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Constitutive and cytokine-stimulated expression of eotaxin by human airway smooth muscle cells

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June 1998

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Science

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ABSTRACT

Airway eosinophilia is a prominent feature of asthma that may be mediated in part through the expression of eotaxin, a potent eosinophil-active chemokine that is highly expressed by epithelial cells and inflammatory cells in asthmatic airways.

The major aim of this study was to determine whether human airway smooth muscle (ASM) cells may be a source of eotaxin in asthma. The evidence presented in this thesis shows constitutive eotaxin gene expression in ASM in vitro that is markedly increased following stimulation with the proinflammatory cytokines TNF-α and IL-1β. Release of eotaxin was confirmed in ASM culture supernatants which contained significant chemoattractant activity for eosinophils that was partly inhibited with antibodies directed against eotaxin or RANTES, and maximally inhibited by a combined blockade of both chemokines. Strong signals for eotaxin immunoreactivity were also observed in vivo in smooth muscle in asthmatic airways.

In conclusion, the results of this study suggest that ASM may contribute to airway inflammation in asthma through the production and release of eotaxin.
ABREGÉ

La présence d'éosinophiles au niveau des voies aériennes est une caractéristique majeure de l'asthme qui pourrait être médiée, partiellement, par l'expression d'éotaxine. En effet, cette puissante chemokine agit sur les éosinophiles et est fortement exprimée par les cellules épithéliales et les cellules inflammatoires des voies aériennes de sujets asthmatiques.

Le but principal de cette étude fut d'examiner, chez l'homme, la capacité des cellules musculaires lisses (CML) des voies aériennes à exprimer l'éotaxine. Nos résultats montrent une expression constitutive du gène codant pour l'éotaxine, dans les CML, expression qui est fortement augmentée à la suite d'une stimulation par le TNF-α et l'IL-1β. La libération d'éotaxine dans les surnageants de culture de CML a été confirmée et possède une activité chemoattractante significative pour les éosinophiles. De plus, cette activité est inhibée partiellement par des anticorps dirigés contre l'éotaxine ou le RANTES, complètement par la combinaison de ces deux anticorps. In vivo, une expression importante d'éotaxine a été démontrée, au niveau protéique, dans les muscle lisse des voies aériennes de sujets asthmatiques.

En conclusion, cette étude suggère que les CML des voies aériennes pourraient contribuer à l'inflammation observée dans l'asthme, à travers la synthèse et la sécrétion d'éotaxine.
PREFACE

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"Candidates have the option of including, as part of the thesis, the text of one of more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must written in such a way that it is more than a mere collection manuscripts; in other words, results of a series of papers must be integrated.

This thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: a table of contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored paper."
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Papers


Abstracts


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHR</td>
<td>airway hyperresponsiveness</td>
</tr>
<tr>
<td>ASM</td>
<td>airway smooth muscle</td>
</tr>
<tr>
<td>B/TSMC</td>
<td>bronchial/tracheal smooth muscle cells</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille Calmette-Guerin</td>
</tr>
<tr>
<td>EAR</td>
<td>early airway response</td>
</tr>
<tr>
<td>ECP</td>
<td>eosinophil cationic protein</td>
</tr>
<tr>
<td>ED&lt;sub&gt;50&lt;/sub&gt;</td>
<td>effective dose for a 50% maximal response</td>
</tr>
<tr>
<td>EDN</td>
<td>eosinophil-derived neurotoxin</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPO</td>
<td>eosinophil peroxidase</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>γIRE</td>
<td>IFN-γ response element</td>
</tr>
<tr>
<td>GlyCAM</td>
<td>glycan-bearing adhesion molecule-1</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte/macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GRE</td>
<td>glucocorticoid response element</td>
</tr>
<tr>
<td>GRO-α</td>
<td>growth related gene-α</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LAR</td>
<td>late airway response</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>leukotriene</td>
</tr>
<tr>
<td>MadCAM-1</td>
<td>mucosal addressin cell adhesion molecule-1</td>
</tr>
<tr>
<td>MBP</td>
<td>major basic protein</td>
</tr>
<tr>
<td>MCP</td>
<td>monocyte chemotactic protein</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet activating factor</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PF4</td>
<td>platelet factor 4</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated on activation, normal T expressed and secreted</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TSMC</td>
<td>tracheal smooth muscle cells</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VLA-4</td>
<td>very late antigen-4</td>
</tr>
</tbody>
</table>
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1.1 ASTHMA

1.1.1 Definition

Osler described asthma as “... [a disease] associated with an exudation, such as might be supposed to come from a turgescent mucosa and which is of very characteristic and peculiar character” (1). Over a century after the publication of his famous textbook, a clear definition of asthma – one capable of satisfying the generalist, specialist, and researcher – has continued to elude us. The difficulty arises because “asthma” represents a spectrum of clinical and pathophysiological features more than a single abnormality. Many of these features can range from undetectable to severe, and there is often considerable variation in such parameters within the normal population (2-5).

Current definitions of asthma evoke three main features in its pathophysiology (2-5): 1. reversible airway obstruction, 2. airway hyperresponsiveness (AHR), and 3. airway inflammation. According to the International Consensus Report on the Diagnosis and Management of Asthma:

Asthma is a chronic inflammatory disorder of the airways in which many cells play a role, in particular mast cells and eosinophils. In susceptible individuals, this inflammation causes symptoms that are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment and causes an associated increase in airway responsiveness to a variety of stimuli (4).

This definition is useful because it incorporates the two features of asthma that are most often targeted by therapeutic interventions, namely its inflammatory nature and its potential reversibility. However, asthma can still be characterized in a number of different ways (6-9), including clinical (wheeze and/or cough; nocturnal symptoms; history of immediate symptoms upon exposure to cold air, exercise, or irritants), physiological (reversibility of airway obstruction, airway hyperresponsiveness), pathological (airway eosinophilia), and in some patients, immunological (positive skin prick tests, elevated serum immunoglobulin (Ig) E levels).

Airway hyperresponsiveness (AHR) was identified as a feature of asthma as long as 50 years ago and provocation tests with substances that induce bronchoconstriction,
although not routinely used in clinical practice, have been an important research tool (7,8). AHR was previously considered to be the fundamental mechanism of asthma (10). However, the advent of fiberoptic bronchoscopy in the 1980s clarified the inflammatory nature of asthma and gave way to the widely held but as yet unproved notion that AHR is a consequence of airway inflammation rather than a primary process (10-12).

Despite general agreement about the relationship between airway inflammation, airway hyperresponsiveness, and reversible airway obstruction, a number of factors complicate the definition of asthma. In particular, chronic uncontrolled asthma is associated with structural changes in the airway wall which lead to a progressive reduction in the reversibility of airflow obstruction (2-5). Thus, patients with severe chronic asthma may show little or no reversibility of airflow obstruction. In addition, airway hyperresponsiveness and airway inflammation are by no means specific for asthma (13,14).

1.1.2 Epidemiology

Asthma has a substantial impact on health and quality of life (15). The total prevalence of asthma is estimated to be 7.2% of the world population, or approximately 100 million individuals, with a prevalence of about 6% in adults and 10% in children. At least 40,000 deaths per year worldwide may be attributed to asthma (16).

Several recent reports have shown that the prevalence of asthma is increasing (16-18). Studies in a number of countries have supported this increase, showing increased self-reporting of asthma, an increase in the number of episodes of asthma requiring a medical consultation, and a progressive increase in the number of prescriptions for asthma therapy (19-24). For example, in Australian childhood studies, the percentage of children ever diagnosed with asthma rose from 12.9% to 29.7% between 1982 and 1992 (25,26). A greater awareness of asthma amongst physicians and patients is unlikely to be the sole explanation for the increase in reported evidence, as the prevalence of wheezing and shortness of breath have also increased (24,25).

1.1.3 Etiology

The increased prevalence of asthma may result from a combination of genetic and environmental influences. Asthma is highly correlated with atopy, and there has been a
recent unexplained increase in the prevalence of atopy in many populations (27,28). Some studies suggest that bronchial hyperresponsiveness and a major locus controlling IgE production are both located on chromosome 5q31-q33 (29). Even in the presence of an inherited disposition, many other factors also appear to affect the development of atopy and its effects in the individual patient. For example, genetic effects on the fetal immune response may be influenced by maternal smoking, nutrition, and disease (30,31).

Other environmental factors have been suggested to influence the development of atopy and/or its manifestation as asthma. These include increased exposure to indoor allergens, especially house dust mite antigens (32). A rise in exposure to cockroach antigens has been implicated in some urban outbreaks of asthma (33), while other cases have cited air pollution as the cause (34). Although atmospheric pollution can trigger exacerbations in patients with established asthma, there is no evidence that pollution can induce asthma in a previously non-asthmatic individual. For example, a comparison between highly polluted Leipzig and less polluted Munich showed a markedly higher prevalence of asthma in Munich (35).

It is possible that respiratory infections in early childhood may induce asthma, but there is also evidence that early exposure to some childhood infections may protect against atopy and asthma (25,27,28,36). A number of studies have revealed that the increase in atopic disposition inversely correlated with a steady decline in the extent to which the population is exposed to major human diseases, such as tuberculosis, measles, whooping cough, and influenza (28,36,37). A common feature of these diseases is that they are associated with a Th1-like immune response (see section 1.2.3). Because Th1-type cytokines are suppressors of pro-allergic Th2 activity, the lack of frequent exposure to infections during development has been speculated to increase the risk of developing atopy. Support for this hypothesis comes from a recent study demonstrating an inverse correlation between atopy and infection with, or exposure to, Mycobacterium tuberculosis (37). This has been substantiated in a murine model of asthma in which intranasal Mycobacterium bovis-Bacillus Calmette Guerin (BCG) infection effectively inhibited allergen-induced pulmonary eosinophilia in large part through a Th1-mediated mechanism (38). Notably however, this BCG-induced protection began to subside within
12 weeks. Other studies have shown that infection of mice with respiratory syncytial virus, which also induces Th1 cytokines, actually increases airway responsiveness and sensitization to allergen (39).

1.1.4 Pathology

Originally, most information on the pathology of asthma was derived from post-mortem studies of patients who had died from asthma and thus had severe disease (40-43). At post-mortem, the medium-sized and small airways are usually occluded by tenacious plugs of exudate and secretions containing eosinophils and epithelial cells. Histologically, the bronchial wall shows regions of denuded epithelium, epithelial regeneration, generalized gross edema, and severe submucosal inflammation with an eosinophilic infiltrate. Macroscopically, the lungs from fatal cases of asthma often show sustained hyperinflation caused by air trapping due to widespread plugging of the airways. In contrast to the appearance of emphysema, there is no destruction of the alveolar walls (1,40-43).

More recently, bronchial biopsy samples have been obtained from patients with asthma of varying grades of severity. Microscopic studies of biopsy samples have revealed the presence of changes in asthma of all grades of severity, including recently diagnosed and mild asthma (44-47). In the early stages of asthma, the inflammatory processes are potentially reversible since no permanent structural changes are discernible (10,48-51). In addition to airway inflammation, edema of the airway wall, shedding of the airway epithelium, and mucus plugging of the airway lumen may found in relatively early stages of asthma (52). Although the structure of the material occluding the airways is referred to as mucus plugs, these plugs consist of an inflammatory exudate that is made up of plasma proteins, epithelial cells that have sloughed from the airway surface, and inflammatory cells, particularly eosinophils. Only a small amount of material filling the lumen is actually mucus (52). These processes may also be associated with severe symptoms but are potentially reversible with anti-inflammatory treatment (48-51).

Chronic asthma is associated with less reversible structural changes, particularly if the disease is left untreated (10,48,52). A relatively early finding is the apparent thickening of the reticular layer of the epithelial basement membrane which may be due to
an increase in the amount of type IV collagen produced by airway epithelial cells that proliferate to replace those shed into the lumen (52). Other pathological features of chronic asthma include goblet cell hyperplasia, new vessel formation and vasodilatation in the smooth muscle layer, hyperplasia and hypertrophy of the airway smooth muscle, mucous gland hypertrophy, and ultimately the development of irreversible fibrosis (10,48,52).

1.1.5 Mechanisms

Many asthmatic subjects develop symptoms in response to allergens such as house dust mites or pollen (53). As such, asthma is usually regarded as an allergic condition. Often however, there are no obvious underlying allergies, particularly in adult patients (10). The recognition that many provoking factors could be involved in the development of asthma symptoms has led to a conceptual model that divides these factors into two main groups: inducers and triggers (53-56). Inducers of asthma include genetic factors, allergies, infections, and other factors related to occupational background or environmental influences. They are believed to act mainly by inducing airway inflammation which could lead to AHR and asthma symptoms. Triggers of asthma are those factors that may cause bronchospasm and asthma symptoms superimposed on a background of pre-existing airway inflammation and airway hyperresponsiveness. These include a wide range of stimuli such as exercise, cold air, irritants, smoke, pollutants, aspirin, and other inhaled or ingested substances.

The “traditional” view of asthma, which was widely held until recently, implicated mast cells as the principal orchestrator of the asthmatic process (10,57-59). According to this view, inhaled allergen specifically interacts with specific IgE bound to cell surface receptors on mast cells. This interaction leads to the release of mediators, such as histamine and leukotrienes, which then act on receptors on airway smooth muscle cells and lead to bronchoconstriction. Although this mechanism may account for some of the immediate features of acute asthma, it is now clear that IgE-dependent mast cell degranulation cannot explain most of the recognized features of chronic, established asthma (10,57-59).
The current view of asthma regards it as a complex condition involving many inflammatory cells which release a panoply of mediators (10,57-59). These mediators, in turn, may then act on a variety of target cells in a self-sustaining cascade that is amplified upon exposure to certain stimuli. These events culminate in smooth muscle contraction, mucus hypersecretion, plasma leakage, edema, and activation of cholinergic reflexes and sensory nerves which can lead to amplification of the ongoing inflammatory response. As mentioned earlier, chronic inflammation also leads to structural changes, such as goblet cell hyperplasia, subepithelial fibrosis, and smooth muscle hypertrophy and hyperplasia (10,48,52).

At the cellular level, the mechanisms of asthma seem to be similar regardless of its cause (60). However, the existence of a subgroup of asthmatic subjects that are not atopic by current conventions has led to the suggestion that asthma may be divided clinically into atopic and non-atopic variants (61). There does not appear to be any fundamental difference between allergic and non-allergic asthma; both involve a combination of inflammation and airway hyperresponsiveness, and both are potentially susceptible to the same pharmacological approaches (61). Moreover, there is some evidence that atopic and non-atopic asthma have similar pathologic mechanisms. For example, increased numbers of activated T cells, eosinophils, high affinity IgE receptor bearing-cells, interleukin (IL)-4 mRNA+ cells, and IL-5 mRNA+ cells are features in bronchial biopsies from both atopic and non-atopic asthmatics (60,62). A recent epidemiological study showed that the incidence of self-reported asthma correlated with total serum IgE concentrations irrespective of atopic status (63). Serum IgE concentrations were also shown to decline with age, consistent with the negative allergen skin test reactivity of non-atopic asthmatics who tend to be older. The molecular immunopathology of atopic and non-atopic asthma is still not clear however, and other studies have shown marked differences between atopic and non-atopic asthmatics in, for example, the numbers of cells in the bronchial mucosa expressing granulocyte/macrophage colony-stimulating factor (GM-CSF) and its receptor (64). Whether these differences are a function of age is not known at present.

1.1.6 Early and late reactions to allergen challenge
Atopic asthmatic patients can be exposed to a single large dose of allergen in an experimental setting (65). This results in an immediate transient decline in lung function as measured by forced expiratory volume in 1 second (FEV$_1$) or the peak expiratory flow. The decline is commonly followed by a late-phase reaction (late airway response, LAR) within 6 or more hours. The immediate or early reaction (early airway response, EAR) is usually reversible by inhaled bronchodilator therapy, but the late reaction is less reversible (10,65). The symptoms of asthma may persist for several days with daily fluctuations in FEV$_1$ values, particularly in the early morning. Over the same period in the late response, there is an increase in airway responsiveness. While mast cells are believed to have a predominant role in the immediate response to allergen challenge, other inflammatory cells (particularly eosinophils) have been implicated in the prolonged late reaction (10,55-59,65).

1.2 INFLAMMATORY CELLS IN ASTHMA

1.2.1 Mast cells

Some studies have reported that the numbers of mast cells in the bronchoalveolar lavage (BAL) of asthmatics are increased compared to controls (66). These findings have not been universal however, and mast cell numbers in bronchial biopsies from asthmatics are unchanged from control values (67-69). Although their numbers may not be increased, histamine release from BAL mast cells is enhanced in asthmatic patients (66,70). In addition, mast cell mediators have been detected in BAL from asthmatics during allergen-induced EARs (70).

Electron microscopic and ultrastructural studies have provided evidence of allergen-induced mast cell degranulation in asthmatic airways (44,48,50,51). The measurement of mediators after allergen challenge in blood and BAL has suggested that the EAR is predominantly due to effects of histamine, prostaglandin (PG) D$_2$, and the cysteinyl leukotriene (LT) C$_4$, LTD$_4$, and LTE$_4$, the latter two being generated from LTC$_4$ extracellulary (10,65). Attenuation of the EAR can be achieved following administration of specific antagonists to the receptors for these compounds (71,72).
The wide range of vasoactive and spasmogenic mediators released during the EAR is believed to originate in large part from mast cells resident in the airway mucosa. When IgE bound to the high affinity IgE receptors on mast cells is cross-linked by allergen, a series of membrane and cytoplasmic events result in the secretion of preformed granule-derived mediators (histamine, heparin, tryptase, and chymase) and the synthesis and release of newly formed lipid products (prostaglandin D$_2$, leukotriene C$_4$, and platelet activating factor) (10,54-59,65,73-75). These mediators may elicit a number of functions including smooth muscle contraction, vasopermeability, increased airway reactivity, airway inflammation, and a further increase in mediator release (54-59,65). For example, tryptase can generate C3 and bradykinin from their protein precursors, and these mediators may act in turn as secondary mediators of smooth muscle contraction and vascular permeability (76).

The observation that sodium cromoglycate, an inhibitor of mast cell degranulation, completely abolishes the early reaction supports the importance of the mast cell in this response (77). Th2-type cytokines (see section 1.2.3) have been strongly implicated in the pathogenesis of the late response and, although mast cells have the ability to synthesize, store, and secrete these cytokines, current evidence suggests that their contribution to the overall cytokine production in the lung is relatively minor (10,54-59,65).

1.2.2 Macrophages

Macrophage numbers have been shown to be increased in baseline asthma and further elevated at 48 h after allergen challenge (78,79). Alveolar macrophages of asthmatic patients can release tissue-damaging mediators including eicosanoids, superoxide anions, lysosomal enzymes, and proinflammatory cytokines by IgE-dependent and independent mechanisms (80-83). These cells are also a prominent source of GM-CSF, tumor necrosis factor (TNF)-α, and IL-1 in asthmatic airways, and macrophages isolated from asthmatic subjects spontaneously produce increased concentrations of these cytokines in vitro (84-86). Macrophages have been suggested to act as antigen-presenting cells in the lungs (87), but conclusive evidence for such a role is lacking.
Likewise, some investigators have suggested that macrophages have a suppressive effect on T cells (88), but there is little evidence that this function is impaired in asthma.

1.2.3 T lymphocytes

By recognizing foreign antigens through their cognate receptors, T lymphocytes play a fundamental role in the initiation and regulation of inflammatory responses. There is now substantial evidence that T lymphocytes orchestrate immune responses largely through the secretion of cytokines, and that the combination of cytokines secreted during the course of an inflammatory response is the critical determinant in the type of inflammatory reaction that results. Initial studies of mouse CD4+ T lymphocyte clones revealed that these could be divided into two basic functional subsets termed Th1 and Th2 (89). Th1 T lymphocytes were characterized by the predominant secretion of IL-2, interferon (IFN)-γ, and TNF-β, while Th2 cells characteristically secreted IL-4, IL-5, IL-6, and IL-10. Other cytokines, such as TNF-α, IL-3, and GM-CSF, were produced by both Th1 and Th2 cell subsets. Seminal work in murine models of intracellular and extracellular infections showed the differing patterns of cytokine secretion by CD4+ T lymphocytes had a profound effect on the activation of distinct immune effector functions (90,91). In general, a predominance of Th1-type cytokines is crucial for the onset of delayed type hypersensitivity reactions, cell mediated responses, and clearance of intracellular pathogens, while Th2-type cytokines favor antibody production, tissue eosinophilia, and productive immune responses to extracellular or helminthic pathogens (92,93). T lymphocyte clones secreting cytokines characteristic of both Th1 and Th2 cells were designated Th0 cells (89,91).

In patients with atopic dermatitis, T lymphocyte clones specific for house dust mite raised from peripheral blood cells secreted IL-4 and not IFN-γ, whereas all clones specific for Candida albicans or tetanus toxoid raised from the same donors secreted an excess of IFN-γ and relatively little IL-4 (94). These and other early studies suggested the existence of distinct Th1 and Th2 subsets in human T lymphocytes. However, an important distinction emerged: polarization of T cell clones was initially studied by enzyme-linked immunosorbent assay (ELISA) of supernatants from bulk cultures of individual clones. This made it impossible to distinguish whether a rise in a certain group
of cytokines was due to a preferential expansion of discrete populations of Th1 or Th2 cells or a shift in the cytokine secretion by the whole population to one extreme along a continuous distribution. Subsequent work using flow cytometry showed a more stochastic acquisition of cytokine profile in clones and primary CD4+ T cells at the single cell level, confirming that cytokine expression is continuously distributed in individual T cells (95,96). This data argued against the existence of distinct "lineages" of Th1- or Th2-differentiated T cells, supporting instead a model in which polarization of a T cell response towards Th1 or Th2 is achieved by shifting the distribution of cytokine titers of the whole population rather than altering the ratio between distinct subsets (96).

Studies have shown an increased number of activated (IL-2 receptor+) T lymphocytes in the blood and airway walls of asthmatics compared to normal controls (97-100). Activated T cells were shown to correlate with AHR, asthma symptom severity, and the numbers of eosinophils in the BAL (101-103). A large body of work has characterized the cytokine profile in asthmatic lungs as predominantly Th2-like. In particular, IL-4 and IL-5 production has been shown to be strikingly elevated in bronchial biopsies and BAL from asthmatics, and T cells have been identified as the predominant source of these cytokines (103-107). Several lines of evidence strongly suggest a pivotal role for IL-4 and IL-5 in the pathogenesis of asthma, including: 1) IL-4 and IL-5 expression increase in parallel with allergen-induced exacerbations of asthma (104), 2) the treatment-related remission of asthma symptoms is associated with an abrogation of IL-4 and IL-5 expression (108), and 3) the selective blockade of IL-4 or IL-5 in animal models of asthma attenuates allergen-induced LARs and airway inflammation (109,110).

IL-4 and IL-5 are thought to elicit their effects through a number of pathways. IL-4 favors the initiation and propagation of a Th2-type cytokine response and counterregulates the development of inhibitory Th1 responses (91). IL-4 also stimulates IgE isotype switching and production (111), upregulates endothelial cell expression of vascular cell adhesion molecule (VCAM)-1 which may be important in eosinophil recruitment (112), and stimulates mucus hypersecretion (113). IL-5 has essential effects on the biology of eosinophils not limited to differentiation, activation, and survival (114-116). In a recent study examining the expression of IL-5 receptor isoforms in asthmatics,
mRNA expression of the membrane-bound type of IL-5 receptor correlated inversely with lung function while expression of mRNA for the soluble receptor, a natural IL-5 receptor antagonist, correlated directly with lung function (117).

The importance of T cells in asthma has been further demonstrated in animal models showing that allergen-induced LARs could be conferred upon non-sensitized rats with the adoptive transfer of CD4+ T cells from sensitized donors (118). More recent studies have shown that a single intravenous infusion of an anti-CD4 monoclonal antibody in severe asthmatics significantly improves lung function (119).

1.2.4 Eosinophils

Circumstantial evidence linking eosinophils and asthma began to accumulate when eosinophilic infiltration of the airways was found to be a regular feature of fatal asthma and of mucosal biopsies obtained from asthmatic patients (40-45). Asthma is now well known to be associated with an elevated eosinophil count in the lungs (120). In contrast, eosinophils are rarely seen in BAL and biopsies of normal subjects. Numbers of eosinophils are dramatically increased in the blood, BAL, sputum, and bronchial biopsies from asthmatic subjects compared to normal controls and are further elevated in asthmatic airways after allergen provocation (98-105,120-122). Increased eosinophil counts in the airways of asthmatics have been correlated with decreases in specific airway conductance, FEV1, and maximum mid-expiratory flow rate, and with increases in bronchial reactivity to histamine and clinical severity scores (97,98,101-105). A role for eosinophils in these processes is supported by efficacy of glucocorticosteroids in reducing airway eosinophilia in parallel with relieving asthma symptoms (108).

Studies in the airways of living asthmatics have been limited to the information provided by mucosal biopsies of the major airways and BAL samples. However, measurements of bronchial pressure and flow, in addition to computational modeling, have established the peripheral airways as an important site of airway obstruction and airway hyperresponsiveness in asthma (123-125). A recent study showed that eosinophilic inflammation in asthma is not limited to the major airways; numbers of total and activated eosinophils are dramatically increased in the small airways of asthmatics compared to controls (126). Interestingly, eosinophil numbers per mm² were found to be
significantly higher in the region between the smooth muscle layer and the adjacent parenchyma compared to the region between the smooth muscle and the epithelium. Therefore, although the presence of eosinophils in asthmatic airways is well recognized, the extent to which these cells are increased cannot be fully appreciated by studies of mucosal biopsies or BAL.

The effector role of the eosinophil in the pathogenesis of asthma may be mediated in part by the release of eosinophil-specific granule proteins. The four principal basic proteins in the secondary granules of the eosinophil are major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) (127,128). Immunohistochemical detection of secreted MBP and ECP at sites of bronchial epithelial cell damage have provided evidence of \textit{in vivo} eosinophil degranulation (129,130). Extracellular MBP and ECP can also be detected in mucus plugs and in necrotic areas beneath the basement membrane in the lung tissue of fatal asthmatics (52). Furthermore, MBP and ECP are detected in BAL fluid of asthmatics at increased concentrations after antigen challenge (131).

MBP is highly cationic, inducing bronchoconstriction and a dose-related increase in AHR when instilled into the airways of rats (132-134). A number of mechanisms have been proposed to account for the increase of bronchial responsiveness by MBP (134). Studies in intact animals and in tracheal explants have led to the hypothesis that MBP exerts its effects either by causing detachment of the epithelium allowing greater entry of agonist in the tissue, or by altering the epithelial function such that increased concentrations of a bronchoconstrictor or decreased concentrations of a bronchodilator are produced (132,133,135). In either case, the ability of MBP to induce AHR can be mimicked by other polycationic proteins such poly-L-arginine and abrogated with polyanionic polymers that neutralize charge (133). MBP may also act as an allosteric inhibitor of M2-muscarinic receptors which are involved in feedback inhibition of vagally-mediated bronchoconstriction (136). ECP can inhibit proteoglycan degradation in fibroblasts, stimulate mucus secretion by airway epithelial cells, and induce basophil histamine release (137-140). Intratracheal instillation of ECP produces epithelial injury in the bronchial tree of rabbits (127,128). A possible mechanism for the cytotoxic action of
ECP is through pore-formation, although this has not been definitively proven (141). MBP, ECP, EPO, and EDN are all capable of stimulating histamine release from mast cells and more variably basophils (127,128,140).

Eosinophils, in addition to mast cells, basophils, and monocytes, are a potential source of leukotrienes in asthmatic lungs (10). Leukotrienes cause bronchoconstriction, mucosal edema, mucus secretion, and BHR (142-145). Inhaled LTC4 and LTD4 are approximately 6000 times more potent than histamine and elicit a more sustained response in asthmatics (144,145). Eosinophils preferentially elaborate LTC4, particularly when stimulated by calcium ionophore, immunoglobulins, and platelet activating factor (PAF) (146). PAF can also be produced by eosinophils (127,128,147). In addition to activating eosinophils, the activities of PAF include the ability to: stimulate chemotaxis of eosinophils and neutrophils, increase airway vascular permeability and promote mucosal edema, inhibit the apoptosis of B cells, induce bronchoconstriction, and increase airway responsiveness (148,149). The application of PAF-activated eosinophils to explanted human bronchi causes contraction, in part through the elaboration of the 5-lipoxygenase pathway (resulting in LTC4 generation) in eosinophils (150).

Eosinophils are also a source of a large number of cytokines, such as IL-1 and TNF-α which, in addition to their potent proinflammatory effects, can induce bronchoconstriction, increase airway responsiveness, and increase vascular permeability (151). Eosinophils may also be an important source of IL-6 and transforming growth factor (TGF)-β, which are profibrotic and immunomodulatory cytokines. In most cases however, the contribution of eosinophils to cytokine-expression in asthmatic lungs is relatively small (107).

1.3 MECHANISMS OF EOSINOPHIL RECRUITMENT IN ASTHMA

Recruitment of eosinophils to asthmatic lungs is considered to occur in a number of steps which include: 1. hematopoiesis, 2. mobilization in the blood, 3. adhesion to vascular endothelium, and 4. extravasation and chemotaxis to inflammatory foci (152). The third step can be further subdivided into margination, tethering, rolling, activation, and firm adhesion (153). In the sequence of events that culminates in tissue eosinophilia,
each step is regulated by complex molecular pathways involving cytokines, chemokines, adhesion molecules, and interactions with other cells. These processes are only beginning to be elucidated.

1.3.1 Role of cytokines.

Normally, eosinophils comprise only 1 to 2% of blood leukocytes in humans (127,128). In spite of this, high numbers of eosinophils are able to accumulate at sites of allergic inflammation. This suggests the existence of mechanisms to acutely increase blood levels of eosinophils. Clinical and experimental evidence indicates that the activation of specific hematopoietic pathways in the bone marrow may contribute to asthma through the increased production of eosinophils and their release in the peripheral blood (154,155). In the bone marrow, mature eosinophils are formed from myeloid progenitors under the influence of IL-3, IL-5, and GM-CSF (127,128,147). While IL-3 and GM-CSF appear to be involved in relatively early stages of megakaryocyte, basophil colony forming unit (CFU), granulocyte-macrophage CFU, and eosinophil CFU production, IL-5 is specific for later stages of eosinophil differentiation. In mild asthmatics exposed to allergen, an increased proportion of progenitor cells in the bone marrow were shown to express the α subunit of IL-5 receptor compared to pre-challenge bone marrow (156). This increase occurred preferentially in “dual responder” asthmatics who develop late bronchoconstrictor responses, AHR, and airway and blood eosinophilia 24 h after allergen inhalation. Although it not known at present whether this is accompanied by elevated bone marrow IL-5 expression in humans, pulmonary allergen challenge of sensitized mice is associated with an increase in IL-5 expression by T cells in the bone marrow (157). Collectively, the information available to date suggests that the generation of an unidentified serum hematopoietic factor during airway allergen challenge can prime the bone marrow to produce increased numbers of eosinophil-committed (i.e. IL-5-responsive) progenitors. Concomitantly, T cells in the bone marrow may express elevated levels of IL-5 which, presumably, results in the terminal differentiation of eosinophils in situ and their subsequent release in the blood. The release of lineage-committed eosinophil progenitor cells for terminal differentiation in the peripheral blood or at distal sites including the lungs may also contribute to the airway eosinophilia
associated with asthma (158). However, the relative contribution of progenitors to the high degree of eosinophilia in asthmatic lungs has barely been explored.

In the inflamed pulmonary vasculature, circulating eosinophils released from the bone marrow undergo margination, a process in which they move from the center to the periphery of the blood vessel. The initial contact of marginating eosinophils with the endothelium manifests as a tethering and rolling motion along the length of the vessel. Next, rolling eosinophils are activated to firmly adhere to the endothelium. Firmly adherent eosinophils may then emigrate out of the vasculature. Each of these events is sequential inasmuch as inhibition of tethering and rolling or activation blocks further adhesion and migration (159,160). In addition, each event is coordinately regulated by specific adhesion molecules and cytokines.

Tethering and rolling of eosinophils is mediated by the interaction of L-selectin and other adhesion molecules on the surface of eosinophils with diverse structures on the endothelium such as glycan-bearing cell adhesion molecule-1 (GlyCAM-1), mucosal addressin cell adhesion molecule-1 (MadCAM-1), CD34, P-selectin, and/or E-selectin (152,159). A recent study of a murine late phase allergic response showed that P-selectin underlies all of the antigen-induced rolling after a brief transient contribution from E-selectin (161). The subsequent induction of firm adhesion is dependent on the activation of eosinophils by locally generated chemokines (see section 1.3.2), the shedding of L-selectin, and the expression of endothelial adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and VCAM-1 (152,159). VCAM-1 has been suggested to be particularly important since circulating eosinophils but not neutrophils constitutively express very late antigen-4 (VLA-4), the ligand for VCAM-1 (112).

Cytokines expressed in asthmatic airways may act at multiple points in the cascade of events leading to eosinophil infiltration. IL-5, in addition to stimulating eosinophil hematopoiesis, increases both the adhesive properties of eosinophils and their responsiveness to chemokines (162). An upregulation of VCAM-1 expression on endothelial cells may be achieved by the actions of TNF-α, IL-1, or IL-4 (112,163,164).

1.3.2 Role of chemokines
The migration of eosinophils from the circulation into the tissue necessitates a specific mode of activation; not only must eosinophils express adhesion molecules that reversibly interact with counter-receptors on the surface of endothelial cells, but they must remodel their cytoskeleton to flatten against the vessel wall and subsequently alter their morphology to transmigrate through the vessel wall (152,153,159). This migration requires eosinophils to detect changes in the concentration of signals in the microenvironment to achieve directionality of movement. Chemokines appear to fit into this general paradigm in a number of ways. It has been shown that several members of the chemokine family can be immobilized on the luminal surface of the endothelium through cell surface proteoglycans (165). This immobilization allows chemokines to be "presented" to the marginating eosinophils. Chemokines also regulate the expression of adhesion molecules on eosinophils; for example, RANTES (short for "regulated on activation, normal T expressed and secreted) causes eosinophil upregulation of CD11b which, when complexed to CD18, can mediate adherence to ICAM-1 (166). Chemokine-induced expression of adhesion molecules is generally rapid, resulting from the membrane translocation of protein from preformed intracellular stores (167). Following interaction with the specific receptors on the eosinophils, chemokines may also induce actin reorganization in the cytoskeleton that allows the cells to flatten and attain cellular polarization (168). In the extravascular space, eosinophils again utilize chemokines as signals that guide them to appropriate regions of the tissue.

1.3.2.1 Classification and generalized function of chemokines

Chemokines represent a unique family of chemotactic cytokines. In excess of 40 chemokines have been identified to date, and these are divided into four families (figure 1) – α (CXC), β (CC), δ (CX3C), and γ (C) – based on the presence and position of conserved cysteine residues (168-170).
### CHEMOKINES

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**Figure 1. Chemokine families.** Chemokines are subdivided into one of four families based on the position of the first two cysteines in a classical four cysteine motif. Chemokines in which only two cysteines are present are represented by the γ family. Some examples of chemokines in each family are shown. Leukocyte subsets attracted by a given family of chemokines are indicated in italics.

In members of the α family, the first two cysteines are separated by another amino acid, while in those of the β family they are placed next to each other. Only two members of the γ family have been identified thus far and both contain one instead of two cysteines in their N-terminus. The δ chemokines comprise the most recently described chemokine family, containing two N-terminal cysteines separated by three amino acids. In the α, β, and δ families, two disulphide bonds are present between the first and third, and the second and forth cysteine residues, respectively (figure 2).
The three dimensional structures of three α chemokines – IL-8, growth related gene-α (GRO-α), and platelet factor (PF4) – and three β chemokines – macrophage inflammatory protein (MIP)-1β, RANTES, and monocyte chemoattractant protein (MCP)-1 – have been solved by multidimensional nuclear magnetic resonance or X-ray crystallography (169). The structures of all of the monomers are similar as expected from the significant degree of sequence identity. Although the quaternary structure of the monomers is fairly well conserved across the chemokine families, the quaternary structures of α and β chemokine dimers are entirely distinct as the dimer interface is formed by a different set of residues. Whereas the IL-8 dimer is globular, the MIP-1β dimer is elongated and cylindrical.
A striking feature of α and β chemokines is their redundancy in receptor binding (171,172). As shown in table I, most chemokine receptors bind multiple ligands within the same family. The differences in quaternary structures between α and β chemokines provide an elegant explanation for the lack of receptor cross-binding between the two subfamilies.

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<th>Chemokine Receptor</th>
<th>IL-8</th>
<th>GROα</th>
<th>NAP-2</th>
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Table I. Chemokine receptors and their ligands. Examples of chemokine receptors and their ligands (+) are shown. CC and CXC chemokines do not share receptors (shaded areas). ND, not done.

While the activity of α chemokines tends to be restricted to neutrophils and lymphocytes, β chemokines are more versatile and act on lymphocytes, monocytes, eosinophils, and basophils (168-170). The α family of chemokines is further subdivided based on the presence or absence of a glutamic acid-leucine-arginine (GLN) sequence near the N terminal region (upstream of the CXC sequence, figure 2 hatched boxes). α chemokines containing the N-terminal GLN are chemotactic for neutrophils, whereas those without the sequence act on lymphocytes (170). Similar to the α chemokines, the N-terminal amino acids preceding the CC residues of β chemokines are critical components in their cell selectivity. For example, the addition of a single amino acid residue at the amino terminal of MCP-1 reduces its biologic activity on monocytes up to 1000 times, and the deletion of a single amino acid converts it from a basophil activator to an eosinophil chemoattractant (173-174). Interestingly, the activity of certain chemokines may be modulated after secretion by the action of peptidases, such as CD26, at the N-terminus. Oravecz et al. recently showed that a truncated form of RANTES generated by CD26 dipeptidase activity lost the capacity of native RANTES to increase cytosolic calcium in monocytes, but retained this activity in colony-stimulating factor-
activated macrophages (175). Other modifications and associations can also alter the biologic activity of chemokines; for example, IL-8 can be converted to a strong eosinophil chemoattractant by associating with the secretory component of IgA (176).

1.3.2.2 Eosinophil-active chemokines and their receptors

The CC chemokines active on eosinophils include RANTES, MIP-1α, MCP-2, MCP-3, MCP-4, eotaxin, and eotaxin-2 (168-170). MIP-1α and MCP-2 are relatively weak eosinophil chemoattractants, while MCP-3, MCP-4, RANTES, and eotaxin are among the most potent with a rank-order of: eotaxin > MCP-3 = MCP-4 = RANTES > MCP-2 = MIP-1α (168,170). The chemoattractant activity of most eosinophil-active chemokines is substantially higher than the activity of conventional chemoattractants. For example, RANTES attracts eosinophils with an effective dose causing a 50% maximal response (ED$_{50}$) of $10^{-8}$ M, whereas the ED$_{50}$ for PAF or CsA is $10^{-7}$ to $10^{-6}$ M (152). As mentioned above, chemokine activity can be modified by enzymatic cleavage and the presence of binding proteins (175,176). In addition, subtle fluctuations in pH and ionic strength can alter the potency of chemokines (177). For these reasons and because of the heterogeneity of human eosinophils from different donors, studies examining the potency of chemokines must be interpreted with caution.

As shown in Table I, the known receptors for eosinophil-active chemokines include: CCR-1, which binds MIP-1α, RANTES, and MCP-3; CCR-2, which binds MCP-3 and MCP-4; CCR-3, which binds eotaxin, RANTES, MCP-3, and MCP-4; and CCR-4 and CCR-5, which bind MIP-1α and RANTES (171,172,178). In contrast to CCR-1, CCR-2, CCR-4, and CCR-5 which are also expressed on lymphocytes and/or monocytes, CCR-3 is expressed predominantly on eosinophils (170,178). The use of an antagonistic monoclonal antibody against CCR-3 was able to reduce, by over 95%, the chemotactic response of IL-5 primed or unprimed eosinophils to eotaxin, RANTES, MCP-2, MCP-3, and MCP-4 (178). These findings suggest that CCR-3 is responsible for the majority of eosinophil chemotactic responses to these chemokines.

1.3.2.3 Eosinophil-active chemokines in asthma

RANTES and MCP-3 mRNA expression have been shown to be increased in bronchial biopsies from allergic and non-allergic asthmatics compared to atopic and non-
atopic controls, respectively (179). Epithelial expression of these chemokines was not detected in this study, however the majority of the asthmatic patients had recently received inhaled steroids. MCP-4 mRNA and protein were also shown to be highly expressed in asthmatic BAL and biopsies compared to controls, and airway epithelial cells were a major source of this chemokine (180). The concentrations of MIP-1α and RANTES in the BAL fluid were found to be elevated in patients with mild asthma (181). Further, an endobronchial allergen challenge increased the concentrations of these chemokines (182). The eosinophil chemotactic activity in BAL fluid from patients with asthma was substantially reduced with antibodies against RANTES, MCP-3, MCP-4, and eotaxin (181,183).

1.4 EOTAXIN

Eotaxin is a CC chemokine that was originally isolated as the predominant eosinophil chemoattractant in lung lavage fluid of allergen-sensitized and challenged guinea pigs (184). The murine homologue of eotaxin was molecularly cloned subsequently (185), and a mouse eotaxin coding probe was used to clone human eotaxin cDNA (186).

1.4.1 Genomic organization and structure

Human eotaxin has been mapped to chromosome 17q21.1-q21.2, near MCP-1, MCP-2, and MCP-4 at 17q11.2-q12 (187). Shared features in the intron-exon structure of eotaxin and the MCPs have led to the hypothesis that these genes arose from a recent gene duplication (170). Each gene consists of three exons and two introns with the location of the splice sites being conserved.

Translation of human eotaxin cDNA gives rise to a 97 amino acid protein containing a 23-amino acid signal peptide (186). Similar to other β chemokines, eotaxin contains two adjacent cysteines near the N-terminus and two non-adjacent cysteines towards the C-terminus. Alignment of the eotaxin amino acid sequence with those of guinea pig, mouse, and human chemokines reveals 58% identity to guinea pig and mouse eotaxin; 49% and 48% identity to mouse MCP-1 and MCP-3, respectively; and 64%, 62%, 69% and 66% identity to human MCP-1, MCP-2, MCP-3, and MCP-4, respectively
The recently identified eotaxin-2 shares only 40% homology with eotaxin at the amino acid level (189).

A number of structural features of eotaxin distinguish it from the other β chemokines. For example, all the MCP family members have an N-terminal glutamine which is postulated to be critical for activity on monocytes (173). Eotaxin lacks an N-terminal glutamine and, interestingly, deletion of this residue converts MCP-1 to an eosinophil chemoattractant (174,186). Other unique structural features of eotaxin include a two amino acid gap upstream of the first cysteine and the presence of a lysine-lysine-lysine near the C-terminus. The functional significance of these features is not known.

1.4.2 Expression and regulation

Eotaxin mRNA is constitutively expressed at high levels in the normal human small bowel and colon, and to a lesser extent in heart, kidney, pancreas, thymus, spleen, liver, lung, prostate, ovary, placenta, and skeletal muscle (186). No signal was observed by northern analysis in the brain, testes, or peripheral blood leukocytes. By in situ hybridization and immunocytochemistry, constitutive expression of eotaxin mRNA and immunoreactivity has also been shown in normal human bronchial and nasal epithelial cells, as well as in keratinocytes in normal skin (190,191).

Early proinflammatory cytokines such as IL-1 and TNF-α, bacterial products such as lipopolysaccharide (LPS), and viral infection are strong stimuli for eotaxin expression (186,192). IFN-γ and IL-4 can synergize with IL-1 and TNF-α in eotaxin induction in bronchial and alveolar epithelial cell lines and in human umbilical vein endothelial cells (186,192), indicating that eotaxin may be upregulated in both Th1- and Th2-type cytokine predominated responses. In human dermal fibroblasts, IL-4 selectively upregulates eotaxin but not RANTES, MCP-3, MCP-4, or MIP-1α (193). Peripheral blood mononuclear cells, neutrophils, and eosinophils do not constitutively express eotaxin mRNA (186). However, significant levels of eotaxin mRNA were observed in eosinophils cultured for 3 days in IL-3. Preliminary studies have also shown that stimulation of eosinophils with IL-5 induces the expression of eotaxin granule-associated immunoreactivity (194). Untreated HL60 cells (a myelocytic tumor line) or “eosinophil-like” (butyric acid-treated) HL60 cells also do not constitutively express eotaxin mRNA,
nor does the Jurkat T cell line or the RM3 B cell line (186). U937 cells (a monocytic tumor line) in contrast, were found to constitutively express eotaxin mRNA (186). TNF-α and IL-1 induction of eotaxin in epithelial cells is suppressed by the steroid dexamethasone (192), which could be related to the efficacy of these drugs in decreasing the airway eosinophilia associated with asthma (108). Nuclear factor κB (NF-κB), glucocorticoid response element (GRE), γ interferon response element (γIRE), GM-CSF element, Sp1, and E2A binding site motifs are well conserved in both the human and mouse eotaxin promoters (187). The presence of a NF-κB binding site and a GRE in the eotaxin promoter might explain the observed regulation of eotaxin by TNF-α, IL-1, and glucocorticoids. NF-κB is activated after stimulation of cells with LPS, IL-1, TNF-α, and other agents, while GRE mediates glucocorticoid regulation of transcription (195). Deletion of GRE in the IL-8 promoter has been shown to abolish dexamethasone-mediated suppression of IL-8 expression in vitro (196).

1.4.3 Potential functions in allergic inflammation

Eotaxin has a number of potential functions in allergic diseases, including the regulation of eosinophil hematopoiesis, chemoattraction, and activation.

1.4.3.1 Hematopoiesis

A number of recent studies have suggested that the bone marrow may be an important target site for eotaxin. The administration of neutralizing antibodies to eotaxin during pulmonary allergic inflammation in mice caused a decrease in the number of myeloid progenitors in the bone marrow (197). This resulted in the reduced traffic of mature myeloid cells from the bone marrow to the blood. Conversely, in vivo administration of eotaxin increased the numbers of myeloid progenitors in the bone marrow. Furthermore, eotaxin acted as an early colony-stimulating factor for murine granulocytes and macrophages in vitro. In human cord blood cells, eotaxin has been shown to synergize with known eosinophilopoietic cytokines in the later stages of eosinophil differentiation (198). Contrasting with human and mice, guinea pig eotaxin does not appear to enhance the differentiation of progenitors but rather triggers the release of mature eosinophils and their progenitors from the bone marrow to the blood (199).
1.4.3.2 Chemoattraction

Eotaxin is a potent eosinophil and basophil chemoattractant with no significant activity on neutrophils, monocytes, macrophages, or lymphocytes (186,200). Recent studies have also shown that eotaxin may selectively attract Th2-type cytokine expressing T cell populations expanded in vitro (201). In mice and guinea pigs, administration of eotaxin intranasally or subcutaneously results in the rapid development of a local infiltrate containing almost exclusively eosinophils (202,203). Importantly, eotaxin-induced eosinophilia in these experiments required high levels of IL-5 achieved by using IL-5 transgenic animals or coadministering recombinant IL-5 intravenously. There is evidence that IL-5 cooperates with eotaxin in promoting tissue eosinophilia by increasing the pool of circulating eosinophils through stimulating eosinophilopoiesis and/or bone marrow release (204). Alternatively, IL-5, a known chemokinetic agent for eosinophils, may prime these cells for enhanced responsiveness to eotaxin (162). Increased responsiveness of human eosinophils to a variety of chemoattractants including eotaxin has been shown after IL-5 stimulation (162,183). Although eotaxin has been demonstrated to act on basophils and Th2 cells in vitro (200,201), all administration studies in guinea pigs, mice, and monkeys have shown a pure eosinophil accumulation (202,203,205-207), though basophils and Th2-type cytokine expressing T cells both lack reliable cell surface markers.

1.4.3.3 Activation

In addition to promoting eosinophil accumulation, eotaxin also elicits a brief cytosolic calcium flux, actin polymerization, CD11b upregulation, increased avidity of integrin adhesion molecules for their ligands, oxidative burst with release of superoxide free radicals, and exocytosis of granule constituents in eosinophils (202,208). Although eotaxin can induce histamine release in cytokine-primed basophils, it’s activity is relatively weak compared to other CC chemokines (200).

1.4.4 Expression in animal models of asthma

Local eotaxin generation increased at 3 h post inhalational challenge in sensitized guinea pigs, paralleling the pulmonary recruitment of eosinophils (192). In mice, eotaxin peaked at 3-6 h after OVA challenge, but remained elevated for at least 48 h (205). Similarly, repeated exposure of animals to intranasal Aspergillus fumigatus antigen
resulted in an approximately 60-fold increase in eotaxin mRNA in the lungs at 18 h after the last antigen dose (210). Recent studies in OVA sensitized and challenged mice rendered genetically deficient in eotaxin revealed a level of BAL eosinophilia that was significantly reduced but not abolished at 18 h post challenge compared to identically-treated wild-type mice (211). Lymphocyte, neutrophil, or macrophage cell counts in the airways did not differ between wild-type and control mice. The effect of the eotaxin deficiency on pulmonary eosinophil numbers was no longer observed at 48 h after antigen challenge. Collectively, these studies suggest that eotaxin is involved in the allergen-induced pulmonary recruitment of eosinophils at a relatively early time point. Other chemoattractants appear to be capable of compensating, to some extent, for the absence of eotaxin.

Maclean et al. (210) investigated the association between T cells, eotaxin, and eosinophils in a murine model of allergic asthma. Treatment of sensitized mice with an anti-CD3 monoclonal antibody prior to allergen challenge inhibited eotaxin but not MIP-1α mRNA expression. The abolition of eotaxin mRNA expression was associated with a significant decrease in eosinophil accumulation in the lungs. The observation that the IgE response and mast cell degranulation after antigen challenge remained unaffected by T cell depletion suggests that IgE-mediated mast cell degranulation is insufficient for the induction of eotaxin and airway eosinophilia in this model. Also, the necessity of T cells for pulmonary eotaxin mRNA expression implicates Th2-type cytokines as possible stimuli for allergen-induced eotaxin upregulation.

1.4.5 Expression in human allergic inflammation

Eotaxin mRNA and immunoreactivity have been shown to be constitutively expressed in normal human nasal and bronchial epithelial cells (183,190). However, this basal expression is markedly increased in the nasal mucosa of allergic and non-allergic subjects with chronic sinusitis (190). Compared to normal controls, subepithelial eotaxin mRNA and immunoreactivity are also significantly increased in the nasal mucosa of chronic sinusitis patients. In the allergic subgroup, eotaxin mRNA and protein expression correlated significantly with numbers of eosinophils in the nasal mucosa. Colocalization studies revealed that the majority of the eotaxin mRNA+ cells in the nasal subepithelium
of non-allergic chronic sinusitis patients were macrophages, with lesser contributions from T cells and eosinophils. In addition, a small proportion of eotaxin mRNA+ cells stained with the anti-mast cell tryptase antibody.

Eotaxin gene expression has also been shown to be significantly increased in the epithelium and submucosa of nasal biopsies from allergic rhinitic subjects 24 h after local allergen but not diluent challenge (190). This allergen-induced increase was paralleled by a selective elevation in eosinophils.

Recent studies have reported substantially higher concentrations of eotaxin in the BAL fluid from stable asthmatics (400-800 pg/ml) compared to normal controls (100-200 pg/ml) (183). In addition, eotaxin mRNA and immunoreactivity were significantly higher in the BAL cells and bronchial biopsies from asthmatics, and colocalization patterns revealed a cellular distribution of eotaxin mRNA that was similar to that observed in nasal inflammation in chronic sinusitis (190). BAL fluid from asthmatics contained chemotactic activity for eosinophils that was inhibited to a greater extent with antibodies against eotaxin than with antibodies against RANTES or MCP-4.

1.5 THE ROLE OF AIRWAY SMOOTH MUSCLE (ASM) IN ASTHMA

There is ample evidence that ASM is a key effector of acute airway narrowing during spontaneous asthma attacks and experimental allergen challenge. The ability to produce symptomatic improvement with the use of bronchodilators and the characteristic airway hyperresponsiveness observed in asthmatic patients indicates that ASM spasm contributes significantly to airway obstruction (10). As alluded to earlier, ASM also contributes to airway wall thickening through hypertrophy and hyperplasia.

1.5.1 ASM hypertrophy and hyperplasia

Whether ASM hypertrophy or hyperplasia predominate in asthmatic airways is not certain. ASM hyperplasia and, to a lesser extent, hypertrophy were recognized as prominent features of asthmatic airways in a number of early post-mortem investigations. Compared with normal airways, these studies reported that the number of smooth muscle cells is increased approximately 2- to 3-fold in asthma (212-214). Recently, advanced morphometric studies in three dimensions have shown that one of two types of
pathological changes may exist, at least in some asthmatics (215,216). In one group, the central airways were associated with marked hyperplasia and some hypertrophy. In the second group, hypertrophy predominated in the whole airway and hyperplasia was only observed in the bronchi. It is not known if these distinct patterns of increased ASM mass have any clinical correlates.

The exact mechanisms involved in ASM growth and/or division in vivo have not been established. However, resident airway cells and those infiltrating the airway tissue of asthmatics are known to produce factors that are mitogenic for rat, guinea pig, canine, bovine, and/or human ASM in vitro (217,218). Potential sources of ASM growth factors include proinflammatory cells (e.g. histamine, 5-hydroxytryptamine, epidermal growth factor, LTD₄, mast cell tryptase, lysosomal hydrolases, thromboxane A₂, IL-1, and TNF-α) and airway epithelial and endothelial cells (e.g. endothelin). Substances present in the blood may also cause ASM mitogenesis (e.g. thrombin, platelet-derived growth factor). A hypertrophic or hyperplastic response has also been suggested to result from mechanical strain or a physical stimulus that would occur with frequent bronchoconstriction and bronchodilatation in the airways of asthmatics. In support of this, premature neonates receiving assisted ventilation showed gross hypertrophy of the airway smooth muscle upon post-mortem examination (219). This hypertrophic response was postulated to result from the increased pressure associated with mechanical ventilation. Similarly, canine airway smooth muscle, when subjected to a stretch-relaxation stimulus, showed an increase in cell number and DNA synthesis (220).

1.5.2 Functional consequences of increased muscle mass

In chronic asthma, the increase in ASM mass may contribute to a more persistent decrease in airway caliber through a "space-occupying" effect (221). However, the extent to which ASM thickening contributes to AHR is the subject of debate. A number of inflammatory cell products are capable of causing ASM hyperreactivity to contractile agonists and/or hyporesponsiveness to relaxants. For example, treatment of human ASM with IL-1 results in decreased responsiveness to β-adrenoceptor-mediated relaxation (222). These and other studies support the idea that AHR can be explained on the basis of increased inflammatory cell products in asthmatic lungs. Nonetheless, ASM could still
have a role in AHR by virtue of its increased mass, which may generate more total force for a given degree of muscle stimulation (223). Other hypotheses to explain AHR have been advanced, including that: 1) asthmatic ASM elicits exaggerated shortening because its load is reduced due to decreased airway elastance or reduced mechanical interaction with lung parenchyma (224,225), 2) thickening of the airway wall as a result of airway remodeling, airway edema, and/or cellular infiltration enhances lumenal narrowing, even for a normal degree of airway smooth muscle shortening (226), or 3) there is a functional abnormality in asthmatic smooth muscle sensitivity or force generation. For smooth muscle biologists, the last possibility has been the most enigmatic. In the few studies of asthmatic smooth muscle, most investigators have reported normal contractile function or even constrictor hyporesponsiveness (227-232). The majority of these studies used isometric force generation (which reflects the number of actin-myosin cross-bridges) as the measure of contractile force. More recent studies in sensitized SJL mice, which exhibit hyperresponsiveness, showed increased maximal force velocity with no difference in isometric force generation compared to unsensitized SJL mice or sensitized or unsensitized mice of a normoresponsive strain (233). In contrast to isometric force generation, shortening velocity is indicative of the rate of cycling of actin-myosin cross-bridges, regardless of number (234). A mechanism by which increased shortening velocity could contribute to AHR was recently proposed by Solway and Fredberg (235).

1.5.3 Role in airway inflammation

Irrespective of whether inflammatory processes, increased ASM mass, or intrinsic abnormalities in asthmatic ASM lead to AHR, it is clear that airway myocytes play a major role in the physiological aspects of asthma. Although the effect of inflammatory cells and their products on the regulation of ASM growth, mitogenesis, and function have been studied extensively, the possibility that ASM can contribute to the regulation of inflammatory responses has hardly been explored. In part, this may be related to the inaccessibility of ASM in superficial mucosal biopsies.

In vitro, ASM can synthesize a number of mediators that may be relevant in asthma. For example, significant concentrations of the ASM mitogen insulin-like growth factor-2 and the regulatory binding protein for insulin-like growth factors are produced
by cultured rabbit ASM (236). In addition, the autologous action of platelet-derived growth factor produced by ASM has been implicated in IL-1-induced ASM proliferation (237). These findings suggest the existence of autocrine mechanisms through which ASM can positively and negatively regulate its growth. ASM has also been shown to produce PGE$_2$ which inhibits cholinergic contraction, mast cell mediator release, eosinophil chemotaxis and survival, and IL-2 and IgE production by lymphocytes (238,239). When given to patients with asthma by inhalation, PGE$_2$ inhibits the airway response to a number of indirect bronchoconstrictor challenges (240,241). ASM may therefore be an important source of bronchoprotective mediators in the lungs.

Among the first studies indicating that ASM may interact directly with inflammatory cells were those of Lazaar et al. (242). These investigators showed that ICAM-1 and VCAM-1 could be upregulated in human ASM by TNF-α, and that this upregulation concomitantly increased the adherence of T cells to ASM. The resulting T cell – ASM interaction was inhibited by antibodies against lymphocyte function associated antigen (LFA-1, a ligand for ICAM-1), ICAM-1, and CD44 (a non-integrin adhesion molecule constitutively expressed by ASM). Furthermore, the ICAM-1 and CD44-mediated binding of T cells to ASM caused DNA synthesis in ASM, suggesting a novel mechanism for hyperplasia. The subsequent observation that human ASM can express class II MHC raises the possibility that airway myocytes could act as antigen-presenting cells in the lungs (243).

Studies in rabbit and human ASM showed that preincubation of these cells with serum from asthmatic subjects results in hyporesponsiveness to β-mediated relaxation and hyperresponsiveness to cholinergic-mediated contraction (222). More recent work established that this activity was due to the presence of IgE immune complexes in asthmatic serum which, when bound to low affinity IgE receptors on ASM, induced the production of IL-1 (244). The autologous action of IL-1, in turn, caused the changes in agonist responsiveness by mechanisms that remain undefined. These findings may have interesting implications on our understanding of asthma. Firstly, they demonstrate a potential link between allergen exposure and AHR. Secondly, these results establish ASM as a possible source of immunoregulatory cytokines.
The latter point poses an important question as to the ability of ASM to contribute to the recruitment of eosinophils to asthmatic lungs. Recent studies have demonstrated the cytokine-inducible expression and release of RANTES in cultured human ASM (245). Whereas unstimulated cells did not express RANTES at detectable levels, TNF-α caused a dose- and time-dependent accumulation of RANTES mRNA and secreted protein. Paradoxically, Th1 cytokines potentiated TNF-α-induced RANTES expression, while Th2 cytokines inhibited this production. Other cytokines and chemokines that have been shown to be expressed by ASM in vitro include MIP-1α, GM-CSF, IL-6, IL-8, and IL-11 (245-248).

1.6 GENERAL HYPOTHESIS
Human airway smooth muscle cells can express eotaxin and this expression can be increased with cytokine stimulation.

1.7 AIMS OF THIS STUDY
1. To investigate the expression of eotaxin mRNA and secreted protein in cultured human airway smooth muscle cells.
2. To examine the dose- and time-dependent effect of proinflammatory cytokines (TNF-α and IL-1β) on eotaxin expression by airway myocytes.
3. To determine whether cytokine-stimulated airway smooth muscle cells produce eosinophil chemoattractant activity in sufficient quantities to elicit eosinophil chemotaxis in vitro.
4. To compare the contribution of eotaxin to that of RANTES in the eosinophil chemoattractant activity produced by cytokine-stimulated airway smooth muscle cells.
5. To examine the expression of eotaxin immunoreactivity in smooth muscle in vivo in asthmatic airways.

1.8 REFERENCES


Increased expression of eotaxin in bronchoalveolar lavage and airways of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation. J. Immunol. 159:4593-601.


Chapter 2. Constitutive and cytokine-stimulated expression of eotaxin by human airway smooth muscle cells

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CONSTITUTIVE AND CYTOKINE-STIMULATED EXPRESSION OF EOTAXIN BY HUMAN AIRWAY SMOOTH MUSCLE CELLS

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Running Title: Eotaxin expression by airway myocytes
2.1 ABSTRACT

Airway eosinophilia is a prominent feature of asthma that is believed to be mediated in part through the expression of specific chemokines such as eotaxin, a potent eosinophil chemoattractant that is highly expressed by epithelial cells and inflammatory cells in asthmatic airways. Recently, airway smooth muscle (ASM) has been identified as a potential source of cytokines and chemokines. The aim of the present study was to examine the capacity of human ASM to express eotaxin. We demonstrate that airway myocytes constitutively express eotaxin mRNA as detected by RT-PCR. Treatment of ASM for 24 h with different concentrations of TNF-α and IL-1β alone or in combination enhanced the accumulation of eotaxin transcripts. Maximal mRNA expression of eotaxin was shown at 12 h and 24 h following IL-1β and TNF-α stimulation, respectively. The presence of immunoreactive eotaxin was demonstrated by immunocytochemistry, and constitutive and cytokine-stimulated release of eotaxin was confirmed in ASM culture supernatants by ELISA. Strong signals for eotaxin immunoreactivity were observed in vivo in smooth muscle in asthmatic airways. In addition, chemotaxis assays demonstrated the presence of chemoattractant activity for eosinophils and PBMC in ASM supernatants. The chemotactic responses of eosinophils were partly inhibited with antibodies directed against eotaxin or RANTES, and a combined blockade of both chemokines caused > 70% inhibition of eosinophil chemotaxis. The results of this study suggest that ASM may contribute to airway inflammation in asthma through the production and release of eotaxin.
2.2 INTRODUCTION

Asthma is a chronic inflammatory disease associated with paroxysmal bronchospasm, bronchial hyperresponsiveness, and the presence of an eosinophilic infiltrate in the airways (1). The development of tissue eosinophilia necessitates processes of eosinophil hematopoietic generation, mobilization into the blood, endothelial adhesion, chemotaxis to inflammatory foci, and survival in the tissues (2,3). IL-3, IL-5, and GM-CSF induce the differentiation of eosinophils from progenitors in the bone marrow, increase the pool of available eosinophils in the circulation, and promote local eosinophil survival (4). In contrast, the adhesion and locomotion of eosinophils is regulated by specific chemoattractants. These include complement factor C5a; the lipid mediators platelet activating factor, leukotriene B4, and 5-oxo-ETE; and the chemokines macrophage inflammatory protein (MIP)1-1α, monocyte chemotactic protein (MCP)-2, MCP-3, MCP-4, RANTES, and eotaxin (5,6). While the Th2-type cytokines IL-4 and IL-5 are produced predominantly by infiltrating T cells (7), a number of eosinophilopoietic cytokines and eosinophil-active chemokines can be produced by resident airway cells such as epithelial cells (8), endothelial cells (9), alveolar macrophages (10), fibroblasts (11), and smooth muscle cells (12,13).

Eotaxin is a C–C (β) chemokine that was identified by the microsequencing of HPLC purified proteins from bronchoalveolar lavage (BAL) fluid of ovalbumin-sensitized and challenged guinea pigs (14). Through the selective expression of its specific receptor, CCR3, human eotaxin is a potent chemoattractant for eosinophils (15,16), basophils (17), and Th2-like T lymphocytes (18), all of which are found in tissues undergoing allergic reactions (1,7,19). Intratracheal instillation of eotaxin in rodents is followed by a marked lung eosinophilia (20-22). Similarly, injection of eotaxin in the skin of animals is associated with the rapid accumulation of eosinophils (14,16,20). In a guinea pig model of allergic airways disease, local eotaxin generation parallels the entry of eosinophils into the lungs (23). Furthermore, compared to sensitized and challenged wild-type mice, eotaxin-deficient mice exhibit a marked decrease in allergen-induced eosinophil recruitment into the airways (24).
Recently, we have shown the increased expression of eotaxin in the lungs of asthmatic subjects compared to normal controls (25). *In vitro* eosinophil chemotaxis by bronchoalveolar lavage fluid from asthmatics was partly inhibited with antibodies against eotaxin to a greater extent than antibodies against RANTES or MCP-4. At sites of inflammation in asthma, allergic rhinitis, and chronic sinusitis, eotaxin immunoreactivity has been localized to various inflammatory cells including monocyte/macrophages, T cells, and eosinophils (25,26). However, the observation that eotaxin is also expressed by epithelial cells (25,26) suggests that structural cells may also contribute to tissue eosinophilia through eotaxin generation in human allergic inflammation. Indeed, TNF-α and IL-1β - cytokines that are increased in allergic inflammation - have been shown to act on bronchial and alveolar epithelial cells lines to upregulate eotaxin synthesis and release (27).

Airway myocytes are recognized as important target cells in asthma owing to their ability to contract in response to inflammatory cell products including histamine, eicosanoids, cytokines, and proteins released from eosinophils (28). Further, through growth and proliferation, airway smooth muscle (ASM) cells have been implicated in lung remodeling, a feature of asthma that can contribute to persistent airway narrowing and bronchial hyperresponsiveness (28). Recent work has suggested that the functions of ASM are not restricted to contractile and growth responses. *In vitro*, ASM has also been shown to express immunoglobulin receptors (29), HLA-DR (30), VCAM-1, ICAM-1 (31), and a limited repertoire of cytokines including RANTES (12), IL-1 (32), IL-6, and IL-11 (33). These observations raise the possibility that ASM has the potential to regulate inflammatory responses.

In asthmatic lungs, eosinophilia can be observed within and around ASM (34). However, the possibility that human ASM cells are a source of eotaxin has not been investigated. In this study, we show that airway myocytes express eotaxin *in vitro* and *in vivo* in subjects with asthma. ASM production of eotaxin was upregulated by TNF-α and IL-1β stimulation, and eotaxin accounted for a significant proportion of the eosinophil chemoattractant activity produced by ASM cells.
2.3 METHODS

2.3.1 Cell culture
Human ASM cells from two sources were used. Bronchial/tracheal smooth muscle cells (B/TSMC) were purchased from Clonetics (San Diego, CA). These cells stained positively for α-smooth muscle actin and negatively for factor VIII, CD45, and CD3. B/TSMC were grown as recommended by the supplier in their optimal medium (SmGM-2; Clonetics) containing 5% FBS at 37°C in a humidified incubator with 5% CO₂. The second source of ASM cells was human tracheas which were obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. Tracheal smooth muscle cells (TSMC) were isolated and purified from the trachealis muscle as described by Panettieri et al. (33,35). TSMC were grown at 37°C with 5% CO₂ in Ham's F12 media supplemented with 10% FBS, penicillin (10³ U/ml), streptomycin (1 mg/ml), NaOH (12 mM), CaCl₂ (1.7 mM), L-glutamine (2 mM), and 25 mM HEPES. These cells retain smooth muscle-specific actin expression and have the requisite receptor/second messenger systems necessary to support both contractile and relaxant responses (35). B/TSMC and TSMC grow with the hill and valley appearance characteristic of smooth muscle in culture, are elongated and spindle-shaped with a central nucleus.

2.3.2 Cell stimulation
Confluent B/TSMC and TSMC in passages 3-9 were growth-arrested by FBS-deprivation for 48 h (31). After serum-deprivation, the cells were stimulated in fresh serum-free medium containing TNF-α and/or IL-1β (R & D Systems, Minneapolis, MN) in a concentration- and time-dependent manner.

2.3.3 Semi-quantitative RT-PCR and southern analysis
Total cellular RNA was extracted from culture flasks using the trizol isolation reagent (Life Technologies, Gaithersburg, MD). Reverse transcription was performed by using 2 µg of total RNA in a first strand cDNA synthesis reaction with the moloney
murine leukemia virus reverse transcriptase (Life Technologies). Comparison was achieved by amplifying the constitutively expressed β-actin gene (a housekeeping gene) and eotaxin under subsaturating conditions in parallel tubes as previously described (25,36). β-actin was used as the standard to control for variations in RNA isolation, cDNA synthesis, and PCR performance. A sample of cDNA was subjected to sequential cycles of amplification (20, 25, 30, 35, and 40 cycles). Samples were amplified at 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min. A 322 bp fragment was generated using specific primers for human eotaxin (25). The optical density obtained for each amplified fragment was plotted against the number of cycles. The amounts of PCR generated bands increase logarithmically up to a certain number of cycles, reaching a plateau thereafter. Under these conditions, it was established when PCR reactions were in the exponential (quantifiable) phase. The quantification was achieved by scanning the band intensities obtained on ethidium bromide-stained agarose gels with an Instant Imager System 2000 (Pharmacia Biotech, Piscataway, NJ). Bands were transferred to nylon membranes and Southern analysis was performed with an internal primer for eotaxin (25) to verify the specificity of the PCR product. Enhanced chemiluminescence detection (Boehringer Mannheim, Mannheim, Germany) was used for Southern blots according to the manufacturer's instructions.

2.3.4 ELISA
Supernatants from serum-deprived B/TSMC (cytokine-stimulated or unstimulated) were collected from culture flasks, centrifuged at 1200 RPM for 7 min at 4°C to remove cellular debris, and stored at -80°C until use. Eotaxin release in supernatants was assayed by a sandwich ELISA as described previously (25,27). Each well of a high-binding efficiency 96-well ELISA plate was coated with a mouse anti-human eotaxin monoclonal antibody (2A12; ref. 25,27) at 400 ng in PBS for 4 hours at room temperature. All incubations were carried out in a humidified atmosphere. Residual binding sites were blocked with 200 μl/well of 3% BSA (Sigma Chemical Co., St. Louis, MO) in PBS with 0.02% azide and incubated overnight at room temperature. After washing with PBS, standard eotaxin solution or B/TSMC supernatants (50 μl/well in PBS containing 3%
BSA) were added in duplicate to the coated wells, incubated for 2 hr at room temperature, and washed again with PBS. 50 µl of rabbit anti-eotaxin affinity purified polyclonal antibody (650 µg/ml) was subsequently added at 1:1000 in 3% BSA/PBS. After a 2 hr incubation at room temperature, the plates were washed three times with PBS; 50 µl of horseradish peroxidase-linked goat anti-rabbit IgG (Kirkegaard & Perry, Gaithersburg, MD) diluted 1/1000 in PBS containing 3% BSA was added to each well and incubated for 90 min at room temperature. After washing, the binding was visualized with 3,3',5,5' tetramethylbenzidine substrate according to the instructions of the manufacturer (Kirkegaard & Perry). The reaction was stopped after 15 min by adding 100 µl of 1M H₃PO₄ per well, and absorbance at 650 nm was measured. Under these conditions, this assay is sensitive to 31 pg/ml.

2.3.5 PBMC and eosinophil purification from peripheral blood

PBMC and granulocytes were purified from peripheral blood of non-atopic volunteers by Ficoll-Paque (Pharmacia Biotech) density centrifugation. After removing the mononuclear cell fraction, granulocytes were obtained by dextran sedimentation. Human eosinophils were further purified by negative selection with anti-CD16 and anti-CD3-coated immunomagnetic microbeads using a Magnetic Cell Sorting system (Miltenyi Biotec, Bergisch-Gladbach, Germany) at 4°C. The degree of purity of eosinophil populations, estimated after staining with Giemsa, was between 92 and 100%. Freshly isolated PBMC and eosinophils were resuspended at concentrations of 2 x 10⁶ cells/ml in RPMI-1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM L-glutamine, and 1 mM sodium pyruvate. Cells were incubated for 1 hour at 37°C in a humidified atmosphere of 5% CO₂ (25). Cells were washed, resuspended, counted, and employed for the chemotaxis assays.

2.3.6 Chemotaxis assay

Experiments were performed with a 48-well micro-chemotaxis chamber (NeuroProbe, Cabin John, MD) and carried out as previously described (15,25). Migration of human PBMC and eosinophils in response to recombinant eotaxin or
B/TSMC supernatant was performed on a polycarbonate filter (5 μm pore size) and a polyvinylpyrrolidone-free polycarbonate filter (3 μm pore size) was used for PBMC. Eosinophils (2 x 10^6 cells/ml) and mononuclear cells (2 x 10^6 cells/ml) were loaded into the chambers and incubated at 37°C, 5% CO₂ (60 min for eosinophils and 90 min for mononuclear cells). The filters were subsequently fixed and stained with a RAL kit (Labonord, France). Only cells morphologically identified as eosinophils or mononuclear cells were counted by microscopy in five high power fields (magnification x 400) as previously described (14-16,25).

For neutralization experiments, B/TSMC supernatants were preincubated with anti-eotaxin, anti-RANTES, or anti-eotaxin + anti-RANTES (at 1/100 dilution) at 37°C for 1 hour as described before (25). Normal rabbit serum was used as a negative control.

2.3.7 Immunocytochemistry

B/TSMC grown on 8 well glass slides (Naige Nunc, Naperville, IL) were serum-deprived and stimulated with cytokines or incubated with media alone. Slides were fixed in 70:30 acetone-methanol, air-dried, and stored at -20°C until use. Immunoreactive eotaxin was detected as described previously (25). Briefly, after treatment with tris-buffered saline containing 10% goat serum and 0.5% BSA for 30 min, slides were incubated with anti-eotaxin antiserum at a final dilution of 1/200 for 90 min at 37°C, followed by incubation for 1 hour at 37°C with 5 μg/ml FITC-labeled swine anti-rabbit IgG. After each incubation with antibody, slides were extensively washed with TBS. Nuclei of cells were stained for 2 min with Hoechst 33258 dye (bisbenzimide; Sigma Chemical Co., St. Louis, MO). Normal rabbit serum (NRS) was used at the same dilution as a negative control. Slides were visualized with a Zeiss Axiophot fluorescence microscope (Carl Zeiss (Oberkochen), Ltd., Welwyn Garden City, U.K.).

2.3.8 Immunocytochemistry on lung sections of asthmatic individuals
To determine whether ASM has the capacity to produce eotaxin in vivo, immunocytochemistry (38) was employed on sections of the major airways from 6 asthmatic subjects using anti-eotaxin polyclonal antibodies (25,26). The airway sections were obtained from 6 asthmatic subjects who have been described previously in detail (34). The subjects were part of the St. Paul’s Hospital Lung Study (Vancouver, Canada) for which ethical approval was obtained. A clinical diagnosis of asthma was made on the basis of an evaluation of the patients’ medical files by a respiratory physician. The clinical criteria used to establish this diagnosis included prior physician diagnosis and treatment for asthma, documented evidence of variable airflow obstruction greater than 15%, and bronchial hyperresponsiveness. Immediately after resection, lung specimens were prepared for immunocytochemistry as described before (34).

2.3.9 Statistical analysis

Statistical significance was determined using a Student’s t test. P values < 0.05 were considered statistically significant.
2.4 RESULTS

2.4.1 Constitutive and cytokine-induced eotaxin mRNA expression
In initial experiments, total RNA purified from TSMC and B/TSMC was reverse transcribed and the cDNA amplified by PCR with specific primers for eotaxin. Gel electrophoresis revealed bands which corresponded to the predicted length of the eotaxin cDNA product (322 bp). The specificity of the PCR was confirmed by Southern analysis using an internal primer for eotaxin (figure 1). Eotaxin mRNA expression was further studied in TSMC cultured in the presence of TNF-α, IL-1β, or media alone by semi-quantitative RT-PCR using the constitutively-expressed β actin as a standard (figure 2). Expression of mRNA for eotaxin was found in cells cultured in media alone and was increased after 24 h stimulation with TNF-α or IL-1β by 11-fold and 7-fold, respectively. Constitutive expression of eotaxin mRNA was also observed in B/TSMC (figure 3). Following 24 h TNF-α or IL-1β stimulation, this was increased by 3-fold and 2-fold, respectively. In B/TSMC, a combination of TNF-α and IL-1β caused a greater increase in eotaxin mRNA expression compared to either cytokine alone.

2.4.2 Time course of cytokine-induced eotaxin mRNA accumulation
Treatment of B/TSMC with 100 ng/ml of TNF-α induced an increase in eotaxin mRNA expression at 6 h reaching a maximal response at 24 h (figure 4A). Levels of eotaxin mRNA remained elevated for the duration of the experiment (up to 48 h). Stimulation with IL-1β (1 ng/ml) also resulted in an increase in eotaxin mRNA by 6 h; this reached a maximum at 12 h and was still elevated at 24 h and 48 h (figure 4B).

2.4.3 Dose-response effect of TNF-α and IL-1β on eotaxin mRNA expression
The addition of increasing doses of TNF-α (1, 10, 25, 50, 100 ng/ml) to the B/TSMC medium 24 h before harvesting was associated with increasing eotaxin mRNA expression up to stimulation with 25 ng/ml TNF-α, after which eotaxin mRNA levels declined modestly but were still higher than in cells incubated with medium alone (figure 5A). IL-
IL-1β also had a dose-dependent effect on eotaxin mRNA induction. At 24 h, the maximal eotaxin mRNA response was observed with 25 ng/ml IL-1β, and all IL-1β concentrations resulted in increased eotaxin mRNA compared to cells cultured in medium alone (figure 5B).

2.4.4 Examination of eotaxin expression in B/TSMC by immunocytochemistry
Immunocytochemistry of B/TSMC with anti-eotaxin antiserum confirmed the expression and localization of eotaxin immunoreactivity in B/TSMC cultured in medium alone and in those stimulated with IL-1β or TNF-α (Plate 1 and data not shown). The expression of eotaxin mRNA assessed by the intensity of the signal was increased following TNF-α or IL-1β stimulation. No positive signal was observed when normal rabbit serum was used instead of the primary antibody.

2.4.5 Measurement of eotaxin release from B/TSMC by ELISA
To confirm the release of eotaxin from smooth muscle, we measured the levels of eotaxin in B/TSMC supernatant following incubation of cells with media alone, TNF-α (1, 10, 25, 50, 100 ng/ml), or IL-1β (1, 10, 25, 50, 100 ng/ml). Eotaxin was detected in supernatants from B/TSMC in the absence of cytokine stimulation. Stimulation for 24 h with TNF-α and IL-1β resulted in a marked increase in eotaxin release at all concentrations tested (figure 6).

2.4.6 Contribution of eotaxin to eosinophil and PBMC chemotaxis mediated by B/TSMC supernatant
As a functional assay of B/TSMC-derived eotaxin, supernatants from cultures stimulated for 24 h with TNF-α or IL-1β were examined for their chemotactic activity on eosinophils. Recombinant eotaxin increased the chemotaxis of eosinophils but not PBMC (not shown). In contrast, B/TSMC supernatants induced the chemotaxis of both eosinophils and PBMC at all concentrations tested (1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64; figure 7). Anti-eotaxin antibodies had a marked inhibitory effect on the chemotactic activity of B/TSMC supernatant for eosinophils but not for PBMC (figure 7, table 1).
We compared the contribution of eotaxin to that of RANTES in eosinophil chemotaxis mediated by supernatant from B/TSMC stimulated with TNF-α or IL-1β. The addition of antibodies to either chemokine alone resulted in significant decreases in eosinophil chemotaxis (table 1), however the difference between anti-eotaxin and anti-RANTES treatment was not significant in IL-1β or TNF-α-stimulated cultures. Anti-eotaxin + anti-RANTES caused an inhibition of eosinophil chemotaxis that exceeded 70% compared to the eosinophil chemotactrant activity of supernatants without the antibodies. This inhibition was significant compared to the inhibitory effect of either antibody alone.

2.4.7 Eotaxin immunoreactivity in ASM of asthmatic individuals

Using immunocytochemistry, eotaxin immunoreactivity was detected in ASM of all 6 asthmatic subjects investigated (Plate 1). As demonstrated previously, eotaxin also localized to the airway epithelium and infiltrating cells in the submucosa.
2.5 DISCUSSION

In this study, we show that cultured human airway myocytes constitutively express eotaxin mRNA and protein. TNF-α and IL-1β stimulation enhanced the accumulation of eotaxin transcripts in a concentration-dependent manner and a combination of the two cytokines had a slightly greater effect than either alone. Distinct kinetics of cytokine-induced eotaxin mRNA upregulation were observed; maximal responses occurred at 12h and 24h following IL-1β and TNF-α stimulation, respectively. In vivo, strong signals for eotaxin immunoreactivity were demonstrated in smooth muscle around asthmatic airways. Furthermore, the release of eotaxin protein from ASM was confirmed in culture supernatants which had a strong chemoattractant activity for eosinophils and PBMC. The chemotactic responses of eosinophils to ASM supernatant were found to be partly inhibited with antibodies directed against eotaxin or RANTES, and a combined blockade of both chemokines caused an inhibition of eosinophil chemotaxis that exceeded 70%. Collectively, our findings demonstrate that human ASM may contribute to airway inflammation in asthma through the production and release of eotaxin. The results of this study complement and extend previous investigations localizing eotaxin to macrophages, T cells, eosinophils, and epithelial cells in bronchial biopsies and BAL from patients with asthma (25), and recent observations that eotaxin is expressed in ASM of the allergen-challenged mouse (39) and guinea pig lungs (40). Our findings further implicate airway myocytes as effector cells with the capacity to regulate inflammatory responses in asthmatic lungs.

A number of processes believed to be important in the pathogenesis of asthma have been ascribed to the activities of TNF-α and IL-1 (41). These cytokines are found at increased levels in lung lavage fluid from asthmatics and their spontaneous release is augmented in alveolar macrophages from adult patients with asthma and wheezy infants (42, 43). Although TNF-α and IL-1 can cause the airway hyperresponsiveness and eosinophilia that characterize asthma, neither is a potent chemoattractant for eosinophils (41). On the other hand, the contribution of IL-1 and TNF-α to eosinophil recruitment has been demonstrated with the use of IL-1 receptor antagonist proteins and soluble TNF receptors in animal models of eosinophilic airway inflammation; inhibition of either
activational pathway significantly decreased allergen-induced eosinophil migration into the airways (2, 41). Together, these observations suggest that effects of IL-1 and TNF-α on eosinophil recruitment to the airways are indirect. Indeed, in addition to IL-4 and IL-13, TNF-α and IL-1 induce VCAM-1 expression on vascular endothelial cells (44), a key step in the recruitment of eosinophils and mononuclear cells to the airways after allergen exposure.

Germaine to the observation that TNF-α and IL-1 upregulate eotaxin production by ASM, mast cells – which also comprise a source of TNF-α and IL-1 – have been isolated from human ASM tissue (45). TNF-α and IL-1 are therefore potentially available to myocytes in vivo, particularly after IgE-mediated triggering of mast cells by allergen. Interestingly, Hakonarson et al. (32) have demonstrated that exposure of ASM tissue or cultured cells to serum from atopic asthmatic individuals induces the elaboration of IL-1β by ASM. Although the factor(s) responsible for this activity have not been definitively identified, the ability of ASM to produce IL-1 suggests an autocrine mechanism by which eotaxin production could be increased. Within the range of concentrations that we found effective in the upregulation of eotaxin in ASM, TNF-α and IL-1β have previously been shown to stimulate the production of eotaxin, RANTES, and MCP-4 by airway epithelial cells (8, 27). The combined effect that we observed between IL-1 and TNF-α in the induction of eotaxin mRNA is consistent with results obtained for eotaxin expression in a bronchial epithelial cell line (BEAS-2B cells; ref. 27).

A number of studies have recently identified ASM as a source of cytokines and chemokines in vitro (12, 32, 33). Pertinent to eosinophilic inflammation in asthma, ASM produces the eosinophilopoietic agent GM-CSF constitutively and this expression can be increased with IL-1 or TNF-α (13). Recently, the induction of RANTES expression by TNF-α in human ASM has been reported (12). In addition to attracting eosinophils, RANTES causes the migration of monocyte/macrophages and T lymphocytes in vitro (5). However, when injected in the skin of the rhesus monkey, RANTES is a less effective eosinophil chemoattractant than eotaxin (16). After cytokine stimulation, the time that we found eotaxin mRNA expression to be maximal (12 to 24 h) in ASM
coincides with the time frame in which increased eosinophils begin appearing in the airways after allergen exposure. Maximal induction of RANTES in ASM was reported at 96 h after cytokine stimulation, suggesting a prominent role for RANTES in sustaining inflammatory cell recruitment over a more prolonged period (12).

We found that both eotaxin and RANTES comprised significant proportions of ASM-derived eosinophil chemoattractant activity. Similar results were obtained using supernatants from TNF-α and IL-1β stimulated ASM, suggesting that like TNF-α, IL-1β can also induce RANTES production by ASM. Interestingly, the blockade of both chemokines caused decrease in ASM derived eosinophil chemotaxis by over 70%. This is in contrast to findings that the eosinophil chemotaxis elicited by asthmatic BAL was inhibited by 25-45% with anti-eotaxin and anti-RANTES antibodies (25). TNF-α- and IL-1β-stimulated ASM appear, therefore, to produce a more restricted profile of eosinophil chemoattractants than what is present in the lungs of subjects with asthma. The remainder of eosinophil chemotactic activity produced by ASM might be attributed to MIP-1α and IL-8, weaker eosinophil-active chemokines that have been shown to be produced by airway myocytes (12, 46). The growing number of cytokines and chemokines that are potentially produced by ASM suggests that these cells may also express other eosinophil chemoattractants in addition to eotaxin, RANTES, MIP-1α, and IL-8.

The receptor for eotaxin, CCR3, was recently reported to be selectively expressed by human Th2 cells (18). Since CCR3+ T cells only comprise approximately 1% of peripheral blood T cells, a low level of CCR3 expression and consequently the high contamination by negative cells might explain our observation that anti-eotaxin antibodies had no effect on PBMC chemotaxis induced by supernatant from stimulated ASM. Eosinophils and basophils are known to release a variety of substances that are directly active on ASM. The attraction of T cells to ASM expressing eotaxin may also be important in the pathogenesis of asthma. Activated T lymphocytes have been demonstrated to adhere to TNF-α-stimulated ASM via integrins and CD44 (31). Such interactions were shown to induce DNA synthesis in ASM, suggesting a role for T cells in airway remodeling. In addition, studies demonstrating the expression of MHC class II
on ASM raise the possibility that airway myocytes could act as antigen-presenting cells in asthmatic airways (30). However, the effect of Th2 versus Th1 T lymphocyte interactions with ASM remain to be investigated.

In conclusion, the results of this study show that ASM has the capacity to produce eotaxin, and may thus contribute to the recruitment of eosinophils, basophils, and Th2 cells in the airways of subjects with asthma.
2.6 ACKNOWLEDGMENTS

The authors thank Elsa Schotman and Serge Seguin for their technical assistance, and Dr. J.G. Martin for his critical review of the manuscript.
2.7 REFERENCES


2.8 FIGURE LEGENDS

**Figure 1.** RT-PCR and Southern blotting demonstrating eotaxin mRNA expression in B/TSMC. RNA purified from B/TSMC was examined for the presence of eotaxin transcripts by RT-PCR (A). For positive controls, RNA from A549 cells, Hep-2 cells, and PBMC was used. Putative eotaxin bands (322 bp) were transferred to nylon membranes and hybridized with a digoxigenin-labeled internal oligonucleotide specific for eotaxin (B) as described in *Materials and Methods* section.

**Figure 2.** Expression of eotaxin mRNA in cytokine-stimulated and unstimulated TSMC by semiquantitative RT-PCR. A, RT-PCR was performed using total RNA purified from confluent TSMC cultured in serum-free media alone or in the presence of 25 ng/ml IL-1β or TNF-α for 24 h. Eotaxin-specific signals of 322 bp were obtained. B, RT-PCR for β-actin. C, Densitometric analysis of semiquantitative RT-PCR for eotaxin mRNA in cytokine-stimulated and unstimulated TSMC.

**Figure 3.** Eotaxin mRNA expression in cytokine-stimulated and unstimulated B/TSMC by semi-quantitative RT-PCR. A, Total RNA purified from confluent B/TSMC cultured in serum-free media alone or in the presence of 1 ng/ml IL-1β, 100 ng/ml TNF-α, or 100 ng/ml TNF-α + 1 ng/ml IL-1β for 24 h was subjected to RT-PCR using specific primers for eotaxin. B, RT-PCR for β-actin. C, Densitometric analysis of semiquantitative RT-PCR for eotaxin mRNA in cytokine-stimulated and unstimulated B/TSMC. IDV, integrated density value.

**Figure 4.** Kinetics of TNF-α- and IL-1β-stimulated accumulation of eotaxin mRNA in B/TSMC by semi-quantitative RT-PCR. Confluent serum-deprived B/TSMC were stimulated with 100 ng/ml TNF-α or 1 ng/ml IL-1β for 6, 12, 24, and 48 h. Eotaxin mRNA expression was examined at each time point after TNF-α (A) and IL-1β (B) stimulation by semi-quantitative RT-PCR. Densitometric assessments of the eotaxin-specific signals at each time point are illustrated below A and B.
Figure 5. Dose-response effect of TNF-α and IL-1β on the expression of eotaxin mRNA in B/TSMC by semi-quantitative RT-PCR. The effect of increasing concentrations of TNF-α (A) and IL-1β (B) on eotaxin mRNA expression in B/TSMC was determined by semi-quantitative RT-PCR. Total RNA was extracted 24 h after the addition of the cytokines to the culture medium. Below A and B are densitometric assessments of the eotaxin-specific signals at each concentration.

Figure 6. Release of eotaxin protein by B/TSMC detected by ELISA. B/TSMC were stimulated for 24 h with increasing concentrations of TNF-α and IL-1β. Supernatants were recovered and eotaxin protein was measured by ELISA. A, TNF-α stimulation, B, IL-1β stimulation. Each point represents the mean ± SD of four measurements. *p < 0.05, **p < 0.01, ***p < 0.001

Figure 7. Cellular migration induced by B/TSMC supernatant in filter assays and inhibition of chemotactic activity with a polyclonal anti-eotaxin antibody. Eosinophils (Eos) or PBMC were incubated in a Boyden chamber with serial dilutions of supernatant (St) recovered from B/TSMC stimulated for 24 h with TNF-α (100 ng/ml) (solid bars). To determine the contribution of eotaxin to eosinophil and PBMC chemotaxis, supernatants were preincubated with polyclonal anti-eotaxin antibodies (hatched bars) or normal rabbit serum. A, Chemotaxis of human eosinophils; B, Chemotaxis of human PBMC. The results of a representative experiment of three experiments are shown and expressed as the mean number of cells ± SD in five fields (magnification X 400) per well (25). Similar results were obtained using supernatants from B/TSMC stimulated with 100 ng/ml IL-1β (table 1). Control NRS had no effect on chemotaxis (not shown). *p < 0.001, **p < 0.01

Plate 1. Expression of eotaxin in ASM in vitro and in vivo. Top panel, Immunofluorescent staining of eotaxin in unstimulated B/TSMC. B/TSMC grown on 8-well glass slides were fixed in acetone-methanol. Slides were incubated with rabbit anti-eotaxin antiserum followed by swine anti-rabbit IgG conjugated to FITC. Middle panel, Cross-section of an intermediate airway of an
asthmatic subject showing eotaxin immunoreactivity in smooth muscle cells (large arrows) and epithelium (small arrow). Cryostat sections were cut from frozen lung tissue from asthmatic subjects. Slides were incubated with anti-eotaxin polyclonal antibodies, the appropriate secondary antibodies, and a tertiary layer of streptavidin peroxidase. Sections were developed with diaminobenzidine and positive cells stained brown. Bottom panel, High power magnification of transverse section of a large airway in an asthmatic subject demonstrating eotaxin immunoreactivity in bundles of smooth muscle (arrows) by streptavidin peroxidase method of immunostaining.
### 2.9 FOOTNOTES

1 Abbreviations used in this paper:

<table>
<thead>
<tr>
<th>Term</th>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>macrophage inflammatory protein-1α</td>
<td>MIP-1α</td>
</tr>
<tr>
<td>monocyte chemotactic protein</td>
<td>MCP</td>
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<tr>
<td>bronchoalveolar lavage</td>
<td>BAL</td>
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<tr>
<td>airway smooth muscle</td>
<td>ASM</td>
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<tr>
<td>bronchial/tracheal smooth muscle cells</td>
<td>B/TSMC</td>
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<td>tracheal smooth muscle cells</td>
<td>TSMC</td>
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Table 1. Contribution of eotaxin and RANTES to eosinophil chemoattractant activity in stimulated B/TSMC supernatants

<table>
<thead>
<tr>
<th>B/TSMC + stimulus</th>
<th>Eotaxin (pg/ml)</th>
<th>Supernatant</th>
<th>Supernatant + anti-eotaxin</th>
<th>Supernatant + anti-RANTES</th>
<th>Supernatant + anti-eotaxin + anti-RANTES</th>
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</thead>
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<tr>
<td>TNF-α (100 ng/ml)</td>
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<td>143</td>
<td>72</td>
<td>77.4</td>
<td>36†</td>
</tr>
<tr>
<td>IL-1β (100 ng/ml)</td>
<td>576</td>
<td>105</td>
<td>43.8</td>
<td>50.8</td>
<td>24.8†</td>
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* Supernatants collected from stimulated B/TSMC were employed for chemotaxis assays as described in the Materials and Methods. The concentration of eotaxin in the supernatants as determined by ELISA is indicated. Supernatants elicited a strong eosinophil chemotactic response that was partly inhibited by preincubation of the supernatants with polyclonal rabbit anti-eotaxin and/or anti-RANTES antibodies. Preincubation of supernatants with normal rabbit serum had no effect on eosinophil chemotaxis (not shown).

† p < 0.0001 compared to eosinophil chemotaxis elicited by supernatant without anti-chemokine antibodies

‡ p < 0.01 compared to eosinophil chemotaxis elicited by supernatant without anti-chemokine antibodies

§ p < 0.0001 compared to eosinophil chemotaxis elicited by supernatant + anti-eotaxin or supernatant + anti-RANTES
Eotaxin
322 bp
A

![Gel Image with Lanes](image1)

- MW
- Media
- IL-1β
- TNF-α
- TNF-α + IL-1β
- No cDNA

B

![Gel Image with 322 bp Marker](image2)

C

![Bar Chart](image3)

- Mean OD (10^5 IDU)

- Media
- IL-1β
- TNF-α
- IL-1β + TNF-α
- No cDNA
Chapter 3. Overall conclusions.

3.1 Expression of eotaxin by human airway smooth muscle cells

3.2 Limitations of this study

3.3 Future studies

3.4 References
3.1 EXPRESSION OF EOTAXIN BY HUMAN ASM CELLS

Infiltration of eosinophils in the airway tissue has been linked to events considered to be central to pathophysiology of asthma, including bronchial hyperresponsiveness, airway smooth muscle constriction, epithelial alterations, and pulmonary remodeling (1,2). The development of pharmacological therapies that could selectively block eosinophil recruitment is therefore desirable, but necessitates an unraveling of the complete repertoire of cells and mediators involved.

The major aim of this study was to examine the capacity of ASM to express eotaxin. The data presented here show for the first time that cultured human airway myocytes constitutively express eotaxin. Although the function of eotaxin expression in normal tissues including the airway epithelium is not certain (3,4), studies in the small bowel of eotaxin-deficient mice suggested that low levels of this chemokine are required for the baseline homing of eosinophils (5). Nonetheless, eosinophils are rarely observed in uninflamed human airways (1). Unlike asthmatic airways however, normal airway tissue is not associated with the expression of eosinophilopoietic and survival-promoting cytokines (6). It is therefore possible that the continual recruitment of small or even undetectable numbers of rapidly turning-over eosinophils is dependent upon the constitutive expression of eotaxin.

Stimulation of ASM with cytokines that are highly expressed in asthmatic airways, TNF-α and IL-1β (6), substantially increased eotaxin mRNA accumulation and protein release. TNF-α and IL-1-induced eotaxin upregulation has also been reported in human bronchial and alveolar epithelial cells in addition to umbilical vein endothelial cells (4,7). In contrast to epithelial cells in which cytokine-induced eotaxin mRNA accumulation was maximal at 4 h, the kinetics of cytokine-stimulated eotaxin gene expression in ASM showed a maximal response at 12-24 h. It is unlikely that these in vitro differences can be simply applied to allergen-induced eosinophil recruitment in vivo, but it is interesting to note that eosinophil migration to the airway lumen of guinea pigs after allergen challenge was shown to be temporally dissociated from the peak of tissue eosinophilia (8). In this recent study, the time-course of eosinophil migration to the airway lumen correlated with the kinetics of eotaxin upregulation in epithelial cells in
Conversely, maximal tissue eosinophilia was congruent with the time by which epithelial eotaxin production had subsided, yet high tissue levels of eotaxin persisted. The authors suggested that a concentration gradient of epithelial-derived eotaxin was responsible for the migration of eosinophils to the lumen (8). This gradient was postulated to diminish at a later time point when epithelial production of eotaxin decreased and eotaxin concentrations in the epithelium were washed out by mucociliary clearance. The continued recruitment and/or retention of eosinophils in the tissue during this later time frame was attributed to the prolonged half-life of epithelial-derived eotaxin associated with extracellular matrix components in the airway mucosa. Based on the distinct kinetics of cytokine-induced eotaxin upregulation in human epithelial and ASM cells however, it is tempting to speculate that continued recruitment and/or retention of eosinophils in the tissue at later time points is associated with the peak of cytokine-induced eotaxin expression by ASM.

Although eosinophils are known to produce an array of mediators that could act on ASM cells (9), most studies attempting to delineate the precise roles of eosinophils in asthma have focused on their potential effects on epithelial cells. Indeed, eosinophils appear to preferentially localize around the epithelium in bronchial biopsies from asthmatics (10). However, the recent finding that eosinophil numbers are significantly higher in the region between the smooth muscle and parenchyma compared to the region between the smooth muscle and epithelium (11) suggest that: 1. specific mechanisms exist to attract eosinophils to ASM, and 2. eosinophil – ASM interactions may be more important than is currently appreciated. ASM culture supernatants were found to contain significant chemoattractant activity for eosinophils that was partly inhibited with antibodies directed against eotaxin or RANTES, and maximally inhibited by a combined blockade of both chemokines. These findings confirm that ASM-derived eotaxin and RANTES have the capacity to elicit eosinophil chemotaxis and may contribute substantially to cytokine-induced eosinophil recruitment to the ASM layer in particular. Although there have been a number of studies investigating the effect of eosinophils on explanted airways (12,13), there are very few that have clearly examined the potential role of direct eosinophil – ASM interactions in co-culture conditions. In addition to
eotaxin and RANTES, ASM can produce levels of GM-CSF that are sufficient to prolong eosinophil survival (14). Equally interesting would be the possibility that eosinophils directly modulate ASM function. The ability of ASM to express VCAM-1 and ICAM-1 raise the prospect that direct cell to cell contact and adhesion could occur with eosinophils (15).

The observation of eotaxin expression in vitro by ASM was extended to asthmatic individuals, as strong signals for eotaxin immunoreactivity were observed in smooth muscle in asthmatic airways. Collectively, the results of this study demonstrate that human ASM may contribute to airway eosinophilic inflammation in asthma through the production and release of eotaxin. These findings complement previous investigations localizing eotaxin to macrophages, T cells, eosinophils, and epithelial cells in bronchial biopsies and BAL from patients with asthma (3), and further implicate airway myocytes as effector cells with the capacity to regulate inflammatory responses in asthmatic lungs.

3.2 LIMITATIONS OF THIS STUDY

The main limitation of this study is related to the inherent difficulties in investigating human ASM. The depth of the smooth muscle layer in human airways precludes any detailed study on biopsy material obtained by fiberoptic bronchoscopy (16). Although we attempted to purify ASM cells from deep biopsies of asthmatic and normal airways, the number of ASM cells and their viability proved insufficient for cell culture.

On the other hand, airway tissue obtained from asthmatic subjects during lung resection was useful for addressing the important question of whether eotaxin is expressed in vivo in ASM, but reliable quantification of immunoreactive signals in asthmatics compared to controls was not possible without introducing multiple sources of potential error. For example, it is not clear how increased smooth muscle mass in asthmatics should be taken into account. Simply quantifying the numbers of eotaxin+ ASM cells may produce a higher result in asthmatics solely because there are more smooth muscle cells. Conversely, expressing the eotaxin signal as a percentage of smooth muscle cells could lend itself to misinterpretation: what is more important, the proportion of eotaxin+ ASM cells or the overall levels of eotaxin produced by ASM? The
issue is further complicated by the notion that ASM can have at least two phenotypes, proliferative and contractile (17). It is possible that eotaxin is differentially expressed among ASM cells having these different phenotypes, in which case inadvertently counting one or the other – which is impossible to discriminate without using a large panel of antibodies (18) – could have a profound effect on the results in different individuals and in different regions of the airway in the same individuals (19,20). In addition, the smooth muscle layer in the airways is diffuse, making it difficult to count positive ASM cells without double staining for α-smooth muscle actin; even then, studies have demonstrated the capacity of fibroblasts to express this marker (18).

For these reasons, we employed cell culture to more precisely examine the potential of ASM to produce eotaxin. Although ASM cultures established from asthmatic cells were not available, we attempted to mimic the microenvironment in asthmatic lungs, particularly during allergen-induced exacerbations, by modulating the levels of TNF-α and IL-1. The limitations of cell culture are obvious and are somewhat compounded in the study of ASM. In contrast to culturing of cells from the peripheral blood in which the relevant growth factors can easily be supplemented, the substances normally present in the milieu of smooth muscle cells remain largely unknown. Furthermore, the effect of anchoring and contact with other cell types in the lung is impossible to replicate in vitro. However, our main results were confirmed in cultured ASM from two sources, one of which (TSMC) was well characterized in previous studies and shown to maintain the requisite receptor/second messenger pathways necessary to support contractile and relaxant responses to physiologically relevant stimuli including histamine, leukotrienes, bradykinin, PAF, substance P, and β agonists (21). Together, the combined use of cultured ASM and airway sections from asthmatics in this study permitted us to balance the weaknesses of each approach with the strengths of the other.

3.3 FUTURE STUDIES

A number of important questions arise from the work in this thesis:

1. Does TNF-α upregulate eotaxin in ASM through IL-1?
In a number of cell types, TNF-α has been shown to induce IL-1 expression (22). The distinct kinetics of TNF-α and IL-1-stimulated eotaxin upregulation, coupled with the observation that ASM can produce IL-1 (23), suggest that TNF-α may mediate its effect by inducing the autocrine action of IL-1. This might account for the time-lag in the maximal effect of TNF-α (24 h) versus IL-1 (12 h) in causing upregulation of eotaxin in ASM. Analysis of the efficacy of TNF-α in upregulating eotaxin mRNA in the presence or absence of exogenously-added IL-1 receptor antagonist could address this question.

2. How do TNF-α and IL-1 increase eotaxin mRNA accumulation in ASM? Is nascent protein synthesis required?

The presence of NF-κB binding sites in the human eotaxin promoter suggests that augmented transcription may contribute to the cytokine-induced increase in eotaxin transcripts (24). Alternatively, the presence of four ATTATA sequences in the human eotaxin gene indicates that eotaxin mRNA may be susceptible to post-transcriptional modification of message stability (4,24,25). To determine the predominant mechanism of TNF-α and IL-1-induced eotaxin mRNA accumulation in ASM cells, experiments could be performed with the addition of actinomycin D at the onset of cell stimulation to stop transcription. Similar studies could be undertaken with cyclohexamide addition prior to cell stimulation to determine whether TNF-α and IL-1-induced eotaxin mRNA accumulation requires de novo protein synthesis.

3. What other stimuli can regulate eotaxin expression and release by ASM?

The effect of other cytokines, particularly Th2-type cytokines, on ASM production of eotaxin could give valuable insight into the regulation of this chemokine in ASM in asthma. The identification of inhibitory mechanisms, either with other cytokines, cytokine antagonists, and corticosteroids would also be of interest. The ability of IgE immune complexes to induce IL-1 release and autologous action on ASM suggests that IgE binding and cross-linking or the binding of IgE-allergen complexes would upregulate eotaxin. Another possibility is that smooth muscle contraction leads to eotaxin generation in ASM. The latter could be tested by examining the effect of a broad range of contractile agonists on eotaxin expression in ASM cultured on a substrate that permits contraction (e.g. silicone derivatives or collagen), and also in ASM cultured in uncoated
plastic wells that preclude contraction. In this way, the effect of contraction may be dissociated from the other potential effects of the contractile agents.

4. **What is the contribution of ASM to the overall eosinophil recruitment in the lung?**

Selectively inhibiting the expression of eotaxin in ASM would be extremely difficult but, in theory, may be possible. One approach would require the identification of a gene that is selectively expressed in ASM but not in other cells including fibroblasts and vascular smooth muscle cells (for example, by differential display or subtractive hybridization). After cloning the promoter for this gene, a construct could be designed containing eotaxin cDNA in antisense orientation or an eotaxin-specific ribozyme downstream of the ASM-specific promoter. This construct could be transferred to lung cells of mice by inhalation or by using a transgenic strategy. The eotaxin antagonist (antisense or ribozyme) would then be selectively expressed in ASM cells and could be used in combination with a murine model of asthma.

### 3.4 REFERENCES


Appendix 1. Role of the candidate

The work for this thesis was performed at the Meakins-Christie Laboratories, McGill University between September 1996 and June 1998. The work was supervised by Dr. Qutayba Hamid and supported by grants to Dr. Hamid from the Medical Research Council of Canada and the Respiratory Health Network of Centers of Excellence. I am grateful for the salary support provided to me by a studentship from Canadian Cystic Fibrosis Foundation.

My involvement in the practical work was as follows:

- Design of studies (with the help of Dr. Hamid and Dr. Bouchaib Lamkhioued).
- Cell culture, stimulation, and harvesting of materials.
- Semi-quantitative RT-PCR and southern analysis (with the help of Dr. Lamkhioued).
- PBMC and eosinophil purification from peripheral blood (with the help of Dr. Lamkhioued).
- Chemotaxis assays (with the help of Dr. Lamkhioued).
- Immunocytochemistry (with the help of Zolfia Allakhverdi).
- The analysis and presentation of the results and writing of the enclosed manuscript.

I am indebted to my colleagues who contributed to the practical work as follows:

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- Dr. Sophie Molet who instructed me in the ASM cell culture.
• Dr. Andrew Luster (Harvard Medical School) who provided the reagents for eotaxin and performed the ELISA on ASM supernatant.
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