NOTE TO USERS

NOVEL CYSTEINYL LEUKOTRIENE BIOLOGY IN HUMAN AIRWAYS

By

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ABSTRACT

Cysteinyl leukotrienes (LTC_4, D_4, E_4) play an important role in the pathophysiology of asthma. They produce bronchoconstriction, microvascular leakage and eosinophil infiltration into the airway mucosa. They mediate the airway responses following allergen inhalation and exercise. This thesis examined new effects of cysteinyl leukotrienes on three cells in human airways: an antigen presenting cell (dendritic cell), a key inflammatory cell (eosinophil) and a structural cell (smooth muscle).

Dendritic cells initiate allergen-induced airway responses by presenting the allergen to lymphocytes. Cysteinyl leukotrienes are necessary for the migration of dendritic cells from tissues to the regional lymph nodes. We observed that they also regulate the recruitment of myeloid dendritic cells from peripheral blood following an allergen inhalation. In a clinical trial, we observed that two weeks of treatment with a leukotriene receptor antagonist prevented the allergen-induced decrease in the number of circulating myeloid, but not plasmacytoid, dendritic cells in atopic asthmatic subjects. This was in keeping with our observation that greater proportion of myeloid dendritic cells than plasmacytoid dendritic cells expressed the $CysLT_1$ receptor.

Esoinophilic infiltration of the airway is a characteristic feature of asthma pathology. We observed that inhalation of cysteinyl leukotrienes caused an increase in the number of eosinophils in airway mucosa and lumen in subjects with atopic asthma. We also observed that leukotriene E_4 caused greater eosinophilia than leukotriene D_4. We further examined bone marrow eosinophilopoiesis as one of the mechanisms by which leukotrienes cause airway eosinophilia. Treatment of atopic asthmatic subjects with a leukotriene-receptor antagonist for two weeks attenuated allergen-induced increase in the number of eosinophil/basophil colony forming units in the bone marrow. This was the first in-vivo observation of a direct role of cysteinyl leukotrienes in regulating eosinophilopoiesis in humans.

The first recognized biological effect of cysteinyl leukotrienes was their ability to contract smooth muscles. We observed that they could also modulate another property of human airway smooth muscle, ie, migration. We observed that human airway smooth muscle cells show chemotaxis towards platelet-derived growth factor. Although leukotrienes by themselves were not chemoattractants, they caused chemokinesis of smooth muscle cells and augmented chemotaxis towards platelet-derived growth factor. A leukotriene receptor antagonist inhibited this. The mechanism was not dependent on increased integrin expression on smooth muscle, or Src-kinase or phosphatidylinositol 3-kinase phosphorylation. Migration of airway smooth muscle cells may be one of the mechanisms of accumulation of smooth muscles in airway submucosa of patients with long-standing asthma.

This thesis has identified three novel aspects of leukotriene biology in the human airway that have therapeutic implications. First, treatment with leukotriene receptor antagonists may be effective in preventing allergies in a susceptible person. Second, long term treatment with leukotriene receptor antagonist, by a direct effect on bone marrow eosinophilopoiesis, may suppress airway eosinophilia refractory to usual therapy. Third, long-term treatment with leukotriene receptor antagonist may prevent smooth muscle accumulation in remodelled airway mucosa.

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TABLE OF CONTENTS

TITLE PAGE i

DESCRIPTIVE NOTE ii

ABSTRACT iii

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS vii

LIST OF TABLES xi

LIST OF FIGURES xii

LIST OF ABBREVIATIONS xiv

PREFACE xvi

CHAPTER 1: INTRODUCTION 1

- Asthma 1
- Cysteinyl leukotrienes 2
  - Formation 2
  - Transport 3
  - Receptors 4
  - Signalling through CysLT receptors 5
- Leukotriene biology in asthma 6
  - Dendritic cells 7
  - Eosinophils 8
  - Smooth muscle 9
- Over-all hypothesis 11
- Specific objectives 11

CHAPTER 2: A ROLE FOR CYSTEINYL LEUKOTRIENES IN ALLERGEN-INDUCED CHANGES IN CIRCULATING DENDRITIC CELL NUMBER IN ATOPIC ASTHMATIC SUBJECTS 13

Abstract 15
Introduction 17
Methods
Subjects 18
Study design 19
Airway responses 19
Allergen inhalation 20
Immunofluorescent staining 20
Flow cytometry 21
Sputum measurements 21
Analysis 22
Results 22
Discussion 24
References 28

CHAPTER 3: INHALED LEUKOTRIENE E_4, BUT NOT LEUKOTRIENE D_4, INCREASED AIRWAY INFLAMMATORY CELLS IN SUBJECTS WITH ATOPIC ASTHMA 43

Abstract 45
Introduction 45
Methods
Study design 45
Clinical methods 45
Statistical analysis 46
Results 46
Discussion 48
References 49

CHAPTER 4: THE EFFECT OF PRANLUKAST ON ALLERGEN-INDUCED BONE MARROW EOSINOPHILOPOIESIS IN SUBJECTS WITH ASTHMA 51

Abstract 54
Introduction 55
Methods
Subjects 57
Design 57
Allergen inhalation 58
Sputum induction and processing 58
Sputum cytochemistry 58
Bone marrow progenitor culture 58
Immunofluorescence staining 58
Flow cytometry 59

Analysis 59
Results 60
Discussion 61
References 67
Online supplemement 76

CHAPTER 5: CYSTEINYL LEUKOTRIENES PROMOTE HUMAN AIRWAY SMOOTH MUSCLE MIGRATION 84

Abstract 86
Introduction 86
Methods
Reagents 86
Smooth muscle culture 86
Smooth muscle migration assay 86
Migration experiments 87
Signal transduction pathways 87
Statistical analysis 87
Results 87
Discussion 87
References 89

CHAPTER 6: EXTRACELLULAR MATRIX PROTEINS REGULATE LEUKOTRIENE-PRIMED HUMAN AIRWAY SMOOTH MUSCLE CELL MIGRATION 92

Abstract 94
Introduction 95
Methods
Reagents 96
Smooth muscle culture 97
Smooth muscle migration assay 97
Migration experiments 98
Adhesion experiments 98
Signal transduction pathways 99
Flow cytometry 99
SDS-Page and Western blot 100
Analysis 100
Results
Effect of extra-cellular matrix on migration 100
Effect of extra-cellular matrix on adhesion 101
The effect of leukotriene priming on adhesion and migration 102

Discussion 102

CHAPTER 7: SUMMARY OF FINDINGS, DISCUSSION AND FUTURE DIRECTIONS

Summary of findings 124
General discussion 125
Study designs 125
Clinical methods
Allergen provocation 126
Sputum induction 127
Bronchoscopy 128
Bone marrow aspiration 128
Laboratory methods
Sputum cell counts 129
Bronchial biopsy 130
Dendritic cell assay 131
Bone marrow progenitor cell assay 132
Airway smooth muscle culture 132
Airway smooth muscle migration 133
Interpretation of results, future direction
Dendritic cell migration 134
Eosinophil infiltration 136
Eosinophilopoiesis 137
Smooth muscle migration 138

CHAPTER 8: BIBLIOGRAPHY 142

LIST OF TABLES

Table 2.1 Baseline subject characteristics 33

Table 2.2 The effect of placebo and pranlukast treatment on RANTES, MIP-1α, MIP-3α in sputum before and after an allergen inhalation 34

Table 2.3 Cys-LT_1 receptor expression on circulating myeloid and plasmacytoid dendritic cells and the effects of placebo and pranlukast treatments 35

Table 3.1 Subject characteristics 46

Table 3.2 Sputum cell counts after leukotriene inhalation 48

Table 4.1 Baseline subject characteristics 79

Table 4.2 Changes in sputum immumostaining for cytokines after placebo and pranlukast treatment 80

Table 4.3 Changes in bone marrow $CD34^+$ cells after placebo and pranlukast treatments 81

LIST OF FIGURES

Figure 2.1 The effect of pranlukast on allergen-induced early and late asthma responses ... 37

Figure 2.2 The effect of pranlukast on allergen-induced airway hyper-responsiveness 38

Figure 2.3 The effect of pranlukast on circulating dendritic cell number 39

Figure 2.4 Cys-LT1 receptor expression on dendritic cell subtypes 40-42

Figure 3.1 The effect of inhaled leukotriene on FEV_1 46

Figure 3.2 The effect of inhaled leukotriene on airway responsiveness to methacholine 47

Figure 3.3 The effect of inhaled leukotriene on eosinophil number in sputum 47

Figure 3.4 Bronchial mucosal biopsy after leukotriene inhalation 47

Figure 3.5 The effect of inhaled leukotriene on eosinophil number in bronchial mucosa 48

Figure 4.1 The effect of pranlukast on allergen-induced changes in eosinophil number in sputum 82

Figure 4.2 The effect of pranlukast on allerge-induced changes in eosinophil/basophil colony forming units in the bone marrow 83

Figure 5.1 Migration of human airway smooth muscle cells towards PDGF 87

Figure 5.2 Chemotactic and chemokinetic responses of airway smooth muscle cells to LTE_4 87

Figure 5.3 Priming effect of LTE_4 on PDGF-induced migratory response 88

Figure 5.4 Effect of PGE2 on airway smooth muscle migration 88

Figure 5.5 The effect of inhibitors of p38 MAPkinase, Rhokinase and phosphoinositol 3-kinase on airway smooth muscle migration 89

Figure 5.6 Photograph of migrated cells on collagen-coated membrane 91

Figure 6.1 The effect of various extra-cellular matri proteins on airway smooth muscle migration towards PDGF 115

Figure 6.2 Concentration-dependent effect of collagen on airway smooth muscle migration 116

Figure 6.3 Chemoattraction towards collagen 1 117

Figure 6.4 Dose-response of PP1 on airway smooth muscle migration 118

Figure 6.5 The effect of Src-kinase on leukotriene-primed migration towards PDGF 119

Figure 6.6 Src-kinase activation by collagen 120

Figure 6.7 Src-kinase activation by various extra-cellular matrix proteins 121

Figure 6.8 Src-kinase activation by leukotriene 122

Figure 6.9 The effect of leukotriene on integrin expression on airway smooth muscle cells ... 123

Figure 7.1 Schema of the airway mucosa in asthma 141

LIST OF ABBREVIATIONS

AHR	Airway hyperresponsiveness
ANOVA	Analysis of variance
ASM	Airway smooth muscle
$cPLA_2$	Cytosolic phospholipase A_2
CysLT	Cysteinyl leukotriene
$CysLT_1R$	Cysteinyl leukotriene 1 receptor
DC	Dendritic cell
EAR	Early asthma response
Eo/B CFU	Eosinophil/Basophil colony forming units
FEV_1	Forced expiratory volume in the first second
FLAP	5-lipoxygenase activating protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
IL	Interleukin
LAR	Late asthma response
5-LO	5-lipoxygenase
LTC_4	Leukotriene C_4
LTD_4	Leukotriene D_4
LTE_4	Leukotriene E_4
MRP1	Multiple drug resistance related protein 1
PC_{20}	Provocative concentration of methacholine causing 20% fall in FEV_1

PGE_2	Prostaglandin E_2
PDGF	Platelet derived growth factor
PI3-kinase	Phosphatidylinositol 3-kinase
RANTES	Regulated upon activation in T cells, expressed and secreted
TNF	Tumour necrosis factor
UDP	Uridine diphosphate

PREFACE

The following manuscripts form the basis of chapters two, three, four, five and six of this thesis:

ROLE FOR CYSTEINYL LEUKOTRIENES IN ALLERGEN-INDUCED CHANGE IN CIRCULATING DENDRITIC CELL NUMBER IN ASTHMA
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I am the first author on four manuscripts and the second author on one manuscript included in this thesis. Three manuscripts are published and two have been accepted for publication. My name is cited as "K. Parameswaran" in the manuscripts. I was involved in the planning and design of the experiments and the development of protocols. I was also involved in recruiting subjects for the studies and obtaining informed consent from them. I performed the bone marrow biopsies and the fiberoptic bronchoscopies and assisted with the bronchial provocation challenges. I was responsible for

processing the bone marrow samples and maintaining the bone marrow cells and smooth muscle cells in culture. I assisted with the processing of the sputum samples and flow cytometry of blood and bone marrow samples. I collated the data from all the studies, analyzed and interpreted them and prepared all the manuscripts for publication.

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CHAPTER 1:

INTRODUCTION

Asthma

Asthma is a disease of the airways which causes episodic symptoms of wheeze, chest tightness or breathlessness. The hallmark of asthma is variable airflow limitation, which is often associated with chronic inflammation of the airways (Scadding 1971). This inflammatory response is characterized by the presence of inflammatory cells in the airway mucosa, increased vascularity, and mucus hypersecretion. Long-standing asthma results in hypertrophy and hyperplasia of the smooth muscles and deposition of fibrous tissue in the lamina propria. These changes, collectively described as "airway remodelling" (Bousquet et al 2000), result in partial loss of reversibility of lung function (Ward et al 2001). A characteristic accompaniment of the inflammatory and structural changes in the airway is hyperresponsiveness of the airways to a number of direct and indirect bronchoconstrictor stimuli (O'Byrne et al 2003).

The inflammatory response in the airways of patients with asthma is a consequence of a complicated interplay between genes and environment (Holgate 1999). Genetic predisposition to atopy or allergy and consequent exposure to allergens in the environment is one of the most important inducers of airway inflammation (Gibson et al 1991; Djukanovic et al 1996). The process is initiated by the presentation of processed allergen to naïve Th-precursor cells by dendritic cells (Holt et al 1999). In the presence of a cytokine milieu comprising of IL-4, IL-5, GM-CSF, IL-9 and IL-13, a selective expansion of Th2-polarized memory cells occurs. They elaborate more Th2 favourable cytokines and promote the production of specific IgE by B cells. Allergen-induced cross-linking of specific IgE antibody bound to mast cells in the airway through high affinity receptors releases a number of

mediators such as histamine and cysteinyl leukotrienes (CysLTs), which cause immediate symptoms by causing contraction of airway smooth muscles and promoting airway mucosal oedema and leakage. These mediators, along with the cytokines and chemokines produced from mast cells, macrophages and lymphocytes, recruit more inflammatory cells including eosinophils, neutrophils and basophils into the airway, perpetuating the inflammatory process (Djukanovic et al 1990; O'Byrne 2000). The cysteinyl leukotrienes are particularly involved in causing bronchoconstriction, microvascular leakage and eosinophil infiltration into the airway mucosa.

Cysteinyl Leukotrienes

Cysteinyl leukotrienes, comprising of leukotriene C_4, D_4, and E_4, are a group of eicosanoids derived from arachidonic acid. They were first isolated from leukocytes and their molecular structure includes a cysteine residue and three conjugated double bonds (triene); hence the name cysteinyl leukotriene. The story of leukotrienes is a remarkable journey of research, perseverance and inter-continental collaboration spanning over seven decades, starting with the description by Feldberg and Kellaway of a substance that could cause an immediate constriction of smooth muscle followed by a slow relaxation (Feldberg et al 1938). This was followed by the demonstration of the slow-reacting substance (SRS) activity following anaphylactic challenge (Kellaway et al 1940; Brocklehurst 1960), and the development of antagonists against the slow reacting substance. The characterization of the enzymatic pathways of their synthesis and their molecular structure resulted in these compounds being renamed leukotrienes (Murphy et al 1979, Samuelsson et al 1979). This monumental research was awarded with the Nobel Prize in Medicine in 1982. The development of antagonists to CysLTs helped toelucidate their roles in health and disease, particularly in asthma.

Formation

The formation of CysLTs from arachidonic acid involves the action of three key enzymes: phospholipase A_2 (PLA_2), 5-lipoxygenase (5-LO) and 5-lipoxygenase activating protein (FLAP). A major advance in our understanding of leukotriene synthesis in the past 10 years has been the precise intracellular localization of these enzymes (Peters-Golden et al 2001). Contrary to previous belief that these events took place at the plasma membrane, arachidonic acid metabolism takes place at the outer nuclear membrane (Peters-Golden et al 1996). Upon activation of a cell, PLA_2 and 5-LO translocate to the nuclear envelope (Peters-Golden et al 1993) to lie in close proximity to FLAP, which is now considered to be an arachidonate transfer protein rather than a 'docking protein' (Abramovitz et al 1993). The translocation of 5-LO follows its phosphorylation by MAP kinase kinase. PLA_2 hydrolyzes nuclear membrane phospholipids to form arachidonic acid, which is then converted in a two-step process by 5-LO, in the presence of calcium and ATP, to an epoxide, leukotriene A_4 (LTA_4) (Borgeat et al 1979). In polymorphonuclear leukocytes, LTA_4 is converted to leukotriene B_4 by LTA_4 hydrolase. In eosinophils, mast cells and monocytes, LTA_4 is conjugated with glutathione by a unique leukotriene C_4 synthase (Yoshimoto et al 1988) and converted to leukotriene C_4 (LTC_4). It is generally believed that only cells of myeloid lineage have 5-LO and therefore have the ability to synthesize CysLTs. However, non-myeloid cells are able to produce CysLTs by the process of "transcellular metabolism" (Lindgren et al 1993). For example, LTA_4 from neutrophils can be supplied to nearby platelets or endothelial or epithelial cells that lack 5-LO, but possess LTC_4 synthase. There is recent evidence that airway smooth muscle cells may also have 5-LO activity (James et al 2001).

Transport

LTC_4 produced deep within the cell near the nuclear membrane is then actively transported outside of the cell for further metabolism. This is achieved by a specific transporter called MRP1 (Lam

et al 1989). The MRP (multidrug resistance related protein) family is comprised of nine related proteins which can transport lipophilic anions and anti-cancer alkylating agents such as anthracycline, vincristine and etoposide to the outside of a cell (Kruh et al 2003). A tumour cell is thus resistant to the alkylating drug as the drug level within the cell is kept low. LTC_4 is a particularly high affinity substrate for MRP1 because of its glutathione and sulphate conjugates. Mice homozygous for the MRP mutant allele, mrp-/-, have decreased secretion of LTC_4, and consequently, an impaired inflammatory response to allergen inhalation (Wijnholds et al 1997). Once transported outside of a cell, the gamma glutamyl residue of the glutathione side chain of LTC_4 is cleaved by gamma glutamyl transpeptidase to form leukotriene D_4 (LTD_4). Cleavage of the peptide bond between the cysteinyl and glycine residues of LTD_4 by a dipeptidase results in the formation of leukotriene E_4 (LTE_4) (Lewis et al 1990). This is prevented by L-cysteine. In the body, LTC_4 is rapidly metabolized. The half life in monkeys is estimated to be about 0.2 minutes. The stable LTE_4 is catabolized in the liver predominantly by N-acetylation and excreted in the urine and bile. The concentration of LTE_4 can measured in urine using sensitive immunoassays and reflects endogenous LTC_4 production (Taylor 1997).

Receptors

CysLTs, transported extracellularly, exert their effects through receptors on the cell surface. The existence of the receptors was proven almost twenty years ago when specific pharmacological agents were observed to inhibit the smooth muscle contraction caused by cysteinyl leukotrienes. FPL-55712 antagonized the effects of both LTC_4 and LTD_4 in human bronchial smooth muscle suggesting that they both act on the same receptor to cause contraction (Buckner et al 1986). The existence of other CysLT receptors in humans was suggested by the observation that specific inhibitors of LTC_4 and LTD_4, such as MK 571 and SKF 104353 failed to inhibit CysLT induced contraction of venous smooth muscle cells

(Labat et al 1992). However, it was inhibited by BAYu9773, a non-selective antagonist of CysLTs. At lower doses, CysLTs have also been reported to cause relaxation of human vascular smooth muscle acting through the $CysLT_2$ receptor (Ortiz et al 1995). In recent years, two receptors in humans, $CysLT_1$ (Lynch et al 1999; Sarau et al 1999) and $CysLT_2$ (Heise et al 2000; Takasaki et al 2000) have been cloned using sequence data bases, computational mining and high throughput screening methodologies. Both are classical G-protein coupled receptors with seven transmembrane regions and about 38% amino acid homology. CysLT receptors also bear some homology to the purinoreceptor P2Y family, and UDP is an agonist of the $CysLT_1$ receptor on human mast cells (Mellor et al 2001). CysLT and UDP have been demonstrated to activate human mast cells through an IL-4 regulated, non IgE dependent mechanism (Mellor et al 2002). Further research is required to fully understand the biological relevance of the similarity between $CysLT_1$ and P2Y receptors. Expression of $CysLT_1$ receptor is mainly in spleen, leukocytes, $CD34^+$ granulocytes and airway smooth muscle, while the largest concentration of $CysLT_2$ receptors are in the Purkinje fibres of the heart, myocardium, placenta, brain, adrenals, spleen and leukocytes, with very little expression in the lungs (Lynch et al 1999; Heise et al 2000; Figueroa et al 2001). $CysLT_2$ receptors have also been recently described on human mast cells. Their stimulation resulted in the production of IL-8 suggesting a role for mast cells and CysLTs in causing neutrophilic airway inflammation (Mellors et al 2003).

Signalling through CysLT receptors

Intracellular signalling pathways of CysLT receptors have not been studied thoroughly. Both $G_{q/11}$ and $G_{i/o}$ class of G-proteins have been described to couple with the CysLT receptors. While calcium mobilization in $CysLT_1$ transfected cells is not inhibited by pertussis toxin (evidence of $G_{q/11}$ coupling), it is partially inhibited in transfected human U937 cells (evidence of $G_{i/o}$ coupling). In human

monocytic leukaemia THP-1 cells, $CysLT_1$ stimulation activates phospholipase C, causes intracellular calcium influx and also activation of the protein kinase C-α-Raf-1 pathway (Hoshino et al 1998). LTD_4 has also been reported to contract human bronchi by a calcium independent pathway involving Rho-kinase (Accomazzo et al 2001). Recent evidences suggest two new features of CysLT receptor biology. There is some speculation that CysLT receptors may form homo- or heterodimers with other G-protein coupled receptors (Hui et al 2002). Second, in cells expressing a constitutively active mutant (N106A) of the human $CysLT_1$ receptor, some of the ligands of the $CysLT_1$ receptor (montelukast, zafirlukast, MK-571) can act as inverse agonists (Dupre et al 2004).

Leukotriene biology in asthma

A role for cysteinyl leukotrienes in the pathogenesis of asthma is evident from two observations: 1) their biologic activities mimic those of clinical asthma and 2) inhibition of their production (5-lipoxygenase inhibitors) or antagonism of their binding to their receptors (leukotriene receptor antagonists) improves asthma control. Leukotrienes impair mucociliary clearance, increase mucous secretion, increase microvascular leakage and attract eosinophils to the airways (Lewis et al 1990). When inhaled, leukotriene C_4 and D_4 are 1000 time more potent than histamine in causing bronchoconstriction (Barnes et al 1984). Their biological relevance in asthma is further confirmed by the presence of increased levels of LTE_4 in the urine of patients following allergen inhalation (Manning et al 1990a), exercise challenge (Kikawa et al 1992), aspirin challenge (Israel et al 1993) and asthma exacerbations (Taylor et al 1989). The development of pharmacological agents to decrease (5-LO inhibitor: Zileuton) or inhibit the effect of leukotrienes (receptor antagonists: Zafirlukast, Pranlukast, Montelukast, MK571) has further confirmed their role in asthma. In randomized, double-blind, clinical trials, these drugs are consistently superior to placebo in attenuating airway responses in many clinical

models of asthma. For example, MK571 (Manning et al 1990b) and Cinalukast (Adelroth et al 1997) inhibited exercise-induced bronchoconstriction by 50-70%. Zileuton attenuated cold-air induced (Israel et al 1990) and aspirin-induced (Israel et al 1993) bronchoconstriction.

The role of cysteinyl leukotrienes in allergen-induced bronchoconstriction is clearly demonstrated by the ability of the receptor antagonists to attenuate allergen-induced early and late asthma responses (Taylor et al 1991; Hamilton et al 1998). In a more recent study where incdices of airway inflammation were measured in induced sputum, a leukotriene receptor antagonist, montelukast, attenuated allergen-induced sputum eosinophilia (Leigh et al 2002). The attenuating effect on airway responses was more pronounced on the early asthmatic response than the late asthmatic response, suggesting a greater role for CysLTs in the early allergen-induced bronchoconstriction. Leukotriene receptor antagonists have also been shown to decrease the number of lymphocytes and basophils in bronchoalveloar lavage fluid after segmental allergen challenge (Calhoun et al 1998). The precise mechanisms of these effects in allergen-challenge models of asthma are not known.

Allergen-induced airway changes are critically dependent on three cells: a) dendritic cells, which are necessary for antigen presentation b) eosinophils, which are important effector cells in the airway mediating the acute inflammatory symptoms and c) smooth muscles, which cause bronchoconstriction. The effect of cysteinyl leukotrienes on these cells is the focus of this thesis.

Dendritic cells

Dendritic cells are the major antigen presenting cells in the airway. They form a tight mesh along the airway epithelium. The MHC Class II bearing dendritic cells capture antigens from the airway mucosa and migrate to the regional lymph nodes, where they acquire the ability to prime naïve T-cells to switch to a predominantly Th2 type (Banchereau at al 2000). The exact mechanism by which this

occurs is not known, but it appears to be related to the expression of CD86 surface antigen (Kuchroo et al 1995) and the ability of dendritic cells to produce IL-10 (Stumbles et al 1998), whereas surface expression of CD80 and secretion of IL-12 appears to favour a Th1 type phenotype. In addition to their role in inducing primary immune response, they are essential for controlling effector T-cell responses in sensitized hosts (Lambrecht et al 2003). They are necessary to cause eosinophilic airway inflammation in sensitized mice on subsequent allergen exposure possibly by activating memory/effector $CD4^+$ Th2 cells (Lambrecht et al 1998).

The role of cysteinyl leukotrienes in airway dendritic cell biology is not well known. The immature dendritic cells from the bone marrow or peripheral blood that migrate to the antigen capture sites such as skin or the airway, express the chemokine receptor, CCR6. Following antigen capture, the immature cells express CCR7 (Cyster et al 1999), which is the ligand for the chemoattractants CCL19 and CCL21 in the regional lymph nodes. This migration is dependent on cysteinyl leukotrienes. It is known that skin DCs express 5-LO (Spanbroek et al 1998). The movement of dendritic cells from the epidermis into lymphatic vessels was greatly reduced in mice deficient in MRP1 and therefore unable to transport LTC_4 to the outside of dendritic cells (Robbiani et al 2000). This was restored by exogenous LTC_4 or LTD_4. DC migration was similarly reduced in 5-LO deficient mice. Human epidermal DCs treated with MK571 (a $CysLT_1$ antagonist and an inhibitor of MRP1) showed less migration towards CCL19, further demonstrating a role of CysLTs in migration of DCs towards the regional lymph nodes and towards CCL19.

Following an allergen inhalation, the number of myeloid dendritic cells in peripheral blood decreases at 3 hours (Upham et al 2002), and they appear in the airway mucosa (Jahnsen et al 2001). Since cysteinyl leukotrienes are elaborated at this time point, it is reasonable to hypothesize that they

may also regulate the migration of dendritic cells from the circulation to the airway.

Eosinophils

Eosinophilic infiltration of the airway mucosa is a characteristic feature of allergic asthma (Busse et al 2001). Allergen inhalation is associated with an increase in the number of eosinophils in the airway and this correlates with the increase in airway hyperresponsiveness (Gibson et al 1991; Pin et al 1992a; Gratziou et al 1996). The importance of eosinophils in asthma pathophysiology has recently been questioned based on an observation that a treatment (humanized anti IL-5 antibody) that decreased blood and airway eosinophil numbers failed to attenuate allergen-induced airway responses (Leckie et al 2000). Problems related to sample size and study design, however, cast doubt on the results of the study (O'Byrne et al 2001). The controversy raised by the study continues and has not been resolved. It may be that the lack of clinical response with anti-IL5 therapy is related to the persistence of activated eosinophils in the airway mucosa (Flood-Page et al 2003). Treatment with corticosteroids has clearly shown a relationship between decrease in airway eosinophils and improvement in asthma control (Green et al 2002).

Eosinophils are produced in the bone marrow and move into peripheral circulation and to local tissues such as the airway under the influence of cytokines such as IL-5 and chemokines such as eotaxin and RANTES. The progenitor cells of the eosinophil, the Eo/B colony forming unit, are also increased in number in bone marrow (Sehmi et al 1997), blood (Denburg et al 1985a) and airways (Dorman et al 2003) of patients with atopy or asthma compared to normal subjects. It is possible that the progenitor cells may also migrate to the tissues such as the nose and the lung and mature *in-situ* into eosinophils.

Cysteinyl leukotrienes may have a role in causing airway eosinophilia for two reasons. First, leukotriene antagonists decrease airway eosinophil number (Pizzichini et al 1999; Leigh et al 2002) and

activation (Obase et al 2002). Second, when inhaled, leukotrienes cause airway eosinophilia (Laitinen et al 1993). Cysteinyl leukotrienes may promote airway eosinophilia by a number of mechanisms. They promote chemotaxis (Spada et al 1994), upregulate adhesion molecule expression on eosinophils (Fregonese et al 2002) and the endothelium (Pedersen et al 1997) and promote eosinophil survival (Lee et al 2000). They may have an additional effect of eosinophilopoiesis as well. In-vitro, LTD_4 augmented the proliferation of Eo/B CFUs in peripheral blood and bone marrow induced by IL-5 (Braccioni et al 2001). This has not been demonstrated *in-vivo*.

Smooth muscle

The exact role of the smooth muscle in a normal airway is not clearly known. It may very well be a vestigial organ with no physiological function (Mitzner 2004). However, in diseased states such as asthma, the role of the smooth muscle has evolved from being simply a contractile cell causing bronchoconstriction. It participates in immunomodulation and in the remodelling process by producing a number of cytokines, chemokines and growth factors (Johnson et al 1997). Increases in the size and number of smooth muscle cells are observed in the submucosa and lamina propria of patients with long-standing asthma (Johnson et al 2001; Woodruff et al 2004).

Cysteinyl leukotrienes modulate airway smooth muscle function in a number of ways (Holgate et al 2003). Human airway smooth muscle cells express $CysLT_1$ receptors and this is upregulated by cytokines such as interferon-gamma (Amrani et al 2001) and IL-13 (Espinosa et al 2003). From their initial description of being potent bronchoconstrictors (Dahlen et al 1980), CysLTs have been shown also to promote human airway smooth muscle cell proliferation (Panettieri et al 1998). Treatment with a leukotriene receptor antagonist decreases the smooth muscle thickness of airways of mice that have been chronically challenged with allergen (Henderson et al 2002). These observations suggest that

leukotrienes, by their effect on smooth muscles, can cause chronic structural changes and airway remodelling. Their effect on cytokine synthesis by airway smooth muscle cells is not known.

Another unexplored effect of CysLTs is on the ability of airway smooth muscle cells to migrate. Following an allergen inhalation, the number of myofibroblasts in the airway mucosa increase after 24 hours (Gizycki et al 1997; Schmidt et al 2003). Airway smooth muscle cells are capable of directional migration similar to vascular smooth muscle cells (Yamboliev et al 2001). It is therefore possible that one of the mechanisms by which airway smooth muscle cells accumulate below the submucosa is by migrating from deeper layers. The effect of CysLTs on airway smooth muscle migration has not been studied.

Overall hypothesis

Cysteinyl leukotrienes play a larger role in asthma pathophysiology than is currently known, particularly on dendritic cells, eosinophils and smooth muscle cells. We hypothesized that they have an effect on circulating dendritic cells, just as they have an effect on the migration of dendritic cells from the tissues to the regional lymph nodes. We also hypothesized that one of the mechanisms by which they promote recruitment of eosinophils into the airway is by a direct effect on the maturation of eosinophils in the bone marrow and their subsequent release into blood. Finally, we hypothesized that, as they promote airway smooth muscle proliferation, they also promote airway smooth muscle migration from the deep sumucosal layer to the superficial lamina propria.

Specific objectives

The specific hypotheses tested in this thesis were:

1) Cysteinyl leukotrienes promote movement of dendritic cells from the circulation to the human airway following an allergen inhalation. Pre-treatment with a CysLT1 receptor antagonist will prevent

the allergen-induced decrease in circulating dendritic cell number.

2) Cysteinyl leukotrienes cause airway eosinophilia. Inhalation of LTD_4 and LTE_4 by asthmatic subjects will cause airway eosinophilia.

3) One of the mechanisms by which cysteinyl leukotrienes cause airway eosinophilia is by a direct effect on the bone marrow by promoting eosinophilopoiesis. Pre-treatment with a CysLT1 receptor antagonist will prevent the allergen-induced increase in eosinophil/basophil colony forming units in the bone marrow of atopic asthmatic subjects.

4) Cysteinyl leukotrienes are chemokinetic agents and promote human airway smooth muscle chemotaxis.

CHAPTER 2:

ROLE FOR CYSTEINYL LEUKOTRIENES IN ALLERGEN-INDUCED CHANGE IN CIRCULATING DENDRITIC CELL NUMBER IN ASTHMA

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Development of protocol
Recruitment of subjects
Clinical evaluation of subjects
Supervision of allergen inhalation
Supervision of methacholine inhalation
Collection of blood
Immunostaining of dendritic cells
Supervision of flow-cytometry
Data collection, tabulation, analysis
Interpretation of data, preparation of manuscript

Role for cysteinyl leukotrienes in allergen-induced change in circulating dendritic cell number in asthma.

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Abstract

Background: Dendritic cells are important antigen-presenting cells. Following an allergen inhalation, their numbers rapidly decrease in circulation and increase in the airway mucosa.

Objective: To investigate whether allergen-induced changes in the number of circulating dendritic cells are mediated by cysteinyl leukotrienes.

Methods: In a randomized, double blind, cross-over study, we examined the effects of two weeks of treatment with pranlukast (a $CysLT_1$ receptor antagonist) 300 mg bid and placebo on allergen-induced changes in airway responses and circulating dendritic cells in 15 subjects with mild asthma. We examined, by flow-cytometry, before, and at 3h and 24h after allergen inhalation, the proportion of myeloid ($CD33^+$) and plasmacytoid ($CD123^+$) dendritic cells ($HLA\text{-}DR^+$, $CD14^-$, $CD16^-$) among all peripheral blood mononuclear cells. The fraction of dendritic cells expressing $CysLT_1$ receptor was also determined.

Results: Compared to placebo, pranlukast significantly attenuated both the maximum early (by 55%) and the late (by 39%) asthma responses, the allergen-induced methacholine airway hyper-responsiveness and increase in MIP-1α and MIP-3α in induced sputum. A significantly greater proportion of $CD33^+$ cells (55%) expressed $Cys\text{-}LT_1$ receptor compared to $CD123^+$ cells (11%). Consistent with this, pranlukast prevented the allergen-induced decrease in $CD33^+$ dendritic cells at 3h post-allergen (mean Δ from baseline +4.4%) compared to placebo (mean Δ –8.4) ($p<0.05$), but not the $CD123^+$ cells.

Conclusion: Pre-treatment with pranlukast attenuated allergen-induced airway responses and the decrease in circulating myeloid dendritic cells, demonstrating a novel role of cysteinyl leukotrienes in dendritic cell trafficking.

Key words: Cysteinyl leukotrienes, pranlukast, dendritic cells, allergen inhalation, asthma

Abbreviations

Cys-LT	Cysteinyl leukotriene
EAR	Early asthma response
LAR	Late asthma response
FEV_1	Forced expiratory volume in the first second
PC_{20}	Provocative concentration causing a 20% fall in FEV_1
MIP	Macrophage inflammatory protein
RANTES	Regulated upon activation, normal T cell expressed and secreted

Inhalation of allergens by atopic asthmatic patients causes an immediate bronchoconstriction (early asthma response, EAR), which is followed in about half the subjects, by a late bronchoconstriction (late asthma response, LAR), and an increase in airway hyper-responsiveness (1). The EAR is due to the release of mediators such as histamine and cysteinyl leukotrienes (2). These mediators, and other cytokines, facilitate an inflammatory cell infiltration of the airway that is responsible for the LAR and changes in airway hyperresponsiveness.

Allergen inhalation is able to trigger this response only if the allergens are initially processed by airway dendritic cells that possess pattern recognition receptors to present them to T-cells following their migration to regional lymph nodes. This migration through the lymphatic system is mediated by chemoattractants such as CCL19 (MIP-3β, Exodus-3) and CCL21 (6Ckine, Exodus-2, secondary lymphoid tissue chemokine), which are ligands for CCR7 receptors expressed on the dendritic cells (3,4). Leukotriene C_4, produced from the dendritic cells themselves or from other inflammatory cells, is necessary for the migration of dendritic cells towards CCL19 (5). It is generally believed that the myeloid dendritic cells ($CD33^+$) are responsible for antigen presentation in the lymph nodes, while the lymphoid or plasmacytoid cells ($CD123^+$/IL-3Rα) are natural interferon producing cells (6).

There is little known about the chemoattractants that mediate the migration of dendritic cells from blood to the airway. The newly described C-C chemokine, MIP-3α (LARC, liver and activated-regulated chemokine. Exodus-1) (7), which is produced by airway epithelial cells (8), is a likely chemoattractant. We have previously observed that

the numbers of myeloid dendritic cells in circulation decrease rapidly within 3h of an allergen inhalation (9). Since cysteinyl leukotriene levels increase at this time in the airway (10) and because cysteinyl leukotriene antagonist can attenuate allergen-induced airway responses (11-13), we hypothesized that leukotrienes may also regulate the disappearance of dendritic cells from blood following an allergen inhalation and that this effect will be more on the myeloid cells than the plasmacytoid dendritic cells. In this study we investigated the proportion of myeloid and plasmacytoid dendritic cells which express the cysteinyl leukotriene-1 (Cys-LT_1) receptor and the effect of pre-treatment with a cysteinyl leukotriene receptor antagonist, pranlukast, on allergen-induced airway responses and dendritic cell chemoattractants and circulating dendritic cell numbers.

Methods

Subjects

The subjects were 16 atopic non-smokers with mild asthma (table 1) who were using short-acting beta-agonists infrequently. Asthma had been previously diagnosed by a physician based on typical episodic symptoms and bronchoconstriction induced by allergen inhalation. Subjects were studied out of their allergy season and had not had any respiratory tract infections for at least six weeks prior to the study. All medications, except the study medication, were withheld for at least 8h before each visit. The subjects refrained from vigorous exercise, and caffeine-containing beverages in the morning before visits to the laboratory. All subjects gave written informed consent and the Research Ethics Committee of Hamilton Health Sciences Corporation approved the study.

Study design

This was a randomized, double-blind, cross-over, two-period study comparing two weeks of treatment with pranlukast 300 mg tablet b.i.d. or identical placebo, with at least 2 weeks of wash-out period between the treatment periods. The subjects underwent a screening allergen inhalation to identify those with a dual asthma response (at least 15% fall in FEV_1 within the 120 min followed by a similar drop between 3 and 7h after allergen inhalation). After 2-4 weeks, they were randomized to one of the two treatment arms using computer-derived codes that were maintained off-site by an independent third party pharmacist. The medications were taken at 8 pm and 8 am starting in the evening of the first day. On the 13^{th} day (±2 days), spirometry and methacholine inhalation test were done, sputum was induced for measurement of chemokines and 5 ml blood was collected in EDTA tube for dendritic cell assay. The next day, an allergen inhalation test was performed. At 3h after allergen inhalation, blood was drawn for dendritic cell measurement, and at 7h sputum was induced for chemokine measurement. The following day, approximately 24h after allergen inhalation, subjects attended the laboratory for spirometry, methacholine inhalation test, and blood sampling for dendritic cell measurement. Compliance was evaluated weekly by pill counting. Any adverse effect was evaluated by self-reported symptoms, physical examination, blood chemistry and urine examination.

Airway responses

FEV_1 was measured using a Collins water-sealed spirometer (Warren E. Collins, Braintree, MA) and kymograph according to the American Thoracic society recommendations (14). Methacholine inhalation test was performed by the tidal breathing

method of Cockcroft et al. (15) by inhaling doubling concentrations of methacholine (Methapharm, Mississauga, ON) from a Wright nebulizer (Roxon Medi-Tech, Montreal, PQ). The concentration of methacholine required to achieve a decrease in FEV_1 of 20% (PC_{20}) was calculated through linear interpolation of percent fall in FEV_1 against the log-transformed methacholine concentration.

Allergen inhalation

Allergen inhalation was performed as previously described (16). The allergen producing the largest-diameter skin wheal was diluted in 0.9% saline for inhalation. Allergens used were extracts of house dust mite (n=7), cat (n=2), ragweed (n=3), alternaria (n=1) and grass (n=2). The concentration of allergen extract for inhalation was determined from a formula described by Cockcroft and colleagues (17). The early bronchoconstrictor response was the largest percent fall in FEV_1 within the first 2 hours after allergen inhalation, and the late bronchoconstrictor response was the largest fall in FEV_1 between 3 and 7h after the allergen inhalation.

Immunofluorescent staining

The following directly conjugated monoclonal antibodies were used: mouse anti-human CD14-FITC, CD16-FITC, CD123-PE, CD33-APC, HLA-DR-CyChrome (Pharmingen, Mississauga, ON, Canada). Cys-LT_1 receptor was stained using goat anti-Cys-LT_1 receptor specific antisera, swine biotinylated anti-goat IgG antibody (1/250 dilution) and Streptavidin-ECD (Beckman Coulter, Mississauga, ON, Canada). Dendritic cells were stained as previously described (18). This method has been shown to be valid, reliable and reproducible. Immunofluorescent staining of dendritic cells for Cys-LT_1 receptor expression was performed as previously described (19) with slight modification.

Briefly, 200 μl of blood was added to each 5-mL polystyrene tube. Red cells were lysed using NH_4Cl-EDTA lysing solution, and remaining leukocytes were stained with the directly conjugated antibodies against CD14, CD16, CD33, CD123 and HLA-DR or by indirectly conjugated antibodies against Cys-LT_1 receptor. Myeloid dendritic cells were identified as $CD33^+$, HLA-DR^+ mononuclear cells which were CD14 and CD16 negative. The myeloid dendritic cells were all $CD11c^+$ (18). Plasmacytoid dendritic cells were identified as $CD123^+$, HLA-DR^+ mononuclear cells that were weakly $CD33^+$. Duplicate tubes were stained with these antibodies for analysis of dendritic cell numbers.

Flow cytometry

Stained and control cell preparations were analyzed within 24h using a 6-colour FACSVantageSE™ flow cytometer equipped with a 488-nm argon laser and a 635-nm helium-neon laser (Becton Dickinson Instrument systems, Mississauga, ON, Canada) and operated with CellQuest™ software. Dendritic cell count was based on total leukocyte count of 300,000 to 400,000 cells of which approximately 100,000 were mononuclear cells. Dendritic cell count for Cys-LT_1 receptor staining was based on total leukocyte count of 700,000 to 800,000 cells of which approximately 400,000 were mononuclear cells. Off-line analysis was performed with WinList software, version 5.0 (Verity Software House Inc, Topsham, Maine), as previously described (9,18).

Sputum measurements

Sputum was induced with hypertonic saline, separated from saliva, and processed as described by Pizzichini and co-workers (20). MIP-1α and MIP-3α in sputum supernatant were measured by ELISA (R&D Systems Inc., Minneapolis, MN).

Analysis

All data, except methacholine PC_{20}, are expressed as mean and standard deviation. Non-normally distributed data was log-transformed for analysis. PC_{20} data is expressed as geometric mean and standard deviation. The differences in maximum early and late allergen responses and airway hyperresponsiveness between pranlukast and placebo treatment were compared by paired t-test. Dendritic cells were expressed as a percentage of the total mononuclear cells. This has been shown to be as repeatable as their absolute numbers (18). The percentage change in dendritic cell number at 3h and 24h after allergen challenge compared to the pre-allergen baseline for the two treatment arms were compared by repeated measures analysis of variance. Correlations between variables were examined using Pearson's coefficient. The proportion of myeloid and plasmacytoid dendritic cells expressing the Cys-LT_1 receptor were compared by unpaired t-test. A p-value of less than 0.05 was considered to be statistically significant. Analysis was performed using the Statistical Package for Social Sciences, version 10.0 (SPSS Inc, Chicago, IL).

Results

One subject was withdrawn from the study after developing an urticarial rash after the first dose of the medication. 15 subjects completed the study without any significant adverse events.

Airway responses

The mean FEV_1 values after placebo and pranlukast treatments (pre-allergen challenge) were identical (3.3L). The mean (SD) maximum EAR was 30.3% (12.1) after placebo treatment and 13.5% (12.2) after pranlukast treatment (mean attenuation 55%,

$p<0.05$). The maximum LAR was 15.2% (10.0) after placebo treatment and 9.3% (8.1) after pranlukast treatment (mean attenuation 39%, $p<0.05$) (figure 1). Allergen inhalation after placebo treatment caused a 0.8-fold increase in PC_{20} at 24h. This was significantly decreased after pranlukast treatment (figure 2).

Effect on circulating myeloid and plasmacytoid dendritic cell numbers

One subject had negligible number of dendritic cells and was therefore excluded from the analysis. This decision was made before the randomization code was broken. Anaysis is thus based on data from 14 subjects. At baseline, the mean ratio of myeloid to plasmacytoid dendritic cells was 1.2. Following placebo treatment, the proportion of myeloid dendritic cells in blood decreased by a mean of 8.4% at 3h following allergen inhalation compared to pre-allergen value. This was abolished by pre-treatment with pranlukast. The mean change was an increase by 4.4% ($p<0.05$). Pranlukast also attenuated the decrease in myeloid cells at 24h. This was not statistically significant (figure 3). The proportion of plasmacytoid cells also decreased 3h after allergen inhalation (mean change 5.8%); however, this was not prevented by pranlukast (mean decrease 7.8%). The correlation between the changes in dendritic cell number, percentage fall in FEV_1 or PC_{20} methacholine were not statistically significant.

Effect on dendritic cell chemoattractants in the airway

Levels of MIP-1α and MIP-3α in sputum increased 7h after allergen inhalation in the placebo treatment arm. This was prevented by pranlukast treatment (table 2).

Effect on Cys-LT1 receptor expression

A significantly greater proportion of the myeloid dendritic cells expressed the Cys-LT_1 receptor (mean 55%) compared to the plasmacyoid dendritic cells (mean 11%

($p<0.05$) (table 2, figure 4). This was not significantly altered by allergen inhalation or pranlukast treatment (table 3).

Discussion

This study confirmed that a cysteinyl leukotriene receptor antagonist, pranlukast, can attenuate allergen-induced early and late asthma responses and airway hyperresponsiveness. The study also showed that circulating dendritic cells express Cys-LT_1 receptor. Pranlukast prevented allergen-induced decrease in circulating myeloid dendritic cells. This was accompanied by a reduction in the levels of dendritic cell chemoattractants, MIP-1α and MIP-3α in sputum. The results suggest a role for cysteinyl leukotrienes in the migration of myeloid dendritic cells from blood, presumably to the airway.

We confirmed previous observations that a cysteinyl leukotriene receptor antagonist can attenuate allergen-induced early and late asthma responses and increase in airway hyperresponsiveness (11-13). The degree of protection is comparable to previous reports. Similar to previous reports we observed a greater attenuation of the early asthma response than the late asthma response. This is consistent with fact that the early response is mainly mediated by cysteinyl leukotrienes and that the late asthma response is caused by a cellular inflammatory response that is less affected by cysteinyl leukotrienes.

Airway dendritic cells play a fundamental role in allergen-induced airway responses and inflammation (21). Inhalation of allergen causes a rapid decrease in their number in peripheral blood (9) and their accumulation in the airway mucosa (22). The migration of dendritic cells has been subject of great interest and investigation (23). Most of the studies have focussed on Langerhans cells in the skin or trafficking in animals.

Dendritic cell precursors are derived from the bone marrow. They migrate through the blood and enter non-lymphoid tissues such as skin, and lung, where they may become resident cells. On contact with an allergen, they migrate to the lymph node, become mature and present the allergens to the lymphocyte subsets to polarize them to either Th1 or Th2 phenotype. Leukotriene C_4 plays a critical role in the migration of immature dendritic cells to the lymph node. Mobilization of dendritic cells from the epidermis and trafficking to lymphatics was greatly reduced in mice deficient in the multi-drug resistance-associated protein (MRP1) which is necessary for the transport of leukotriene C_4 from inside a cell to its outside. This was restored by exogenous leukotriene C_4 and D_4 (5). Our experiments suggest that leukotrienes may also be involved in the mobilization of immature dendritic cells from blood following an allergen inhalation. One of the mechanisms by may be by increasing the levels of dendritic cell chemoattractants such as RANTES (23), MIP-1α (24) and MIP-3α (25). Consistent with this, we observed that allergen-induced increase in the levels of RANTES (26) and MIP-1α and -3α were prevented by pranlukast treatment. CysLTs may also have a direct effect on dendritic cells by upregulating the CCR6 receptors that are necessary for their migration towards MIP-3α (25). Alternatively, cysteinyl leukotrienes produced following by inflammatory cells following an allergen inhalation may augment the chemotactic activity of MIP-3α, similar to its potentiating effect on alveolar macrophage derived MIP-1α (27).

There is an apparent difference in the ability of the myeloid and plasmacytoid cells to migrate. While more myeloid cells appear in the bronchial mucosa following an allergen inhalation (22), more plasmacytoid cells seem to migrate to the nasal mucosa following nasal allergen challenge (28). The reason for this difference is not known. In-

vitro, plasmacytoid cells show markedly less chemotaxis than myeloid dendritic cells towards classical agonists (29). One of the reasons may be because most of the chemokine receptors expressed on the plasmacytoid cells are not functional in circulating cells (29). In keeping with these observations, we noted a significant decrease in circulating myeloid dendritic cells than plasmacytoid cells immediately after the allergen inhalation. Also, the effect of pre-treatment with a Cys-LT_1 receptor antagonist was only observed in myeloid cell numbers. A possible explanation is the greater expression of Cys-LT_1 receptor expression on myeloid cells than on plasmacytoid cells. Although, immature dendritic cells express 5- and 15-lipoxygenase activity (30), very little is known about the regulation of Cys-LT_1 receptor expression on circulating dendritic cells.

The present study does not provide information on the destination of the cells that disappear from circulation. Based on previous evidence (9, 22), we believe that they migrate to the site of allergic inflammation, i.e. the airway mucosa. It is also plausible that they may move back into the bone marrow or into the lymphatic system. Similar to the observations of Jahnsen et al. (22), and Upham et al. (9), we did not observe a significant correlation between the changes in circulating dendritic cell number and airway responses. This may be a reflection of the complex interplay between dendritic cells and various inflammatory and structural cells in orchestrating the airway inflammatory responses. The study also does not confirm that the effects of pranlukast are unique to dendritic cells. In this study we did not enumerate the numbers of lymphocytes and monocytes in all the subjects at 3h after allergen inhalation. However, we had previously observed that the number of monocytes in blood is unchanged following an allergen inhalation (9).

Despite these limitations, this study has important clinical implications. A prevention of mobilization of dendritic cells from blood to the airway may prevent the initiation of the allergic process. Although inhaled corticosteroids have also been shown to decrease the number of dendritic cells in the airway (31), there is concern about increased IgE synthesis with long-term corticosteroid therapy (32). Cysteinyl leukotrienes have recently shown to regulate the vesicular-mediated, extracellular transport of the B-cell switch-regulating cytokine, IL-4, from eosinophils (33). This effect, together with their effect on dendritic cell mobilization from blood to the airway, make them attractive target for preventing the development of allergy. Long-term clinical trials similar to the study of cetirizine (34) are necessary to identify whether cysteinyl leukotriene antagonists are effective in the prevention of allergies.

In conclusion, the cysteinyl leukotriene antagonist, pranlukast attenuated allergen-induced airway responses and the allergen induced decrease in circulating myeloid dendritic cells demonstrating a novel role for cysteinyl leukotrienes in dendritic cell trafficking.

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Table 1

	Mean (min, max)
Age, yr	27 (19, 53)
FEV_1, L	3.3 (2.3, 4.6)
FEV_1, % predicted	91 (71, 105)
PC_{20} methacholine, mg/ml*	2.4 (0.1, 18.4)

*geometric mean

Table 2

Chemoattractant mean (SD)	Placebo		Pranlukast	
	Pre-allergen	Post-allergen	Pre-allergen	Post-allergen
MIP-1α (pg/ml)	3.4 (4.8)	8.6 (11.2)	4.5 (8.1)	1.5 (1.5)
MIP-3α (pg/ml)	121 (231)	176 (182)	122 (162)	108 (226)

Table 3

% dendritic cells with Cys-LT_1R (mean, SD)	Placebo			Pranlukast		
	Pre	+3h	+24h	Pre	+3h	+24h
Myeloid	55 (27)	39 (22)	39 (27)	38 (29)	43 (36)	28 (25)
Plasmacytoid	11 (6.8)	10 (10)	8 (11)	13 (17)	18 (32)	5 (4)

Legends for figures

Figure 1: The effect of pranlukast on allergen-induced early and late asthma responses.

Figure 2: The effect of pranlukast on allergen-induced airway hyperresponsiveness.

Figure 3: Effect of pranlukast on circulating dendritic cell number.

Figure 4: Representative data of $CysLT_1$ receptor expression on two dendritic cell subsets in blood. A. Two-colour analysis of gated ($HLA\text{-}DR^+$, $CD14/16^-$) blood mononuclear cells for CD33 (y-axis) and CD123 (x-axis). The myeloid $CD33^+$ dendritic cells are identified within the R3 region ($CD33^+$, $CD123^-$) and the plasmacytoid dendritic cells are identified within the R4 region ($CD33^-$, $CD123^+$). B. All cells identified as myeloid dendritic cells in A were further analyzed for expresssion of the $CysLT_1$ receptor (dark histogram) compared to background control stain (light histogram). C. All cells identified as plasmacytoid dendritic cells in A were further examined for expression of the $CysLT_1$ receptor (light histogram) compared to background control stain (dark histogram).

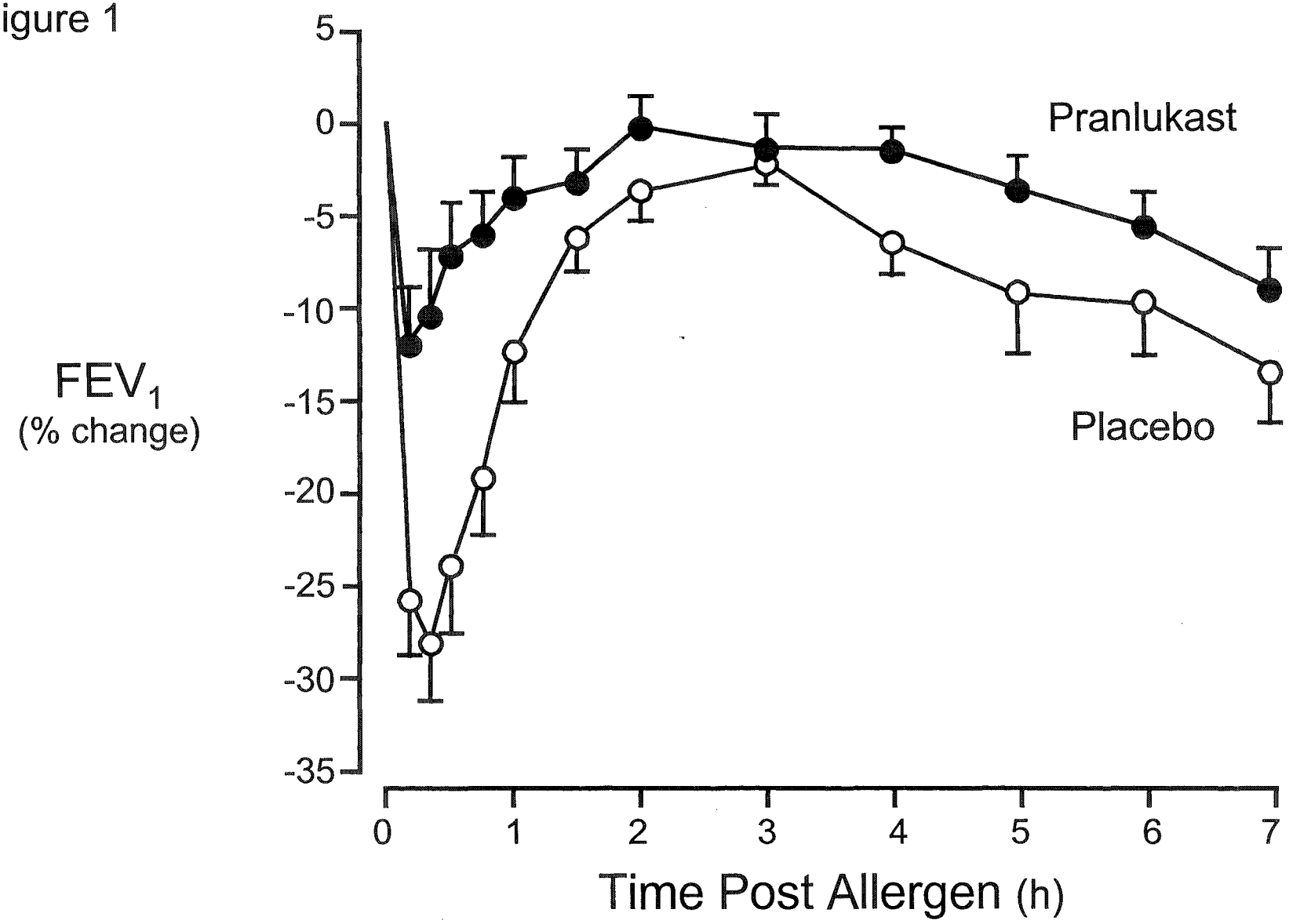
Legends for tables

Table 1: Baseline subject characteristics.

Table 2: The effect of placebo and pranlukast treatment on RANTES, MIP-1α and MIP-3α in sputum before and after an allergen inhalation.

Table 3: Expression of $CysLT_1$ receptor on circulating myeloid and plasmacytoid dendritic cells and the effects of placebo and pranlukast treatment.

Figure 1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FEV1
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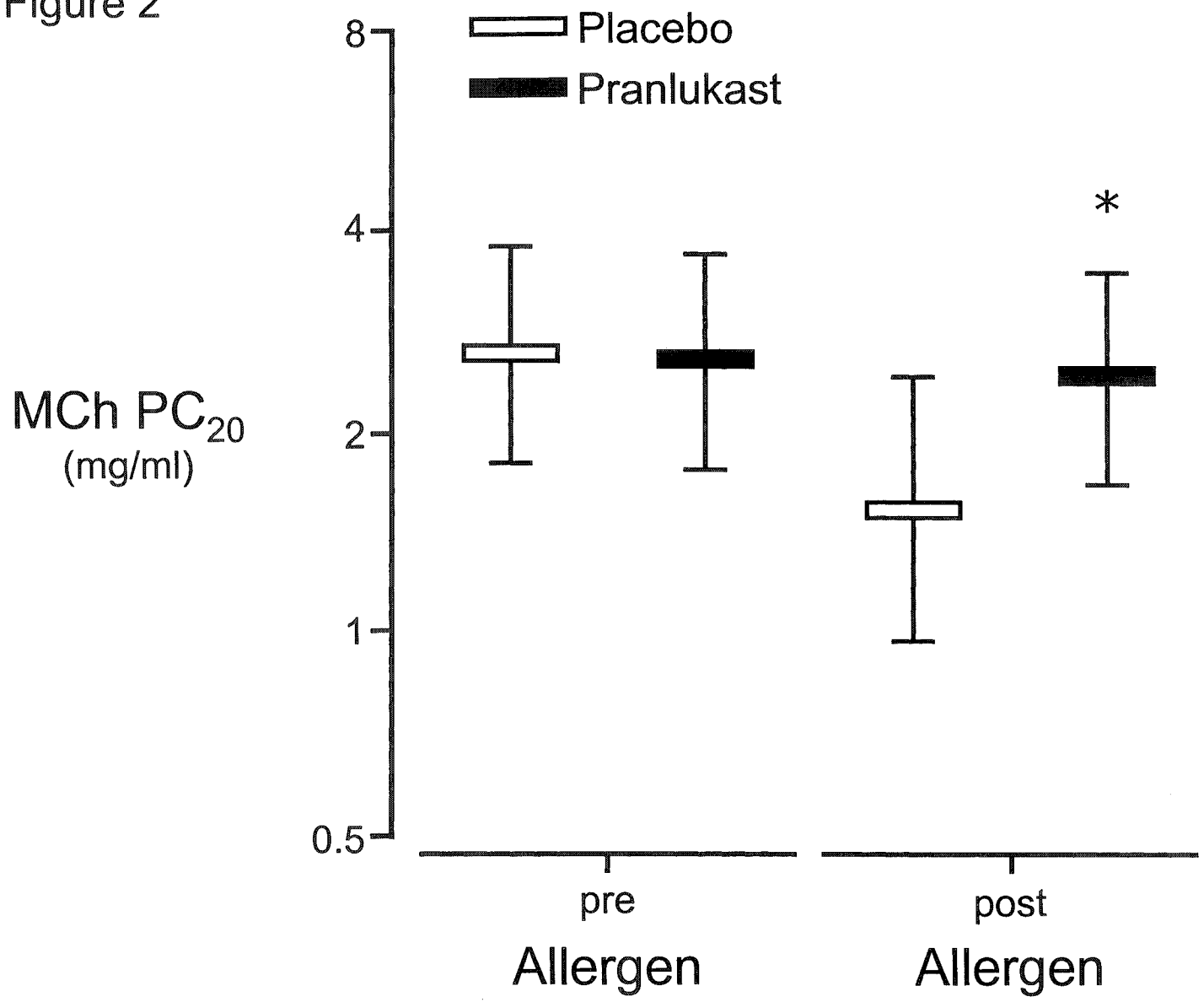
7
Time Post Allergen (h)

Figure 2


Placebo
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Allergen
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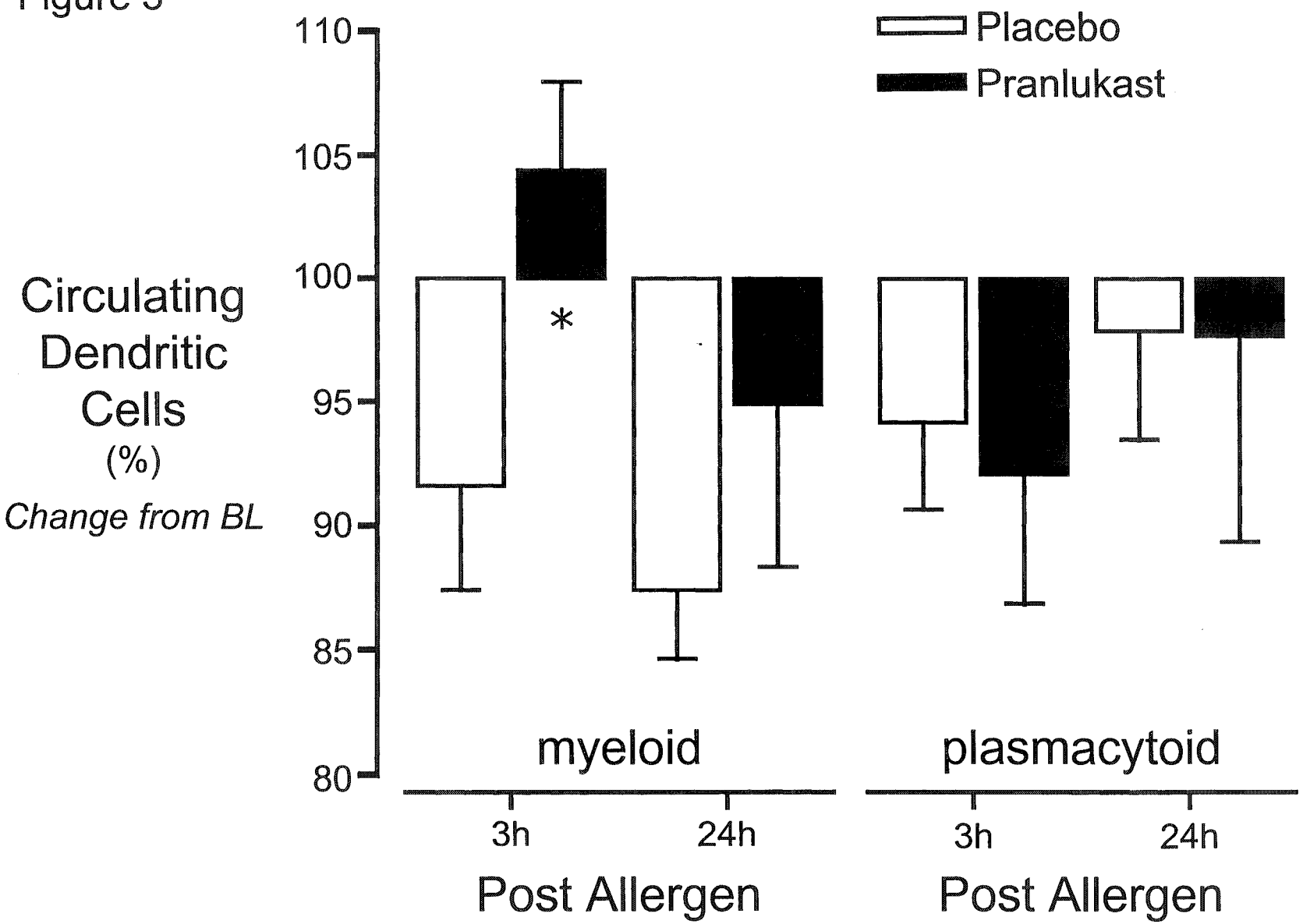
Figure 3


Placebo
Pranlukast
Circulating Dendritic Cells
(%)
Change from BL
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105
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myeloid
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24h
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Post Allergen
Post Allergen

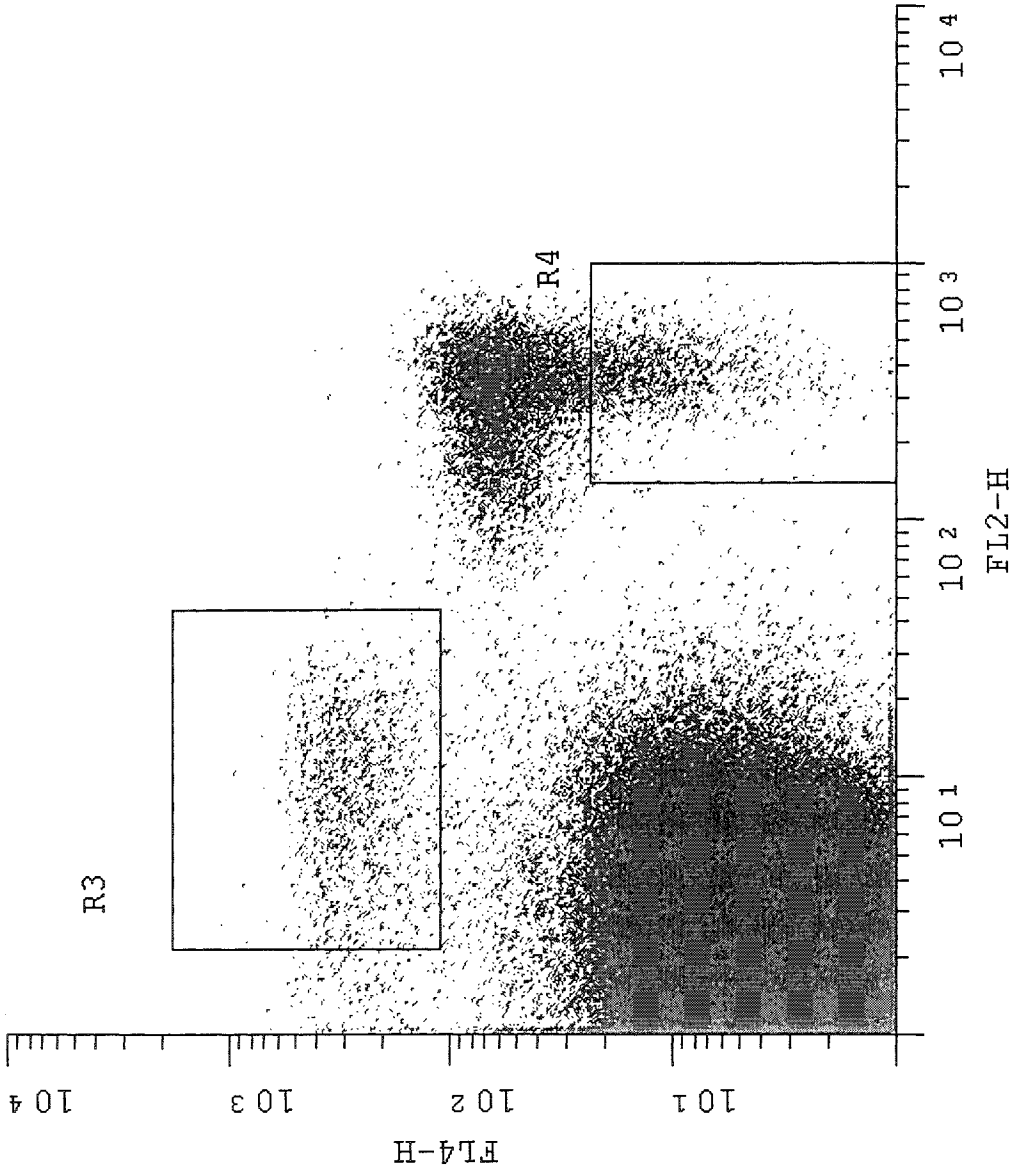

Figure 4A

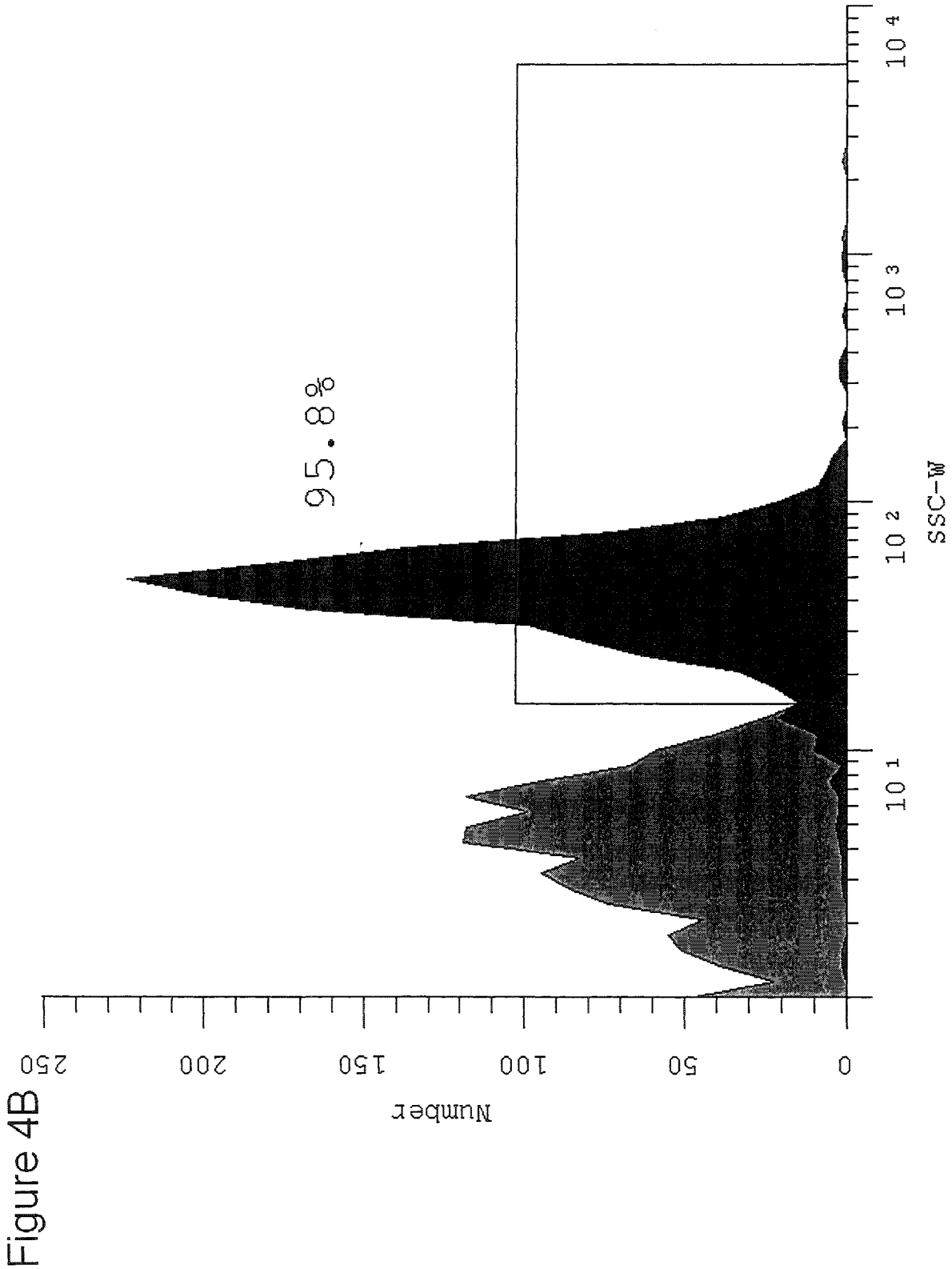

Figure 4B

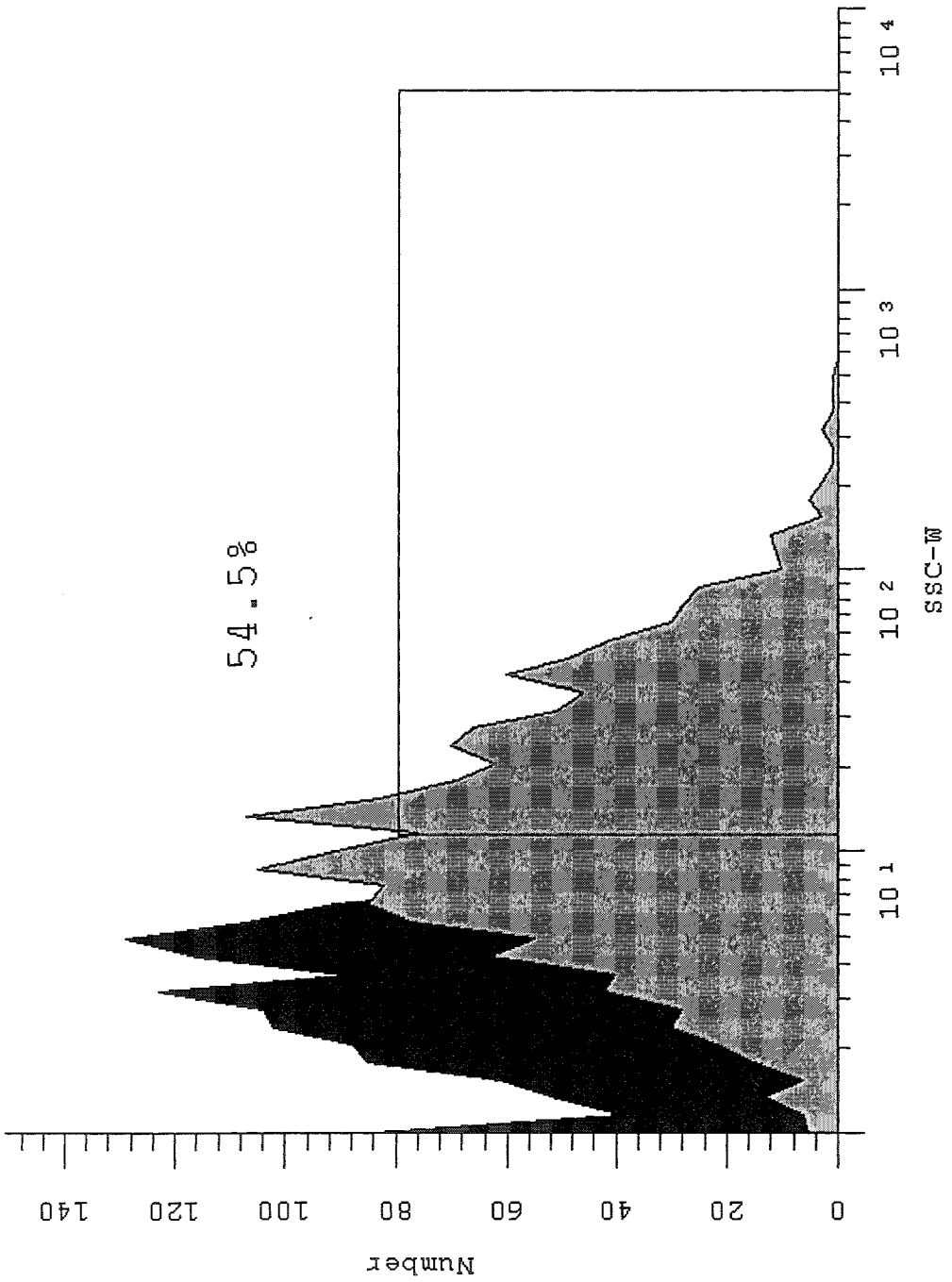

Figure 4C

CHAPTER 3:

INHALED LEUKOTRIENE E_4, BUT NOT LEUKOTRIENE D_4, INCREASED AIRWAY INFLAMMATORY CELLS IN SUBJECTS WITH ATOPIC ASTHMA

G.M. Gauvreau, K. N. Parameswaran, R. M. Watson, P. M. O'Byrne

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Dr. Parameswaran's contribution

Study design
Recruitment of subjects
Clinical evaluation of subjects
Bronchoscopy and biopsy
Interpretation of data
Preparation of manuscript

St. Joseph's Healthcare Hamilton

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45

Inhaled Leukotriene E_4, But Not Leukotriene D_4, Increased Airway Inflammatory Cells in Subjects with Atopic Asthma

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Allergen-induced late airway responses are associated with increased numbers of airway eosinophils and basophils. The purpose of this study was to compare and contrast the effects of inhaled cysteinyl leukotrienes LTD_4 and LTE_4, which are released during allergen-induced airway responses, and allergen, on airway inflammatory cells. Fifteen subjects with atopic, mild asthma inhaled diluent, LTD_4, LTE_4, and allergen. Spirometry was performed for 7 h, and sputum inflammatory cells were measured before, 7 h, and 24 h after challenges. The maximum early percent fall in FEV_1 was 23.6 ± 1.4%, 21.6 ± 2.3%, 29.3 ± 2.4%, and 4.0 ± 1.1% after LTD_4, LTE_4, allergen, and diluent, respectively. Only inhaled LTE_4 and allergen significantly increased sputum eosinophils at 7 h and 24 h, and sputum basophils at 7 h. Six additional subjects underwent airway biopsies 4 h after inhalation. There were significantly more eosinophils in the lamina propria after inhalation of LTE_4 compared with LTD_4 and diluent ($p < 0.05$). These results suggest cysteinyl leukotrienes play a role in eosinophil migration into the airways in allergic asthma, and for the same degree of bronchoconstriction, inhaled LTE_4 causes more tissue and airway eosinophilia than LTD_4.

Keywords: airway inflammation; allergen inhalation; cysteinyl leukotrienes; eosinophil; mediators

Allergen inhalation by sensitized subjects with atopic asthma results in the development of bronchoconstriction, which can be accompanied by airway hyperresponsiveness and an increased number of airway inflammatory cells, including eosinophils, basophils, and mast cells (1, 2). These cells are sources of the cysteinyl leukotrienes LTC_4, LTD_4, and LTE_4 (3), which are lipid mediators known to cause bronchoconstriction in human airways (4–6) and play a role in the pathogenesis of asthma (7). Endogenous cysteinyl leukotrienes produced after allergen inhalation (8) are partly responsible for allergen-induced bronchoconstriction, as leukotriene synthesis inhibitors or cysteinyl LT_1-receptor antagonists inhibit both allergen-induced early responses and late responses (9–11).

The role of cysteinyl leukotrienes in the development of allergen-induced airway hyperresponsiveness and airway inflammation is less well understood. Only a limited number of studies have described the effects of inhaled cysteinyl leukotrienes on airway inflammatory cells. Inhaled LTD_4 has been shown to increase sputum eosinophils in subjects with asthma in one study (12), but not in another (13). Inhaled LTE_4 has been shown to elevate the numbers of eosinophils measured in bronchial biopsies (14, 15), and treatment with a cysteinyl LT_1-receptor antagonist reduced both eosinophils and basophils in BAL after segmental allergen challenge (16).

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Internet address: www.atsjournals.org

To date, there is no direct comparison of inhaled LTD_4, LTE_4, or allergen in their ability to cause airway inflammation and airway hyperresponsiveness. This randomized, cross-over study has compared the effects of inhaled LTD_4 and LTE_4 and allergen on the recruitment of airway eosinophils, mast cells, and basophils measured in induced sputum and bronchial biopsies, and on airway hyperresponsiveness in a group of subjects with mild, stable allergic asthma.

METHODS

Study Design

Twenty-one nonsmoking subjects with mild atopic asthma with a forced expiratory volume in 1 s (FEV_1) greater than 70% of predicted (> 70%pred) were recruited for the study (Table 1). These subjects were selected because they had previously demonstrated that they were able to develop allergen-induced early airway responses of at least a 15% fall in FEV_1, and allergen-induced increase in sputum eosinophils at 7 and 24 h after challenge. Subjects used no medication other than inhaled β_2-agonists in the last 4 wk before and during the study. Medication was withheld for at least 8 h before each visit, and subjects were instructed to refrain from rigorous exercise, tea, or coffee in the morning before visits to the laboratory. Subjects did not have asthma exacerbations or respiratory tract infections for at least 4 wk before entering the study, and were studied out of season for their allergies. The study was approved by the ethics committee of the McMaster University Health Sciences Center (Hamilton, ON, Canada), and subjects gave signed consent.

Clinical Methods

The study was double-blinded, diluent-controlled, randomized, with a cross-over design. Subjects underwent skin testing and allergen challenge (13) to document allergen-induced responses. Fifteen subjects were randomized to 3 treatment periods for inhalation of diluent, LTD_4, and LTE_4 (Cayman Chemical, Ann Arbor, MI) (13), using a Wright nebulizer, with 7 d between inhalations. If methacholine responsiveness or sputum eosinophils had not returned to baseline value, the washout period was extended. LTD_4 and LTE_4 were supplied in ethanol, stored at −70° C, and diluted in physiological saline in 2-fold concentrations immediately before use. Challenges were started at a concentration of leukotriene chosen on the basis of methacholine responsiveness, and the concentration of leukotriene was increased in doubling concentrations until at least a 20% fall in FEV_1 from preinhalation baseline was achieved (Table 1). Diluent challenge consisted of inhaling three doubling concentrations of ethanol diluted to the highest concentration used for leukotriene challenges. Spirometry was performed for 7 h after inhalation, and then sputum was induced and processed (17). Sputum could not be induced earlier because pretreatment with bronchodilator would interfere with subsequent measurement of FEV_1. The methacholine PC_{20} (provocative concentration causing a 20% fall in FEV_1) (18) and sputum cells were measured 1 d before and 24 h after challenge. Sputum cell differential counts and immunostaining for mast cells, basophils, and the activated form of human eosinophil cationic protein (ECP), EG2, were performed (2). Six subjects were randomized as described above, and underwent three bronchoscopic procedures 14 d apart. Biopsies were obtained 4 h after inhalation, as a previous report demonstrated significant increases in tissue eosinophils after inhalation of LTE_4 at this time point (14). Fiberoptic bronchoscopy was carried out with an

46

TABLE 1. CHARACTERISTICS OF SUBJECTS UNDERGOING INHALATION CHALLENGES WITH SPUTUM (A), AND BRONCHIAL MUCOSA SAMPLING (B)

					Inhalation Challenge Maximum Fall in FEV_1			Highest Inhaled Concentration of:		Cumulative Inhalations of:		PC_{20} Molar Concentration		
Subject	Sex	Age (yr)	MCh PC_{20} (mg/ml)	Predicted FEV_1 (%)	LTE_4 (%)	LTD_4 (%)	Diluent (%)	LTE_4 (μM)	LTD_4 (μM)	LTE_4 (no.)	LTD_4 (no.)	LTE_4 (μM)	LTD_4 (μM)	MCh (mM)
1-A	F	22	1.4	99.6	−15.9	−22.8	0	58	0.8	3	2	39	0.32	7.15
2-A	F	23	2.5	80.4	−27.4	−19.4	−1.7	115	0.4	2	4	33	2.08	12.8
3-A	F	22	15.5	95.1	−7.1	−22.9	1.5	14	6.3	5	5	77.8	2.72	79.2
4-A	M	25	12.1	93.3	−18.9	−19.7	2.7	115	1.6	2	4	52.1	0.8	61.8
5-A	M	30	8.0	85.8	−19.4	−18.0	−5.3	230	25	2	4	102.5	17.5	40.8
6-A	F	22	0.1	80.3	−25.5	−36.9	−13.7	1.8	0.2	2	1	0.55	0.07	0.5
7-A	M	22	0.8	75.5	−19.6	−26.9	−6.2	58	1.6	4	2	27.4	0.43	4.08
8-A	M	51	1.7	94.5	−30.9	−25.4	−3.0	115	3.1	1	2	50.0	3.1	8.69
9-A	F	23	16.0	85.3	−18.4	−18.0	0	230	3.1	3	2	108.1	1.9	81.8
10-A	F	24	1.13	84.3	−17.3	−19.2	−5.9	115	6.3	2	2	18.5	3.41	5.77
11-A	M	22	3.9	89.3	−16.3	−16.3	−6.1	230	6.3	2	3	140.8	9.36	19.9
12-A	F	22	0.7	96.5	−44.8	−27.8	−5.0	58	3.1	2	2	8.32	1.06	3.58
13-A	F	23	14.7	84.3	−12.7	−23.2	−1.8	460	25	4	3	403.9	10.9	75.1
14-A	M	21	0.4	118.0	−30.9	−26.0	−11.1	14	0.8	2	2	3.57	0.24	2.04
15-A	F	31	1.1	78.6	−18.4	−31.3	−4.3	115	3.1	3	3	59.9	0.98	5.62
Mean		25.5	2.3*	89.4	−21.6	−23.6	−4.0	72.3*	2.6*	2.6	2.7	33.7*	1.5*	11.6*
1-B	M	23	2.5	71.0	−21.9	−25.8	−3.2	115	1.6	3	3	44.3	0.57	12.8
2-B	M	51	1.7	94.5	−52.9	−27.9	−7.6	230	0.78	2	2	34.3	0.26	8.69
3-B	M	34	1.2	83.0	−20.3	−23.5	−5.4	115	1.56	2	3	81.9	1.14	6.13
4-B	F	27	1.6	95.1	−25.0	−23.0	−1.8	14	0.39	2	2	5.3	0.32	8.18
5-B	M	30	8.0	85.8	−27.8	−28.8	−4.4	230	3.1	3	4	46.6	0.66	40.9
6-B	F	27	1.5	102.7	−21.2	−27.7	−3.1	115	1.6	2	3	44.2	0.6	7.66
Mean		32.0	2.2*	88.7	−28.2	−26.1	−4.3	102.0*	1.3*	2.3	2.8	21.3*	0.9*	7.7*

Definition of abbreviations: F = female; FEV_1 = forced expiratory volume in 1 s; LT = leukotriene; M = male; MCh = methacholine chloride; PC_{20} = concentration resulting in a 20% fall in FEV_1.

* Values expressed as geometric means.

Olympus (Lake Success, NY) 1T20D fiberoptic bronchoscope according to a standardized protocol (19). Specimens were fixed in 4% paraformaldehyde, mounted in paraffin, and stained with Congo red for eosinophils and toluidine blue for mast cells and basophils. Cells were enumerated in the lamina propria, to a depth of 115 μm. The area and depth of lamina propria in which the counts were performed were measured by computerized image analysis (microscope: Olympus BX40; camera, Sony 3CCD Power HAD video camera; software, Northern Eclipse; Empix Imaging, Mississauga, ON, Canada), and results are expressed as the number of cells per millimeter squared of lamina propria.

Statistical Analysis

Summary statistics for measurements of FEV_1 are expressed as means and SEM. Values of methacholine PC_{20} and inflammatory cells were log-transformed before analysis and are reported as geometric means (+geometric SEM [GSEM]). Two-way repeated measures analysis of variance (ANOVA) was used to examine the effects of leukotriene and time. Because allergen inhalation was not randomized into the study design, its effect was not included in the ANOVA. Provocation data were not analyzed with regard to sequence effects. Statistical analyses were performed with computer software (Statistica 4.5; StatSoft, Tulsa, OK).

RESULTS

Inhaled LTD_4 and LTE_4 caused bronchoconstriction, which began to resolve by 10 min after inhalation, and returned to within 10% of baseline by 1 h (Figure 1). The maximum percent fall in FEV_1 and the area under the curve at 0–1 h (AUC_{0-1h}) after LTD_4 and LTE_4 inhalation were not different from each other ($p < 0.17$). A 40-fold higher concentration of LTE_4 (median) was required to elicit the same degree of bronchoconstriction as with LTD_4 (Table 1). Allergen-induced bronchoconstriction demonstrated a slower onset than LTD_4 and LTE_4, was maximal at 20 min, and more prolonged returning to only 14% of the baseline value by 1 h (Figure 1). The percent fall in FEV_1 between 3 and 7 h after LTD_4 and LTE_4 was 3.1 ± 0.8 and 4.0 ± 1.0%, respectively, which was significantly smaller than the allergen-induced maximum late percent fall in FEV_1 of 18.8 ± 3.1% ($p < 0.0002$) (Figure 1).

There was no effect of inhaled diluent, LTD_4, or LTE_4 on methacholine PC_{20} measured 24 h after inhalation challenge ($p = 0.79$) (Figure 2). By contrast, inhaled allergen caused a fall in methacholine PC_{20} from 2.1 mg/ml (+GSEM, 1.0) before allergen inhalation to 0.5 mg/ml (+GSEM, 0.3) 24 h after allergen inhalation ($p < 0.00004$).

There was no significant difference in the number of sputum eosinophils at baseline before inhalation of diluent, LTD_4,

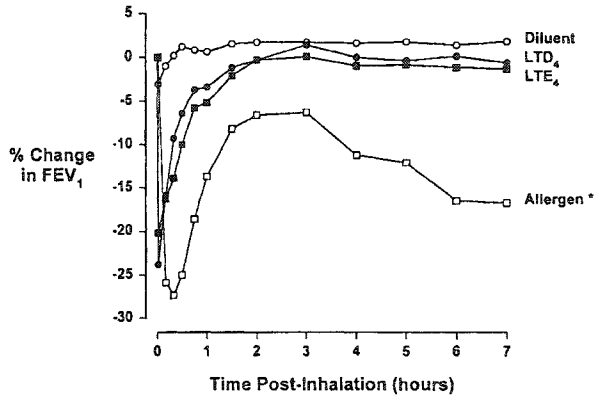

Figure 1. Mean percent change in FEV_1 for 7 h after inhalation of diluent (*open circles*), LTD_4 (*solid circles*), LTE_4 (*solid squares*), and allergen (*open squares*). *$p < 0.05$ early (0–2 h) and late (3–7 h) asthmatic responses compared with diluent control. The SEM has been omitted for clarity, but did not exceed 2.8 at any time point shown.

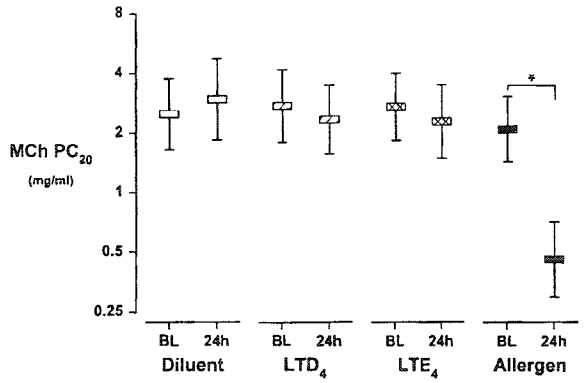

Figure 2. Methacholine PC_{20} (geometric mean and geometric SEM) at baseline (BL) and 24 h after inhalation of diluent (*open bars*), LTD_4 (*hatched bars*), LTE_4 (*crossed bars*), and allergen (*solid bars*). *$p < 0.05$ compared with baseline.

and LTE_4 (Figure 3). The number of sputum eosinophils increased from 2.6×10^4/ml (+GSEM, 1.4) at baseline, to 14.7×10^4/ml (+GSEM, 5.8) 7 h after inhalation of LTE_4 ($p = 0.004$), and to 19.1×10^4/ml (+GSEM, 5.3) at 24 h after inhalation of LTE_4 ($p = 0.005$). This increase in sputum eosinophils was significantly higher than after diluent or LTD_4 ($p < 0.05$). There was no significant difference in the number of sputum eosinophils between diluent and LTD_4 inhalation challenge (Figure 2). The number of eosinophils in the lamina propria of the airway biopsies 4 h after inhaled LTE_4 was higher than after inhaled LTD_4 or diluent ($p < 0.05$) (Figures 4 and 5). There was no statistically significant change in the number of activated sputum eosinophils (EG2-positive cells) after inhalation of LTD_4 or LTE_4 (Table 2).

Inhalation of LTE_4 increased the number of sputum basophils at 7 h ($p = 0.048$) but not at 24 h compared with diluent ($p > 0.05$) (Table 2). There was no increase in the number of sputum mast cells after inhalation of diluent, LTD_4, or LTE_4 ($p > 0.05$). The number of metachromatic cells (mast cells and basophils) measured in the lamina propria was significantly greater 4 h after inhalation of LTE_4 compared with LTD_4 and diluent ($p = 0.05$); however, there was no effect of diluent or LTD_4 on the number of metachromatic cells measured in the lamina propria ($p > 0.05$) (Figure 5).

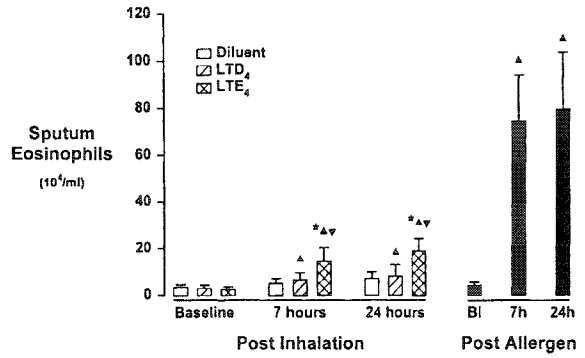

Figure 3. Mean number of sputum eosinophils (+GSEM) at baseline, 7 h, and 24 h after inhalation of diluent (*open columns*), LTD_4 (*hatched columns*), LTE_4 (*crossed-hatched columns*), and allergen (*solid columns*). †$p < 0.05$ compared with preinhalation baseline (*solid triangles*); *$p < 0.05$ compared with diluent control, ‡$p < 0.05$ compared with LTD_4 (*open triangles*). Allergen was not compared with diluent, LTD_4, or LTE_4.

The sputum total cell count increased after inhalation challenge with diluent, LTD_4, and LTE_4 ($p = 0.01$), leading to a significant effect of time on the number of sputum mast cells (tryptase-positive cells) ($p < 0.0009$) despite no change in the mast cell differential ($p = 0.15$). The number of sputum neutrophils also increased after inhaled diluent, LTD_4, and LTE_4

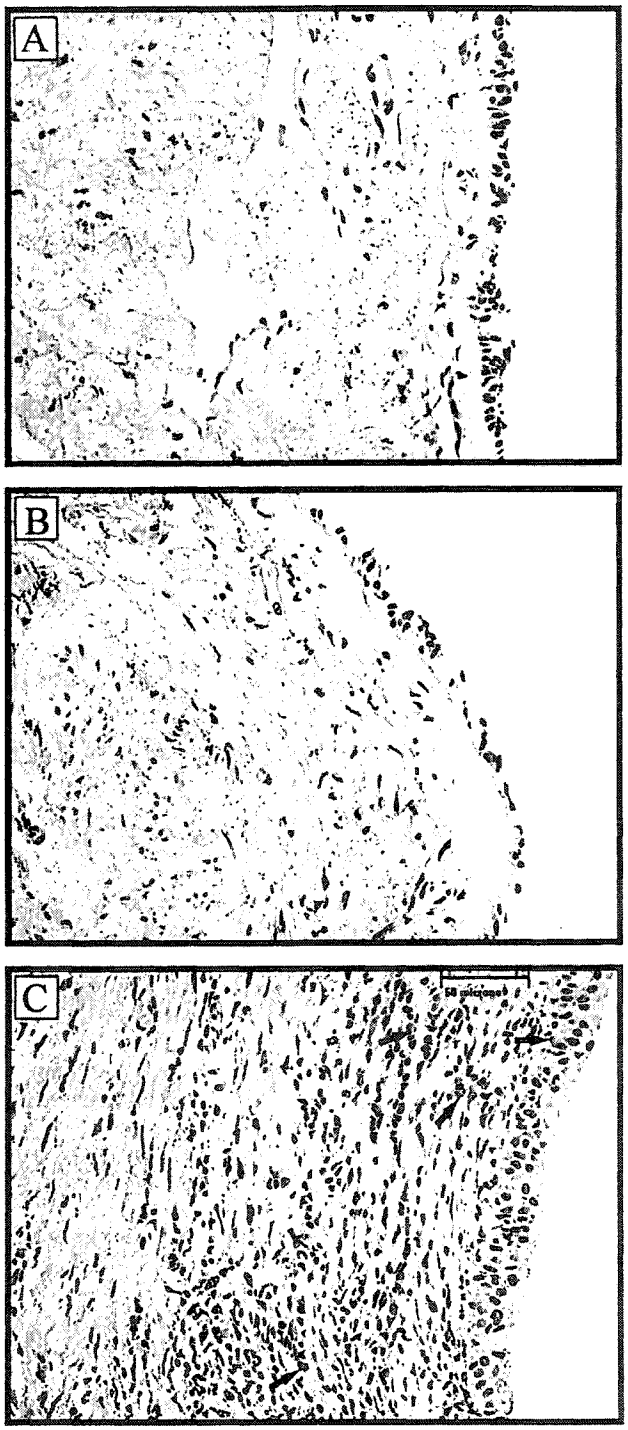

Figure 4. Eosinophils (*arrows*) in bronchial tissue sampled 4 h after inhalation of diluent (*A*), LTD_4 (*B*), and LTE_4 (C) in a representative subject. Original magnification ×400.

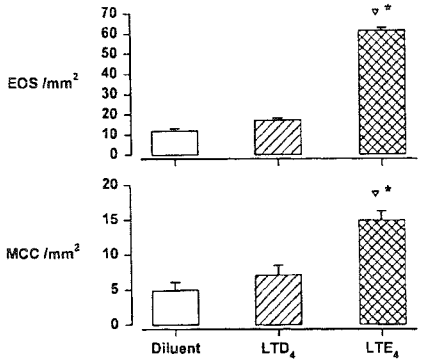

Figure 5. Geometric mean number of eosinophils (*top*) and metachromatic cells (*bottom*) per millimeter squared in the lamina propria 4 h after inhalation of diluent (*open columns*), LTD_4 (*hatched columns*), and LTE_4 (*crossed columns*). *p < 0.05 compared with diluent control; open triangle = p < 0.05 compared with LTD_4.

(p < 0.005) as a result of an increase in the neutrophil differential (p = 0.0007) and sputum total cell count (p = 0.01). There was, however, no difference in the number of sputum neutrophils or mast cells between any of the inhalation challenges (p = 0.07 and p = 0.16).

DISCUSSION

We have demonstrated that inhalation of LTE_4 by subjects with mild asthma increases the number of airway eosinophils measured in bronchial lamina propria 4 h after challenge, and in sputum 7 and 24 h after challenge, suggesting that inhaled LTE_4 induces a prolonged inflammatory event. However, inhaled LTD_4, causing the same degree of bronchoconstriction as inhaled LTE_4, did not cause sputum or tissue eosinophilia. The magnitude of the effect of inhaled LTE_4 was significantly less than the sputum eosinophilia caused by inhaled allergen in the same subjects, and it was not associated with the development of methacholine airway hyperresponsiveness, as occurred after inhaled allergen.

Increased numbers of inflammatory cells, including eosinophils and neutrophils, have been found in bronchial biopsies performed 4–6 h after LTE_4 inhalation (14, 15), and small increases in sputum eosinophils have been measured in 50% of subjects with asthma 4 h after LTE_4 inhalation (20). The results from this study, demonstrating increased numbers of both eosinophils and basophils in sputum after LTE_4 inhalation, support the current concept that the cysteinyl leukotrienes may be a cause of the airway eosinophilia in asthma.

There have been few studies investigating the effects of antileukotrienes on allergen-induced airway inflammation. Pretreatment with a cysteinyl LT_1 antagonist has been shown to significantly inhibit allergen-induced lymphocytes and basophils, but not eosinophils, measured in bronchoalveolar lavage (BAL) 48 h after segmental allergen challenge (21). However, when higher doses of cysteinyl LT_1 antagonist were administered, these investigators were able to demonstrate a partial effect on eosinophils (16). In a separate study, Diamant and coworkers were unable to see any effect of cysteinyl LT_1 antagonist on allergen-induced sputum eosinophils or other inflammatory cells measured in sputum, despite significant inhibition of the early and late asthmatic responses (22). This lack of effect may have been a result of additional mechanisms leading to accumulation of inflammatory cells after allergen inhalation.

The cysteinyl leukotrienes are thought to be the main cause of allergen-induced bronchoconstriction, as inhibitors of leukotriene synthesis or cysteinyl LT_1-receptor antagonists have produced almost complete inhibition of allergen-induced early responses and partial inhibition of the late response (9–11). Although cysteinyl leukotrienes have been shown to be the main cause of allergen-induced early bronchoconstriction, histamine, and possibly neural reflexes, also contribute to the early phase of bronchoconstriction. Despite a similar degree of bronchoconstriction to inhaled LTE_4 to theoretically achieve approximately the same airway levels of leukotrienes as after allergen inhalation (at least on the cysteinyl LT_1 receptor on airway smooth muscle), other mediators/neural reflexes as well as prolonged leukotriene generation may contribute to the enhanced responses observed after allergen inhalation. We induced a similar degree of bronchoconstriction to inhaled allergen, LTD_4, and LTE_4; there were different effects of LTD_4 and LTE_4, and both were different than allergen inhalation. The early airway response to allergen was more prolonged than to LTD_4 or LTE_4, and neither inhaled LTD_4 nor LTE_4 caused a late response. The degree of sputum eosinophilia after LTE_4 was approximately one-quarter of that measured after allergen challenge (Figure 3) and considerably fewer sputum basophils and mast cells were detected than previously measured after allergen challenge (2). The LTE_4-induced airway inflammation was not associated with the development of methacholine airway hyperresponsiveness, which was measured 24 h after challenge. Subjects may have developed LTE_4-induced airway hyperresponsiveness to methacholine earlier than 24 h, as earlier studies have shown airway hyperresponsiveness peaks 3 to 7 h after inhalation of LTE_4 (23). Our experiments, however, cannot explain this apparent discrepancy between the presence of eosinophilic inflammation without the concurrent airway hyperresponsiveness 24 h after LTE_4 inhalation. It is possible that LTE_4-induced sputum eosinophilia and basophilia

TABLE 2. SPUTUM CELL MEASURED AT BASELINE, 7 h, AND 24 h AFTER INHALATION OF DILUENT, LTD_4, AND LTE_4*

	Diluent			LTD_4			LTE_4		
	Baseline	7 h	24 h	Baseline	7 h	24 h	Baseline	7 h	24 h
Basophils ($\times 10^{-6}$/ml)	0.80 (0.64)	0.33 (0.14)	1.41 (1.00)	0.48 (0.33)	0.97 (1.02)	1.82 (2.12)	0.22 (0.20)	3.70 (4.90)†,‡	2.60 (2.93)†
Mast cells ($\times 10^{-6}$/ml)	0.23 (0.05)	0.31 (0.01)†	0.47 (0.06)†	0.21 (0.08)	0.34 (0.46)†	0.38 (0.14)†	0.22 (0.03)	1.11 (0.96)†	0.83 (0.53)†
EG2 positive ($\times 10^{-4}$/ml)	4.5 (3.9)	2.2 (3.1)	2.2 (2.5)	2.3 (2.3)	1.8 (2.3)	6.1 (7.3)	2.2 (2.0)	9.6 (4.4)	14.7 (3.8)
Neutrophils ($\times 10^{-4}$/ml)	106.1 (24.8)	132.7 (43.0)†	180.7 (41.9)†	48.5 (13.9)	117.2 (39.5)†	163.6 (40.2)†	78.2 (20.5)	105.5 (34.4)†	90.1 (19.0)†

* Values are shown as geometric means (+geometric SEM).
† p < 0.05 difference from baseline.
‡ p < 0.05 difference from diluent.

49

is not sufficient to cause the same magnitude of airway inflammation that is observed in association with allergen-induced late responses. On the other hand, allergen inhalation resulting in airway hyperresponsiveness induces the release of many proinflammatory mediators. Upregulation of the eosinophilic cytokines after allergen inhalation (24) may enhance (i.e., interleukin 5 [IL-5] and eotaxin) or prolong (i.e., granulocyte-macrophage colony-stimulating factor [GM-CSF]) the allergen-induced airway eosinophilia and basophilia, resulting in late airway responses and methacholine airway hyperresponsiveness.

This study has confirmed previous observations that inhaled LTD_4 resulting in submaximal (13) or maximal (12) bronchoconstriction does not increase sputum eosinophils in asthmatic airways when compared with control challenge (diluent or methacholine). The results of this study, that inhaled LTE_4, but not LTD_4, caused significant increases in the number of sputum eosinophils, was a surprising finding, because it is thought that both LTD_4 and LTE_4 act on the cysteinyl LT_1 receptor. Studies using allergic guinea pigs have demonstrated eosinophil influx into the airway after LTD_4 challenge (25), and LTD_4 appears to be a chemoattractant for isolated human peripheral blood eosinophils *in vitro* (26). We have also shown a direct, albeit small, chemotactic effect of LTD_4 and LTE_4 on peripheral blood eosinophils (27). There are, however, other mechanisms whereby leukotrienes may cause elevated airway eosinophil levels, such as leukotriene-induced survival of eosinophils (28), and leukotriene-induced production of eosinophil chemoattractants such as eotaxin (29).

Different effects of LTD_4 and LTE_4 may be explained by the stability of each metabolite, as well as the relative concentration of each leukotriene that was inhaled. It has been shown that LTC_4, and presumably LTD_4, are quickly metabolized in the airway, as more than half of the LTC_4 instilled into the asthmatic airway is converted to LTE_4 within 15 min (30). This suggests that inhalation of LTD_4 may actually supply LTE_4, as well, to the airways. If inhalation of LTD_4 is indirectly supplying LTE_4 to the airways through rapid metabolism, higher concentrations of inhaled LTD_4 may reproduce what has been observed after inhalation of LTE_4. This is supported by observations that both LTD_4 and LTE_4 have direct effects on eosinophil accumulation *in vivo* (31) and cause eosinophil migration *in vitro* (32). We chose to deliver leukotrienes into the airways at concentrations resulting in a physiological response that could be immediately measured. We were unable to match the concentration of inhaled LTD_4 to the concentration of inhaled LTE_4 causing a physiological response, as this would result in severe bronchoconstriction, given the greater potency of LTD_4 than LTE_4 on airway smooth muscle. LTE_4, being a less potent bronchconstrictor agent than LTD_4, was delivered at a 40-fold higher concentration to achieve the same degree of bronchoconstriction. If LTD_4 and LTE_4 share a common cysteinyl LT_1 receptor (33), as suggested by the inhibitory effects of an LT-receptor antagonist to LTE_4-induced responses *in vivo* (15), the relative potency of the two cysteinyl leukotrienes may be different in their effects on airway smooth muscle and eosinophils, or any other upstream event leading to eosinophil accumulation. Evidence that LTE_4 is less potent than LTD_4 in causing changes in vascular permeability despite similar contractile potencies when tested *in vitro* (34) supports the hypothesis that differences in both vascular responses and cellular responses may contribute to the different bronchoconstrictor potencies observed *in vivo*.

There is, however, evidence of selective binding of LTE_4 to only a subset of LTD_4 receptors (35). Although it has been generally thought that LTD_4 and LTE_4 act on the same receptors, pharmacological reversal has been found to be different between the two leukotrienes, suggesting these leukotrienes may not necessarily bind the same receptors (31). In agreement, molecular dynamics simulations have demonstrated that LTE_4 conformation spans the LTD_4 and LTC_4 types and therefore may occupy both of these receptors (36).

Whether cysteinyl leukotrienes contribute to cell activation is unclear, as we did not observe a statistically significant increase in EG2-positive cells (activated eosinophils). In another study of LTD_4- and LTE_4-challenged conjunctiva, there was no observed tissue damage despite a dose-dependent eosinophilia (25). The primary action of leukotrienes, therefore, may be amplification of inflammation rather than activation of inflammatory cells. The increase in sputum neutrophils observed after all the inhalation challenges is likely a result of the sputum induction procedure, as repeated induction of sputum has been shown to cause elevations of sputum neutrophils (13). The increase in sputum mast cells observed after all the inhalation challenges is a result of an effect of time on the total sputum cell count, and a low between-subject variability in sputum mast cell numbers. In contrast to the sputum neutrophil differential, the sputum mast cell differential was not affected by time ($p = 0.15$).

We have provided evidence that inhaled LTE_4, but not inhaled LTD_4, administered in concentrations to match the bronchoconstriction achieved with inhaled allergen, attracts eosinophils into the airways of subjects with atopic asthma. This accumulation of eosinophils was relatively small when compared with that after allergen inhalation, and this effect did not result in the development of late responses or methacholine airway hyperresponsiveness. These studies suggest that the cysteinyl leukotrienes are important not only in causing allergen-induced bronchoconstriction, but may also directly contribute to accumulation of airway eosinophils. The magnitude of eosinophil accumulation *in vivo* and *in vitro* is small. We suggest that although cysteinyl leukotrienes may induce primary effects on eosinophils, their role as priming agents may have a greater overall effect on eosinophil accumulation during an allergic event such as allergen inhalation.

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CHAPTER 4:

THE EFFECT OF PRANLUKAST ON ALLERGEN-INDUCED BONE MARROW EOSINOPHILOPOIESIS IN ASTHMATIC SUBJECTS

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Dr. Parameswaran's contribution

Study design and protocol development
Recruitment of subjects
Supervision of allergen challenge
Bone marrow aspiration
Bone marrow culture and colony assay
Supervision of bone marrow flow cytometry
Maintaining case record forms
Collection, tabulation, analysis of data
Data interpretation, preparation of manuscript

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The effect of pranlukast on allergen-induced bone marrow eosinophilopoiesis in asthmatic subjects

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Abstract

We investigated the mechanisms by which leukotriene receptor antagonists decrease airway eosinophil number. In a randomized, double blind, cross-over study, we examined the effects of two weeks of treatment with pranlukast 300 mg b.i.d. or placebo on allergen-induced changes in airway eosinophil number and bone marrow eosinophil progenitors in 15 subjects with mild asthma. Pranlukast treatment for two weeks decreased mean sputum eosinophil count from $0.15x10^6$/g (5.3% of cells) before treatment to $0.02x10^6$/g (0.7% of cells) after treatment ($p<0.05$), while placebo did not. Pranlukast also decreased the eosinophil count (5.6% at 7h and 7.5% at 24h) ($p<0.05$) after allergen inhalation compared to placebo (13.8% at 7h and 15.3% at 24h). There was similar trend for sputum cells immunostaining for EG2, eotaxin, IL-5 and RANTES. Pranlukast also significantly attenuated the allergen-induced increase in the number of bone marrow eosinophil/basophil colony forming units (mean 0.3) at 24h compared to placebo (mean 6.2). The proportion of $CD34^+$ cells expressing the eotaxin receptor CCR3, 24h after allergen inhalation, were also reduced by pranlukast. We conclude that, the cysteinyl leukotriene receptor antagonist, pranlukast, attenuates allergen-induced increase in airway eosinophils by decreasing bone marrow eosinophilopoiesis and airway chemotactic and eosinophilopoietic cytokines.

Abstract word count: 195

Key words: leukotriene receptor antagonist, pranlukast, asthma, allergen challenge, sputum eosinophil, bone marrow eosinophil progenitor

Introduction

Allergen inhalation by patients with asthma causes, within 7 hours, an increase in the number of inflammatory cells in the airway, notably eosinophils and basophils (1). Eosinophilic inflammation, which is a hallmark of allergic inflammation, is associated with the late asthma response and an increase in airway hyperresponsiveness (2,3). This cellular infiltration is facilitated by a number of cytokines such as Interleukin (IL)-5, chemokines such as eotaxin, and regulated upon activation, normal T-cell expressed and secreted (RANTES), and mediators such as cysteinyl leukotriene C_4, D_4, and E_4. While IL-5 promotes eosinophil proliferation, differentiation, priming and survival (4), eotaxin (5) and RANTES (6) induce chemotaxis of eosinophils to the airway and their activation.

There is strong evidence to suggest that the bone marrow plays an important role in the allergen-induced eosinophilic airway inflammation (7). Following an allergen inhalation, eosinophil lineage-committed progenitor cells expressing the membrane bound isoform of IL-5 receptor α-subunit ($CD34^+IL5\text{-}R\alpha^+$) and the IL-5 responsive eosinophil/basophil colony-forming units (Eo/B CFU) increase in the bone marrow (8-10) and in the peripheral blood (11). Progenitor cells are also observed in the airway mucosa of subjects with asthma (12). It is likely that allergen inhalation increases the number of eosinophil progenitors in the bone marrow, which migrate to the airways either as mature eosinophils or as immature cells and undergo local differentiation. An increase in the eotaxin receptor, CC chemokine receptor 3 (CCR3) on the bone marrow progenitor cells following an allergen inhalation may facilitate the progenitor cell mobilization from the bone marrow to the peripheral circulation (13).

Cysteinyl leukotrienes promote airway eosinophilic inflammation. Inhalation of LTD_4 and LTE_4 cause sputum and tissue eosinophilia (14) and cysteinyl leukotriene receptor antagonists can decrease sputum eosinophilia (15). Cysteinyl leukotrienes can cause airway eosinophilia by a number of possible mechanisms (16). They can promote eosinophil chemotaxis into the airway (17), increase the surface expression of adhesion molecules on eosinophils and blood vessels facilitating their migration (18), can prolong eosinophil survival (19), and can up-regulate gene expression of various cytokines/chemokines such as IL-4, IL-5, IL-13, eotaxin and GM-CSF that can promote eosinophil production and survival (20). We have previously shown that bone marrow eosinophil/basophil colony forming unit cultures grown in the presence of IL-5 were significantly increased by LTD_4 and this was inhibited by montelukast (21). We therefore hypothesized that one of the mechanisms by which cysteinyl leukotrienes promote airway eosinophilia is by promoting eosinophilopoiesis in the bone marrow.

We investigated this by studying the effect of a cysteinyl leukotriene-1 receptor antagonist, pranlukast, on allergen-induced changes in bone marrow derived eosinophil-lineage committed $CD34^+$ cells and IL-5 responsive eosinophil/basophil colony forming units. In addition, we studied the effect of treatment on CCR3 receptor expression on the progenitor cells. We also studied the effect of pranlukast treatment on the numbers of total and activated eosinophils in sputum and the immunoreactivity in sputum cells for EG2+, IL-5, eotaxin and RANTES.

Some of the results of these studies have been previously reported in the form of an abstract (22).

Methods

Word count: 492

Subjects

The subjects were 16 atopic non-smokers with mild asthma (table 1) using short-acting beta-agonists infrequently. All subjects gave written informed consent and the Research Ethics Committee of Hamilton Health Sciences Corporation approved the study.

Design

This was a randomized, double-blind, cross-over, two-period study comparing two weeks of treatment with pranlukast 300 mg tablet b.i.d. or matching placebo, with at least 2 weeks of wash-out period between the treatment periods. The subjects underwent a screening allergen inhalation to identify those with a dual asthma response (greater than 15% fall in FEV_1 within 120 min, followed by a similar drop between 3 and 7h after allergen inhalation). After 2-4 weeks, they had spirometry, sputum induction and a methacholine inhalation test. They were then randomized to one of the two treatment arms using computer-derived codes that were maintained off-site by an independent third party pharmacist. The medications were taken at 8 pm and 8 am. The first dose was in the evening of the first day and the last dose was on the morning of day 15. On the 8^{th} day (± 2 days), they visited the laboratory for a physical examination to evaluate for adverse effects and to evaluate compliance. On the 13^{th} day (± 2 days), subjects attended the laboratory for spirometry, sputum induction and an iliac bone marrow aspiration. The next day, an allergen inhalation test was performed. At 7h after allergen inhalation, sputum was induced. The following day, approximately 24h after allergen inhalation, subjects attended the laboratory for spirometry, sputum induction and another bone marrow

aspiration. Compliance was evaluated weekly by pill counting. Any adverse effect was evaluated by self-reported symptoms, physical examination, blood chemistry and urine examination.

Allergen inhalation

FEV_1 was measured using a Collins water-sealed spirometer (Warren E. Collins, Braintree, MA) and kymograph according to the ATS recommendations (23). Allergen inhalation was performed as previously described (2, 24).

Sputum induction and processing

Sputum was induced with hypertonic saline, separated from saliva, and processed as described by Pizzichini and co-workers (25).

Sputum cytochemistry

Sputum cytospins were prepared on Aptex (Sigma Chemical Co., Mississauga, ON) coated slides, fixed for 10 min in periodate-lysine-paraformaldehyde and stained as described by Gauvreau et al (1).

Bone marrow progenitor culture

5 ml of bone marrow sample were aspirated into heparinized (1000 U/ml) syringes from the iliac crests after freezing the skin and periosteum with 2% lidocaine. Low-density mononuclear cells were isolated by sedimentation on Percoll density gradients (specific gravity 1.08) and cultured in the presence of IL-5 (1 ng/ml) as previously described (8-10).

Immunofluorescence staining

Non-adherent mononuclear cells were stained with saturating amounts of biotin-conjugated anti-IL-5Rα, and anti-CCR3, or the isotype-control antibody in 100 μl of ice-cold fluorescence-activated cell sorter staining buffer for 30 min at 4^0C (8,9,12,13).

Flow cytometry

The stained non-adherent mononuclear cells were analysed using a FACScan flowcytometer equipped with an argon laser (Becton Dickinson Instrument Systems, Mississauga, ON, Canada) using the CELLQUEST programme. $CD34^+$ blast cells were identified as previously described (8,13).

Analysis

Demographic data was summarized using descriptive statistics. Sputum eosinophil counts, expressed in absolute terms and as a percentage of the total cell count, and the immunopositive cells were analyzed using repeated-measures ANOVA. Treatment (pranlukast or placebo), time (baseline, pre-allergen, 7h post-allergen, 24h post-allergen), and period (first or second treatment) were the within-subject factors. The differences between pranlukast and placebo on allergen-induced changes in bone marrow progenitor colony counts and receptor expression were analyzed using paired t-test. All analyses were performed using the Statistical Package for Social Sciences, version 10 (SPSS Inc., Chicago, IL). P-values of less than 0.05 were considered to be statistically significant.

Results

One subject developed a facial urticaria after one dose of the study medication and was withdrawn from the study. 15 subjects completed the study. One subject had transient, mild and reversible elevation of liver enzymes that was not considered due to pranlukast treatment. The over-all compliance with treatment was 90%.

Sputum cell counts

Two weeks of treatment with pranlukast decreased mean sputum eosinophil count from $0.15x10^6$/g (±0.24) before treatment to $0.02x10^6$/g (±0.02) after treatment ($p<0.05$) (Figure 1), while placebo did not ($0.11x10^6$/g (±0.16) before treatment and $0.10x10^6$/g (±0.15) after treatment).

Allergen inhalation increased sputum eosinophil numbers and this effect was attenuated by pranlukast. After placebo treatment, sputum eosinophils increased to $0.72x10^6$/g (±1.04) at 7h and $0.54x10^6$/g (±0.59) at 24h. After pranlukast treatment, the increase in sputum eosinophil was significantly less ($0.21x10^6$/g at 7h (±0.38) and $0.33x10^6$/g (±0.38) at 24h) ($p<0.05$) (figure 1). There were no significant changes in the total sputum cell counts or other cell counts with either allergen inhalation or pranlukast treatment (data not shown).

Sputum cytochemistry

Similar to the effect on eosinophil counts, two weeks of treatment with pranlukast decreased $EG2^+$ cells and attenuated the allergen-induced increase in $EG2^+$ cells (table 2). The effect at 7h was statistically significant compared to the placebo treatment. A similar pattern was observed on sputum cells stained for IL-5, RANTES and eotaxin. While placebo pre-treatment caused a 2.4 fold increase in IL-5 positive cells, and a 1.7 fold and 2.0 fold increase in RANTES and eotaxin-positive cells at 7h following allergen challenge, the corresponding numbers after pranlukast treatment were 1.5 fold, 1.0 fold and 1.2 fold. However, this difference was not statistically significant (table 2).

Bone marrow eosinophil/basophil colony forming units

After two weeks of treatment with pranlukast, the number (meanSD) of IL-5 responsive Eo/B colonies forming units in the bone marrow was 17.36.3. This was not different from placebo treatment (17.3 6.1). Allergen inhalation increased the bone marrow Eo/B colony forming units during placebo treatment to 23.66.2. This effect was significantly decreased during pranlukast treatment to 17.77.4 ($p<0.05$) (figure 2).

Bone marrow progenitor cell receptor expression

We examined the number of bone marrow cells expressing CD34 and the numbers of $CD34^+$ cells expressing CCR3. Allergen inhalation preceded by two weeks of placebo treatment did not cause a significant increase in $CD34^+$ cells. However, pranlukast significantly reduced the allergen-induced increase in $CD34^+CCR3^+$ cells compared to placebo ($p<0.05$) (table 3).

Discussion

This study confirms previous findings that treatment with a leukotriene receptor antagonist, pranlukast, decreases the number of total and activated eosinophils in the airway and attenuates their increase following an allergen inhalation. The study also showed for the first time that this was associated with a decrease in the numbers of IL-5 responsive eosinophil colony forming units and $CD34^+CCR3$ cells in the bone marrow. This suggests that one of the mechanisms by which leukotriene antagonists decrease allergen-induced airway eosinophil number is by a direct effect on allergen-induced eosinophilopoiesis in the bone marrow.

The ability of specific antagonists of the Cys-LT_1 receptor to decrease airway eosinophil number is well recognized. Six weeks of treatment with montelukast decreased sputum eosinophil number in patients with mildly uncontrolled asthma (14). Four to six weeks of

treatment with pranlukast (26), montelukast (27) and zafirlukast (28) decreased allergen-induced increase in airway eosinophil number. We observed a similar effect on eosinophil number and activation after two weeks of treatment with pranlukast and following an allergen inhalation. We hypothesized, for a number of reasons, that one of the mechanisms would be a direct effect on eosinophilopoeisis and egress of the progenitor cells from the bone marrow. First, allergen inhalation results in increased production of cysteinyl leukotrienes in the airway (29). Second, $CD34^+$ granulocytic precursors express Cys-LT_1 receptors on their surface (30). Third, we had previously demonstrated, in-vitro, an increase in IL-5 responsive eosinophil/basophil colony forming units when non-adherent mononuclear cells from bone marrow of atopic subjects were treated with leukotriene D_4 (21). Finally, leukotriene D_4 evoked calcium fluxes and actin polymerization in $CD34^+$ cells derived from bone marrow of normal subjects, and promoted chemotaxis towards it that was inhibited by a Cys-LT_1 receptor antagonist (31).

Consistent with our hypothesis, we observed two novel effects of the Cys-LT_1 receptor antagonist, pranlukast, on eosinophil progenitor cells. First, the increase in the number of IL-5 responsive colony forming units following an allergen inhalation was significantly decreased by two weeks of treatment with pranlukast, which did not seem to have an effect on the baseline number compared to the placebo treatment arm. The study did not investigate the mechanism of this effect. The effect of a leukotriene receptor antagonist may be direct or indirect. Since cysteinyl leukotrienes may be involved in mediating some of the biological effects of IL-5 (32), pranlukast may interfere with the ability of IL-5 to promote eosinophilopoiesis. This seems unlikely in this study because the non-adherent mononuclear cells from the pranlukast-treated subjects were grown in-vitro in the presence of optimal concentration of IL-5, unless pranlukast

is able to modulate IL-5 signal transduction pathways and make the cells unresponsive or less responsive to the effect of IL-5. There are two major signalling pathways of IL-5 in eosinophils. IL-5 activates Lyn, Syk and JAK2 and propagates signals through the Ras-MAP-kinase and JAK-STAT pathways (33). It is not known whether cysteinyl leukotrienes are involved in either of these pathways. Based on recent evidence that they may be upstream of STAT6 signalling in the IL-13 signalling mechanism (34), this is a likely possibility that needs further investigation. The effect of pranlukast on eosinophilopoiesis may also be indirect. Since IL-5 can upregulate Cys-LT_1 receptor expression on HL-60 cells differentiated into eosinophils (35), they may also increase the expression of Cys-LT_1 receptors on $CD34^+$ cells. Pranlukast may directly prevent IL-5 responsive eosinophil differentiation of the $CD34^+$ cells with increased Cys-LT_1 receptor expression.

The second novel observation in this study was that pranlukast attenuated the allergen-increased increase in the number of $CD34^+$ cells in the bone marrow expressing the eotaxin receptor, CCR3. We confirmed previous observation of CCR3 expression on $CD34^+$ cells (13); however, we did not examine the localization of CCR3 to $CD34^+$ cells that also express IL-5Rα. The $CD34^+$ cells expressing CCR3 show increased chemotaxis towards eotaxin (13) and this is augmented in the presence of IL-5. It is likely, therefore, that the allergen-induced increase in the levels of IL-5 and eotaxin in the airways of patients with dual asthma response causes the migration of pluripotent undifferentiated hemopoeitic cell from the bone marrow to the airway (36), where it can undergo local maturation into eosinophils. Our results suggest that cysteinyl leukotrienes are involved in the expression of CCR3 receptors on the progenitor cells. The mechanism was not investigated in this study. Since IL-5 is known to increase CCR3 expression

on leukemic cell lines (37), and since treatment with leukotriene receptor antagonists can decrease airway IL-5 levels in murine models of asthma (38,39), we postulate that the effect of pranlukast may be indirect by decreasing airway or perhaps bone marrow IL-5 levels. In deed, airway and bone marrow IL-5 levels increase following an allergen inhalation (40), and in the present study, pranlukast treatment caused nearly 50% attenuation in the number of sputum cells staining positive for IL-5, 7 hours after an allergen inhalation, compared to placebo. Two weeks of treatment with pranlukast (pre-allergen) also seemed to cause a trend towards increasing the number of $CD34^+$ cells and $CD34^+CCR3^+$ cells. Since the bone marrow contains a dynamic progenitor cell pool and since we did not do bone marrow aspiration before the start of treatment, it is difficult to interpret whether this increase represents an increase in bone marrow production or whether it is a reflection of the ability of pranlukast to prevent them from exiting the bone marrow. The latter seems to be the likely possibility.

The effect of pre-treatment with a leukotriene antagonist on allergen-induced bone marrow responses seem to be different from that of pre-treatment with an inhaled corticosteroid. Seven days of treatment with budesonide decreased IL-5 responsive eosinophil/basophil colony forming units in asthmatic subjects; however, unlike in this study, it did not prevent the allergen-induced increase in the numbers of the colony forming units (41). In other words, while an allergen inhalation was able to overcome the inhibitory effect of inhaled corticosteroid in the growth of IL-5 responsive bone marrow progenitor cells, a leukotriene antagonist seems to be able to prevent it. The most logical explanation is that cysteinyl leukotriene levels increase significantly following an allergen inhalation and may contribute to the stimulation of the bone marrow. It is also possible that allergen may increase the expression of $CysLT_1$ receptor on the

progenitor cells making them more responsive to a leukotriene antagonist. The lack of significant effect of two weeks treatment on pre-allergen Eo/B CFUs was surprising considering that the sputum eosinophil counts were decreased with two weeks of treatment. It is possible that the role of cysteinyl leukotrienes in eosinophilopoiesis is modest in patients with mild stable asthma and that other mechanism such as eosinophil chemoattraction and effect of cytokines are more pronounced. Although it is known that leukotrienes are produced in the bone marrow (42), it is not known whether their levels are increased following an allergen inhalation. We did not measure cysteinyl leukotrienes in the bone marrow in this study, but we plan to do it in future studies. Similar to the previous study (41), we did not find the total number of $CD34^+$ cells to change significantly with allergen inhalation or with treatment.

We also examined the effects of pranlukast treatment on three cytokines/chemokines that are relevant in causing airway eosinophil infiltration. Compared to placebo, pranlukast treatment attenuated the increase in sputum cells staining for IL-5, RANTES and eotaxin 7h after an allergen inhalation. The difference however was not statistically significant. One of the reasons is that this was not a primary outcome measure and the study was not powered to show this difference. Secondly, the variability in the total cell counts in sputum was high, similar to previous reports (1), increasing the noise to signal ratio. However, our observations are consistent with previous reports that have shown leukotriene receptor antagonists to decrease airway IL-5 (38,39,43) and RANTES (44) levels in murine models of allergic sensitization.

In summary, the cysteinyl leukotriene receptor antagonist, pranlukast, decreases allergen-induced increase in airway eosinophil by decreasing both eosinophil progenitor cells in the bone marrow and the levels of eosinophilopoietic and chemotactic cytokines in the airway. The

reduction in the numbers of IL-5 responsive eosinophil colony forming units and $CD34^+$ cells expressing CCR3 in the bone marrow suggest a role for cysteinyl leukotrienes in IL-5 signal transduction pathway.

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Legends for figures

Figure 1: Changes in sputum eosinophil count. Two weeks of placebo treatment did not have any effect on the eosinophil count (expressed as percentage of total cell count), while treatment with pranlukast decreased it significantly. The increase in eosinophil count at 7h and 24h after allergen inhalation was also significantly decreased by pranlukast treatment, but not by placebo. (*$p<0.05$)

Figure 2: Changes in eosinophil/basophil colony forming units (Eo/B CFU) in the bone marrow. Allergen inhalation after two weeks of treatment with placebo caused a significant increase in the number of CFU (per 2.5 x 10^5 non-adherent mononuclear cells in the bone marrow). This was completely attenuated by two weeks of treatment with pranlukast (*$p<0.05$). However, pranlukast treatment did not have an effect on the baseline number of CFU.

Legends for tables

Table 1: Baseline subject characteristics

Table 2: Changes in sputum cells immunostaining for cytokines. Immunocytochemistry on sputum cells were performed for EG2, eotaxin, IL-5 and RANTES at baseline (B), after two weeks of treatment before the allergen inhalation (pre) and 7h and 24h after allergen inhalation. Pre-treatment with pranlukast decreased the allergen-induced increase in positively staining cells compared to placebo. Statistical significance was observed only for EG2 staining. The numbers indicate mean and standard deviation.

Table 3: Changes in bone marrow progenitor cells. Cells expressing CD34 and CD34CCR3 on non-adherent mononuclear cells in the bone marrow were enumerated by flow cytometry. There was no effect of allergen or pranlukast on the total number of $CD34^+$ cells. The allergen-induced

increase in $CD34^{+}CCR3^{+}$ cells observed after placebo treatment was attenuated by pranlukast treatment (*$p<0.05$). The data is presented as mean and standard error of the mean.

Online data supplement

The effects of pranlukast on airway eosinophils and bone marrow eosinophil progenitors in asthmatic subjects

Krishnan Parameswaran, Richard Watson, Gail M. Gauvreau, Roma Sehmi, and Paul M. O'Byrne.

Methods

Subjects

The subjects were 16 atopic non-smokers with mild asthma (table 1) using short-acting beta-agonists infrequently. The subjects were eligible if they had a physician diagnosis of asthma and a 15% drop in FEV_1 induced by allergen inhalation. Subjects were studied out of their allergy season and had not had any respiratory tract infections for at least six weeks prior to the study. All medications, except the study medication, were withheld for at least 8h before each visit. All subjects gave written informed consent and the Research Ethics Committee of Hamilton Health Sciences Corporation approved the study.

Allergen inhalation

FEV_1 was measured using a Collins water-sealed spirometer (Warren E. Collins, Braintree, MA) and kymograph according to the ATS recommendations (22). Allergen inhalation was performed as previously described (2, 23). The allergen producing the largest-diameter skin wheal was diluted in 0.9% saline for inhalation. The concentration of allergen extract for inhalation was determined from a formula described by Cockcroft and colleagues (23). The starting concentration of allergen was chosen to be 3 doubling doses below that predicted to cause a 20% fall in FEV_1. The early bronchoconstrictor response was the largest percent fall in FEV_1 within the first 2 hours after allergen inhalation, and the late

bronchoconstrictor response was the largest fall in FEV_1 between 3 and 7h after the allergen inhalation.

Sputum cytochemistry

Sputum cytospins were prepared on Aptex (Sigma Chemical Co., Mississauga, ON) coated slides and fixed for 10 min in periodate-lysine-paraformaldehyde as described by Gauvreau et al. (1). Slides were stained with murine monoclonal antibodies to the activated form of human eosinophil cationic protein (ECP) at 1.0 μg/ml (EG2) (Kabi Pharmacia, Uppsala, Sweden); to IL-5 and RANTES at 30.0 μg/ml each; and to eotaxin at 100 μg/ml (R&D Systems, Minneapolis, MN). Positively immunoreactive cells were identifed by counting 400 cells under light microscopy and graded on a scale from zero to three based on the intensity of positive staining in comparison to the negative control. The number of cells staining more intensely than the controls were counted and expressed as the total number of immunopositive cells per gram of sputum.

Bone marrow progenitor culture

5 ml of bone marrow sample were aspirated into heparinized (1000 U/ml) syringes from the iliac crests after freezing the skin and periosteum with 2% lidocaine. Low-density mononuclear cells were isolated by sedimentation on Percoll density gradients (specific gravity 1.08) and cultured in the presence of IL-5 (10 ng/ml) as previously described (8-10). Cultures were incubated for 14 days at 37^0C and 5% CO_2 after which colonies were identified as Eo/B-CFU according to previously described criteria and expressed as Eo/B-CFU per $2.5x10^5$ NAMC plated.

Immunofluorescence staining

Non-adherent mononuclear cells were stained with saturating amounts of biotin-conjugated anti-IL-5Rα, and anti-CCR3, or the isotype-control antibody in 100 μl of ice-cold fluorescence-activated cell sorter staining buffer for 30 min at 4^0C (8,9,12,13). The washed cells were then stained with steptavidin-conjugated peridinin chlorophyll protein (PerCp) together with saturating concentration of FITC-CD45 IgG1 (anti-HLE1) and PE-CD34 IgG1 (HPCA-2) for 30 min at 4^0C as previously described (8,9,13).

Flow cytometry

The stained non-adherent mononuclear cells were analysed using a FACScan flow cytometer equipped with an argon laser (Becton Dickinson Instrument Systems, Mississauga, ON, Canada) using the CELLQUEST programme. Progenitor cells were identified based on their unique cell size, granularity and immunofluorescence characteristics. Off-line analysis was performed using the PC LYSIS software. True $CD34^+$ blast cells were identified as cells with $CD34^{high}/CD45^{dull}$ staining and low side scatter. Within the true $CD34^+$ population, specific staining of PerCp-linked cytokine/chemokine receptor mAbs or control antibody was detected, and the data were collected as numbers of cells at the 99% confidence limit (8,13).

Table 1

	Mean (min, max)
Age, yr	27 (19, 53)
FEV_1, L	3.3 (2.3, 4.6)
FEV_1, % predicted	91 (71, 105)
PC_{20}methacholine, mg/ml*	2.4 (0.1, 18.4)

* Geometric mean

Table 2

	Placebo				Pranlukast			
($x10^6$/g)	B	pre	7h	24h	B	pre	7h	24h
EG2+	0.04 (0.05)	0.08 (0.10)	0.51 (1.20)	0.24 (0.33)	0.11 (0.21)	0.02 (0.02)	0.10* (0.18)	0.16 (0.23)
IL5	2.16 (1.80)	1.77 (1.33)	4.16 (4.17)	2.87 (2.70)	2.49 (2.82)	1.14 (1.10)	1.71 (1.45)	2.13 (2.16)
Eotaxin	2.02 (1.98)	1.65 (1.21)	3.38 (3.61)	1.89 (1.73)	3.48 (2.47)	1.24 (1.23)	1.51 (1.30)	2.00 (3.05)
RANTES	0.64 (0.86)	0.50 (0.90)	0.85 (1.51)	0.68 (1.06)	0.65 (0.92)	0.89 (1.93)	0. 92 (1.89)	0.18 (0.30)

Table 3

Receptor (/2.5x10^5 NAMNC)	Placebo		Pranlukast	
	Pre-allergen	Post-allergen	Pre-allergen	Post-allergen
CD34	4557 (±2110)	4777 (±2214)	6568 (±5097)	6452 (±4712)
CCR3	63 (±42)	79 (±65)	93 (±66)	57 (±40) *

Figure 1

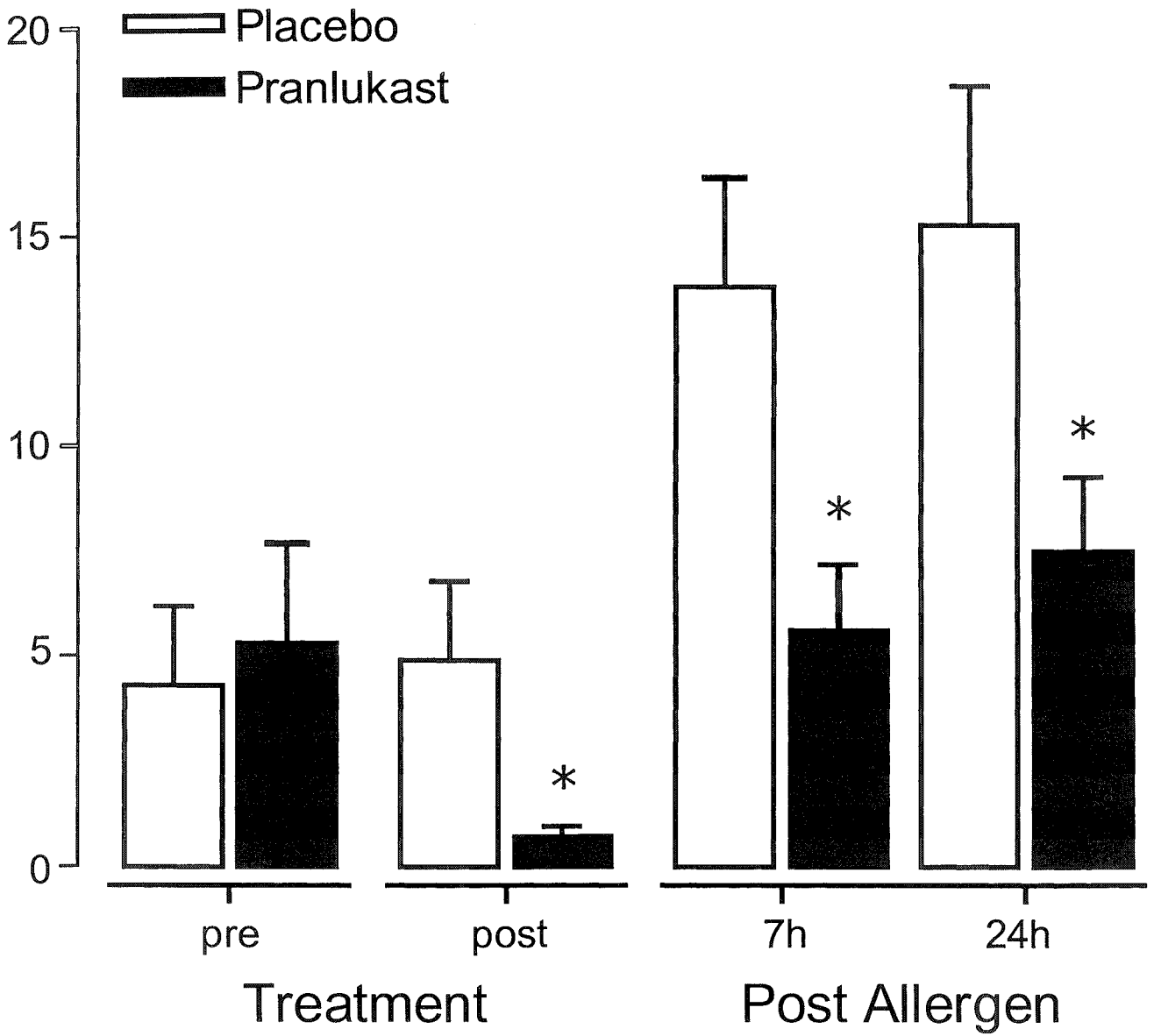
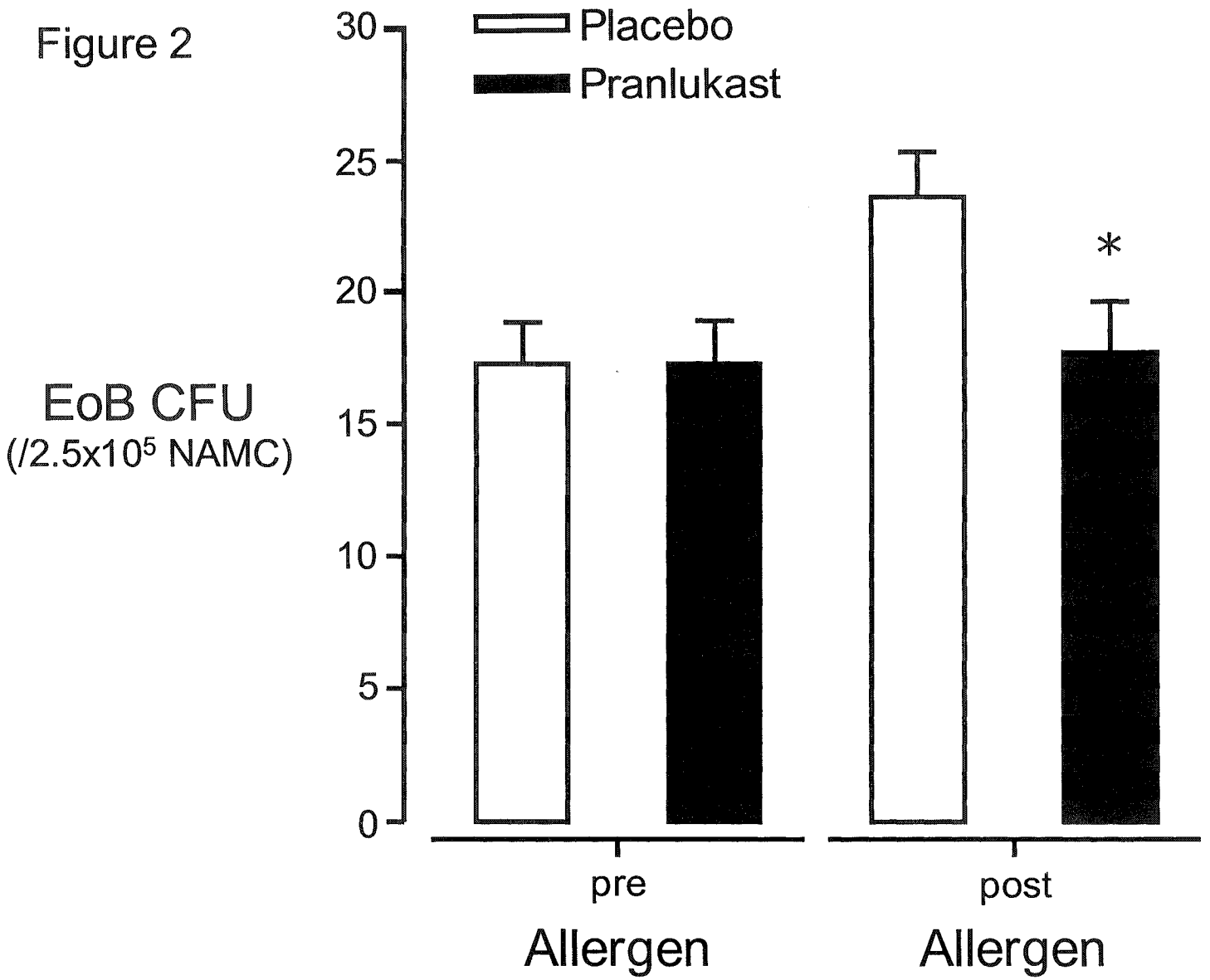

Placebo
Pranlukast
20
15
10
5
0
Sputum Eosinophils (%)
*
*
*
pre
post
7h
24h
Treatment
Post Allergen

Figure 2


Placebo
Pranlukast
30
25
20
15
10
5
0
EoB CFU
(/2.5x10^5 NAMC)
*
pre
Allergen
post
Allergen

CHAPTER 5:

CYSTEINYL LEUKOTRIENES PROMOTE HUMAN AIRWAY SMOOTH MUSCLE MIGRATION

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Dr. Parameswaran's contribution

Development of the concept
Study design and protocol development
Smooth muscle culture
Migration experiments
Analysis of data
Data interpretation, Preparation of manuscript

St. Joseph's Healthcare Hamilton

Firestone Institute for Respiratory Health

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Cysteinyl Leukotrienes Promote Human Airway Smooth Muscle Migration

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Cysteinyl leukotrienes promote airway smooth muscle (ASM) contraction and proliferation. Little is known about their role in ASM migration. We investigated this using cultured human ASMs (between the second and fifth passages) obtained from the large airways of resected nonasthmatic lung. Platelet-derived growth factor-BB (1 ng/ml) promoted significant (3.5-fold) ASM migration of myocytes across collagen-coated 8-μm polycarbonate membranes in Transwell culture plates. Leukotriene E_4 (10^{-7}, 10^{-8}, 10^{-9} M) did not demonstrate a chemotactic effect; it did promote chemokinesis. Priming by leukotriene E_4 (10^{-7} M) significantly augmented the directional migratory response to platelet-derived growth factor (1.5-fold, $p < 0.05$). This was blocked by montelukast (10^{-6} M), demonstrating the effect to be mediated by the cysteinyl leukotriene receptor. The "priming effect" was also partially attenuated by prostaglandin E_2 (10^{-7} M). Whereas both the chemokinetic and the chemotactic "primed" responses were equally attenuated by a p38 mitogen-activated protein kinase inhibitor (SB203580, 25 μM) and by a Rho-kinase inhibitor (Y27632, 10 μM), the chemotactic response showed greater inhibition than chemokinesis by a phosphatidylinositol-3 kinase inhibitor (LY294002, 50 μM). These experiments suggest that cysteinyl leukotrienes play an augmentary role in human ASM migration. The phosphatidylinositol-3 kinase pathway is a key signaling mechanism in the chemotactic migration of ASM cells in response to cysteinyl leukotrienes.

Keywords: cysteinyl leukotrienes; airway smooth muscle migration; chemotaxis; montelukast; phosphatidylinositol-3 kinase

The inflammatory process that characterizes the pathophysiology of asthma involves a number of cells and mediators. The cysteinyl leukotrienes (Cys-LTs) C_4, D_4, and E_4 have a number of inflammatory effects relevant to the pathophysiology of asthma (1). Inhalation of leukotriene (LT) E_4 causes eosinophil influx into the airway wall (2). *In vitro*, both LTD_4 and LTE_4 by themselves are weak chemoattractants for eosinophils, but they augment the chemoattractant effects of other factors such as eotaxin (3). The effects of Cys-LTs on the migrational response of other inflammatory cells, in particular airway smooth muscle (ASM), are less well known. Over the past 10 years, the role of ASM in asthma has been recognized to extend beyond bronchoconstriction (4). They participate in the inflammatory and remodeling processes by producing an array of cytokines, chemokines, and matrix proteins (5). An increase in the size and number of ASMs has been reported in asthma (6). Cys-LTs facilitate this process by promoting ASM proliferation (7). Electron microscopic studies of biopsies obtained during the late response to allergen challenge indicate that muscle cells may dedifferentiate in response to allergen to form motile, contractile cells with a myofibroblast phenotype (8). It is likely that these cells migrate away from the original blocks of deep ASMs to sites close to the disrupted reticular basement membrane, similar to the process of ASM migration and remodeling described in atheromatous plaques and postangioplasty stenosis (9). The migrational property of ASM cells in response to platelet-derived growth factor (PDGF) has recently been demonstrated (10). No information is available on whether Cys-LTs promote ASM migration.

We hypothesized that Cys-LTs are also chemoattractants for ASM and promote the migration of ASM. This effect is likely to be mediated through the G-protein–coupled Cys-LT1 receptor. The objectives of this study were to investigate whether human ASM cells migrate in response to Cys-LTs and to investigate the signaling mechanism of this migratory response.

METHODS

Reagents

Roswell Park Memorial Institute (RPMI)-1640 culture medium, fetal calf serum, bovine serum albumin, and PDGF-BB were purchased from Invitrogen Canada Inc. (Burlington, ON, Canada). Vitrogen collagen solution (3.2 mg/ml) was purchased from Cohesion Technologies Inc. (Palo Alto, CA) and was diluted to 1.5 mg/ml with sterile deionized water. LTE_4 and prostaglandin E_2 (PGE_2) were purchased from Cayman Chemicals (Ann Arbor, MI). Inhibitors of p38 mitogen-activated protein (MAP) kinase (SB203580), Rho-kinase (Y27632), and phosphatidylinositol-3 (PI-3) kinase (LY294002) were purchased from Sigma (Oakville, ON, Canada). Diff-Quik Wright Giemsa solution was purchased from VWR International (Mississauga, ON, Canada). Montelukast was a generous gift from Merck Frosst Canada (Kirkland, PQ, Canada).

Smooth Muscle Culture

Portions of human lungs that were resected at St. Joseph's Healthcare (Hamilton, ON, Canada) were obtained with the cooperation of the Division of Thoracic Surgery, after obtaining approval from the institutional review board. Smooth muscle tissue was isolated from macroscopically disease-free areas of human bronchi. ASM cells were grown to confluence, as described before (11), in RPMI medium containing 10% fetal calf serum and penicillin-streptomycin (100 U/ml–100 μg/ml). The confluent cell growth exhibited the typical "hill and valley" appearance under light microscopy and also had caveolae and gap junctions by electron microscopy (data not shown). The cells were passaged between two to five times and were used for the migration assay.

Smooth Muscle Migration Assay

Migration experiments were performed using a 6.5-mm Transwell culture plate with an 8.0-μM pore polycarbonate membrane separating the inner and the outer chambers (Fisher Scientific Limited, Nepean, ON, Canada). Both chambers of the culture plate were treated overnight (at 4°C) with Type 1 collagen solution, which was then aspirated, and the chambers dried for 4 hours in a laminar flow hood. The inserts were then washed thoroughly with sterile deionized water before using them for the experiments. Bovine serum albumin-RPMI medium was added to both chambers 30 minutes before treatments and was aspirated

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87

immediately before the experiment. Confluent cells were maintained in growth-factor free medium for 24 hours before the experiments. The cells were then harvested with trypsin (0.05% and 0.53 mM ethylenediaminetetraacetic acid), counted, centrifuged (1,000 r.p.m. for 10 minutes), and resuspended at 8.0×10^5 cell/ml in 0.3% bovine serum albumin-RPMI medium. The cells (vol 100 μl) were then plated on the upper side the membrane. The chemoattractants (vol 600 μl) were added to the lower wells. After 5 hours of incubation at 37°C, the membranes were peeled off, and the cells on the upper face of the membranes were scraped using a cotton swab. Cells that migrated to the lower face of the membrane were fixed with 3.7% formaldehyde and were stained with Diff-Quik. The number of migrated cells on the lower face of the filter was counted in four random fields under ×20 magnification (microscope, Olympus BX40; camera, Sony 3CCD Power HAD video camera, Japan; software, Northern Eclipse, Empix Imaging, Mississauga, ON, Canada). Assays were done in duplicate using tissues from six to eight different lung specimens except for the experiment investigating the effect of PGE_2, where only four different specimens were used.

Migration Experiments

The migratory response to different doses of PDGF-BB (0.1, 1, and 10 ng/ml) was initially studied by adding PDGF to the lower wells to identify the optimal dose to be used as a positive control. Based on our previous studies, which demonstrated a greater effect of inhaled LTE_4 compared with LTD_4 on human bronchial mucosal eosinophil infiltration (2), all of the LT experiments were performed with LTE_4. Chemokinesis (nondirectional or random migration) to LTE_4 (10^{-7}, 10^{-8}, 10^{-9} M) was studied by adding it to both the upper and lower wells. Chemotaxis (directional migration) was studied by adding the doses only to the lower wells. To study whether LTE_4 could augment or "prime" the migratory response to PDGF, the smooth muscle cells were incubated with LTE_4 (10^{-7} M) for 30 minutes before the migration assay. To investigate whether the priming effect of LTE_4 was mediated through the Cys-LT receptor, montelukast, a specific Cys-LT receptor antagonist (10^{-6}, 10^{-8}, 10^{-10} M), was added to the upper well along with the smooth muscle cells and LTE_4 during the "priming" experiments. Similarly, the effect of PGE_2 on the chemotactic response to LTE_4 and PDGF was studied by adding it (10^{-7} M) to the upper well along with the smooth muscle cells and LTE_4. All the chemicals were diluted in 0.3% bovine serum albumin-RPMI buffer. LTE_4 and PGE_2 were extracted from solutions of methanol using compressed nitrogen gas. The RPMI-bovine serum albumin buffer solution was used as the control for all of the assays.

Signal Transduction Pathways

The influence of the p38 MAP kinase pathway, the Rho-kinase pathway, and the PI-3 kinase pathway on smooth muscle migration was studied using selective pharmacologic inhibitors to the kinases. The p38 MAP kinase inhibitor (SB203580, 25 μM), the Rho-kinase inhibitor (Y27632, 10 μM), and the PI-3 kinase inhibitor (LY294002, 50 μM) were added to the upper wells along with the smooth muscle cells with and without LTE_4 (depending on whether the experiments were to study chemokinesis or chemotaxis) 30 minutes before the migration assay and remained in contact with the cells for the entire 5 hours of the migration assay.

Statistical Analysis

Data were summarized using means and SDs. The means of migrated smooth muscle cells were compared for each of the experiments by factorial analysis of variance statistics using the Statistical Package for Social Sciences (SPSS for Windows, version 10.0, Chicago, IL). The source of significant variation was identified by Student–Newman–Keuls test. Statistical significance was accepted for p values of less than 0.05.

RESULTS

ASM cells showed significant migratory response to 1 and 10 ng/ml doses of PDGF-BB (Figure 1). A 1-ng/ml dose (an approximate 3.5-fold response compared with the negative control) was selected as the optimal dose for all further experiments. In comparison, ASM cells did not show any chemotactic response to any of the doses of LTE_4. However, they showed a dose-dependent weak chemokinetic response to LTE_4 (Figure 2).

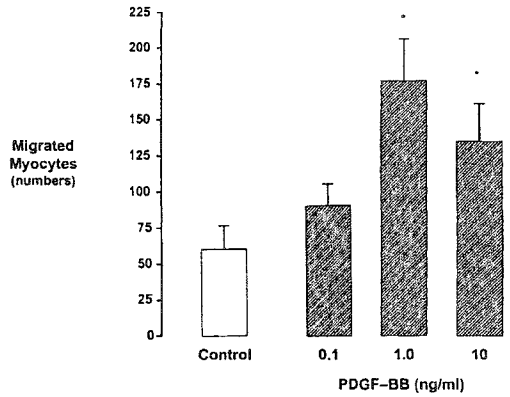

Figure 1. Dose-dependent migratory response of cultured human ASM cells to PDGF-BB. *$p < 0.05$ compared with control.

LTE_4 augmented the directional migratory response of ASM cells to PDGF by approximately 1.5-fold. This was almost completely inhibited by montelukast 10^{-6} M, but not by doses of 10^{-8} and 10^{-10} M (Figure 3). PGE_2 (10^{-7} M) also attenuated the migratory response to PDGF alone ($p < 0.05$), and the "LTE_4-primed" response to PDGF ($p < 0.05$). The magnitude of inhibitory effect was greater on the primed response (Figure 4).

All of the three kinase inhibitors significantly attenuated the directional migratory response to PDGF, without any significant differences between their effects (Figure 5). In contrast, the PI-3 kinase inhibitor caused a greater inhibitory response on the LTE_4-primed directional migration than the inhibitory effects of the MAP kinase inhibitor and the Rho-kinase inhibitor. The PI-3 kinase inhibitor had little effect on the chemokinetic response to LTE_4, which was significantly attenuated by the MAP kinase inhibitor and the Rho-kinase inhibitor.

DISCUSSION

The results of this study show that LTE_4 does not promote directional migration of nonasthmatic human ASM cells, but

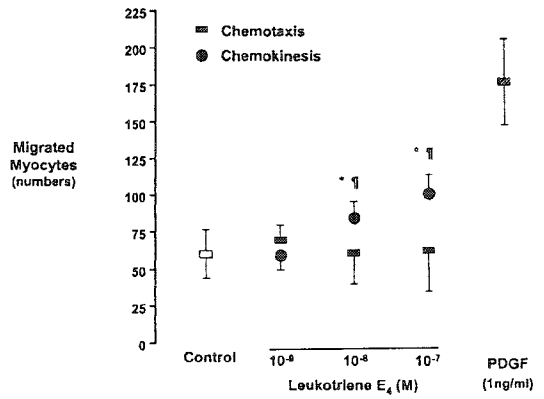

Figure 2. Chemotactic and chemokinetic responses of cultured human ASM cells to LTE_4. LTE_4 caused significant chemokinesis, but not chemotaxis. *$p < 0.05$ compared with control; ¶$p < 0.05$ compared with corresponding chemotactic response.

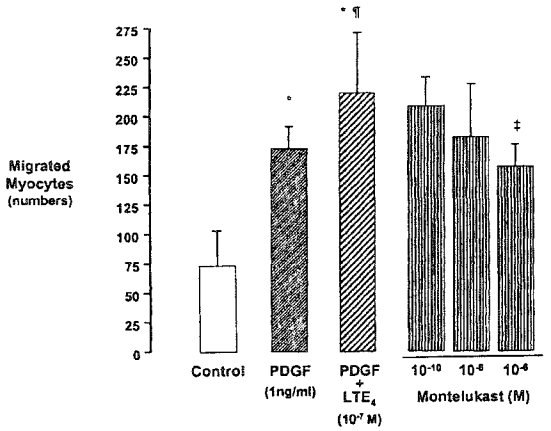

Figure 3. Priming effect of LTE_4 on PDGF-induced migratory response. The augmented response of LTE_4 pretreated smooth muscle cells to PDGF was inhibited by montelukast. *$p < 0.05$ compared with control; ¶$p < 0.05$ compared with unprimed response to PDGF; ‡$p < 0.05$ compared with a primed response to PDGF.

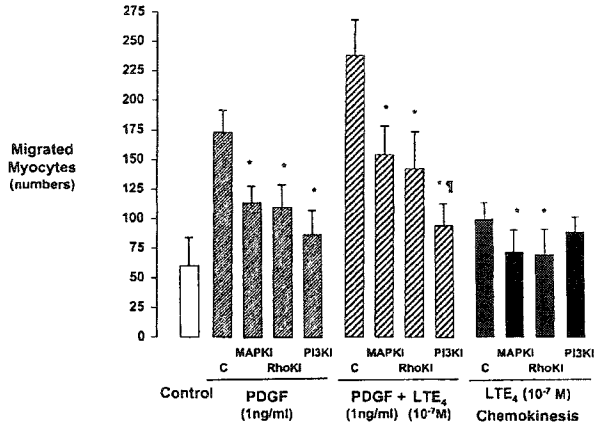

Figure 5. The effect of protein kinases involved in the signal transduction pathways of human ASM chemotaxis and chemokinesis to PDGF and LTE_4. Inhibitors of MAP kinase (MAPKI), Rho-kinase (RhoKI), and the phosphatidylinositol-3 kinase (PI3KI) attenuated PDGF-induced migration of primed and unprimed smooth muscle cells. PI3KI did not attenuate the chemokinetic response to LTE_4, whereas MAPKI and RhoKI did. *$p < 0.05$ compared with the respective control experiments; ¶$p < 0.05$ compared with the inhibitory effect of MAPKI and RhoKI.

it causes nondirectional movement or chemokinesis. However, LTE_4 augments the migratory response to a growth factor, an effect that is mediated through the Cys-LT1 receptor. The PI-3 kinase signaling pathway plays an important role in this chemotactic response, which may be relevant in the remodeling process in airways that are asthmatic.

The mitogenic and contractile response of cultured human ASM cells has been studied more than their migratory response. This study demonstrated the ability of the human ASM cell to migrate in response to a chemoattractant gradient, as has been previously shown with nonhuman (10) and non-ASM cells (12). The methods employed to culture the cells were similar to previously published reports (13). It is possible that the cells may have included some fibroblasts and myofibroblasts and that the culture may have represented a composite of "airway structural cells." The clinical or biologic relevance of this, however, is not known. The study confirmed previous observations that PDGF is a chemoattractant for tracheal smooth muscle cells of dogs (10); however, the magnitude of response was less than previously reported. This may represent differences in the species studied or study conditions.

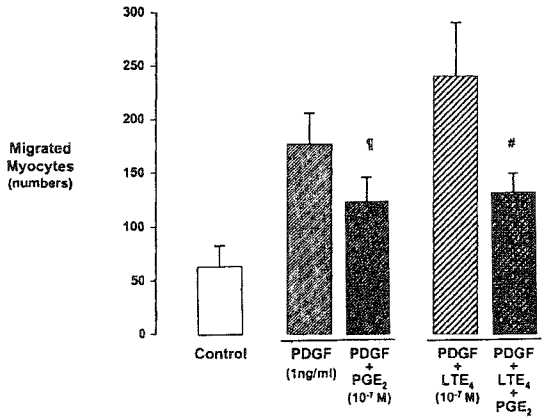

Figure 4. The effect of PGE_2 on human ASM migration. PGE_2 attenuated PDGF, and LTE_4-induced ASM migration. ¶$p < 0.05$ compared with PDGF-induced migration; #$p < 0.05$ compared with the LTE_4-primed migratory response to PDGF.

Cys-LTs have various biologic effects on smooth muscles mediated through the G-protein–coupled Cys-LT1 receptor (14) and perhaps through an uncharacterized receptor (7). Although LTD_4 and LTE_4 are believed to act through the same Cys-LT receptor, we observed greater eosinophil cell infiltration into airway mucosa after LTE_4 inhalation than after LTD_4 inhalation in mildly atopic patients with asthma (2). Therefore, in this series of experiments, we employed LTE_4 to study the migratory response of smooth muscle cells. The Cys-LT1 receptor-mediated signal transduction is mostly coupled through the $G\alpha_i$ (inhibition of cAMP) and the $G\alpha_q$ (phospholipase C, protein kinase C, and calcium-mediated) pathways (15) to cause smooth muscle contraction (16). LTD_4 can cause actin cytoskeleton reorganization, a critical component of smooth muscle adhesion, spreading, and movement by coupling with a pertussin toxin–sensitive G protein, Rho-guanosine triphosphatases, and tyrosine phosphorylation pathways (17). In keeping with this observation, we observed that LTE_4 causes a significant chemokinetic response in ASM cells (Figure 2), which was attenuated by inhibitors of the Rhogr-kinase and the p38 MAP kinase pathways. The PI-3 kinase pathway (17), unlike the Rho-kinase pathway (18), was not critically important in this nondirectional movement, although LTD_4 has been observed to induce a translocation of PI-3 kinase to a membrane fraction in human intestinal epithelial cells (19). However, the Class 1b isoform of PI-3 kinase γ, which is activated by the $\beta\gamma$ subunits of heterotrimeric G proteins, has not been demonstrated in ASMs (20).

Unlike the significant chemokinetic response, ASM did not show a chemotactic response to LTE_4. Directed movement along a concentration gradient of chemical attractants is a complex event mediated by chemoattractant receptors belonging to the seven-transmembrane helix receptor family (21). After binding to their ligands, these receptors transmit their signals to heterotrimeric G proteins, which then dissociate into α and $\beta\gamma$ subunits. The latter bind and activate target enzymes such as phospholi-

89

pase C, PI-3 kinase, and adenylyl cyclase. The PI-3 kinase is critically involved in maintaining a balance of migration-promoting and migration-suppressing activities of Rho-guanosine triphosphate (GTP)-ase activating protein (GAP), particularly to PDGF, to drive chemotaxis (22, 23). Indeed, we observed that the "LT-primed" chemotactic response to PDGF showed greater inhibition by the PI-3 kinase inhibitor than the inhibitors of the p38 MAP kinase pathway and the Rho-kinase pathway. LY294002 is a competitive inhibitor of the ATP site of PI-3 kinase (24). In most biologic systems, evidence for an involvement of PI-3 kinase is obtained by treating cells with 5 to 20 μM of LY294002 (25). The dose that we used (50 μM) may have been high enough to cause some nonspecific effects but has been previously reported to inhibit PI-3 kinase–mediated human smooth muscle migration (26) and proliferation (27). We would have expected to see a similar response on PDGF-induced cell migration, which is also predominantly chemotactic and regulated by PI-3 kinase (28). Although the PI-3 kinase inhibitor caused a greater inhibition on PDGF-induced cell migration than the MAP kinase and the Rho-kinase inhibitors, this was not statistically significant. Perhaps the migration of human smooth muscle cells (29) and their signal transduction pathways is different from that in Chinese hamster ovary 602 cell lines (21) and rat ELT3 cell lines (23).

Similar to the chemotactic effect on peripheral blood eosinophils (3) and the proliferative effects on cultured ASMs (7), we observed that LTE_4 itself, although not having a chemotactic effect on ASM cells, augmented the chemotactic effects of a growth factor, that is, PDGF. This effect was mediated through the Cys-LT receptor as it was abolished by montelukast, a specific Cys-LT receptor antagonist. However, we do not know the precise signal transduction pathway linking the G-protein–coupled receptor pathway and the receptor tyrosine kinase pathway. Because the priming effect was attenuated by the Rho-kinase, p38 MAP kinase, and the PI-3 kinase inhibitors, the interaction is likely to be upstream of these enzymes. Moreover, given that activated Gαq (through phospholipase C and protein kinase C) and activated Gβγ and receptor tyrosine kinases (through PI-3 kinase γ, Src-family kinases signaling to Shc homology proteins) can stimulate Ras and in turn activate the MAP kinase pathway (30), it is possible that the interaction may be at the level of Ras or Src kinases. Indeed Src-family kinases are involved in migration of cultured vascular smooth muscle cells (12), and they regulate PI-3 kinase and protein kinase B activation to modulate neutrophil chemotaxis (31). It is unlikely that Cys-LTs directly stimulate PI-3 kinase in human ASM to augment PDGF-stimulated chemotaxis, but rather via activation of Src kinases. Alternatively, the priming effect may simply reflect the increased motility of cells triggered by LT-induced cytoskeletal reorganization. This needs further investigation.

The inhibitory effect of PGE_2 on growth factor–induced ASM cell migration is probably similar to the E-prostanoid 1 and 2 (EP1 and EP2) receptor–coupled, cAMP-dependent, protein kinase A–mediated, inhibitory effect on fibroblast chemotaxis (32) and human ASM relaxation (33). The inhibitory effect on LTE_4-primed chemotactic response may represent an interaction between the prostanoid (EP) receptor signaling cascade and the Cys-LT1 receptor signaling cascades, by an inhibitory effect on the Rho-kinase pathway. This needs further investigation. PGE_2 may provide an endogenous mechanism to check the uninhibited facilitatory effects of Cys-LTs in promoting airway inflammation and airway hyperresponsiveness in patients with asthma. Indeed, inhaled PGE_2 has been shown to mitigate human ASM proliferation (34) and allergen-induced airway responses and airway inflammation (35).

ASM plays an important role in the process of airway remodeling in patients with asthma (5). Cys-LTs, albeit causing a modest inflammatory effect when inhaled, play important roles in promoting airway remodeling in murine models of asthma (36). They may do so by augmenting the proinflammatory and mitogenic effects of other cytokines and chemokines relevant to the pathophysiology of asthma. Pretreatment with LT receptor antagonists attenuates an allergen-induced increase in airway hyperresponsiveness (37). This experiment, despite the relatively small magnitude of the migratory response, provides another potential mechanism for how Cys-LTs may contribute to the accumulation of smooth muscle in the airway and airway remodeling. It needs to be proven whether the migratory responses observed in this study are enhanced in smooth muscle from patients with asthma. Drawing on the analogy of the migration of vascular smooth muscles and the inhibitory effects of statins in preventing vascular remodeling in atherosclerosis (38), prevention of ASM migration provides an attractive therapeutic intervention for preventing airway remodeling in asthma.

In conclusion, this study provides evidence that ASM cells show a chemotactic response to PDGF. LTE_4, although it is not directly a chemoattractant, augments this response, which is inhibited by montelukast. The PI-3 kinase pathway is a key signaling mechanism of ASM chemotaxis.

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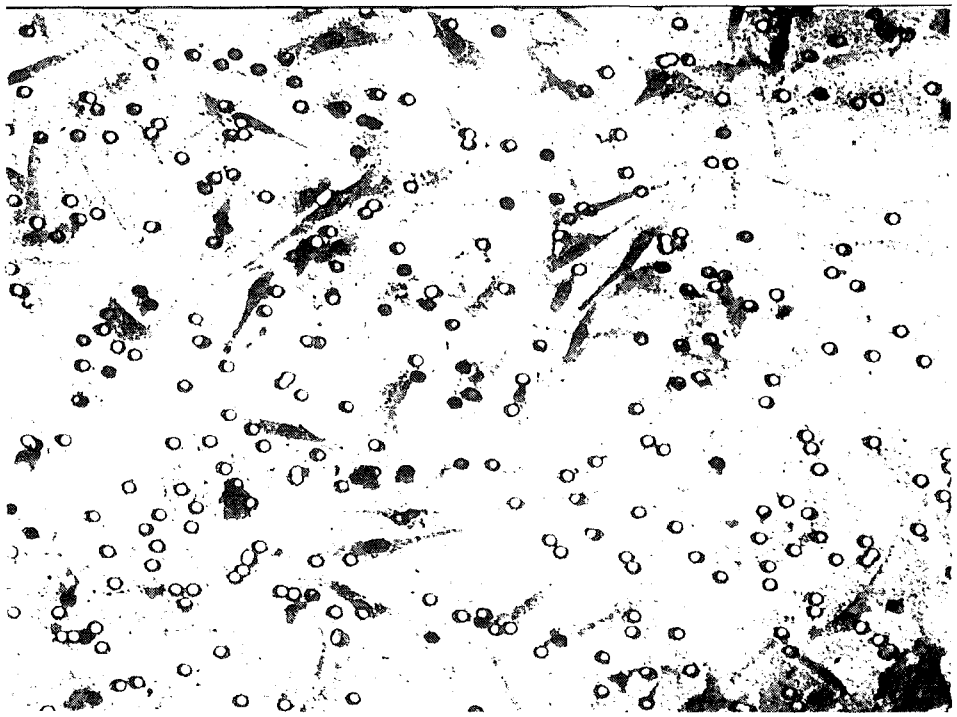
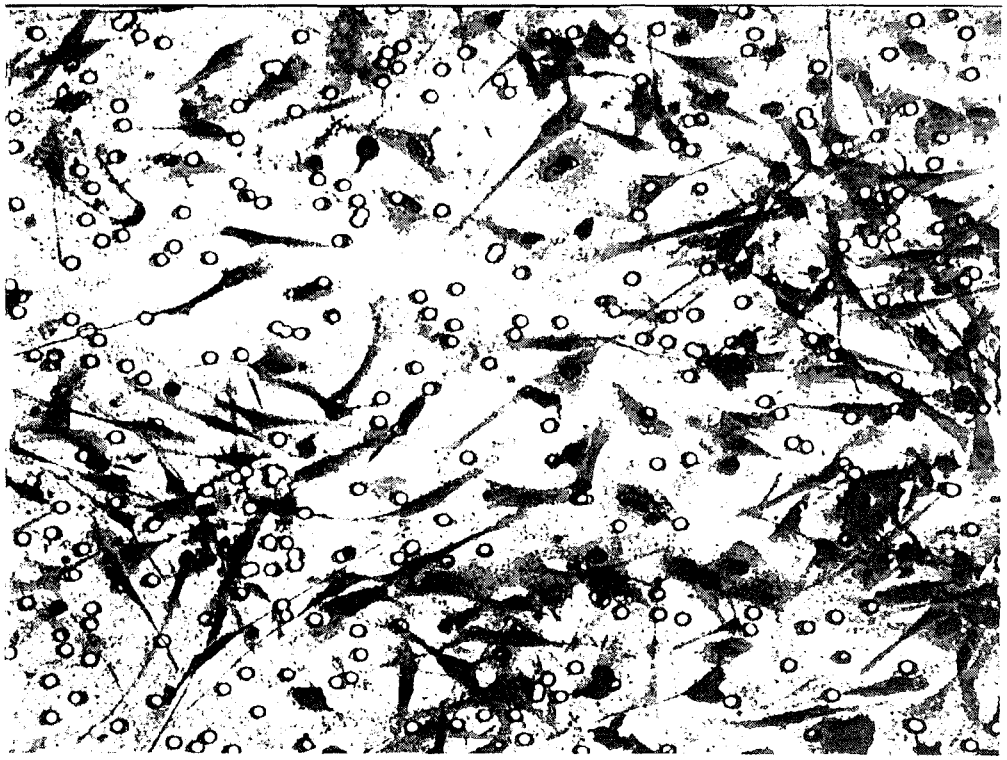


Cells migrated towards control (0.3% BSA in RPMI)



Cells migrated towards PDGF (1ng/ml) in 5 hours. The collagen-I coated polycarbonate membranes were stained with DiffQuik stain.

CHAPTER 6:

EXTRA-CELLULAR MATRIX REGULATES HUMAN AIRWAY SMOOTH MUSCLE CELL MIGRATION

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Dr. Parameswaran's contribution

Development of protocol
Smooth muscle cell culture
Coating of plates
Migration experiments
Western blot
Flow cytometry
Analysis of data
Data interpretation, preparation of manuscript

Extra-cellular matrix regulates human airway smooth muscle cell migration

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Running title: ECM and human airway smooth muscle migration
Key words: extra-cellular matrix, integrins, Src-kinase, cysteinyl leukotrienes, human airway smooth muscle, chemotaxis

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Abstract

Extra-cellular matrix proteins regulate survival and proliferation of smooth muscle cells. Their effect on airway smooth muscle cell migration is not known.

We investigated their role in leukotriene-primed (leukotriene E_4, 0.1 μM) chemotaxis of cultured human airway smooth muscle cells towards platelet-derived growth factor BB (1 ng/ml).

Migration of cells was greater on membranes coated with collagen III, V and fibronectin compared to collagen I, elastin and laminin (all at 10 μg/ml) ($p<0.05$). A concentration-dependent promotion of migration was observed on collagen I (1000>10 μg/ml), which was associated with increased phosphorylation of Src-kinase. This was not observed on laminin or elastin. The role of Src-kinase was further confirmed by demonstrating that its inhibitor, PP1-analog (1 μM) inhibited chemotaxis. Collagen I itself was not a chemoattractant; however, haptokinesis was observed when cells were primed with leukotriene E_4, and haptotaxis was observed when cells were primed with platelet-derived growth factor. The priming effect of leukotrienes on chemotaxis was not by promoting adhesion, increasing the surface expression of β_1, α_v and α_5 intergrins or Src-kinase phosphorylation.

These experiments demonstrate that extra-cellular matrix, along with growth factors and cysteinyl leukotrienes, can regulate human airway smooth muscle migration. This may be relevant in the remodelling process in chronic airway diseases such as asthma.

Word count of abstract: 208

Introduction

Smooth muscles and extra-cellular matrix proteins are important components of the lamina reticularis in the remodelled airway mucosa of patients with asthma (1). It is generally believed that the inflammatory cells such as eosinophils, mast cells, macrophages and lymphocytes, which infiltrate the airway mucosa, initiate the remodelling process. They interact with the resident structural cells and lay down fibronectin, elastin, laminin and other proteins that constitute the extra-cellular matrix (2,3). However, smooth muscle cells, which are seen in great numbers and size immediately beneath the epithelium in asthma, are not normally resident in this area, but in the area below the sub-mucosa (4). Little is known about the processes that regulate the accumulation of airway smooth muscles in the area of the airway immediately beneath the mucosal epithelial cells. These cells, like other inflammatory cells, are capable of directional migration (5,6) and have been demonstrated to migrate close to the submucosa in the airways of asthmatic subjects following allergen inhalation (7,8).

Since extra-cellular matrix proteins are able to regulate the survival (9), proliferation (10) and cyto-skeletal reorganization (9) of airway smooth muscle cells, and the migration of vascular smooth muscle cells (11), we investigated their role in the regulation of human airway smooth muscle cell adhesion and migration. We hypothesized that the matrix proteins that are deposited in greater quantities in asthmatic ariways, ie collagen I, III, V, fibronectin and laminin would support migration more than matrix proteins such as elastin and collagen IV which are less conspicuous in asthmatic airways. Since we had previously demonstrated that cysteinyl leukotrienes augment the chemotaxis of human airway smooth muscle cells (5), we also investigated whether they modulate the effect of extra-cellular matrix proteins. The precise signal

transduction mechanisms involved in the chemotaxis of airway smooth muscle cells are not clearly known (12). Adhesion to a matrix surface via integrins is an essential first step. The Rho-Rhokinase pathways modulate reorganization of actin cytoskeleton. Directional migration is likely to involve the phosphatidylinositol-3 kinase (PI3K) and the mitogen-activated protein kinase pathways (MAPK). Since integrins activate Src-kinase (SrcK) through the FAK complex, which in turn can activate the PI3K and MAPK pathways (13), we hypothesized that the matrix proteins may facilitate chemotaxis of smooth muscle cells by activation of SrcK.

Therefore, the specific questions that we asked were 1) do the different matrix proteins support migration differently? 2) is this effect concentration-dependent? 3) are matrix proteins chemoattractants? 4) do leukotrienes promote adhesion just as they augment migration? 5) are integrins, PI3K and SrcK involved in adhesion and migration?

Methods

Reagents

Roswell Park Memorial Institute (RPMI)-1640 culture medium, fetal calf serum (FCS), bovine serum albumin (BSA), trypsin, phosphate buffered saline (PBS), penicillin/streptomycin and PDGF-BB were purchased from Invitrogen Canada Inc. (Burlington, ON, Canada). Human collagen I, III, V, fibronectin, and laminin were purchased from Calbiochem (San Diego, CA). Human elastin was purchased from Elastin Products Inc. (Owensville, MO). Rabbit polyclonal Anti-phospho-Src (pY^{418}) antibody was obtained from BioSource International (Camarillo, CA). Monoclonal antibodies against integrins (β_1, α_v and α_5) were purchased from Beckman Coulter Canada Inc (Mississauga, ON, Canada). The inhibitor of PI3K (LY294002) was obtained from Sigma (Oakville, ON, Canada) and the inhibitor of SrcK, 4-amino-1-tert-butyl-3-(1-naphthyl)

pyrazolo[3,4-d] pyrimidine (PP1-Analog) was obtained from Calbiochem (San Diego, CA). Electrophoresis reagents were obtained from Bio-Rad (Mississauga, ON, Canada) and chemiluminescence reagents from Amersham Canada (Oakville, ON, Canada). Leukotriene E_4 was purchased from Cayman Chemicals (Ann Arbor, MI). Recombinant human tumour necrosis factor-α was purchased from PeproTech Canada Inc (Ottawa, ON, Canada). Diff-Quik Wright Giemsa Solution was purchased from VWR International (Mississauga, ON, Canada).

Smooth muscle culture

Human lungs that were resected at St. Joseph's Healthcare, Hamilton were obtained after obtaining approval from the Institutional Review Board and the consent of the patients undergoing the resection. Smooth muscle tissue was isolated from disease-free areas of bronchi. ASM cells were grown to confluence in RPMI culture medium containing 10% FCS and penicillin-streptomycin (100 unit/ml-100μg/ml). The confluent cell growth exhibited the typical 'hill and valley' appearance under light microscopy, and also had caveolae and gap junctions by electron microscopy (data not shown). The cells were passaged between 2-5 times.

Migration assay

Confluent cells were maintained in growth-factor free medium for 24 hours prior to the experiments. The cells were then harvested with trypsin (0.05%), counted, centrifuged and re-suspended at 8.0 x 10^5 cell/ml in 0.3% BSA-RPMI medium. The cells (vol 100μl) were then plated on the upper side of a matrix-treated, polycarbonate membrane (8.0μM pore) separating two chambers of a 6.5-mm Transwell culture plate (BSA-RPMI medium was added to both chambers 30 min before treatments). The chemoattractants (vol 600μl) were added to the lower wells. After 5 hours, the membranes were peeled off and the cells on the upper face of the

membranes were scraped using a cotton swab. Cells that migrated to the lower face of the membrane were fixed with 3.7% formaldehyde and stained with Diff-Quik. The number of migrated cells on the lower face of the filter was counted in four random fields under 20x magnification (microscope: Olympus BX40; camera: Sony 3CCD Power HAD video camera, Japan; software: Northern Eclipse, Empix Imaging, Mississauga, ON, Canada). Assays were done in duplicate using cells from six different resected lung tissues.

Chemotaxis/chemokinesis

Chemotactic response of airway smooth muscle cells were studied using PDGF-BB (1 ng/ml) (based on previous experiments) (5) or collagen I (1000μg/ml) (haptotaxis) as chemoattractants in the lower well. Haptokinesis to collagen I was studied by adding 1000μg/ml of collagen to both the inner and outer wells. The regulatory effect of matrix proteins was studied by coating the polycarbonate membrane of the Transwell insert with 10 μg/ml of the various matrix proteins. The membrane was treated overnight with the matrix solutions, which were then aspirated, and the chambers dried for 4 hours in a laminar flow hood. The inserts were washed thoroughly with deionized water before using them for the experiments. In order to study whether the regulatory effect of the matrix proteins was concentration-dependent, the membranes coated with 10, 100 and 1000 μg/ml of collagen I, elastin and laminin solution. The 'priming effect' of leukotriene E_4 (LTE_4, 10^{-7}M) or PDGF (1 ng/ml) were studied by adding them to the smooth muscle cells 30 min before the haptotaxis experiment towards collagen I.

Adhesion experiments

Smooth muscle cells were harvested from the 75-cm^2 plates, washed in PBS and transferred in RPMI-10%FCS into 24-well plates pre-coated with collagen I (in concentrations of 0.1, 1, 10, 100 and 1000 μg/ml) at a seeding density of 20,000 cells/well. At 30, 60 and 120 min,

the plates were removed from the incubator and the overlying medium removed by gentle aspiration. After washing in 0.5ml PBS, the remaining adherent cells were removed by trypsin/EDTA and counted using a hemocytometer. The 'priming effect' of leukotriene was studied by adding LTE_4 (0.1 μM) to the smooth muscle cells 30 min before the experiment.

Signal transduction pathways

We studied the integrin-mediated signalling of airway smooth muscle adhesion and migration by using specific pharmacological antagonists to PI3K and SrcK. The PI3K inhibitor (LY294002, 50μM) and the SrcK inhibitor (PP1, 0.1, 1.0, 10, 30 μM) were added to the smooth muscles cells 30 min prior to the adhesion and migration assays and remained in contact with the cells for the entire duration of the study. In order to study the effect of integrins on airway smooth muscle adhesion to matrix, blocking antibodies to β_1, α_v and α_5 integrins (50μM each) were added to the smooth muscle cells 30 min prior to seeding on culture plates.

Flow cytometry

The effect of pre-treatment with LTE_4 (0.1 μM) for 30 min on integrin expression by airway smooth muscle cells was studied by flow-cytometry. Treatment with TNF-α (100U/ml for 24h) was used as positive control. Cells were serum starved for 24 hours, trypsinized (the exposure was kept as short as possible, ie 30s), washed with 1% PBS + 0.5%BSA, and incubated with 2μg primary antibody against β_1, α_v and α_5 integrins per 10^5 cells for 30 min in the dark at room temperature. After washing with PBS+BSA, cells were incubated with 2μg/10^5 cells secondary antibody for another 30 min before two further washes. Samples were suspended in fluorescence activated cell sorting fix and acquired and analyzed using a FACScan flow cytometer equipped with an argon ion laser (Becton Dickinson Instrument Systems, BDIS, Mississauga, Canada), and PClysis software supplied by BDIS.

SDS-PAGE and Western blot

Airway smooth muscle cells, starved for 24 hours in serum-free medium, were seeded onto plates coated with different concentrations of different matrix proteins. After 2 hours, the cells were lifted with trypsin (30 seconds), rinsed with ice-cold PBS, lysed with 1 ml of lysis buffer (50mM TRIS, 150mM NaCl, 10% glycerol, 1% Triton X-100, 5mM EDTA, 100μM sodium orthovanadate, 1mM β-glycerophosphate, 1mM NaF, 15U aprotinin/ml), and clarified by centrifugation. Protein concentration was determined by the Bradford method, and equal amounts (20μg) were resolved by 10% SDS-PAGE, transferred to 0.22μM pore nitrocellulose filters (<12.6 Nitrogen content), and probed with monoclonal antibody that recognized activated Src (pY^{418}). Proteins were recognized using a secondary antibody conjugated to horseradish peroxidase and enhanced chemiluminescence.

Analysis

Statistical analysis was performed using the repeated measures analysis of variance test (Statistical Package for Social Sciences, version 10.0, Chicago, IL) using the different time points or the experimental conditions as within subject factors. The source of significant variation was identified by pre-defined contrasts. $P \leq 0.05$ was considered statistically significant.

Results

Effect of extra-cellular matrix on migration

a) type of matrix-coating on the migrating surface

There were significant differences in the number of smooth muscle cells migrating towards PDGF across membranes coated with the different matrix proteins. At concentration of 10μg/ml, migration was maximum on collagen III, V, and fibronectin; less on laminin; and absent on collagen I and elastin ($p<0.05$) (Figure 1).

b) concentration of matrix-coating on the migrating surface

Chemotaxis of smooth muscle cells towards PDGF did not occur across uncoated membrane. With increasing concentrations of collagen I solution coating the membranes (10, 100 and 1000 μg/ml), more smooth muscle cells migrated towards PDGF ($p<0.05$) (Figure 2). This was not observed with high concentration (1000 μg/ml) of laminin or elastin.

c) matrix proteins as chemoattractants

Collagen I (1000μg/ml) solution was not a chemoattractant for human airway smooth muscle cells (Figure 3). Pre-treatment of smooth muscle cells with PDGF increased the haptotaxis and haptokinesis towards collagen I. However, cell migration was significantly less than migration towards PDGF.

d) signal transduction

Migration across collagen-coated membrane was inhibited by inhibitors of PI3K ($p<0.05$) and SrcK (IC_{50}=1μM) ($p<0.05$) (Figures 4a, 4b). The role of SrcK was further demonstrated by higher levels of phosphorylated SrcK in airway smooth muscle cells seeded on culture plates coated with 1000μg/ml (concentration that supported chemotaxis) than 10μg/ml (concentration that does not support migration) (Figure 5a). However, we found Src-phosphorylation in airway smooth muscle cells coated on elastin and laminin as well although they did not support migration to the same extent as fibronectin (Figure 5b), suggesting that Src-kinase is associated with chemotaxis, but not the only pathway involved in the process.

Effect of extra-cellular matrix on adhesion

Adhesion to collagen I was maximum at 2 hours after seeding. Adhesion was observed at lower concentrations of collagen I than required for migration (Figure 2). Adhesion was inhibited by blocking antibodies to the α_5, α_V and β_1 sub-units of integrin; however, unlike the effect on

chemotaxis, adhesion was not inhibited by inhibitors of PI3K and SrcK (Figure 4).

The effect of leukotriene-priming on adhesion and migration on different matrix

Pre-treatment (priming) with LTE_4 did not increase adhesion to surface coated with collagen I, although it augmented the chemotaxis of smooth muscles towards PDGF. Consistent with this observation, LTE_4 did not increase the intensity and number of integrins (α_5, α_V and β_1) expression on the surface of airway smooth muscle cells (Figure 6). In contrast TNF-α caused a 1.7 fold increase in the intensity of β_1 integrin. Also, priming with LTE_4 did not overcome the inhibitory effect of antibodies against α_5, α_V and β_1 on adhesion of airway smooth muscle cells to collagen I. A 'priming' response on chemotaxis was observed on all the matrix proteins that supported migration. An increase in phosphorylation of Src-kinase was not observed in airway smooth muscle cells treated with the concentration of LTE_4 that augmented the migratory response to PDGF (Figure 5c).

Discussion

The study shows that extracellular matrix proteins can modulate chemotaxis of human airway smooth muscle cells. Chemotaxis is influenced by the type and quantity of matrix proteins. The effect is partly dependent on PI3K and SrcK. Cysteinyl leukotrienes augment chemotaxis on all the matrix surfaces that facilitate migration; however they do not augment adhesion to matrix. These observations are relevant to the remodelling observed in the airways of patients with inflammatory diseases such as asthma. The deposition of matrix in the sub-epithelial region may by itself promote the migration of airway smooth muscle cells towards the lamina reticularis.

We examined the effect of extracellular matrix on airway smooth muscle migration for two reasons. First, extracellular matrix proteins such as collagen, fibronectin, laminin and

thrombospondin regulate migration of vascular smooth muscle cells (11,14). Some matrix proteins (collagen I, IV) promote vascular smooth muscle migration more than others (laminin) (14). There is very little information on their regulation of airway smooth muscle cell movement. Second, in airway diseases such as asthma, there is excessive accumulation of various extracellular matrix components and smooth muscle cells in the lamina reticularis. The matrix proteins that are commonly observed in the lamina reticularis are collagen I, III, V, and fibronectin; and less frequently laminin. Elastin and collagen IV are rarely observed (15). These different proteins have different effects on various aspects of smooth muscle function such as survival, proliferation and actin cyto-skeletal reorganization. For example, fibronectin and collagen I promote the proliferation of cultured airway smooth muscle cells in a dose-dependent fashion, whereas laminin inhibited growth (10). When survival of cells were studied, cells plated on a surface coated with elastin survived less than cells plated on surfaces coated with fibronectin, collagen I, V, laminin and vitronectin (9). These experiments led us to hypothesize that such a differential effect may be observed in the ability of matrix proteins to support chemotaxis as well. In deed, the morphololgy of smooth muscle cells grown on surfaces coated with fibronectin and laminin show more polarization of the actin-cytoskeletal system suggesting that these matrix surfaces may promote more migration (9).

We observed that collagen V and fibronectin supported migration more than collagen I, III and laminin, whereas elastin, which is not usually observed in asthmatic lamina reticularis, had no effect. We also observed that collagen I, not laminin or elastin, supports migration in a concentration-dependent manner. The matrix solutions were prepared to provide a monomeric coating to the surfaces, as fibrillar matrix may be non-permissive to migration (16). These observations suggest that the type and quantity of matrix deposited in the lamina reticularis may

influence the migration of airway smooth muscle cells towards it and their further survival and proliferation, contributing to the increased smooth muscle mass in patients with long-standing asthma. We also tested whether non-soluble collagen can act as a chemoattractant to smooth muscle cells (haptotaxis and haptokinesis), similar to the effect of matrix proteins on vascular smooth muscle cells (17) and fibroblasts (18,19). Similar to the effects on vascular smooth muscle cells (14,20), we observed that while collagen itself was not a chemoattractant, haptotaxis was observed when the cells were pre-treated with PDGF. The precise mechanism was not examined, but it is likely that PDGF upregulated integrin expression on the airway smooth muscle cells (21) promoting adhesion and downstream signalling. It is also possible that matrix-induced calcium release within the airway smooth muscle cells (22) contributes to cell motility.

We also investigated some of the signalling pathways in the regulation of airway smooth muscle migration by matrix proteins. Although the signal transduction pathways are not clearly defined, it is suggested that the binding of the integrin sub-units on the surface of the smooth muscle to the arginine-glycine-aspartate (R-G-D) sequence of aminoacids on the matrix can trigger an "outside-inside" signalling event in the smooth muscle. This leads to activation of the focal-adhesion kinase and the 60-kDa c-Src kinase. This leads to a further cascade of phosphorylation including that of paxillin, PI3K and p38 MAPK, ultimately resulting in actin remodelling and chemotaxis (23, 24). Consistent with this, we observed that blocking antibodies to integrin subunits β_1, α_v and α_5, which are the integrins most frequently expressed on human airway smooth muscle cells (9), inhibited both adhesion and migration. The contributions of individual integrin subunits were not studied. Pharmacological antagonists of SrcK and PI3K both inhibited chemotaxis, confirming their roles in mediating this activity. Neither antagonist had any effects on adhesion to matrix similar to observations in vascular smooth muscle (25)

suggesting that they are upstream of integrin-matrix assembly. The results may be limited by the lack of specificity of the pharmacological antagonists. For example, PP1 which has an IC_{50} of 0.5 μM for SrcK (26) may inhibit stress-activated protein kinases at high doses. We used a concentration of 1 μM (figures 4a &b) which is unlikely to have caused non-specific inhibition (26). The concentration of LY294002 that we used (50μM) was 5-fold of the reported IC_{50} in non-human cell lines (27). The high dose was chosen because it has been reported to decrease human vascular smooth muscle migration (28) and airway smooth muscle proliferation (29). Further evidence for the role of SrcK in chemotaxis was provided by the demonstration of increased phosphorylation of Src and increased chemotaxis when the muscle cells were seeded on surfaces coated with increasing concentrations of collagen I. The role of SrcK is consistent with previous observations in fibroblasts lacking Src family kinases, where over expression of Src promoted tyrosine phosphorylation, cell spreading and migration (30). We expected less Src-phosphorylation in smooth muscle cells plated on surfaces that promoted less migration. However, we observed the same degree of phosphorylation, again suggesting that SrcK while important in chemotaxis is not the only regulator of chemotaxis. A possible alternate mechanism of increasing cell motility and chemokinesis by Src is via regulating phosphoinositol biphosphate and intracellular calcium levels (31).

We had previously demonstrated the ability of cysteinyl leukotrienes to augment chemotaxis of smooth muscle cells towards PDGF (5). We wished to study whether this was as a result of increased adhesion or migration. In contrast to the effect on chemotaxis, we did not observe a 'priming' effect of leukotriene E_4 on adhesion at the same dose that promoted migration. The lack of priming is probably because LTE_4 does not increase the surface expression of integrins that are necessary for adhesion. This was further supported by the

observation that priming with LTE_4 did not overcome the inhibitory effect of anti-integrin antibodies on adhesion. Since we did not study a range of doses of LTE_4 over a range of time of exposure, we cannot conclude that cysteinyl leukotrienes do not activate surface integrins. However, we observed that priming with leukotrienes increased the haptokinesis produced by collagen I. Given that cysteinyl leukotrienes can augment growth factor stimulated synthesis of proteoglycans such as versican by airway smooth muscle cells (32), and that it can promote haptokinesis towards matrix and chemokinesis towards growth factors, they may play a significant role in airway remodeling.

The interaction between extracellular matrix and airway smooth muscle has important clinical implications in airway diseases such as asthma. They are the most important constituents of a remodelled airway and the least responsive to currently available treatments for asthma (33-35). It is likely that the matrix proteins are synthesized by the resident cells and the inflammatory cells infiltrating the airway in patients with asthma. There is now increasing evidence that, once deposited, the matrix, in addition to its mechanical properties, can contribute to airway narrowing by promoting the accumulation of airway smooth muscle cells. They may also decrease the anti-mitogenic effects of anti-inflammatory treatment such as corticosteroids on airway smooth muscle cells (36). Our results suggest that they may facilitate more accumulation of airway smooth muscle cells in the lamina propria by chemotaxis. Collectively, these observations suggest that extracellular matrix and airway smooth muscle migration should be considered as primary therapeutic targets in patients with chronic asthma.

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Figure Legends

Figure 1: Migration of smooth muscle cells on surfaces coated with different matrix proteins. At concentration of 10μg/ml, fibonectin, collagen III and collagen V supported migration compared to control (*$p<0.05$). The data is from 4 separate experiments performed in duplicate and presented as mean and standard deviation.

Figure 2: The effect of the concentration of collagen I coating on migration and adhesion. A concentration-response on migration and adhesion is observed, with at least 100μg/ml of collagen being required for migration towards PDGF, and 10μg/ml for adhesion. The data is from 3 separate experiments performed in duplicate and presented as mean and standard deviation.

Figure 3: Chemoattraction towards collagen I. Collagen I did not induce haptotaxis or haptokinesis on its own. When cells were pre-exposed to leukotriene E_4, it promoted haptokinesis. When cells were pre-exposed to PDGF, it promoted both haptotaxis and haptokinesis. However this response was less than chemotaxis towards PDGF (*$p<0.05$ compared to control). The data is from 4 separate experiments performed in duplicate and presented as mean and standard deviation.

Figure 4a: Effect of PP1 (a pharmacological antagonist of Src-kinase) on human airway smooth muscle cell migration. 1μM of PP1 caused 50% inhibition of migration towards PDGF. The data is from 3 separate experiments performed in duplicate and presented as mean and standard deviation.

Figure 4b: Signalling pathways in migration and adhesion on collagen I: Leukotriene-priming increases migration towards PDGF (*$p<0.05$), but does not increase adhesion. Inhibitors of SrcK (SrcKI) (PP1, 1μM) and PI3K (PI3KI) (LY294002, 50μM) attenuated migration but not adhesion (¶$p<0.05$ compared to the maximum response). Blocking antibodies to the α_5, α_V and β_1 sub-units

Figure 5c

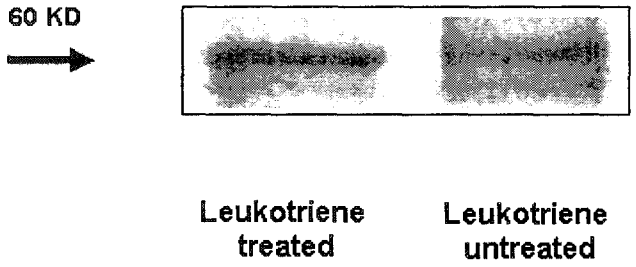
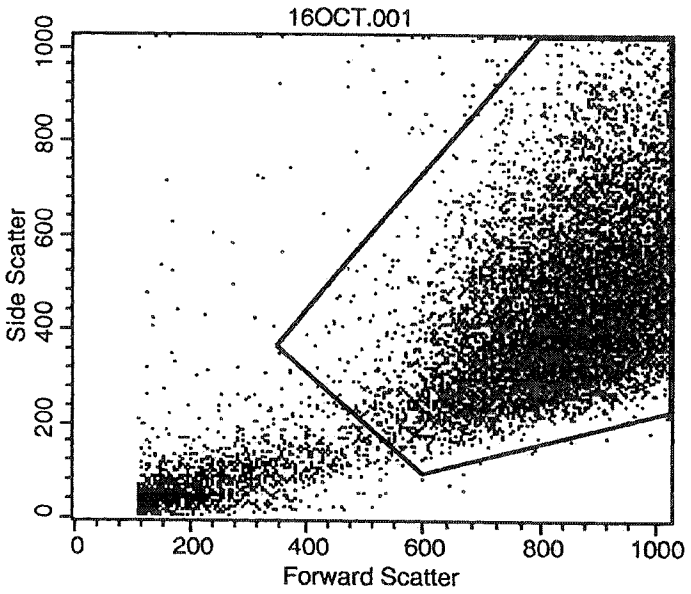
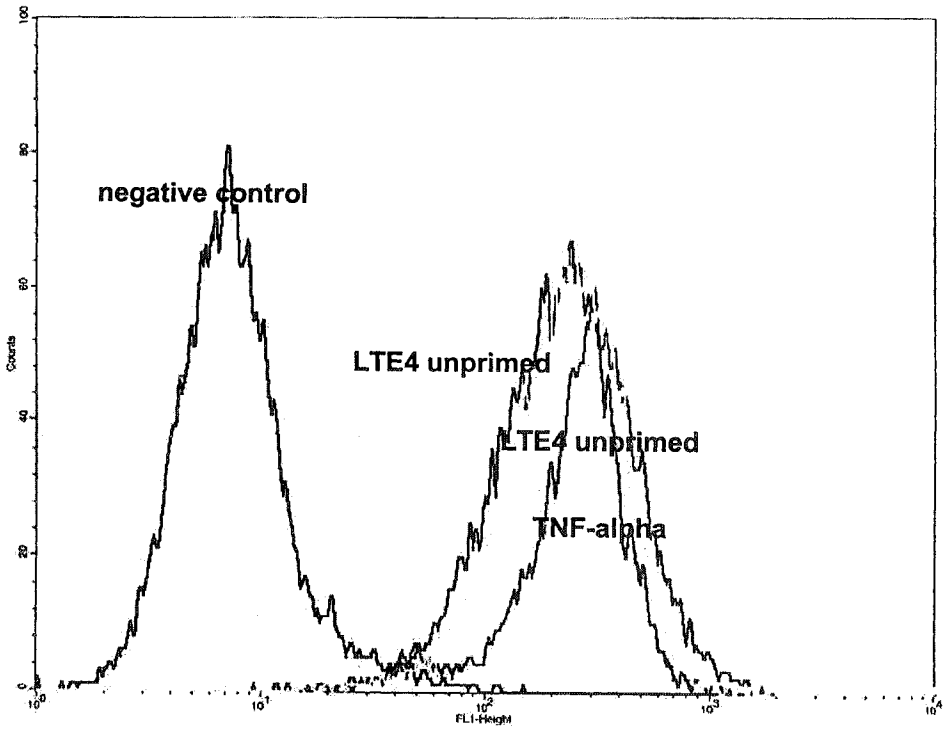


Figure 6





	% surface integrin expression (mean)			intensity of integrin expression (mean)		
	control	+LT	+TNF	control	+LT	+TNF
alpha-v	94	91	99	127	110	184
alpha-5	80	75	98	63	66	128
beta-1	97	92	100	310	219	512

CHAPTER 7:

SUMMARY OF FINDINGS, GENERAL DISCUSSION, FUTURE DIRECTIONS

Summary of findings

The observations in this thesis describe three new biological effects of cysteinyl leukotrienes in the human airway. First, they regulate the recruitment of myeloid dendritic cells from peripheral blood following allergen inhalation. Second, they cause airway eosinophilia. One of the mechanisms is by a direct effect on the bone marrow, promoting eosinophilopoiesis. Third, they promote airway smooth muscle migration, which may be relevant in airway remodeling.

Myeloid dendritic cells are the antigen presenting cells in the human airway. We have demonstrated that they express $CysLT_1$ receptor and that their recruitment from blood following an allergen inhalation is inhibited by a $CysLT_1$ receptor antagonist. Although we have not demonstrated their destination, it is reasonable to assume based on other studies that these dendritic cells would have migrated to the airway. This observation has important clinical significance. Treatment with a leukotriene receptor antagonist, in patients who are susceptible to develop allergies, may prevent it.

Our second important observation was that cysteinyl leukotrienes have a direct effect on eosinophilopoiesis in the bone marrow. This may be one of the mechanisms by which leukotrienes cause airway eosinophilia. Treatment with a leukotriene antagonist prevented allergen-induced increase in eosinophil colony forming units in the bone marrow without having an effect on base line number of eosinphil colonies. This effect is opposite to that described for inhaled corticosteroids. An effect on eosinophilopoiesis suggests that long-term treatment may be able to suppress airway eosinophilia significantly. We also observed a different effect of leukotriene D_4 and leukotriene E_4 on airway

eosinophils, suggesting the existence of separate receptors in human airways.

Our third novel observation was the effect of cysteinyl leukotrienes on human airway smooth muscle migration. We observed that they cause chemokinesis of airway smooth muscles, not chemotaxis. However, they primed the chemotactic response towards platelet-derived growth factor. The exact mechanism of this was not clear. It was not by up-regulating integrin expression on airway smooth muscles or by activating Src-kinase. Extra-cellular matrix also plays an important role in regulating airway smooth muscle cell migration. This has important clinical significance. Long-term treatment with a leukotriene antagonist may be able to decrease the smooth muscle mass seen in the remodelled airway of patients with chronic asthma.

General discussion

The aim of the thesis was to study novel biological roles of leukotrienes in the human airways. Therefore all the experiments were conducted *in-vivo* in human subjects or *ex-vivo* using human tissue. The methods employed included randomized clinical trials and *ex-vivo* experiments. The following discussion will address the validity of the experimental designs used in this study, safety of the clinical procedures, methodological issues and limitations of the laboratory methods, the clinical relevance of the results of the studies, and future directions of research.

Study designs

We employed three different strategies to investigate the role of cysteinyl leukotrienes in human airways. First, the effect of leukotrienes on airway eosinophilia was studied directly by exposing asthmatic subjects to inhaled leukotrienes. Second, the effects of leukotrienes on circulating dendritic cells and on bone marrow eosinophilopoiesis were studied indirectly by observing the effects of

treatment with a specific $CysLT_1$ receptor antagonist in a randomized, placebo-controlled, clinical trial. Third, the effect of leukotrienes on airway smooth muscle migration was studied *ex-vivo* using cultured smooth muscles obtained from patients undergoing lung resection at our hospital.

Allergen challenge was used as a model of allergic asthma. It is a valid and reliable method of mimicking the airway physiological and inflammatory responses in asthma (O'Byrne et al 1987, Gauvreau et al 1999a), and to study the effects of pharmacological agents. It is safe and can be repeated in subjects in cross-over study designs.

The clinical trials had a randomized, double-blind, placebo controlled, cross-over design. The randomization codes were maintained off-site by an independent third party. These minimized selection and allocation biases. The cross-over design minimized the effects of within-subject variability in our measurements. The laboratory measurements were always made blinded to the clinical outcomes and subject details. The results of the experiments are therefore reliable. The external validity or generalizability of the results is limited by the fact that all the subjects had relatively mild asthma, and all had dual airway response to an allergen inhalation. The results may not be true for patients with more severe asthma and those who are not atopic. They may not also be extrapolated to asthmatic subjects who do not have a late asthmatic response to an allergen inhalation (ie. isolated early responders).

Clinical methods

Allergen provocation

Inhalation of nebulized allergen is a well-established model to study the pathophysiology of asthma. The method employed in this study delivers a constant and reproducible amount of allergen each time. The dose of the allergen delivered is calculated to cause not more than 20% drop in FEV_1,

and is therefore safe when performed by experienced personnel under close supervision (Crapo et al 2000). This high dose allergen challenge model may not mimic real life repeated exposure to low doses of allergen (Sulakvelidze et al 1998), but provides an opportunity to study the physiological and inflammatory responses in patients with asthma. This method was employed to study the effect of cysteinyl leukotrienes on dendritic cells and eosinophils for a number of reasons. First, the bronchoconstriction that occurs during the early asthmatic response is largely due to allergen-induced release of cysteinyl leukotrienes. The cellular inflammatory response during the late asthmatic response may also be partly mediated by cysteinyl leukotrienes (O'Byrne et al 2000). Second, dendritic cells are essential for allergen-induced airway eosinophilia (Lambrecht et al 1998) and allergen inhalation causes a rapid recruitment of dendritic cells from circulation (Upham et al 2002). Third, allergen inhalation causes a sustained eosinophilic airway inflammation (Gauvreau et al 1999a), which is associated with an increase in eosinophilopoiesis in the bone marrow (Wood et al 1998). Thus, it provides the opportunity to study the mechanism of agents such as cysteinyl leukotrienes in causing eosinophilic airway inflammation.

Sputum induction

Sputum induction with hypertonic saline provides a relatively non-invasive and safe method (Vlachos-Mayer et al 2001) to sample airway secretions to study airway inflammation. It is also possible to sample sputum repeatedly from the same patient. The technique was developed and validated in our laboratory (Pin et al 1992b; Popov et al 1995; Pizzichini et al 1996) and has been employed in over 500 research studies in over 20 countries world-wide (Parameswaran et al 2002). We employed this technique to collect sputum and measured the cell counts in the cellular phase and the cytokines and chemokines of interest in the fluid phase. The technique was used in two studies in the

thesis. It was done 135 times (at baseline, 7h and 24h after placebo, LTD_4 and LTE_4 inhalation in 15 subjects) in the leukotriene inhalation study. Adequate quantity of sputum was obtained in all but 5 sputum inductions. In the study investigating the role of leukotrienes in eosinophilopoiesis, sputum was induced 120 times and was obtained at 112 attempts. There were no untoward effects or excessive bronchoconstriction caused by inhalation of hypertonic saline.

Bronchoscopy

The effects of leukotriene inhalation in asthmatic subjects were assessed both in the lumen and in the mucosa of the airway. The former was assessed by examination of induced sputum and the latter by airway mucosal biopsies. Both compartments of the airway were assessed because the correlation between the numbers of inflammatory cells, particularly eosinophils, in both compartments is modest (Erjefalt et al 2000). We followed international recommendations regarding ethical, safety, procedural and laboratory considerations (ERS/ATS guidelines 1992; Jeffery et al 2003). The procedure was approved by the Institutional Review Board and all subjects gave informed consent. Fiberoptic bronchoscopies were performed by the same experienced bronchoscopist according to recommended guidelines, with adequate monitoring and oxygenation. Sedation and anaesthesia did not exceed standard recommendations (Langmack et al 2000; ERS/ATS guidelines 1992; Jeffery et al 2003). Endobronchial biopsies were taken under direct vision from the proximal airways from the subsegmental carinae and not from the later

al walls. Reusable forceps was not used for more than four biopsies. None of the bronchoscopies took longer than 20 minutes. Each subject underwent three bronchoscopies approximately 3-4 weeks apart. There were no adverse events in the 21 procedures.

Bone marrow biopsy

Eosinophils play important roles in the clinical manifestation of asthma. Our laboratory has pioneered the use of bone marrow aspirations in understanding the role of eosinophil progenitors in asthma pathophysiology. Over the past decade, the methodology and safety of bone marrow aspirations in clinical studies was confirmed in a number of publications (Sehmi et al 1996; Sehmi et al 1997; Wood et al 1998; Wood et al 1999; Braccioni et al 2002; Wood et al 2002; Dorman et al 2004a; Dorman et al 2004b). Bone marrow aspiration was done according to the standard clinical guidelines after obtaining approval from the Research Ethics Board and informed consent from the subjects. All the procedures were performed by the same physician who was well experienced in the procedure. All the bone marrow samples were obtained from the posterior superior iliac crest, under topical anaesthesia. 5 ml of bone marrow sample was collected into heparinized syringes and processed within 30 minutes. Each subject underwent four bone marrow aspirations. Two procedures were done 48 hours apart from the right and left iliac crests and repeated approximately six weeks later. All sixty procedures were well tolerated without any adverse events.

Laboratory methods

Sputum cell counts

Sputum cytopsins, separated from saliva (Pizzichini et al 1996a) and dispersed with DTT (Efthimiadis et al 1997) yield repeatable and valid differential cell counts (Pizzichini et al 1996b; In't Veen et al 1996; Spanevello et al 1997). The method has been employed in our laboratory to investigate the response to allergen inhalation (Gauvreau et al 1996; Gauvreau et al 1999a, Parameswaran K et al 2000; Inman et al 2001; Gauvreau et al 2003) and various therapeutic interventions, and has been found to be responsive to change and is repeatable (Gauvreau et al 1999b). Sputum was processed according to a standard procedure within an hour of collection and the cell counts were made by an observer who

was blinded to the clinical details of the subjects or their treatment allocation.

Cytokine expression in the airway was studied by immunocytochemistry of sputum cells using previously reported and validated methods (Gauvreau et al 1999a). The success of staining depends on getting adequate quantity of sputum cells, proper preparation of Aptex coated slides, and specificity of the monoclonal antibodies used to identify the proteins. For the identification of eosinophil activation, a double staining with FITC-labelled antibody against the cleaved portion of ECP (EG2) was used. However, we did not confirm the cellular source of the cytokines identified. Also, we were not able to identify whether the cytokine identified was expressed, secreted or receptor-bound. Rather, the semi-quantitative method that we employed only represented the immunolocalization of the protein of interest in sputum cells. Since the total cell count in sputum is less repeatable than the differential cell count, the variability of the cells staining for the cytokines was also high. We did immunostaining to identify EG2, IL-5, eotaxin and RANTES at eight time points in 15 subjects. Thus a total of 480 slides were stained in duplicate. Our over-all success rate was 80%. The failures were due to a number of reasons including inadequate sputum cells on the slides, poor quality of Aptex coating of slides, and technical problems such as positive staining on a negative control slides.

Bronchial biopsy

The bronchial mucosal biopsy samples were processed according to international guidelines (Jeffery et al 2003). The quality of tissue obtained was checked immediately by an assistant. Specimens were fixed in 4% paraformaldehyde, mounted in paraffin, and stained with Congo red for eosinophils and toludine blue for mast cells and basophils. Paraffin embedding is easy to perform and has the advantage of preserving tissue architechture and prevents diffusion of antigen outside the tissue. However, it has the disadvantage of causing some tissue shinking and cross-linking of fixative with

some antigens. Cells were enumerated in the lamina propria to a depth of 115μm. The area and depth of the lamina propria counted was calculated by image analysis software.

Dendritic cell assay

Identification of dendritic cells is difficult because the morphology, surface antigen expression and function vary depending on the tissue site and degree of maturation of the cell. Also, there are significant differences between cultured and freshly isolated dendritic cells as there are differences in the expression of surface markers in mice and human dendritic cells (Shortman et al 2002). In contrast to many studies on mouse dendritic cells, there are few studies on mature dendritic cells isolated from human tissues. Most of the information is obtained from studies of blood which has immature and precursor dendritic cells, which do not usually express CD1a, CD1c or CD11. Functionally, there appears to be two distinct subtypes of human dendritic cell precursors. The myeloid cells are monocytoid in appearance, express CD33 surface antigen and possess the most potent antigen processing capability. They also express on their surface GM-CSF receptors and Toll-like receptors. The second type of cells possess a plasmacytoid morphology, express CD123 (receptor for IL-3) and CD45RA, and depend on IL-3 for their survival and differentiation. They produce interferon α and β, but do not participate in antigen presentation.

We used a previously validated method to identify the circulating dendritic cells as a distinct population of whole blood leukocytes by four-colour flow-cytometry (Upham et al 2000). Dendritic cells were identified as $CD14^-$ and $CD16^-$ (using antibodies conjugated with FITC), HLA-DR^+ (using antibody conjugated with CyChrome). Myeloid dendritic cells were identified as $CD33^+$ (using antibody conjugated with APC) and plasmacytoid cells as $CD123^+$ (using antibody conjugated with PE). The method was shown to be reproducible, with an intra-class correlation coefficient of 0.95. It

was easy to perform, required only 5 ml of whole blood and the dendritic cell count did not vary significantly over 7 hours.

Bone marrow assay

Bone marrow samples were obtained 24 hours after allergen inhalation because a maximal response on Eo/B colony forming units is observed at this time point (Dorman et al 2003). Bone marrow cells were processed, within 30 min, according to well established procedures established in our laboratory over the past 10 years (Denburg et al 1985b; Inman et al 1996, Sehmi et al 1997; Wood et al 1998; Dorman et al 2004a). Non-adherent mononuclear cell fraction that includes the progenitor cells were identified by percoll gradient separation and incubation in plastic jars. They may also include a very small proportion of lymphocytes. The cells were cultured in the presence of optimal concentration of IL-5. Eo/B colony forming units were identified based on morphological characteristics and not by immunochemistry. The errors introduced by the contamination with lymphocytes and lack of immunochemistry are considered negligible and systematic and are unlikely to have altered the results in a cross-over study design. The pluripotent haemopoietic progenitor cell in the bone marrow was also identified by the expression of CD34 surface antigen, a monomeric, transmembrane, O-sialylated glycophosphoprotein (Sehmi et al 1997). We also examined the proportion of $CD34^+$ cells that also expressed CCR3, the receptor for eotaxin using a previously validated method of flow-cytometry. Flow-cytometric detection of $CD34^+CCR3^+$ cells was confirmed by co-localization of CCR3 to CD34 cells by in situ hybridization and immunocytochemistry (Sehmi et al 2003).

Airway smooth muscle cell culture

The effect of cysteinyl leukorienes on airway smooth muscle migration was studied using

cultured human airway smooth muscle cells (Hirst 1996). None of the cells were frozen and thawed before use. Lung tissue was obtained from patients undergoing resection of lung tumours. Macroscopically normal looking portions of tissue were selected. Since most of the patients may have been smokers, it is possible that cigarette smoke may have altered the biology of the tissue. The exact effect of cigarette smoke on phenotypic modulation of cultured human airway smooth muscle is not known. Muscle tissue was obtained from portion of the large airway and was explanted into tissue culture plates. It is not known whether digesting the cells with collagenase and elastase before plating gives rise to a different smooth muscle phenotype. All the cells were used between the 2^{nd} and 5^{th} passages in order to get sufficient number of cells for the experiments. Five cycles of cell division may have changed the smooth muscles from a "contractile" phenotype to a "proliferative phenotype (Halayko et al 1996) and is also known to change the properties of certain ion channels such as the conductance of K^+ channels (Snetkov et al 1996). The effect on receptor expression, in particular, leukotriene expression, is not known. We tried to minimize this effect by serum deprivation of cells for 48 hours which restores the content of contractile proteins to some extent and synchronizes cells to the G_o phase of the cell cycle. Since cell density can affect the synthetic and contractile phenotypes (Bowers et al 1993), all the experiments were performed with cells that had grown to confluence.

Airway smooth muscle migration

Cell migration was studied in 6.5 mm Transwell culture plates with an inner (insert) and an outer well. The cells were seeded on to matrix-coated polycarbonate membranes of the insert. The membranes had pores of 8.2 μm in diameter and were approximately 100 μm in thickness. The chemoattractants were added to the outer wells. Cells migrating through the pores to the lower surface of the membranes were stained and counted. The technique, in addition to allowing studies of

chemotaxis and chemokinesis, also enables to study the effect of different matrix proteins on cell migration. This is achieved by coating the polycarbonate membrane with various extra-cellular matrix proteins. The assay, while easy to use, is not without problems. Peeling of the membranes and staining them is time consuming. The cells on the inner surface of the membrane may interfere with counting under light microscopy. This is minimized by counting only those migrated cells whose nuclei are clearly seen on the outer surface. The assay does not allow studying more than one chemotactic gradient at a time, or the speed or path of cell migration. It also does not allow real-time observations to be made. In adequate results may be obtained due to improper coating of the polycarbonate membrane, contamination of the media with lipopolysaccharide. Both these can prevent smooth muscle cells from migrating. Activation of cells by jarring of cells or vibration can also interfere with migration. Care should be taken to prevent the cells from getting activated by preparing them at 4°C in calcium-free medium.

Interpretation of results, future directions

Dendritic cell migration

Our observation of a role for cysteinyl leukotrienes in allergen-induced changes in circulating dendritic cell number is new. The effect is more on myeloid cells presumably because more myeloid than plasmacytoid cells express $CysLT_1$ receptor on their surface. One of the mechanisms is by increasing the levels of dendritic cell chemoattractants such as RANTES, MIP-1α and MIP-3α. This was attenuated by a $CysLT_1$ receptor antagonist, pranlukast.

A major strength of the data is that it was an in-vivo experiment in subjects with asthma using a specific antagonist of the $CysLT_1$ receptor. There are a number of weaknesses to our arguments. First, the magnitude of allergen-induced decrease in circulating dendritic cells in this experiment (9%) was

less than previously reported (approximately 25%). This does not seem to be related to the magnitude of early or late asthma responses or the quantity of allergen delivered. The method of identification of dendritic cells was identical in both studies. It may represent differences between subjects recruited for the studies. Second, we do not know the ultimate destination of the dendritic cells that disappear from circulation. We assume based on previous evidence that they migrate to the site of allergen delivery. However, they may move back to the bone marrow or perhaps even to the regional lymph nodes. Third, we did not investigate all the mechanism by which cysteinyl leukotrienes promote dendritic cell migration. It may be a direct chemoattractant effect or an indirect effect by increasing the production of other chemoattractants. Pranlukast treatment decreased allergen-induced increase in sputum cells staining for RANTES, MIP-3α (LARC, liver and activated-regulated chemokine, Exodus-1, which can be produced by airway epithelial cells) and MIP-1α. Leukotrienes may also be involved in two different ways. They may upregulate CCR6 (receptor for MIP-3α) on dendritic cells. Alternatively, cysteinyl leukotrienes produced following by inflammatory cells following an allergen inhalation may augment the chemotactic activity of MIP-3α, similar to its potentiating effect on alveolar macrophage derived MIP-1α.

A further aspect of dendritic cell biology that needs to be further explored is the role of MRP-1. There is good evidence that lack of MRP-1 prevents the migration of dendritic cells from tissue to the regional lymph nodes. It is not known whether this is true in vivo. Also, it is not known whether a subset of population may have over-expression of MRP-1 making them vulnerable to increased cysteinyl leukotriene activity. I propose to examine MRP-1 expression in the various cells that produce cysteinyl leukotrienes, such as the eosinophils and mast cells, in patients with asthma who show increased responsiveness to cysteinyl leukotriene antagonists and in situations such as aspirin

sensitivity which are largely mediated by leukotrienes. It may provide a pharmacogenomic method to identify responders to cysteinyl leukotriene antagonist treatment.

Our observation has important clinical significance. If cysteinyl leukotrienes may prevent the recruitment of dendritic cells to the airway from blood, long term treatment with the receptor antagonists may prevent the development of allergies in susceptible children. The potential difficulties of organizing a placebo-controlled, clinical trial are the long duration of therapy and follow-up and the potential side effects of leukotriene receptor antagonists in children. The recommended dosage of 4 mg daily does not cause any significant clinical or biochemical adverse effects. The role of cysteinyl leukotrienes in normal biology in children is not clearly known. There is one report of an infant who presented with muscular hypotonia, psychomotor retardation, failure to thrive, and microcephaly and died aged 6 months. The infant was unable to synthesize leukotriene C_4 presumably due to a deficiency of leukotriene C_4 synthase enzyme (Mayatepek et al 1998). Such a complication has not been reported with treatment with a leukotriene receptor antagonist.

Eosinophil infiltration

We confirmed the observation that cysteinyl leukotrienes can cause airway eosinophilia. In addition, we made two novel observations. First, inhalation of LTE_4 caused a greater degree of airway eosinophilia than LTD_4. This was surprising because both leukotrienes are believed to act through the same $CysLT_1$ receptor and LTD_4 is more potent than LTE_4, at least on airway smooth muscles (Samhoun et al 1989). The rank order of potency on other cells such as the eosinophils has not been investigated. In order to achieve the same degree of immediate bronchoconstriction, we administered approximately 40 fold greater concentration of LTE_4 than LTD_4 in the airway. This may have been responsible for the greater degree of eosinophilia caused by LTE_4. A second possibility is that LTD_4

and LTE_4 may act through different CysLT receptors. There is evidence of selective binding of LTE_4 to only a subset of LTD_4 receptors in guinea pig trachea (Aharony et al 1989). Also, pharmacological reversal has been found to be different between the two leukotrienes, suggesting these leukotrienes may not necessarily bind the same receptors (Silbaugh et al 1989). In agreement, molecular dynamics simulations have demonstrated that LTE_4 conformation spans the LTD_4 and LTC_4 types and therefore may occupy both of these receptors (Herron et al 1995). This needs further investigation.

The second novel observation was that the magnitude of airway eosinophilia caused by leukotriene inhalation was modest compared to that caused by allergen inhalation, despite comparable immediate bronchoconstriction. This suggests that although cysteinyl leukotrienes may induce primary effects on eosinophils, their role as priming agents may have a greater overall effect on eosinophil accumulation during an allergic event such as allergen inhalation.

Eosinophilopoiesis

We provided the first *in-vivo* evidence of an effect of cysteinyl leukotrienes on eosinophil progenitor cells in the bone marrow. The formation of IL-5 responsive Eo/B colony forming units in the bone marrow induced by an allergen was inhibited by pranlukast. This suggests that one of the mechanisms by which a leukotriene antagonist decreases airway eosinophilia is by suppressing eosinophil formation in the bone marrow. There are a number of unanswered questions. First, it is not known whether the allergen-induced increase in leukotrienes in the airway is accompanied by an increase in the bone marrow as well causing a direct stimulation of the marrow cells. Second, the mechanism by which leukotrienes augment IL-5 induced eosinophilopoiesis is not known. IL-5 activates Lyn, Syk and JAK2 and propagates signals through the Ras-MAP-kinase and JAK-STAT pathways (Adachi et al 1998). It is not known whether cysteinyl leukotrienes modulate these pathways

(Elias et al 2003).

We also observed that the expression of the eotaxin receptor, CCR3, on $CD34^+$ progenitor cells induced by inhaled allergen was decreased by pranlukast, suggesting that they prevent the egress of the pluripotent cell from the marrow to the local site of inflammation. This is also likely to be mediated through IL-5. Allergen inhalation increases IL-5 levels in the bone marrow (Wood et al 2002). IL-5 has been reported to increase CCR3 expression in leukemic cell lines (Tiffany et al 1998). It is possible that pranlukast, by antagonising IL-5 production or signalling, decreases CCR3 expression on $CD34^+$cells. These observations suggest new roles for cysteinyl leukotrienes in IL-5 signalling, similar to their possible role in IL-13 signalling (Elias et al 2003). This needs to be investigated.

The effect of leukotrienes on the bone marrow, in addition to providing mechanistic information, has clinical relevance. Two weeks of treatment in our studies did not have any effect on baseline eosinophilopoiesis, but prevented allergen-induced eosinophilopoiesis. Treatment for longer duration may suppress eosinophilia to a greater extent. Indeed, whereas short term treatment studies have not shown leukotriene receptor antagonists to be as effective as add-on therapies to inhaled corticosteroids as long-acting beta-agonists (Nelson et al 2000), the only published 12 month treatment study showed both treatment strategies to be equally effective (Bjermer et al 2003).

Smooth muscle migration

The first observed biological effect of cysteinyl leukotrienes was bronchoconstriction of smooth muscle. We observed that they can also augment airway smooth muscle cell migration, which is an under-appreciated phenomenon in airway disease. Human airway smooth muscle cells were observed to migrate towards platelet-derived growth factor. This may be analogous to the migration of vascular smooth muscle cells in the pathogenesis of atherosclerosis and post-angioplasty stenosis. Cysteinyl

leukotrienes, although by themselves were not chemoattractants, caused chemokinesis and augmented the migration towards PDGF. This was inhibited by montelukast, suggesting that it was mediated through the $CysLT_1$ receptor. We were not able to confirm the precise mechanism by which leukotrienes promoted chemotaxis towards PDGF. The increase in migration was not due to increased integrin expression on airway smooth muscle cells. Since an interaction between receptor tyrosine kinases (PDGF receptors), G-protein coupled receptors (CysLT receptors) and integrins (extracellular matrix signalling) appeared likely, we expected an activation of the Src-kinase pathway through activation of focal-adhesion kinases. We did not examine for FAK activation, but we did not observe increased Src-kinase phosphorylation in smooth muscle cells. The inhibition of chemotaxis by inhibitors of both Src kinase and PI3 kinase suggests a role for both enzymes in chemotaxis. The mechanism by which leukotrienes augment migration towards PDGF may be by activation of PI3-kinase directly. It is also possible that the 70-kDa ribosomal S6 kinase or the Elongation Factor-2 protein, which are downstream of PI3-kinase (Ammit et al 2001), may be directly activated.

We also identified that the type and concentration of the extra-cellular matrix proteins can regulate smooth muscle chemotaxis. Smooth muscle cells showed more chemotaxis towards PDGF on surface coated with extra-cellular matrix proteins such as collagen I and fibronectin (which are present in large quantities in the airways of patients with long-standing asthma) than on elastin and laminin (which are present in lesser quantities). We also observed that chemotaxis was dependent on the concentration of the collagen I solution which was used to coat the migrating surface. Further, collagen I, although by itself was not a chemoattractant, caused chemokinesis and increased the chemotaxis towards PDGF. These results suggest that the extracellular matrix in remodelled airway mucosa, by itself may promote migration of smooth muscle cells towards the submucosa.

These effects on airway smooth muscle migration have potentially important clinical implications. Evidence from Ebina and colleagues (Ebina et al 1993) and more recently from Woodruff and colleagues (Woodruff et al 2004) suggest that the increased smooth muscle mass in the airway of patients with asthma is more due to hyperplasia (increased number) than hypertrophy (increased size) of smooth muscle cells. One of the mechanisms of increased smooth muscle accumulation in the airway mucosa in patients with asthma may be by migration from deeper layers (figure 7.1). Deposition of extra-cellular matrix may further increase smooth muscle chemotaxis. The relative roles of matrix proteases (MMPs) and tissue inhibitors of matrix proteases (TIMPs) on smooth muscle chemotaxis are currently not known. None of the currently available treatments for asthma effectively reduce matrix or smooth muscle accumulation in the airway submucosa. Extrapolating from the evidence from animal studies (Henderson et al 2002), long-term treatment with leukotriene antagonist may prevent or reverse these changes by preventing the accumulation and hypertrophy of smooth muscle cells.

Thus, the data presented in this thesis provide three new biological effects of cysteinyl leukotrienes, which can lead to new strategies for managing allergic asthma. First, treatment with leukotriene receptor antagonists may be effective in preventing allergies in a susceptible person. Second, long term treatment with leukotriene receptor antagonist, by a direct effect on bone marrow eosinophilopoiesis, may suppress airway eosinophilia refractory to usual therapy. Third, long-term treatment with leukotriene receptor antagonist may prevent smooth muscle accumulation in remodelled airway mucosa. As novel biological effects of cysteinyl leukotrienes continue to be unravelled (Beller et al 2004), it is becoming increasingly clear that their effects extend beyond simply contraction of smooth muscles. They play an important role in antigen presentation, perpetuating airway inflammation and causing the structural changes associated with chronic asthma.

Airway in asthma

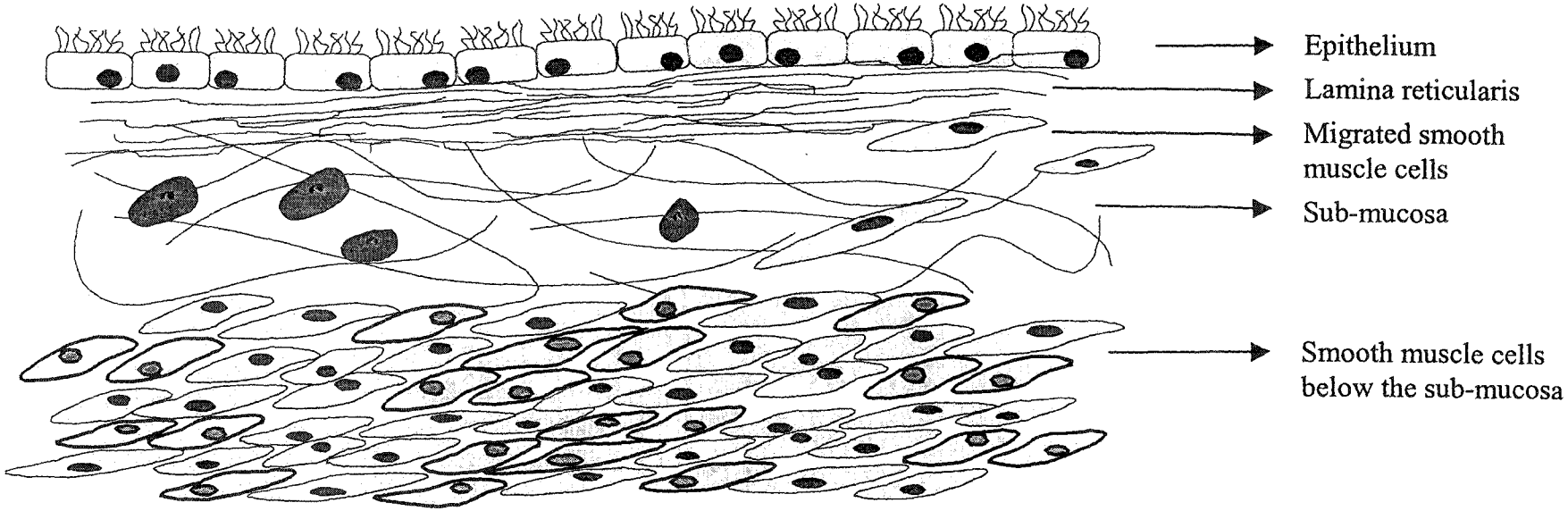


Figure 7.1

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