* 262, L713-24.

Yu, Z., Ahmad, S., Schwartz, J. L., Banville, D., and Shen, S. H. (**1997**). Protein-tyrosine phosphatase SHP2 is positively linked to proteinase- activated receptor 2-mediated mitogenic pathway. *J Biol Chem* 272, 7519-24.

Yukawa, T., Read, R. C., Kroegel, C., Rutman, A., Chung, K. F., Wilson, R., Cole, P. J., and Barnes, P. J. (**1990**). The effects of activated eosinophils and neutrophils on guinea pig airway epithelium in vitro. *Am J Respir Cell Mol Biol* 2, 341-53.

Zacharias, U., Xu, Y., Hagege, J., Sraer, J. D., Brass, L. F., and Rondeau, E. (**1995**). Thrombin, phorbol ester, and cAMP regulate thrombin receptor protein and mRNA expression by different pathways. *J Biol Chem* 270, 545-50.

Zehr, B. B., Casale, T. B., Wood, D., Floerchinger, C., Richerson, H. B., and Hunninghake, G. W. (**1989**). Use of segmental airway lavage to obtain relevant mediators from the lungs of asthmatic and control subjects. *Chest* 95, 1059-63.