

**EOSINOPHILS AND CYTOKINES IN MILD ASTHMA  
AND ALLERGEN-INDUCED ASTHMA**

**By**

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## **EOSINOPHILS AND CYTOKINES IN ASTHMA**

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## ABSTRACT

Asthma is a chronic respiratory disease, characterized by variable airflow obstruction, airway hyperresponsiveness and airway inflammation. Prior to starting this thesis, information on the role of inflammatory cells and cytokines in asthma was limited.

The aim of this thesis was to determine the roles of eosinophils, an inflammatory cell believed to be important in asthma, and GM-CSF and IL-3, cytokines shown to regulate eosinophils in vitro, in mild and allergen-induced asthma. Fiberoptic bronchoscopy was performed on each subject and blood, bronchoalveolar lavage and bronchial biopsy samples were obtained.

In comparison to non-asthmatics, eosinophil number and activity and GM-CSF levels were increased in asthmatics. GM-CSF levels correlated with eosinophil number and activity and airway responsiveness correlated with eosinophil number and activity and GM-CSF levels. IL-3 was detected in both asthmatics and non-asthmatics, with no difference apparent between the groups. These findings indicate that eosinophils, potentially regulated in vivo by GM-CSF, contribute to airway inflammation in mild asthma.

In comparison to the control diluent inhalation, allergen inhalation caused increases in eosinophil number and activity and GM-CSF levels. IL-3 levels did not change after allergen. Eosinophil number and activity correlated with GM-CSF and the severity of the late asthmatic response correlated with the number and activity of eosinophils. These findings indicate that an increase in eosinophil number and activity, possibly due to GM-CSF, contributes to allergen-induced asthma.

This thesis has demonstrated cellular and cytokine involvement in mild and allergen-induced asthma. For the first time, the presence of GM-CSF and IL-3 cytokines, at the protein level, have been demonstrated in the airways of subjects with mild and allergen-induced asthma. Eosinophils, potentially regulated by GM-CSF, appear to play an important role in the disordered airway function associated with asthma.

## ACKNOWLEDGEMENTS

Kakadu elder: "Wior kudua numberaba palebi ero bali malanila ngia"

English translation: "One long run is the same as many small walks"

Reference: Spencer, B. (1914) Native tribes of the Northern Territory of Australia. MacMillan and Co., Ltd., London.

When I came to McMaster I started a PhD and started to run. When I leave McMaster I will have finished my PhD and have run a marathon. Aspects of my PhD have been analogous to my marathon. At the start of the race, I really was just warming up - not really knowing where I was running to or what the race would bring. I was suitably overwhelmed. By the middle of the race, however, I found myself running at a good, steady pace with a very strong and supportive team. Mutual benefits were shared all round. At the end of the race though, I really had to depend on myself, trusting that my commitment would help me finish. Thankfully, memory for pain is short once you cross the line. While I ran the marathon and completed my PhD myself, I did have one partner from start to finish - my husband and best friend, Mark. It is a great pleasure to thank him for all that he gives me.

The other men in my life that I am most pleased to thank are my supervisors, Drs Paul O'Byrne, Manel Jordana and Jack Gauldie. Each ranks as a "must have" in the graduate student's little black book of supervisors. To have all three supervise me during my PhD was true good fortune.

People have often commented on Paul's diplomacy. If the secrets of diplomacy are to make people feel that their concerns are important and offer genuine reassurance, then Paul is the Cardiorespiratory Unit's top attachè. Paul always made me feel that he would do whatever he could to facilitate my learning and enjoyment. For Paul, a charming personality and scientific excellence are not mutually exclusive descriptors.

My experiences with Manel were typified by his Wednesday morning meetings. Morning coffee and Tim Horton doughnuts (a Canadian tradition) jump-started enthusiastic, insightful and engaging discussions. I hope that Manel never

switches to decaf and that some of his exuberance follows me back to Australia.

Jack was the first of my supervisors that I met and I recall being somewhat intimidated. Its funny how quickly first impressions can change. Despite my somewhat circuitous route in selecting a PhD topic, Jack managed to keep one step ahead of me. I heeded his "words of the wise" and thoroughly enjoyed his rogue sense of humour.

On the financial side, I extend my gratitude to the Commonwealth Scholarship and Fellowship Plan. To receive a Commonwealth Scholarship was an honor and I believe that the many learning experiences that I have had in Canada (academic and non-academic) reflect the spirit of the award.

Of course, a PhD is rarely completed without the support and comic relief provided by one's fellow "research associates" (a global term to encompass an assorted lot!). During the less pleasant times (few though they were...), it was comforting to know that my fellow graduate students were facing similar anguish. At times, we were proof that misery loves company... However, no one could be morose for too long when surrounded by members of the infamous BH club. Although their identities cannot be revealed, they know who they are and I thank them for being just that.

I must also acknowledge and thank those responsible for my least expected, but often times most challenging, learning experiences at Mac. Noon hour each day heralded yet another run and the opportunity to experience the "hidden curriculum". I have gained valuable insight into the hallowed ground of male machismo (many amusing anecdotes on this topic - but I am sealed to secrecy) and, despite considerable antagonism at the start, I leave feeling that I have also taught "the boys" a few things about "the girls" (a year's sabbatical in ten years should complete their education).

Last, but not least, I thank my family for keeping my spirits high during my time away with their phone calls, letters, gift packages and visits. My family's contribution to this thesis cannot be underestimated as I am convinced that my productivity increased each time I had news from home. Their tempting descriptions of great surf and balmy sunshine strengthened my commitment to finish my thesis and return to the land of Oz.

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## LIST OF ABBREVIATIONS

BAL: Bronchoalveolar lavage

DPBS H + S: Dulbecco's phosphate buffered saline, Hepes buffer and saponin

EAR: early asthmatic response

ECG: electrocardiogram

ECP: eosinophil cationic protein

EDTA: ethylenediaminetetraacetate

FEV<sub>1</sub>: forced expiratory volume in one second

GM-CSF: granulocyte-macrophage colony-stimulating factor

Ig: immunoglobulin

IL: interleukin

LAR: late asthmatic response

PAF: platelet activating factor

PBS: phosphate buffered saline

PC<sub>20</sub>: provocative concentration causing a 20% fall in FEV<sub>1</sub>

PLP: periodate-lysine-paraformaldehyde

Rs: Spearman's rank correlation coefficient

TNF $\alpha$ : tumour necrosis factor alpha

VLA<sub>4</sub>: very late antigen four

## PREFACE

The following studies have been submitted for publication and form the basis of chapters three, four, five and six of this thesis:

Granulocyte-macrophage colony-stimulating factor, eosinophils and eosinophil cationic protein in mild asthmatics and non-asthmatics.

K.L. Woolley, E. Adelroth, M.J. Woolley, R. Ellis, M. Jordana and P.M. O'Byrne.

Submitted to: European Respiratory Journal

Effects of allergen challenge on granulocyte-macrophage colony-stimulating factor, eosinophils and eosinophil cationic protein in mild asthmatics.

K.L. Woolley, E. Adelroth, M.J. Woolley, R. Ellis, M. Jordana and P.M. O'Byrne.

Submitted to: American Journal of Respiratory Cell and Molecular Biology

Increases in airway eosinophils, metachromatic cells and eosinophil cationic protein, but not interleukin-3, in mild asthmatics compared to non-asthmatics.

K.L. Woolley, E. Adelroth, M.J. Woolley, M. Sabry, M. Jordana and P.M. O'Byrne.

Submitted to: Journal of Clinical Investigation

Allergen challenge increases airway eosinophils, metachromatic cells and eosinophil cationic protein, but not interleukin-3, in mild asthmatics.

K.L. Woolley, E. Adelroth, M.J. Woolley, M. Sabry, M. Jordana and P.M. O'Byrne.

Submitted to: Journal of Clinical Investigation

These studies are the first fiberoptic bronchoscopy studies completed by the Asthma Research Group in Hamilton, Canada. The initiation of bronchoscopy studies required input from many people. Dr. Paul O'Byrne provided and organized much of the financial support and laboratory space to set up and equip the bronchoscopy suite. Dr. Ellinor Adelroth, a well-recognized bronchoscopist, came from Sweden to perform the procedure. Dr. Manel Jordana offered considerable expertise and laboratory space to analyze the collected samples. Mark Woolley, Russ Ellis and Mona Sabry provided necessary technical support.

I am first author on the papers which form the basis of this thesis. Prior to the start of these studies I was involved in the planning and design of the experiments and submitted proposals justifying the studies and outlining their purpose, methods and analysis. I prepared materials for and assisted Dr Adelroth with each bronchoscopy. I was responsible for all of the subsequent assays on the samples collected. This required conducting pilot studies to adapt Dr Jordana's techniques to the bronchial samples and then completing each of the modified assays. I collated the data and completed the required statistical analyses for each study. The figures, tables and photos included in each paper are my own work and, although feedback was provided by the other authors of the papers, it was my responsibility to write each paper.

## CHAPTER 1: INTRODUCTION

### Asthma

The term asthma has been commonly applied by the vulgar, and even by many writers on the Practice of Physic, to every case of difficult breathing...they have introduced a great deal of confusion into their treatises on this subject.

Cullen, W. (1784) quoted in Brewis, R.A.L. (1990)

### Definition and Diagnosis

For many years, asthma has been difficult to define precisely due to its heterogenous nature and unknown etiology (Scadding, 1993). Therefore, asthma has been described in terms of its characteristic features which include episodic wheezing, reversible airflow obstruction and airway hyperresponsiveness. More recently, airway inflammation has been recognized as an important characteristic of asthma (Arm et al., 1992). The operational definition proposed by members of the International Asthma Project in 1992 encompasses these characteristics:

Asthma is a chronic inflammatory disorder of the airways in which many cells play a role, including mast cells and eosinophils. In susceptible individuals this inflammation causes symptoms which 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 various stimuli.

International Asthma Project Members (1992)

The diagnosis of asthma is based on a careful clinical history and confirmed with objective measures of airway function (International Asthma Project Members, 1992; Williams et al., 1993). In the clinical history, symptoms such as cough, wheeze, breathlessness and chest tightness may be described (McFadden et al., 1992; Williams et al., 1993). Symptom chronology is important, with nocturnal and episodic exacerbations often apparent in asthma (McFadden et al., 1992; Scadding, 1993). Due to the episodic nature of symptoms, however, asthmatic patients may be symptom-free at the time of the clinical history. Objective tests are used, therefore, to confirm the diagnosis. These tests include spirometry for assessing airflow obstruction and airway responsiveness tests to demonstrate airway hyperresponsiveness (Expert Panel, 1991).

### Epidemiology

Epidemiological studies of asthma in the last decade suggest an increase in asthma prevalence, severity and mortality (Sears, 1991; Gregg, 1983). Whether these increases are true, however, is not certain due to difficulties in the definition of asthma and method differences between studies (Gregg, 1983; Weiss et al., 1993). Despite these limitations, the most consistent findings are that asthma prevalence is increasing among certain populations, particularly migrants, and that asthma severity and mortality are increasing in many countries (McFadden et al., 1992; Sears, 1991; Weiss et al., 1993). These findings have provided much impetus for investigating the pathophysiological mechanisms underlying asthma.

### **Asthma Pathophysiology**

Adverse physiological changes associated with asthma include airway obstruction and airway hyperresponsiveness. Airway obstruction underlies many of the symptoms of asthma (Pride, 1983). Furthermore, when airway hyperresponsiveness is present, airway obstruction can occur more frequently and with increased severity (Cockcroft et al., 1991).

### Airway Obstruction

Airway obstruction refers to narrowing of the airways, with a resultant increase in airway resistance and decrease in airflow (Williams, 1993). In asthma, the increase in airway resistance is believed to occur primarily in the central airways, where airflow is turbulent and the total cross-sectional area, relative to more peripheral airways, is lower (Pride, 1983; West, 1985). Factors contributing to airway obstruction include contraction of airway smooth muscle, edema of the airway wall and mucus within the airway lumen (Pride, 1983; Expert Panel, 1991). Airway obstruction can be assessed indirectly by measuring flow rates with pulmonary function tests (Williams, 1993). The ratio of the forced expiratory volume in one second (FEV<sub>1</sub>) to the forced vital capacity provides a simple and useful index for determining the severity of airway obstruction (Expert Panel, 1991).

### Airway Hyperresponsiveness

Airway responsiveness refers to the degree to which airways constrict in response to non-sensitizing physical or chemical stimuli (Cockcroft et al., 1991). Therefore, airway hyperresponsiveness is defined as an increased responsiveness of the airway to non-sensitizing physical or chemical stimuli (Cockcroft et al., 1991). The mechanisms causing airway hyperresponsiveness are not clearly established. Inflammation, humoral mediators and abnormalities in smooth muscle and neural

control have been implicated (Cockcroft et al., 1993). Airway responsiveness is commonly measured with histamine or methacholine inhalation challenges. Results are expressed as the provocative concentration required to cause a 20% fall in the FEV<sub>1</sub> (PC<sub>20</sub>). Although airway responsiveness is distributed in a continuous manner, airway hyperresponsiveness is regarded as being present when the PC<sub>20</sub> is less than 8 mg.ml<sup>-1</sup> (Dolovich et al., 1992).

## **Asthma Pathology**

### Normal Anatomy and Histology of the Lower Respiratory Tract

The lower respiratory tract extends from the larynx to the visceral pleura (Jeffery et al., 1984). The right and left lung are each enclosed within a pleural cavity. The right lung has three lobes (upper, middle and lower) and the left lung has two lobes (upper and middle) (Krahl, 1964; Jeffery et al., 1984). The lungs consist of successively branching airways which become more narrow, short and numerous with each division (West, 1985). The anatomical sequence of the lower respiratory tract is: larynx, trachea, right and left main bronchi, lobar bronchi, segmental bronchi, terminal bronchioles, respiratory bronchioles, alveolar ducts and alveolar sacs (Lopez-Vidriero et al., 1983). The conducting, central or larger airways extend from the larynx to the terminal bronchioles while the respiratory,



peripheral or smaller airways extend from the respiratory bronchioles to the alveolar sacs (Lopez-Vidriero et al., 1983; Laitinen et al., 1991). The conducting airways serve to conduct air while the respiratory airways facilitate gas exchange (Hogg, 1993).

As most changes in asthma are believed to occur within the larger airways (Aalbers et al., 1993; Aalbers et al., 1993; Hogg, 1993), the major anatomical features of the bronchi will be reviewed. Histological sections show that the bronchial wall consists of epithelium, connective tissue, glands, muscle, nerves, blood vessels and cartilage. The epithelium is ciliated, pseudo-stratified and columnar. Epithelial cell types include basal cells, goblet or mucus cells and ciliated cells (Jeffery et al., 1984). The connective tissue beneath the epithelial basement membrane is comprised of elastin, collagen and reticular fibers (Krahl, 1964). Together, these fibers form a relatively flexible, lattice framework which helps to maintain the structural integrity of the bronchial wall (Krahl, 1964). Submucosal glands are tubular-alveolar in structure and composed of a ciliated duct, a collecting duct and mucus and serous tubules (West, 1985; Lopez-Vidriero et al., 1983). In health, secretions from the submucosal glands keep the respiratory epithelium moist, enabling ciliated epithelial cells to move particles toward the larynx (Lopez-Vidriero et al., 1983; Krahl, 1964). Bronchial smooth muscle is arranged as two opposing spirals around the bronchi, internal to the cartilage (West, 1985). Due to this spiral arrangement, smooth muscle contraction

causes both constriction and shortening of the bronchi (West, 1985; Lopez-Vidriero et al., 1983). Nerve fibers are found throughout the bronchial wall, located within the epithelium and near submucosal glands, blood vessels and muscle fibers (Lopez-Vidriero et al., 1983; Laitinen et al., 1991). Neural pathways influence secretion from glands, the permeability and blood flow in the bronchial circulation and the tone of airway smooth muscle (Laitinen et al., 1991). Afferent receptors, located in the epithelium and submucosa, include cough or irritant receptors and stretch receptors (West, 1985; Lopez-Vidriero et al., 1983). The blood vessels supplying the bronchi with oxygenated blood arise from the bronchial artery. Venous blood from the large bronchi drains primarily into bronchial veins while venous blood from the smaller bronchi drains into the pulmonary veins (Krahl, 1964; Lopez-Vidriero et al., 1983; Laitinen et al., 1991).

#### Pathological Changes in Asthma

I could not attend at the dissection, but was informed by the gentlemen who were present, that the vessels of the pleura, on the surface of the lungs, and of the trachea, were turgid, and seemed obstructed, that the parts had a livid appearance, resembling that which is observed, when an inflammation terminates in a gangrene, and that the bronchial vessels were filled with a white tough gelatinous substance.

Millar, J. (1769) quoted in Brewis, R.A.L. (1990)

Autopsy findings from patients dying of asthma have provided considerable information on the pathological changes associated with asthma. Observations have included plugging of airways with thick, gelatinous material ("mucus plugs"), enlargement of submucosal glands, goblet cell hyperplasia, marked eosinophilic infiltration, smooth muscle hypertrophy, sub-epithelial collagen deposition ("thickened basement membrane"), edema and shedding of the epithelium (Laitinen et al., 1991; Hogg, 1993; Reid et al., 1989; Lopez-Vidriero et al., 1983).

Concerns have been raised, however, that findings from fatal asthma may not relate to the pathological changes that might occur in asthma which is less chronic or less severe (Reid et al., 1989; Laitinen et al., 1991). Recently, the technique of fiberoptic bronchoscopy has enabled bronchial biopsy tissue to be obtained from living asthmatics (Laitinen et al., 1991; Laitinen et al., 1991; Hogg, 1993).

Biopsy samples from asthmatics with ongoing asthma reveal changes in the epithelium, mucus-secreting cells and glands, cellular infiltrate and connective tissue. In the epithelium, alterations include epithelial shedding, widening of intercellular spaces, hyperplasia and metaplasia (Beasley et al., 1989; Jeffery et al., 1989; Reid et al., 1989; Bousquet et al., 1990). While not universal, goblet cell hyperplasia and glandular enlargement have also been observed (Jeffery et al., 1989; Laitinen et al., 1991; Bousquet et al., 1990). Variable degrees

of inflammatory cell infiltrate have been reported. Mast cells and eosinophils have been the most consistent cell type observed, with lymphocytes and, less frequently, neutrophils also observed (Roche et al., 1989; Beasley et al., 1989; Laitinen et al., 1991; Reid et al., 1989; Lozewicz et al., 1988). Increased deposition of collagen beneath the epithelium has been a relatively common finding in asthma biopsy tissue (Roche et al., 1989; Beasley et al., 1989; Jeffery et al., 1989). In bronchial blood vessels, an increase in endothelial gaps have been reported (Laitinen et al., 1991; Laitinen et al., 1991).

In general, pathological changes noted in bronchial tissue from asthmatics with mild, ongoing disease have been similar, but less marked, than the changes observed in fatal asthma (Jeffery et al., 1989; Beasley et al., 1989; Laitinen et al., 1991).

## References

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## CHAPTER 2: AIRWAY INFLAMMATION IN ASTHMA

### Airway Inflammation

Asthma...is a special form of inflammation of the smaller bronchioles.

Osler, W. (1892)

Inflammation is a homeostatic process necessary for species survival (Weissman, 1988; Gauldie et al., 1992). Inflammatory responses, elicited by trauma or infection, are highly-orchestrated molecular, cellular and physiological events designed to terminate tissue injury and initiate tissue repair (Gauldie et al., 1992). The cardinal signs of inflammation, recognized for centuries, are calor (heat), rubor (redness), tumor (swelling), dolor (pain) and functio laesa (loss of function) (Weissman, 1988; Barnes, 1993).

In asthma, the cardinal signs of inflammation are evident as redness of the airway mucosa (rubor), swelling of the airway wall due to tissue edema (tumor), irritation from cough and secretions (dolor) and altered airway function due to obstruction and hyperresponsiveness (functio laesa) (Barnes, 1993; Rennard et al., 1992). Chronic inflammation is believed to underlie the structural changes often observed in asthmatic airways, including goblet cell hyperplasia,



submucosal gland hypertrophy, epithelial cell metaplasia, smooth muscle hypertrophy and excessive sub-epithelial deposition of collagen by myofibroblasts (Brewster et al., 1990; Hogg et al., 1991).

Airway inflammation in asthma appears to involve a complex series of interactions between inflammatory cells, tissue structural cells, neural reflexes, lipid mediators and cytokines (McFadden et al., 1992). This review, however, will focus on the eosinophil, a cell which has had a long historical association with asthma (Ellis, 1908) and the cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3), whose potential roles in asthma have been examined much more recently (Barnes, 1993).

## Eosinophil

The origin and significance of the eosinophile cells...often found in the sputum and pulmonary tissues in cases of bronchial asthma is a widely discussed and still unsettled question.

Ellis, A.G. (1908)

Since the turn of the century, the debate on the role of the eosinophil in asthma has continued. Until relatively recently, the eosinophil was viewed as having an anti-inflammatory role in asthma because it contained enzymes capable

of inactivating mast cell mediators (Butterworth et al., 1981). In the last decade, however, as eosinophils have been shown to release numerous inflammatory mediators, a pro-inflammatory role for the eosinophil in asthma has been proposed (Bousquet et al., 1990). Although the precise role of eosinophils in asthma remains undefined, considerable progress has been made in understanding eosinophil biology. This review will summarize information regarding the development and distribution of eosinophils, their structure, products and function.

#### Development and distribution

Eosinophils develop from promyelocyte precursor cells in the bone marrow (Zucker-Franklin, 1990; Weller, 1991). Under the influence of cytokine growth factors, the promyelocyte differentiates into an eosinophil granulocyte (Clutterbuck et al., 1989; Rothenberg et al., 1988). Mature eosinophils are stored in the bone marrow for several days and then released into the bloodstream with a circulating half life of 13 to 18 hours (Parwaresch et al., 1976). Eosinophils then migrate through the vascular endothelium to reside in the tissue for an unknown period of time (Weller, 1991). Eosinophils are primarily tissue dwelling cells with an estimated 500 tissue eosinophils for every circulating eosinophil (Wardlaw et al., 1993). In a healthy, non-atopic adult, eosinophils comprise 1 to 3 percent of the circulating leukocyte population, with the number of eosinophils usually less than 400 per  $\mu\text{L}$  of blood (Zucker-Franklin, 1990).

### Structure

Mature eosinophils are 8 to 17  $\mu\text{m}$  in diameter and are characterized by a bilobed nucleus and eosin-staining specific granules in the cytoplasm (Miller et al., 1966; Zucker-Franklin, 1990) (Figure 2.1). Eosinophils contain approximately 20 specific granules which are spherical or ovoid in shape and 0.5 to 1.0  $\mu\text{m}$  in diameter (Wardlaw et al., 1993). The granules have an electron dense core which contains major basic protein (Peters et al., 1986). The core is surrounded by a less electron dense matrix, containing eosinophil cationic protein, eosinophil-derived neurotoxin and eosinophil peroxidase (Peters et al., 1986). Eosinophils also have small granules which contain the enzymes aryl-sulfatase and acid phosphatase (Parmley et al., 1974). Additional cytoplasmic structures in the eosinophil include mitochondria, Golgi apparatus, ribosomes, endoplasmic reticulum, glycogen and lipid bodies (Wardlaw et al., 1993; Weller, 1991). Charcot-Leyden crystals which are distinctive of eosinophils and contain lysophospholipase, are formed from a hydrophobic protein localized to the eosinophil plasma membrane (Weller et al., 1980). Eosinophils express many surface receptors, enabling them to interact with the extra-cellular environment. Receptors for immunoglobulins (Ig) (Hartnell et al., 1990), complement (Hamada et al., 1987), lipid mediators (Kroegel et al., 1989), cytokines (Lopez et al., 1991) and adhesion molecules (Lopez et al., 1986) have been reported.



Figure 2.1. Electron micrograph of an eosinophil. Secondary granules, with an electron dense core and less dense matrix, can be seen. Photo courtesy of Dr. M. Jordana.

## Products

Activated eosinophils can release many pre-formed or newly generated potent products. Pre-formed products include the specific granule proteins, major basic protein, eosinophil cationic protein, eosinophil-derived neurotoxin and eosinophil peroxidase (Peters et al., 1986; Ayers et al., 1985; Gundel et al., 1991; Henderson et al., 1980). Secretion of these granule products is energy dependent and involves mobilization and fusion of the granule with the plasma membrane prior to exocytosis (Tai et al., 1990; Tai et al., 1981). Newly generated mediators released from eosinophils include platelet activating factor (PAF) (Lee et al., 1984) and mediators synthesized from arachidonic acid, including leukotriene C<sub>4</sub> and D<sub>4</sub> (Weller et al., 1983) and prostaglandins E<sub>1</sub> and E<sub>2</sub> (Foegh et al., 1986). In addition, eosinophil peroxidase enables eosinophils to generate oxygen radicals, including superoxide and hydrogen peroxide (Henderson et al., 1980). Recently, the capacity of eosinophils to produce cytokines, small glycoprotein molecules, has been examined. Eosinophils have been found to localize the protein or express the mRNA for an increasing number of cytokines, including GM-CSF (Ohno et al., 1991; Moqbel et al., 1991), transforming growth factor  $\beta$  (Ohno et al., 1992; Wong et al., 1991), IL-3 (Kita et al., 1991) and IL-5 (Desreumaux et al., 1992; Broide et al., 1992). However, the production of these cytokines in vivo and their biological significance is not yet clearly defined.

### Function

The traditional function ascribed to eosinophils has been to defend the host against large, non-phagocytatable organisms such as helminth parasites (Zucker-Franklin, 1990). After opsonization of the helminth with complement, IgG or IgE, eosinophils adhere to and, via the release of cationic proteins, kill the parasite (Butterworth et al., 1981). The preferential distribution of eosinophils at epithelial surfaces and their ability to bind IgA also suggests that they may participate in normal mucosal immunity (Weller, 1991).

The beneficial functions of eosinophils, however, have been overshadowed recently by the current interest in the pro-inflammatory nature of eosinophils. The eosinophil has been implicated as an important effector cell in asthma due to the potential links between eosinophil products and the pathophysiological characteristics of the disease (Arm et al., 1992; Chung, 1993). The ability of cationic proteins to damage pulmonary epithelium, for example, may account for the shedding of the epithelium often observed in asthma (Ayers et al., 1985). Furthermore, increased exposure of afferent nerves to various stimuli following epithelial shedding may contribute to airway hyperresponsiveness (Wardlaw et al., 1988; Beasley et al., 1989). Newly generated mediators have also been implicated in asthma. Eosinophil-derived sulphido-peptide leukotrienes and PAF, for example, may contribute to the vascular leakage, mucus hypersecretion and bronchoconstriction that occurs in asthma (O'Byrne et al., 1992; Kroegel et

al., 1992; Wardlaw et al., 1993).

## **Cytokines**

Communication between cells is essential in both health and disease. Cytokines are now known to have a fundamental role in inter-cellular communication (Calhoun et al., 1993; Barnes, 1993). Although only discovered in the 1960's, interest and information about cytokines is growing rapidly.

Cytokines are generally small (7 to 40 kDa) peptide or glycoprotein molecules (Gauldie et al., 1992). They are exceptionally potent molecules, active at picogram concentrations ( $10^{-12}$  mol/L) (Owen et al., 1987; Lopez et al., 1986; Oppenheim, 1991). Cytokines communicate with their target cells by binding to high-affinity cell surface receptors that have extra-cellular, trans-membrane and intra-cellular domains (Calhoun et al., 1993; Barnes, 1993). Most nucleated cells express receptors for a variety of cytokines allowing them to alter their activity in response to different cytokines. In addition, cytokines can exert multiple effects by binding to a variety of cell types. Cytokines can act in a paracrine, autocrine or occasionally endocrine manner and regulate many processes, including hemopoiesis, tissue growth and inflammation (Calhoun et al., 1993; Clemens, 1991). The effects of cytokines are often dependent upon the presence of other

cytokines (Gauldie et al., 1992; Vilcek et al., 1991). Synergism or antagonism between cytokines is increasingly evident (Lopez et al., 1992; Moore et al., 1990; Fiorentino et al., 1991; Vilcek et al., 1991). As the effects of cytokines are mediated through transcriptional regulation of DNA, the response time of cytokines is normally within hours (Clemens, 1992; Calhoun et al., 1993).

Increasing attention is being directed toward the roles that cytokines may have in asthma (Hoigate, 1993). This interest has been heightened by the finding that certain cytokines have the ability to influence the development, recruitment, activation and survival of the inflammatory cells found in asthmatic airways (Calhoun et al., 1993). The cytokines GM-CSF and IL-3 have been of particular interest due to their influences on eosinophils (Barnes, 1993; Warringa et al., 1993).

### GM-CSF

GM-CSF is a 127 amino acid protein and, depending upon the degree of glycosylation, has a molecular mass of 15-30 kDa (Garland, 1991). GM-CSF is produced by a variety of cell types, including eosinophils (Ohno et al., 1991; Moqbel et al., 1991), macrophages (Broide et al., 1991), epithelial cells (Mattoli et al., 1992), T lymphocytes (Broide et al., 1991), mast cells (Burd et al., 1989) and fibroblasts (Vancheri et al., 1989). GM-CSF binds to high affinity GM-CSF receptors (Lopez et al., 1992). There are between 200 and 400 GM-CSF receptors



per cell (Garland, 1991). The receptor for GM-CSF is comprised of two chains, the  $\alpha$  chain is unique for GM-CSF while the  $\beta$  chain is shared with IL-3 and IL-5 (Lopez et al., 1992).

GM-CSF has been shown in vitro to enhance eosinophil progenitor growth (Sonoda et al., 1989) and eosinophil survival (Lopez et al., 1986; Vancheri et al., 1989; Anwar et al., 1993), effects which could result in increased eosinophil numbers. GM-CSF may also stimulate eosinophil function. In vitro studies have shown GM-CSF to enhance or induce eosinophil chemotaxis (Warringa et al., 1991), adhesion molecule expression (Lopez et al., 1986; Czech et al., 1993), cytotoxicity (Owen et al., 1987), phagocytosis (Lopez et al., 1986) and mediator release (Owen et al., 1987; Tai et al., 1990).

### IL-3

IL-3 is a 133 amino acid protein and, because of several potential glycosylation sites, may have a molecular mass between 14 and 36 kDa (Schrader, 1991; Garland, 1991). The structure of the IL-3 receptor is similar to that of the GM-CSF receptor, with an  $\alpha$  chain unique for the ligand and a  $\beta$  chain common to receptors for GM-CSF and IL-5 (Lopez et al., 1992). Approximately 100 to 1000 high affinity IL-3 receptors are present on IL-3 responsive cells (Garland, 1991). While production of IL-3 was initially thought to be restricted to T lymphocytes (Niemeyer et al., 1989), IL-3 has now been shown to be produced by several cell

types, including eosinophils (Kita et al., 1991; Anwar et al., 1993) and mast cells (Burd et al., 1989). Similar to GM-CSF, IL-3 has been shown to promote eosinophil growth and differentiation (Sonoda et al., 1989) and survival in vitro (Anwar et al., 1993). IL-3 may also upregulate eosinophil function by enhancing mediator release (Tai et al., 1990), chemotaxis (Warringa et al., 1992; Warringa et al., 1991) and the expression of cell adhesion molecules (Czech et al., 1993).

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### CHAPTER 3: EXPERIMENTAL INVESTIGATION OF ASTHMA

While asthma is a chronic disease, acute exacerbations do occur (McFadden et al., 1992). To investigate the pathogenesis of this disease, two models of study have been utilized. The ongoing, chronic component of the disease has been examined with baseline comparison studies of stable, asthmatic patients and non-asthmatic controls. To investigate the mechanisms underlying acute increases in disease severity, allergen challenge models have been employed.

Determination of the cellular and molecular processes which may contribute to the chronic and acute components of asthma necessitates examination of asthmatic airways. The use of fiberoptic bronchoscopy has enabled researchers to examine this site directly (Holgate et al., 1992; Rennard et al., 1992; Dolovich et al., 1992). Furthermore, this technique has enabled bronchoalveolar lavage and bronchial biopsy samples to be collected and used for more detailed study. This review will summarize the major features of the models and techniques used in the experimental investigation of asthma.

## Models of Study

### Baseline comparison studies

Comparisons between asthmatic and non-asthmatic subjects have been made to identify differences between the two groups that might account for the presence of disease. Subjects in baseline comparison studies are usually matched for age and gender, with exclusive criteria set for non-asthmatic medications, smoking history, respiratory infections and other concomitant diseases. An accurate diagnosis of asthma is required for each asthmatic subject studied and details should be given on symptoms and medication use. For both asthmatics and non-asthmatics, subject characteristics should be provided, including atopic status, airway function and airway responsiveness.

Even with mild asymptomatic asthma, airway function may differ from that of non-asthmatics with respect to airway obstruction or airway responsiveness. Airway inflammation is thought to contribute to these pathophysiological features of ongoing chronic disease. Recent research, therefore, has focussed on inflammatory events that may distinguish asthmatics from non-asthmatics (Adelroth et al., 1990; Djukanovic et al., 1990; Bousquet et al., 1991; Hamid et al., 1991; Robinson et al., 1992; Bentley et al., 1992; Robinson et al., 1993). The hypothesis that chronic, ongoing asthma is due to airway inflammation has been strengthened

by evidence, albeit circumstantial, of significant relationships between airway inflammation and airway physiology.

#### Allergen challenge studies

The patient was tested by ingestion of the relevant alcoholic beverage, in this case, 95 ml of "Coolabah" Australian white wine, sipped over 15 minutes.

Pepys, J. et al., (1974)

While various forms of allergen challenge may be used to cause an exacerbation of asthma, allergen inhalation is one of the most commonly used models (O'Byrne et al., 1987; Busse et al., 1993). Sensitized subjects who inhale allergen may develop an early asthmatic response (EAR); bronchoconstriction which occurs within 10 minutes and resolves within 1 to 3 hours (O'Byrne et al., 1987). Three to four hours after challenge, approximately half of the subjects with an early response develop a late asthmatic response (LAR), with airway narrowing persisting for several hours (O'Byrne et al., 1987). The late response is usually associated with an increase in airway responsiveness and is thought to result from an influx of inflammatory cells and the release of pro-inflammatory mediators (De Monchy et al., 1985; Brusasco et al., 1990; Rossi et al., 1991; Gibson et al., 1991; Aalbers et al., 1993).

Due to circadian variation in airway function, allergen inhalation studies should incorporate a diluent inhalation control day (Sterk et al., 1993; O'Byrne et al., 1987). Standardization of testing procedures is essential with careful attention paid to allergen doses and solutions, aerosol generation, protocol timing and the calculation of results (Sterk et al., 1993).

While the allergen inhalation model is a useful way to study acute asthma, it does have limitations. For example, subjects may have a late asthmatic response without an increase in airway responsiveness (Gibson et al., 1991) and allergen-induced increases in airway responsiveness may occur before the late response is evident (Aalbers et al., 1993). Nevertheless, the allergen inhalation model has enabled airway inflammation to be induced and examined in a laboratory setting. In this regard, allergen challenge has provided a valuable and relatively convenient instrument to investigate mechanisms involved in the pathogenesis of asthma.

### **Flexible fiberoptic bronchoscopy**

Flexible fiberoptic bronchoscopy was introduced in 1968 for the investigation of cancer (Rennard et al., 1992). In the last decade, however, this technique has been used increasingly by investigators researching airway

inflammation in asthma (Kendall, 1993; Holgate et al., 1992; Rennard et al., 1992). The technique, which does not require general anesthesia and can be performed outside an operating room, involves passing a flexible fiberoptic bronchoscope, either trans-nasally or orally, into the major bronchi of the lungs (Kendall, 1993) (Figure 3. 1a).

Although the use of fiberoptic bronchoscopy has provided considerable insight into the inflammatory events associated with asthma, the technique does have limitations. To date, findings from bronchoscopy studies have not provided researchers with a precise way to define or diagnose asthma (Marone et al., 1993). Inflammatory changes have been observed but the specificity of these changes for asthma has not been clearly determined (McFadden et al., 1992; Holgate et al., 1992). Use of the technique also raises safety and ethical concerns with respect to testing subjects repetitively or when they may be symptomatic, obstructed or hyperresponsive (Workshop Committee, 1985; Kendall, 1993; Barnes, 1993).

The use of fiberoptic bronchoscopy as a research tool, however, has been facilitated by guidelines designed to ensure the safety of subjects and protocol standardization (Workshop Committee, 1985). These guidelines provide essential information for researchers with respect to subject selection, potential contraindications and hazards, technical considerations and pre- and post-procedure evaluation requirements (Workshop Committee, 1985).

For asthma researchers, one of the greatest advantages of the

fiberoptic bronchoscopy technique has been its ability to permit direct examination of airway inflammation. Prior to this technique, direct examination of airway inflammation was only possible in autopsy studies. Evaluation of airway inflammation in living asthmatics relied on indirect signs, such as the degree of airway obstruction or airway hyperresponsiveness (Rennard et al., 1992). Direct examination of asthmatic airways with bronchoscopy, however, has provided a means by which non-fatal airway inflammation can be evaluated in both qualitative and quantitative terms. Qualitative descriptions of the edema, secretions, erythema and tissue damage observed during bronchoscopy have been made (Thompson et al., 1988). In addition, various markers of inflammation have been quantified in the bronchoalveolar lavage and bronchial biopsy specimens that have been obtained with bronchoscopy. Brief descriptions of these sampling methods are outlined below.

#### Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) is a technique used to recover cells, fluid and proteins from the airway lumen (Rennard et al., 1992). A lavage is performed by gently impacting or "wedging" a bronchoscope against a bronchus, instilling warm sterile aliquots of saline into the airway and then carefully aspirating the fluid back (Walters et al., 1991) (Figure 3. 1b). Technical aspects of BAL, such as the size and number of aliquots, aspiration time and pressure and the method of

processing fluid can influence results and, therefore, must be standardized in research studies (Walters et al., 1989).

Although relatively simple to perform, difficulties can arise with the interpretation of BAL results. Statements regarding the source of cells and proteins recovered in BAL are limited as the areas sampled by BAL are not well localized (Holgate et al., 1992; Barnes, 1993; Bousquet et al., 1991). Radiographic studies do suggest, however, that both central and peripheral airways contribute to the BAL return (Kelly et al., 1987). Interpretation of the levels of cytokines and mediators recovered in BAL fluid is also difficult as dilution effects are not yet clearly understood (Kelly et al., 1988; Walters, 1992; Walters et al., 1991). Extrapolating findings from BAL to the tissue is also questionable as inflammatory events in the airway lumen may not reflect inflammatory events in the mucosa (Holgate et al., 1992; Barnes, 1993; Rennard et al., 1992; Walters et al., 1992).

Despite these limitations, however, BAL has proven itself as a useful tool in asthma research. Identification and quantification of inflammatory cells and mediators has been achieved with BAL and these inflammatory indices have been related to airway function and asthma severity (De Monchy et al., 1985; Metzger et al., 1986; Kirby et al., 1987; Adelroth et al., 1990; Kelly et al., 1989; Brusasco et al., 1990; Broide et al., 1992; Robinson et al., 1993). These findings have highlighted the potential importance of inflammation in asthma and provided the impetus for further investigations.



### Bronchial biopsies

Most recently, the use of fiberoptic bronchoscopy in asthma research studies has included obtainment of bronchial biopsy samples. Although the presence of inflammatory cells and mediators in the airway lumen had been demonstrated in BAL studies, biopsy studies were required to examine these inflammatory events in the tissue.

To obtain bronchial biopsy specimens, a bronchoscope is positioned across the subcarinae of the bronchi and biopsy forceps are then passed through the bronchoscope to extract a 1 to 2 mm<sup>2</sup> sample of tissue (Holgate et al., 1992) (Figure 3. 1c). The limitations associated with bronchial biopsies include the small size of the sample, the restriction of sampling the central airways only and the technical artifacts (eg. structural damage) that may occur during extraction (Rennard et al., 1992; Bousquet et al., 1991; Barnes, 1993).

Despite these limitations, however, bronchial biopsies from asthmatic subjects have provided evidence for the importance of inflammatory processes in asthma (Djukanovic et al., 1990; Azzawi et al., 1990; Bentley et al., 1992; Hamid et al., 1991; Broide et al., 1992; Aalbers et al., 1993; Bentley et al., 1993).

Further insight into the pathogenic mechanisms involved in asthma will require additional experimental investigation. Examination of both the acute and chronic components of asthma may reveal interactions between the processes which initiate and maintain airway inflammation. When possible, both BAL and

biopsy tissue should be examined. Evaluation of airway inflammation based on both samples would probably be preferable to evaluation based on either sample alone (Bousquet et al., 1991).



Figure 3.1. Flexible fiberoptic bronchoscopy technique. (a) top, trans-oral insertion of bronchoscope. (b) middle, collection of instilled BAL fluid. (c) bottom, insertion of forceps through the bronchoscope to extract bronchial tissue.

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## CHAPTER 4: GRANULOCYTE-MACROPHAGE COLONY-STIMULATING-FACTOR, EOSINOPHILS AND EOSINOPHIL CATIONIC PROTEIN IN MILD ASTHMATICS AND NON-ASTHMATICS

### Abstract

Increasing evidence implicates the eosinophil as an important effector cell in asthma but little is known regarding its regulation *in vivo*. Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been shown to regulate eosinophil function *in vitro*. We investigated the *in vivo* role of eosinophils and GM-CSF in mild asthma. We compared the number and function of eosinophils and the presence of GM-CSF in blood, bronchoalveolar lavage (BAL) and biopsy tissue obtained from 8 mild, atopic asthmatics and 10 non-asthmatics. Eosinophils were significantly increased in the blood, BAL and biopsy tissue from asthmatics. Activated eosinophils, assessed by immunostaining for the secreted form of eosinophil cationic protein, were also increased in asthmatic BAL cells and biopsy tissue. Significant increases in GM-CSF in BAL cells and biopsy tissue from asthmatics were also evident. Correlations between GM-CSF in BAL and biopsy tissue and eosinophils in BAL and biopsy tissue were significant. Airway



responsiveness was significantly correlated with eosinophil number and activation and with GM-CSF. These results demonstrate that eosinophils are more numerous and more activated and GM-CSF is increased in patients with mild asthma. Furthermore, GM-CSF is correlated with eosinophil number and function in vivo and these indices are significantly correlated with airway function. These findings emphasize the importance of eosinophils, potentially regulated in vivo by GM-CSF, in contributing to the disordered airway function evident even in mild asthma.

## Introduction

Airway inflammation is a characteristic feature of asthma, even in its mild form (O'Byrne, 1990). While many different cell types may contribute to airway inflammation, increasing evidence suggests that the eosinophil may be particularly important. Mediators and proteins released from eosinophils are able to contract airway smooth muscle (Weller et al., 1983), damage the bronchial epithelium (Ayers et al., 1985) and may induce airway hyperresponsiveness (Gundel et al., 1991). Increases in the number of eosinophils and levels of eosinophil-derived proteins have been reported in the blood (Durham et al., 1989), sputum (Gibson et al., 1989) and bronchoalveolar lavage (BAL) fluid (Kirby et al., 1987) of patients with asthma, compared to non-asthmatics. More recently, eosinophils have been

examined in asthmatic bronchial tissue. Increases in the number and state of activation of eosinophils have been reported in some (Azzawi et al., 1990; Beasley et al., 1989) but not all (Jeffery et al., 1989; Lozewicz et al., 1988) studies. Examination of bronchial biopsies may be particularly important as eosinophils are predominantly tissue dwelling cells (Weller, 1991) and inflammatory events occurring within the airway wall may be most relevant to the disordered airway function associated with asthma (Djukanovic et al., 1990).

The increased number of eosinophils in asthmatic airways may reflect an increase in eosinophil recruitment and/or survival. The cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF), may be involved in these processes. In vitro, GM-CSF has been shown to be chemotactic for eosinophils (Warringa et al., 1992) and can enhance the activation (Lopez et al., 1986) and survival (Owen et al., 1987) of peripheral blood eosinophils. Limited information is available, however, concerning the presence and role of GM-CSF in the pathogenesis of asthma. GM-CSF mRNA has been detected in BAL cells from asthmatics (Broide et al., 1992) and increased GM-CSF protein has been reported in the supernatant from cultured BAL cells (Rotondetto et al., 1993) and bronchial epithelial cells (Mattoli et al., 1992) from asthmatics with moderate to severe disease. To date, no studies have directly investigated the presence of GM-CSF protein in BAL cells or in bronchial tissue from mild asthmatics. Identification of GM-CSF protein in the airways of patients with mild, ongoing asthma would

support the hypothesis that this cytokine is involved in the local regulation of eosinophil function in asthma.

The purpose of this study was to compare the numbers of eosinophils, the amounts of eosinophil-derived cationic protein (ECP), an indicator of eosinophil activation, and GM-CSF in the blood, BAL fluid, BAL cells and bronchial tissue of mild asthmatics and non-asthmatics. We hypothesized that eosinophil numbers would be increased in the asthmatic subjects. We also sought to examine whether the presence of GM-CSF protein was associated with eosinophil number and function. To evaluate the physiological significance of these markers of inflammation, we examined the relationships between eosinophil numbers, ECP and GM-CSF and methacholine airway responsiveness.

## **Methods**

### Subjects

Eight patients with mild asthma and ten non-asthmatic subjects were selected for study (Table 4. 1). The study was approved by the Ethics Committee of McMaster University Medical Center and all subjects provided written informed consent. All asthmatics and five non-asthmatics were atopic, as indicated by one or more positive weal and flare responses to a battery of 16 skin prick tests to

common antigens. All subjects were non-smokers and none had experienced a respiratory infection during the four weeks prior to the study. The asthmatic subjects were stable at the time of study, requiring only intermittent use of inhaled  $\beta_2$ -agonists and having baseline FEV<sub>1</sub> values > 70% predicted.

### Study Design

Subjects attended the laboratory on two occasions. At the initial visit, subjects completed a respiratory questionnaire and a full medical history, physical examination, skin prick tests and an electrocardiogram (ECG) was performed. A blood sample was taken to measure white blood cell count, platelet count, prothrombin time and partial thromboplastin time. Baseline spirometry and a methacholine inhalation test were then performed. Within one week, subjects returned to the laboratory to complete a short follow-up questionnaire and perform spirometry. Providing the FEV<sub>1</sub> was within 5-10% of the baseline value, fiberoptic bronchoscopy was performed. After bronchoscopy, subjects were observed until they had recovered from the procedure.

Table 4.1: Characteristics of asthmatic and non-asthmatic subjects

Asthmatic							Non-Asthmatic						
Subject No.	Age (yrs)	Sex	Atopy	FEV <sub>1</sub> (% pred)	MCh PC <sub>20</sub> (mg ml <sup>-1</sup> )		Subject No.	Age (yrs)	Sex	Atopy	FEV <sub>1</sub> (% pred)	MCh PC <sub>20</sub> (mg ml <sup>-1</sup> )	
1	40	M	+	82	3.27		1	35	F	+	111	> 16	
2	30	F	+	86	1.56		2	31	M	+	99	> 16	
3	49	F	+	90	1.00		3	41	M	+	108	> 16	
4	40	F	+	98	1.28		4	27	F	+	106	> 16	
5	21	M	+	100	13.40		5	29	F	+	97	> 16	
6	42	M	+	108	2.20		6	31	M	-	110	> 16	
7	22	F	+	93	2.20		7	35	M	-	96	> 16	
8	25	M	+	89	1.45		8	44	F	-	101	> 16	
							9	28	M	-	92	> 16	
							10	47	F	-	97	> 16	
Mean	34			93	2.2*		Mean	35			101		
± SD	10.5			8.4	1.33		± SD	7.0			7.0		

\* Geometric mean and %SEM

### Methacholine Inhalation Test

To determine the degree of airway responsiveness, a methacholine inhalation challenge was performed as described by Cockcroft et al., (1987). Subjects inhaled doubling concentrations of methacholine chloride from a Wright nebulizer, for two minutes by tidal breathing. The test was continued until a fall in FEV<sub>1</sub>  $\geq$  20% of the baseline value occurred. The results were expressed as the provocative concentration causing a 20% fall in FEV<sub>1</sub> (PC<sub>20</sub>).

### Fiberoptic Bronchoscopy

Fiberoptic bronchoscopy was performed according to the recommendations of the National Institutes of Health (Workshop Committee, 1985). Subjects arrived at the research bronchoscopy suite at McMaster University Medical Center at 8:00 am, having fasted since midnight. An intravenous line was secured and blood samples obtained. Premedication with atropine (0.6 mg subcutaneous) was given 30 minutes prior to bronchoscopy. If sedation was required, midazolam (0.07 mg·kg<sup>-1</sup>) was given intravenously. Asthmatic subjects inhaled 400 µg of salbutamol, administered via a spacer device, before topical anesthesia with lidocaine (Xylocaine) was applied to the upper airways. The bronchoscope (Olympus BF-1T20D; Olympus Optical Co., Tokyo, Japan) was inserted via a mouthpiece under further anesthesia to suppress cough. During the

entire procedure, oxygen at 4 L·min<sup>-1</sup> was administered via a nasal cannula and subjects were monitored with ECG and ear oximetry. No complications were observed in any subjects during or following bronchoscopy.

### Blood Samples

Two venous blood samples were obtained from each subject. Samples were collected in either ethylenediaminetetraacetate (EDTA)-treated tubes, for total and differential white blood cell counts, or non-treated tubes, to obtain serum. Total cell counts were performed using a hemocytometer and differential cell counts were made using blood smears stained with a modified Wright-Geimsa (Diff-Quik, Baxter, McGraw Park, IL). Duplicate cell counts (200 cells counted per slide) were performed by one investigator blinded to the subject tested. Cells were classified as neutrophils, eosinophils, lymphocytes, monocytes and basophils using standard morphological criteria. To obtain serum, venous blood was stored at room temperature for two hours, centrifuged twice (1350 g, 10 min) and then stored at -70°C.

### Biopsy Samples

Following insertion of the bronchoscope into the left lung, four mucosal biopsies were obtained from the lobar carinae and carinae of the basal segments using a separate forceps (FB-20C) for each biopsy. The position of the biopsy site

and the appearance of the mucosal surface were carefully recorded. Two biopsies were placed immediately in glutaraldehyde for electron microscopy analysis and one biopsy was fixed in 4% paraformaldehyde for future studies. The biopsy sample for immunohistochemistry was placed immediately in periodate-lysine-paraformaldehyde (PLP) fixative and stored at 4°C for four hours. The tissue was then transferred through four solutions (phosphate buffered saline, 10%, 20% and 30% sucrose phosphate buffer), each for 4-6 hours at 4°C. The tissue was then frozen in Tissue-Tec OCT compound (Miles, Elkhart, In.) in -70°C isopentane (Aldrich, Milwaukee, Wi.) and stored at -70°C. Serial cryostat sections (6 µm) were cut onto slides coated with 3-aminopropyltriethoxysilane (Sigma, St. Louis, Mo.).

#### Bronchoalveolar Lavage

The bronchoscope was subsequently inserted into the middle lobe of the right lung and wedged into a segment or sub-segment. Five 20 ml aliquots of pre-warmed (37°C), sterile normal saline were infused and gently aspirated back into polypropylene tubes kept on ice. The fluid was then centrifuged at 200 g for 10 minutes at 4°C. The supernatant was decanted and stored at -70°C. The cell pellet was washed in phosphate buffered saline (PBS) and a total cell count was performed using a hemocytometer. The cells were then diluted with PBS to a concentration of  $1 \times 10^6$  cells·ml<sup>-1</sup>. Cytocentrifuge slides were prepared and stained with a modified Wright-Geimsa (Diff-Quik, Baxter, McGraw Park, IL) for a



differential cell count (400 cells counted). Duplicate cell counts were performed by one investigator blinded to the subject tested. Cells were classified as macrophages, neutrophils, eosinophils and lymphocytes using standard morphological criteria. Total and differential cell counts were expressed as the number of cells per milliliter of fluid recovered. Cytocentrifuge slides prepared for immunocytochemistry were air dried for five minutes, fixed in PLP for 10 minutes at 4°C, placed in a 15% sucrose phosphate buffer solution for 10 minutes at 4°C and air dried overnight. Slides were then wrapped in pairs in foil and stored at -70°C.

#### Tissue Eosinophils

Paraffin-embedded sections, prepared from the biopsy sample fixed in 4% paraformaldehyde, were stained with Chromotrope 2R (Sigma) to enumerate eosinophils in the tissue (Lendrum, 1944). Slides were coded prior to evaluation. For each section, a count was made of the number of positively staining cells within intact epithelium and another count made of the positive cells in the lamina propria to a depth of 115  $\mu\text{m}$  (delineated by a squared eyepiece graticule) was counted. A computerized and calibrated graphics tablet (Apple IIe) was used to determine the length of the epithelium and the area of the lamina propria in which counts were made. Results were expressed as the number of eosinophils per unit length of epithelium (1 mm) and area of lamina propria (1  $\text{mm}^2$ ).

### Immunocytochemistry

Immunocytochemistry was performed on BAL cytopins and tissue sections. To remove remaining OCT compound and sucrose and to enhance permeability and block non-specific reactions, slides were incubated with Dulbecco's phosphate buffered saline (GIBCO), supplemented with 0.01 M HEPES buffer (Boehringer Mannheim Canada Ltd.) containing 0.1% saponin (Sigma) (DPBS H + S) for five minutes at room temperature, twice. To further block non-specific binding, slides were incubated at room temperature with DPBS H + S containing 75% heat inactivated human AB sera for one hour and then with DPBS H + S containing 25% heat inactivated normal rabbit serum (Zymed, San Francisco, Ca.) for one hour. Slides were washed briefly with DPBS H + S before application of the monoclonal antibodies. The monoclonal antibodies used were EG2 (Pharmacia, Sweden) which recognizes the cleaved form of human ECP and is thought to represent eosinophil activation (Tai et al., 1984) and a mouse monoclonal antibody which reacts with human GM-CSF protein (Genzyme, Boston, Ma.). EG2 and GM-CSF antibodies were diluted in DPBS H + S containing 0.1% bovine serum albumin (Zymed) at final concentrations of  $1 \mu\text{g}\cdot\text{ml}^{-1}$  and  $3 \mu\text{g}\cdot\text{ml}^{-1}$ , respectively. Slides were incubated overnight at 4°C and then washed in DPBS H + S. Labelling of the monoclonal antibodies was detected by the alkaline phosphatase anti-alkaline phosphatase method (APAAP Kit, Dako, Santa Barbara, Ca.). Slides were washed in distilled water, lightly counterstained with Mayer's hematoxylin

(Sigma) and mounted. Appropriate system and specific controls were included in each assay.

#### Immunochemistry Quantification

All slides were coded before evaluation. For BAL cytospins, the cell pellet was divided into four equal sections, delineated with a squared eyepiece graticule. At least 100 cells were counted in each section. Results were expressed as the percentage of positively stained cells. The intra-class correlation coefficient, a stringent test of reproducibility, for repeated counts was 0.99 for EG2 and 0.99 for repeated counts of GM-CSF. Assessment of staining for the biopsy specimens was performed with a semi-quantitative method, previously described for evaluating immunohistochemical staining (Leung et al., 1989). Briefly, two observers graded the extent and intensity of positive staining for each biopsy specimen on a scale from 1 (negative) to 5. Intra-class correlation coefficients between observers were 0.81 for EG2 and 0.92 for GM-CSF.

#### ECP and GM-CSF Assays

Levels of ECP in serum and BAL fluid were determined using a double-antibody radioimmunoassay (Kabi Pharmacia Diagnostics AB, Sweden), with results expressed in micrograms per liter. Assay sensitivity for ECP was  $2 \mu\text{g}\cdot\text{L}^{-1}$ . Measurement of GM-CSF in serum and 20-fold concentrated (Amicon-10, Amicon

Corp., Danvers, Ma) BAL fluid was performed using an enzyme-linked immunosorbent assay (Quantikine, Research and Diagnostics System, Minneapolis, Mn), with results expressed in picograms per milliliter. Assay sensitivity for GM-CSF was  $2.8 \text{ pg}\cdot\text{ml}^{-1}$  for serum and  $1.5 \text{ pg}\cdot\text{ml}^{-1}$  for BAL fluid. In all assays, samples were analyzed in duplicate.

### Statistical Analysis

Statistical analyses were performed using the CSS Statistica computer software program. Data distributions were checked for normality using Kolmogorov-Smirnoff and Chi-Square analyses. As most distributions were significantly different from normal, non-parametric statistics were used. Given the a priori hypothesis that eosinophils would be increased in asthmatic compared to non-asthmatic subjects, one-tailed Mann-Whitney U tests for unpaired samples were used for eosinophil counts. Differences in indices of eosinophil activation (ECP levels and EG2 staining) and GM-CSF between asthmatics and non-asthmatics were examined using two-tailed Mann-Whitney U tests. Spearman's rank correlation coefficient test ( $R_s$ ) was used to examine the association between inflammatory parameters and the physiological measurement of airway responsiveness. A p value of  $< 0.05$  was accepted as statistically significant.

## Results

Eosinophils were increased in the blood, bronchoalveolar lavage and biopsy tissue from mild asthmatic subjects. The percentage of circulating eosinophils was significantly higher in the asthmatic subjects, with a median of 5% (range: 3 - 13%), than in non-asthmatic subjects, with a median of 3% (range: 1 - 9%) ( $p = 0.019$ ). The absolute number ( $\times 10^4 \cdot \text{ml}^{-1}$ ) of circulating eosinophils was also significantly higher in asthmatics, with a median of 36 (range: 16 - 100), than in non-asthmatics, with a median of 17 (range: 6 - 52) ( $p = 0.008$ ). The percentage of BAL eosinophils was also significantly increased in asthmatic subjects, with a median value of 1.4% (range: 0.0 to 5.0%), compared to non-asthmatics, with a median of 0.5% (range: 0.0 - 2.0%) ( $p = 0.034$ ) (Table 4. 2).

Table 4.2: Bronchoalveolar lavage cell counts for subjects

Asthmatics						
Subject No.	Return (ml)	Total ( $\times 10^4 \cdot \text{ml}^{-1}$ )	M	N	L	E
			(% )			
1	55	5.59	92.0	1.8	4.8	1.5
2	66	8.35	95.0	0.0	3.5	1.3
3	49	3.88	92.0	1.0	2.0	5.0
4	67	4.85	95.5	2.5	2.0	0.0
5	55	9.09	95.3	1.0	2.5	1.3
6	57	1.39	94.5	2.5	0.8	2.3
7	69	2.87	93.5	4.3	1.5	0.8
8	67	7.99	85.3	1.0	12.3	1.5
Median	62	5.22	94.0	1.4	2.3	1.4
Non-Asthmatics						
Subject No.	Return (ml)	Total ( $\times 10^4 \cdot \text{ml}^{-1}$ )	M	N	L	E
			(% )			
1	65	4.23	89.0	10.3	0.3	0.5
2	47	3.15	89.0	6.5	3.5	1.0
3	62	8.61	97.3	0.0	0.8	2.0
4	65	4.42	93.3	2.3	4.5	0.0
5	62	9.64	93.0	3.3	2.3	1.5
6	68	13.16	87.0	7.8	5.0	0.3
7	67	23.06	97.3	1.8	1.0	0.0
8	51	6.72	94.0	1.8	4.3	0.0
9	71	11.83	98.3	0.3	0.5	1.0
10	76	9.70	94.8	2.5	2.3	0.5
Median	65	9.13	93.7	2.4	2.3	0.5
p value*	0.59	0.08	0.86	0.27	0.59	0.03

M, macrophage; N, neutrophil; L, lymphocyte; E, eosinophil.

\* p value for comparison between asthmatics and non-asthmatics.

In bronchial biopsy tissue, eosinophils were again significantly higher in asthmatic than non-asthmatic subjects (Figure 4. 1). In areas of intact epithelium, intra-epithelial eosinophils could be seen in asthmatics, with a median of  $1.05 \cdot \text{mm}^{-1}$  (range: 0.67 - 3.18) but were never observed in non-asthmatic subjects ( $p = 0.002$ ). Within the lamina propria, eosinophils were increased in asthmatics, with a median of  $17.02 \cdot \text{mm}^{-2}$  (range: 7.41 - 78.95), compared to non-asthmatics, with a median of  $1.41 \cdot \text{mm}^{-2}$  (range: 0.00 - 10.81) ( $p = 0.003$ ).

Immunocytochemistry on BAL cells demonstrated that asthmatics had a significantly increased number of cells positive for EG2 and GM-CSF (Figure 4. 2). The median value of EG2-positive cells was 2.03% for asthmatics and 0.10% for non-asthmatics ( $p = 0.008$ ). The median value of GM-CSF-positive cells was 36% for asthmatics and 0% for non-asthmatics ( $p = 0.037$ ). In BAL cells from asthmatics, staining for EG2 was present in the cytoplasm and was also observed external to the cell surface, suggestive of ECP secretion (Figure 4. 3). Occasionally, staining for EG2 was apparent in alveolar macrophages, suggesting engulfment of secreted ECP. Staining for GM-CSF in asthmatic BAL cells was evident in the cytoplasm of a variety of cell types which, based on size and morphology, resembled eosinophils, alveolar macrophages and lymphocytes (Figure 4. 4). In non-asthmatics, few BAL cells were positive for either EG2 or GM-CSF. No staining was present in the slides stained with IgG<sub>1</sub> (negative control).

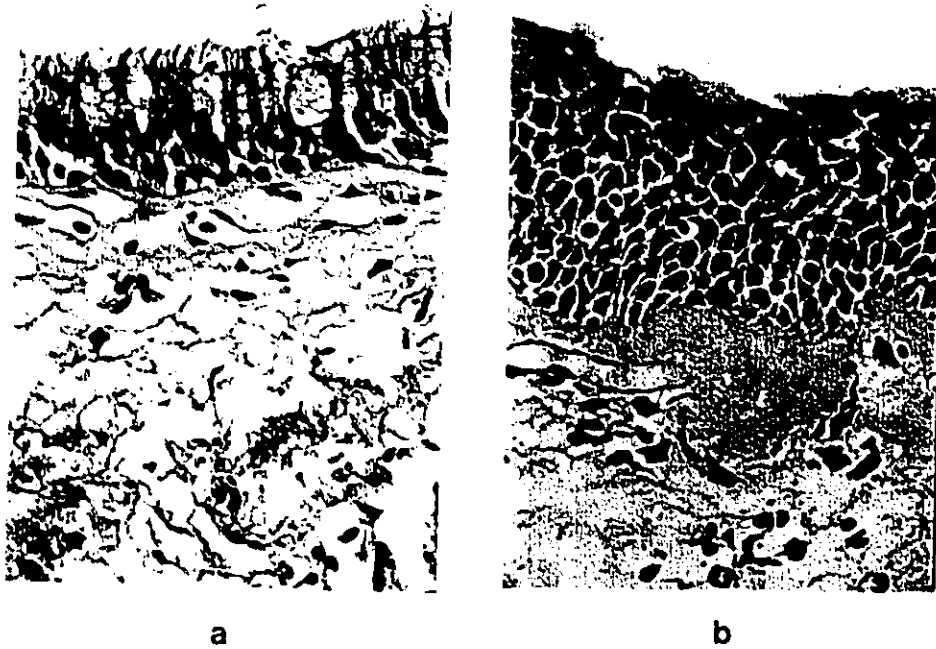


Figure 4.1. Staining for chromotrope 2R within bronchial biopsy tissue from a (a) non-asthmatic and (b) asthmatic subject (scale bar = 50  $\mu\text{m}$ ). Asthmatics had significantly increased numbers of eosinophils in the epithelium and lamina propria compared to non-asthmatics. Positively (red) stained eosinophils can be seen in the lamina propria and within the epithelium of the asthmatic tissue.



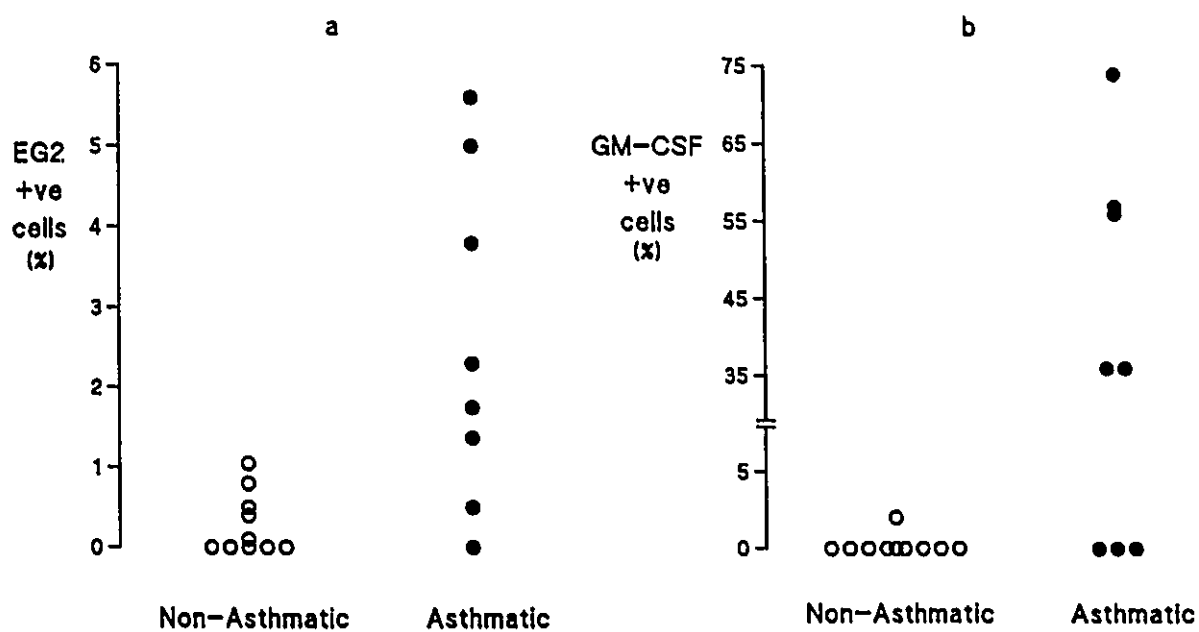


Figure 4.2. Percentage of BAL cells positive for (a) EG2 and (b) GM-CSF in non-asthmatic and asthmatic subjects. Asthmatics had significantly increased numbers of BAL cells positive for EG2 and GM-CSF, compared to non-asthmatics.

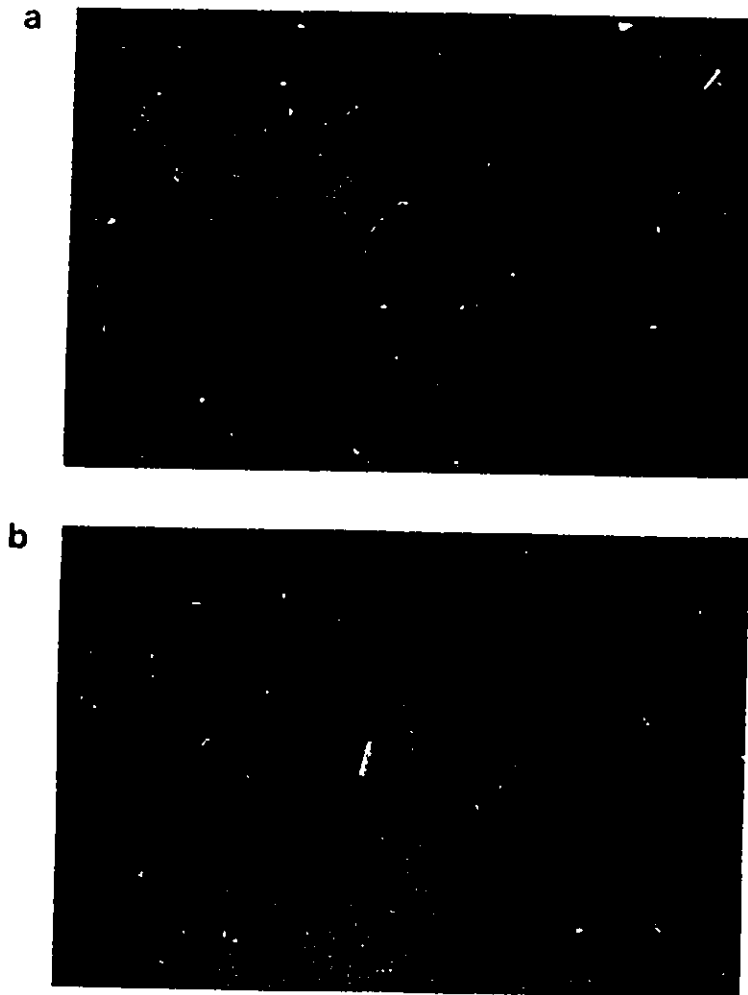


Figure 4.3. Immunocytochemistry with EG2 in BAL cells from a (a) non-asthmatic and (b) asthmatic subject (scale bar = 50  $\mu\text{m}$ ). Staining is not evident in cells from the non-asthmatic subject. Positive (red) staining with EG2 is seen in the cytoplasm, with fainter staining also seen surrounding the eosinophil from an asthmatic subject.

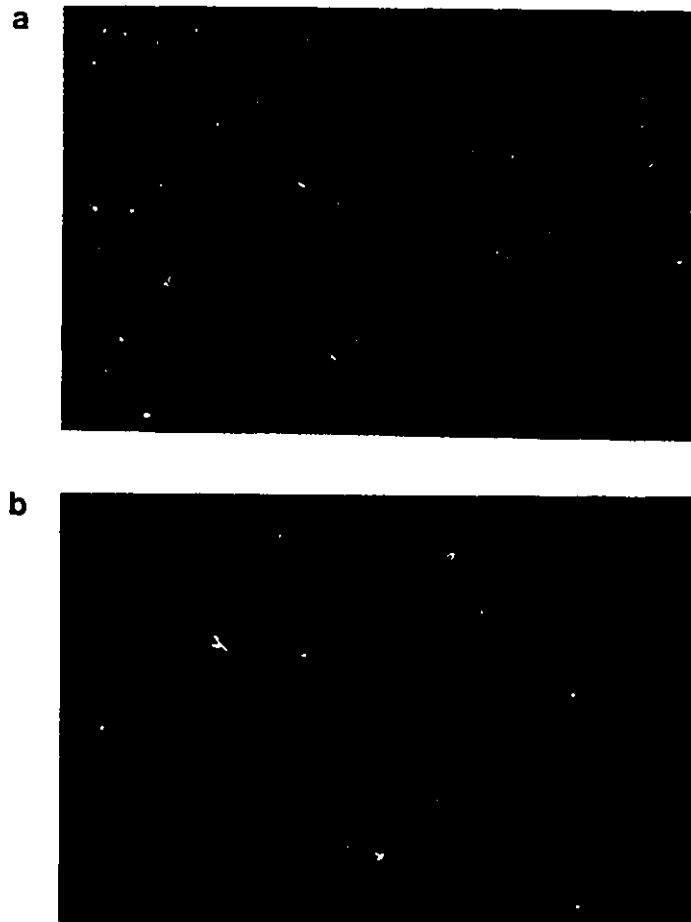


Figure 4.4. Immunocytochemistry for GM-CSF in BAL cells from a (a) non-asthmatic and (b) asthmatic subject (scale bar = 50  $\mu$ m). In cells from the non-asthmatic, no staining is evident. In BAL cells from an asthmatic, positive (red) staining for GM-CSF is seen in the cytoplasm of a cell which, based on morphology, appears to be an eosinophil.

Immunohistochemistry on biopsy tissue demonstrated that asthmatics had significantly increased staining for EG2 and GM-CSF (Figure 4. 5). The median value of EG2-staining was 2.25 for asthmatics and 1.25 for non-asthmatics ( $p = 0.026$ ). For GM-CSF staining, the median value was 3.13 for asthmatics and 1.38 for non-asthmatics ( $p = 0.015$ ). In asthmatic tissue, staining for EG2 was mainly intra-cellular and, although localized mainly beneath the basement membrane, intra-epithelial EG2-positive cells were observed in some asthmatics (Figure 4. 6). Intra- and extra-cellular staining was observed for GM-CSF in asthmatic tissue and was apparent both in the lamina propria and epithelium (Figure 4. 7). In non-asthmatic tissue, minimal staining was observed for either EG2 or GM-CSF. Within the non-asthmatic group, the greatest staining for EG2 and GM-CSF was observed in the atopic subjects. No staining was present in the negative control slides.

In the serum and BAL supernatant, median concentrations of ECP and GM-CSF were not significantly higher in asthmatic compared to non-asthmatic subjects. In the serum, the median concentration ( $\mu\text{g}\cdot\text{L}^{-1}$ ) of ECP was 10.25 for asthmatics (range: 5.10 - 45.50) and 9.30 (range: 1.70 - 32.80) for non-asthmatics ( $p = 0.594$ ). For serum GM-CSF, the median concentration ( $\text{pg}\cdot\text{ml}^{-1}$ ) was 16.94 (range: 6.33 - 38.25) for asthmatics and 9.98 (range: 2.63 - 35.23) for non-asthmatics ( $p = 0.424$ ). In lavage fluid from asthmatics, the median concentration

( $\mu\text{g}\cdot\text{L}^{-1}$ ) of ECP was 4.05 (range: 1.30 - 16.20) and was 3.35 (range: 1.30 - 9.70) in lavage fluid from non-asthmatics ( $p = 0.790$ ). The median concentration of GM-CSF ( $\text{pg}\cdot\text{ml}^{-1}$ ) in concentrated lavage fluid, was 13.7 (range: 8.0 - 25.1) for asthmatics and 10.0 (range: 2.5 - 33.3) for non-asthmatics ( $p = 0.594$ ).

Staining for GM-CSF in lavage cells and biopsy tissue was correlated to eosinophil number and activation. The percentage of BAL cells positive for GM-CSF was significantly correlated to EG2 staining in biopsy tissue ( $R_s = 0.59$ ;  $p = 0.01$ ). Staining for GM-CSF in biopsy tissue was significantly correlated to the percentage of BAL eosinophils ( $R_s = 0.53$ ;  $p = 0.02$ ) and the number of eosinophils in the epithelium ( $R_s = 0.68$ ;  $p = 0.02$ ) and the lamina propria ( $R_s = 0.59$ ;  $p = 0.02$ ).

Methacholine airway responsiveness was related to eosinophil number and activation and to GM-CSF. Significant correlations were found between MCh  $\text{PC}_{20}$  and the number of circulating eosinophils ( $R_s = -0.53$ ;  $p = 0.02$ ), the number of eosinophils in the epithelium ( $R_s = -0.95$ ;  $p < 0.00$ ) and the number of eosinophils in the lamina propria ( $R_s = -0.71$ ;  $p < 0.00$ ). Significant correlations were also observed between MCh  $\text{PC}_{20}$  and staining for EG2 in BAL cells ( $R_s = -0.48$ ;  $p = 0.046$ ) and biopsy tissue ( $R_s = -0.65$ ;  $p = 0.004$ ). Staining for GM-CSF in BAL cells was also significantly correlated with MCh  $\text{PC}_{20}$  ( $R_s = -0.81$ ;  $p = 0.000$ ) and a strong trend was evident between MCh  $\text{PC}_{20}$  and GM-CSF staining in biopsy tissue ( $R_s = -0.46$ ;  $p = 0.056$ ).

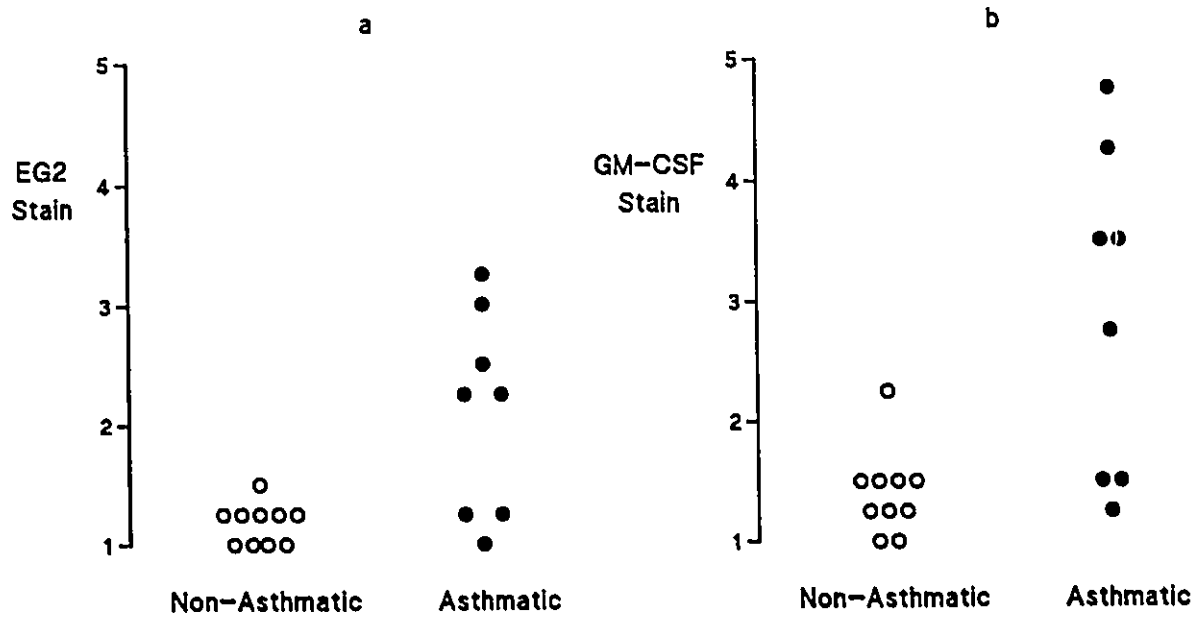


Figure 4.5. Staining of biopsy tissue for (a) EG2 and (b) GM-CSF in non-asthmatic and asthmatic subjects. Biopsy tissue from asthmatics exhibited significantly greater staining for EG2 and GM-CSF, compared to non-asthmatics.

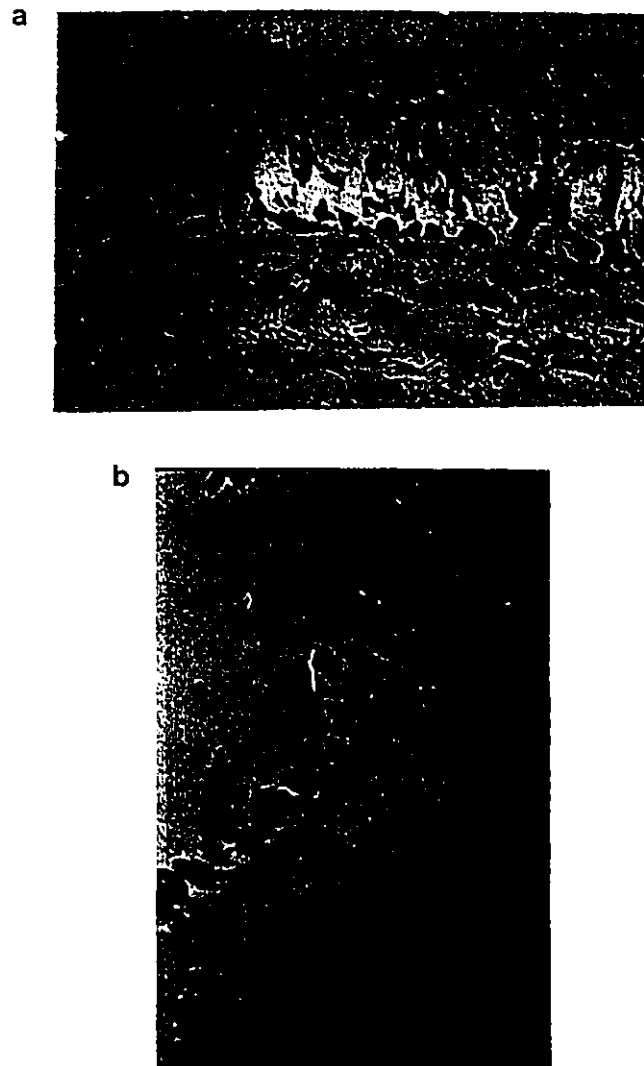


Figure 4.6. Immunohistochemistry for EG2 on bronchial biopsy tissue from a (a) non-asthmatic and (b) asthmatic subject (scale bar = 50  $\mu\text{m}$ ). No staining is evident in tissue from the non-asthmatic subject. In the asthmatic tissue, intra- and extra-cellular positive (red) staining for EG2 can be seen.

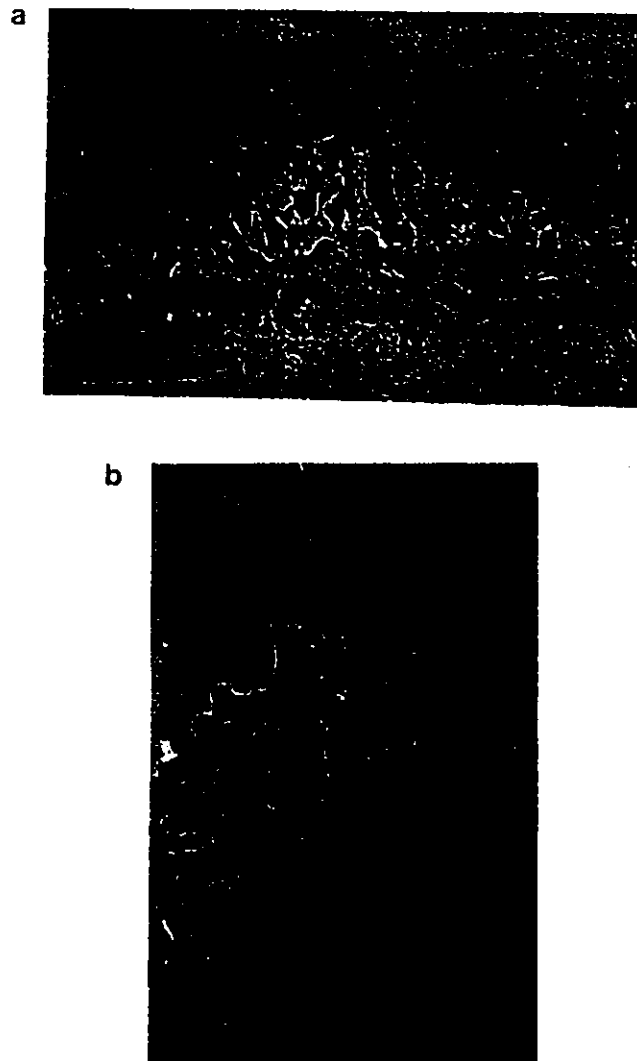


Figure 4.7. Immunohistochemistry for GM-CSF on bronchial biopsy tissue from a (a) non-asthmatic and (b) asthmatic subject (scale bar = 50  $\mu\text{m}$ ). No staining is evident in tissue from the non-asthmatic subject. In the asthmatic tissue, positive (red) staining for GM-CSF is seen in the lamina propria and epithelium.



## Discussion

This study demonstrates that stable asthmatic subjects with mildly increased airway hyperresponsiveness have ongoing airway inflammation, as indicated by increased numbers of activated eosinophils in BAL and biopsies and increases in the eosinophil regulatory cytokine, GM-CSF. We found relationships between the number and function of eosinophils and GM-CSF and, furthermore, these markers of airway inflammation were related to airway function, based on the measurement of airway responsiveness. These findings imply that the eosinophil contributes to ongoing airway inflammation, even in mild asthma and that airway hyperresponsiveness may be a consequence of eosinophilic inflammation.

A pro-inflammatory role for the eosinophil in asthma is suggested by the increased presence and activation of eosinophils in asthmatics. We found an increased number of eosinophils in the blood, bronchoalveolar lavage and biopsy tissue of the mild asthmatics studied. Peripheral blood eosinophilia, observed in asthmatics since the turn of the century (Ellis, 1908), has been confirmed in later studies (Durham et al., 1989; Bousquet et al., 1990). Subsequent investigations, using bronchoalveolar lavage to sample the airway lumen, have documented a significant increase in eosinophils in asthmatic airways (Kirby et al., 1987; Adelroth

et al., 1990) but this finding has not been universal (Beasley et al., 1989). Most recently, eosinophil numbers have been investigated in asthmatic and non-asthmatic bronchial biopsy tissue, with an increase (Azzawi et al., 1990) or no difference (Jeffery et al., 1989) reported in asthmatic tissue. The sometimes inconsistent increase in eosinophil numbers in BAL and biopsy samples in asthmatics may reflect the mild state of disease in the subjects studied.

Within the airways, eosinophil activation may be of greater importance than eosinophil number (Busse et al., 1993). Based on detection of the secreted form of ECP with EG2 antibody, we found an increased number of activated eosinophils in the bronchoalveolar lavage cells and biopsy tissue of mild asthmatics. Increased EG2-positive lavage cells in asthmatics compared to non-asthmatics is a novel finding. This result implies that eosinophils within the bronchial lumen may continue to secrete ECP, potentially causing damage to pulmonary epithelial cells. In contrast to some investigators (Broide et al., 1992; Bousquet et al., 1991), we were not able to detect significant differences in the concentration of ECP in lavage fluid between asthmatics and non-asthmatics. However, higher local concentrations of ECP within asthmatic airways may have existed due to the increased number of activated BAL eosinophils. Such a difference may not have been detected due to dilution effects from the lavage instillate. Confirming findings from recent studies (Azzawi et al., 1990; Djukanovic et al., 1990; Bousquet et al., 1990), we found increases in the secreted form of

ECP in asthmatic biopsy tissue. Our findings demonstrate, for the first time, that eosinophils in both BAL and biopsy tissue are activated in mild asthmatics. As these sites presumably sample both peripheral and central airways, our results imply that eosinophil activation occurs throughout asthmatic airways.

The increased presence of GM-CSF protein in asthmatic lavage cells and biopsy tissue is a new finding which provides direct evidence for the involvement of this cytokine in asthma airway inflammation. Detection of GM-CSF protein in lavage cells extends previous findings of lavage cells positive for GM-CSF mRNA (Broide et al., 1992). Our findings imply that mRNA translation occurs, enabling the protein to exert its biological activities in asthmatic airways. In agreement with mRNA findings, we observed GM-CSF within cells which, based on size and morphology, included eosinophils, lymphocytes and macrophages. The presence of GM-CSF protein within eosinophils is of particular significance, suggesting the possibility of autocrine regulation of eosinophil function (Moqbel et al., 1991). Detection of GM-CSF protein within the airway mucosa and epithelium also points toward the involvement of GM-CSF in the local regulation of airway inflammation.

The relationships between GM-CSF and eosinophil number and function observed in the present study provide some validation for results from in vitro studies documenting GM-CSF regulation of eosinophils. The correlation between the presence of GM-CSF in biopsy tissue and eosinophils in BAL and biopsy

tissue suggests that GM-CSF could be involved in the recruitment and/or survival of airway eosinophils. This suggestion is supported by in vitro studies which have shown GM-CSF to be chemotactic for eosinophils (Warringa et al., 1992) and to prolong their survival (Owen et al., 1987). The correlations between the presence of GM-CSF in lavage cells and EG2 staining in biopsy tissue suggests that GM-CSF may contribute to activation of eosinophils within airway tissue. In vitro studies have clearly shown that GM-CSF can enhance the release of inflammatory mediators, such as leukotrienes, from eosinophils (Owen et al., 1987). Importantly, however, these correlations do not imply cause and effect. One alternative explanation for our findings could be that a common mechanism co-regulates eosinophil activation and GM-CSF production. Recently, several investigators have attributed such an "orchestrating" role to the T lymphocyte (Bentley et al., 1992; Robinson et al., 1993). Another reason for the relationship we observed between GM-CSF and eosinophils may relate to eosinophil autocrine regulation. It is conceivable that the continued production of GM-CSF by eosinophils, with their subsequent activation, would lead to a self perpetuating inflammatory process (Busse et al., 1993). This autocrine mechanism could be an important mechanism in the chronicity of asthma airway inflammation, even in subjects with mild disease.

The physiological importance of increases in eosinophil number and function and GM-CSF in asthmatics is highlighted by the significant relationships we observed between these inflammatory markers and airway responsiveness.

These findings support the involvement of the eosinophil, potentially activated by GM-CSF, in mediating the airway hyperresponsiveness associated with asthma. It is quite likely, however, that other cells and other cytokines, such as IL-3 and IL-5, contribute to the pathophysiology of asthma. Examination of individual subject values for the numerous inflammatory markers that have been investigated in other studies (Azzawi et al., 1990; Djukanovic et al., 1990; Bousquet et al., 1990; Bentley et al., 1992), highlights the remarkable variability observed in asthmatic subjects. In our own study, a wide range of values were observed for GM-CSF and eosinophil number and function (Figure 4. 1 and 4. 4; Table 4. 2). This variability may reflect the presumably complex and multifactorial etiology of asthma.

In summary, we have found that mild asthmatics demonstrate increases in eosinophils, eosinophil cationic protein and GM-CSF protein compared to non-asthmatics. Relationships were evident between GM-CSF and eosinophil number and function and, furthermore, these inflammatory markers were related to airway responsiveness. These findings imply that the eosinophil and GM-CSF, a cytokine regulating eosinophil function, play an important role in the chronic airway inflammation and airway hyperresponsiveness evident even in mild asthma.

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## CHAPTER 5: EFFECTS OF ALLERGEN CHALLENGE ON GRANULOCYTE-MACROPHAGE COLONY-STIMULATING-FACTOR, EOSINOPHILS AND EOSINOPHIL CATIONIC PROTEIN IN MILD ASTHMATICS

### Abstract

Allergen inhalation challenge is associated with increases in eosinophil number and activation and provides a useful model for investigating airway inflammation in asthma. Limited information, however, is available on the effect of allergen challenge on cytokines regulating eosinophil function. We investigated allergen-induced changes in eosinophil number and activation and granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine known to regulate eosinophil function *in vitro*. Seven, mild, atopic asthmatics, with late asthmatic responses, completed diluent and allergen inhalation challenges. Blood, bronchoalveolar lavage (BAL) and biopsy samples were collected 24 hours after challenge. Allergen inhalation caused a significant increase in eosinophils in BAL and biopsy samples. Eosinophil activation, assessed by secretion of eosinophil cationic protein, and GM-CSF were significantly increased in BAL fluid and BAL cells. Allergen inhalation did not cause a significant change in eosinophil activation in biopsy tissue but did result in a significant decrease in GM-CSF in

biopsy tissue. Significant correlations were demonstrated between the concentration of GM-CSF in the BAL fluid and the percentage of BAL eosinophils ( $R_s=0.75$ ,  $p=0.05$ ) and the severity of the late asthmatic response and the number of BAL eosinophils ( $R_s=0.82$ ,  $p=0.02$ ). A trend was seen between the late response and the concentration of GM-CSF in the BAL fluid. These results suggest that GM-CSF regulates eosinophils in vivo and that these inflammatory indices contribute to allergen-induced decreases in airway function.

## Introduction

Asthma is a disease characterized by variable airflow obstruction and airway hyperresponsiveness (Arm et al., 1992). Recently, increased attention has been directed toward the relationship between airway inflammation and the changes in airway function associated with asthma. This relationship has been investigated in the laboratory using allergen challenge. The late asthmatic response, which occurs in some sensitized asthmatics three to eight hours following allergen inhalation, is characterized by the development of airway obstruction and an increase in airway responsiveness (Beasley et al., 1989; Brusasco et al., 1990). These physiological changes are thought to be a consequence of allergen-induced inflammatory events occurring within asthmatic

airways (Beasley et al., 1989).

Although a variety of inflammatory cells and mediators are likely to contribute to the late asthmatic response, the eosinophil has been implicated as an important effector cell (Chung, 1993). Increases in the number of eosinophils and their progenitors in blood (Booij-Noord et al., 1972; Cookson et al., 1989; Gibson et al., 1991) and increases in eosinophils in bronchoalveolar lavage (BAL) fluid (Brusasco et al., 1990; De Monchy et al., 1985; Metzger et al., 1986; Aalbers et al., 1993) and induced sputum (Pin et al., 1992) in asthmatics with a late asthmatic response have been reported. Eosinophil activation is also believed to occur, with increases documented in the levels of preformed and newly generated mediators in the serum (Dahl et al., 1978; Durham et al., 1989) and BAL fluid (De Monchy et al., 1985) of asthmatics with late asthmatic responses. Not all studies, however, have confirmed these findings (Beasley et al., 1989).

Most recently, investigators have examined bronchial biopsy tissue to obtain information on inflammatory events occurring within the airway wall. To date, however, results have been inconsistent with an increase (Jarjour et al., 1993) or no change (Beasley et al., 1989; Crimi et al., 1991) reported in eosinophil numbers. Limited information is also available concerning eosinophil activation in the bronchial mucosa. Beasley et al., (1989) reported no changes in eosinophil activation following allergen challenge based on the absence of changes in the ultrastructural appearance of eosinophil granules. However, Bentley et al., (1993)

and Aalbers et al., (1993) reported increases in eosinophil activation based on increased immunostaining for eosinophil cationic protein (ECP) following allergen challenge.

In subjects developing a late asthmatic response, the recruitment and activation of airway eosinophils may involve the release of the cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF), which has been shown *in vitro* to be chemotactic for eosinophils (Warringa et al., 1992) and can enhance eosinophil survival and activation (Vancheri et al., 1989; Lopez et al., 1986). The role of GM-CSF in allergen-induced eosinophil recruitment or activation, however, is yet to be established. After local allergen challenge, an increase in the number of cells expressing mRNA and protein for GM-CSF in BAL (Broide et al., 1992) has been reported and, after allergen inhalation, an increase in the number of cells expressing GM-CSF mRNA in biopsy tissue (Bentley et al., 1993) was observed. Currently, no studies have reported GM-CSF protein in asthmatic airway cells or tissue after allergen inhalation. In addition, no studies have investigated the relationship between allergen-induced changes in GM-CSF and lung function.

The purpose of this study, therefore, was to examine the effect of allergen inhalation on eosinophil number, levels of ECP, an indicator of eosinophil activation, and the presence of GM-CSF in the blood, BAL fluid, BAL cells and bronchial tissue of mild asthmatics. We hypothesized that allergen inhalation by

subjects who develop allergen-induced late asthmatic responses would be associated with increases in airway eosinophils. We also sought to examine whether changes in eosinophil number or activation were associated with changes in levels of GM-CSF. To evaluate the physiological significance of these markers of inflammation, we examined the relationships between eosinophil numbers, levels of ECP and GM-CSF and allergen-induced airway responses.

## **Methods**

### Subjects

Seven patients with mild asthma were selected for study (Table 5. 1). The study was approved by the Ethics Committee of the McMaster University Medical Center and all subjects provided their written informed consent prior to starting the study. Subjects were atopic, as indicated by one or more positive weal and flare responses to skin prick tests. Subjects were non-smokers and none had experienced a respiratory infection during the four weeks prior to the study. Asthmatics were stable at the time of study, only requiring intermittent use of inhaled  $\beta_2$ -agonists and had baseline FEV<sub>1</sub> values > 70% predicted.

Table 5.1: Characteristics of subjects completing diluent and allergen challenges

Subject No.	Age (yrs)	Sex	FEV <sub>1</sub> (% pred)	MCh PC <sub>20</sub> (mg·ml <sup>-1</sup> )	Allergen Inhaled	EAR (% fall FEV <sub>1</sub> )	LAR (% fall FEV <sub>1</sub> )
1	40	M	82	3.27	DP	32	33
2	30	F	86	1.56	DP	26	38
3	40	F	98	1.28	DF	30	32
4	21	M	100	13.40	RW	38	54
5	42	M	108	2.20	Cat	26	21
6	22	F	93	2.20	Cat	19	11
7	25	M	89	1.45	Cat	41	18
Mean	31		94	2.5*		30	30
± SD	9.1		9.0	0.36		7.5	14.3

DP, Dermatophagoides pteronyssinus; DF, Dermatophagoides farinae; RW, Ragweed; EAR, early asthmatic response; LAR, late asthmatic response

\* Geometric mean and %SEM.

### Study Design

Subjects attended the laboratory on six occasions. At the initial visit, subjects completed a respiratory questionnaire and a full medical history, physical examination, skin prick test, ECG and chest x-ray were performed. A blood sample was taken to determine white blood cell count, platelet count, prothrombin time and partial thromboplastin time. Baseline spirometry and a methacholine inhalation test were performed. Within one week, subjects returned to the laboratory to complete a short follow-up questionnaire, perform spirometry and

undertake a diluent inhalation challenge. Fiberoptic bronchoscopy was performed 24 hours after the diluent challenge. After a period of three to four weeks, subjects returned to the laboratory to perform spirometry and complete a methacholine inhalation test and skin prick test titration test with the relevant allergen. Within one week, subjects returned to the laboratory to complete a short follow-up questionnaire, perform spirometry and undertake an allergen inhalation challenge. Fiberoptic bronchoscopy was again performed 24 hours after the allergen challenge. After each bronchoscopy, subjects were observed closely until the local anesthesia had worn off.

#### Methacholine Inhalation Challenge and Skin Test

Methacholine inhalation challenge was performed as described by Cockcroft et al., (1987). Subjects inhaled doubling concentrations of methacholine chloride from a Wright nebulizer, for two minutes by tidal breathing. Increasing concentrations of methacholine were administered until a fall in FEV<sub>1</sub>  $\geq$  20% of the baseline value occurred. Results were expressed as the provocative concentration causing a 20% fall in FEV<sub>1</sub> (PC<sub>20</sub>). For the skin prick titration test, the allergen used in the allergen inhalation challenge was administered in doubling dilutions, in duplicate. The dilution giving a two millimeter weal response at 15 minutes was used in the calculation of the predicted PC<sub>15</sub> of allergen.



### Diluent Inhalation Challenge

Three doses of normal saline were given at ten minute intervals. The aerosol was generated by a Wright nebulizer with an output of  $0.13 \text{ ml}\cdot\text{min}^{-1}$ . The aerosol was inhaled for two minutes by tidal breathing using a Hans-Rudolph valve box and mouthpiece with a filter attached to the expiratory route and the nose clipped. FEV<sub>1</sub> measurements were made after each inhalation. After the final inhalation, spirometry was performed at 10, 20, 30, 45, 60, 90 and 180 minutes and then every hour for seven hours after the final inhalation.

### Allergen Inhalation Challenge

Results from the methacholine inhalation challenge and the skin prick titration test were used to predict the allergen PC<sub>15</sub> according to the formula described by Cockcroft et al. (1987). Doubling concentrations of allergen were inhaled at ten minute intervals until a fall of 15% or more occurred in the subject's FEV<sub>1</sub>. Measurements of FEV<sub>1</sub> were made in the same manner as for the diluent challenge. From the predicted allergen PC<sub>15</sub>, the starting concentration of allergen was chosen to be three doubling doses below the predicted value.

### Fiberoptic Bronchoscopy

Fiberoptic bronchoscopy was performed according to the recommendations of the National Institutes of Health (Workshop Committee, 1985).

Subjects arrived at the research bronchoscopy suite at McMaster University Medical Center at 8:00 am, having fasted since midnight. An intravenous line was secured and blood samples obtained. Premedication with atropine (0.6 mg subcutaneous) was given 30 minutes prior to bronchoscopy. If sedation was required, midazolam (0.07 mg·kg<sup>-1</sup>) was given intravenously. Subjects inhaled 400 µg of salbutamol administered via a spacer device before topical anesthesia with lidocaine (Xylocaine; Astra Pharma Inc., Mississauga, Canada) was applied to the airways. The bronchoscope (Olympus BF-1T20D; Olympus Optical Co., Tokyo, Japan) was inserted via a mouthpiece under further anesthesia to suppress cough. During the entire procedure, oxygen at 4 L·min<sup>-1</sup> was administered via a nasal cannula and subjects were monitored with ECG and ear oximetry. No complications were observed in any subjects during or following bronchoscopy.

### Blood Samples

Two venous blood samples were obtained from each subject. Samples were collected in either EDTA-treated tubes for total and differential white blood cell counts or untreated tubes to obtain serum. Total cell counts were performed using a hemocytometer and differential cell counts were made from blood smears stained with Diff-Quik. Cell counts were performed in duplicate by one investigator blinded to the type of challenge undertaken by the subject. Cells were classified as neutrophils, eosinophils, lymphocytes, monocytes and basophils using standard

morphological criteria. To obtain serum, venous blood was stored at room temperature for two hours, centrifuged twice (1350 g, 10 min) and then stored at -70°C.

### Biopsy Samples

The bronchoscope was inserted into the left lung and a separate forceps (FB-20C) was used for each biopsy. Four mucosal biopsies were obtained from the lobar carinae and carinae of the basal segments. The position of the biopsy site and the appearance of the mucosal surface were carefully recorded. Two biopsies were placed immediately in glutaraldehyde for electron microscopy analysis and one biopsy was fixed in 4% paraformaldehyde for future studies. The biopsy sample for immunohistochemistry was placed immediately in periodate-lysine-paraformaldehyde (PLP) fixative and stored at 4°C for four hours. The tissue was then transferred through four solutions (phosphate buffered saline, 10%, 20% and 30% sucrose phosphate buffer), each for 4-6 hours at 4°C. The tissue was then frozen in Tissue-Tec OCT compound (Miles, Elkhart, In.) in -70°C isopentane (Aldrich, Milwaukee, Wi.) and stored at -70°C. Serial cryostat sections (6 µm) were cut and positioned on slides coated with 3-aminopropyltriethoxysilane (Sigma, St. Louis, Mo.).

### Bronchoalveolar Lavage

The bronchoscope was inserted into the middle lobe of the right lung and wedged into a segment or sub-segment. Five 20 ml aliquots of pre-warmed (37°C), sterile normal saline were infused and gently aspirated back into polypropylene tubes kept on ice. The fluid was then centrifuged at 200 g for 10 minutes at 4°C. The supernatant was decanted and stored at -70°C. The cell pellet was washed in PBS and a total cell count was performed using a hemocytometer. The cells were then diluted with PBS to a concentration of  $1 \times 10^6$  cells·ml<sup>-1</sup>. Cytocentrifuge slides were prepared and stained with Diff-Quik for a differential cell count (400 cells counted). Cell counts were performed in duplicate by one investigator blinded to the type of challenge undertaken by the subject. Cells were classified as macrophages, neutrophils, eosinophils and lymphocytes using standard morphological criteria. Total and differential cell counts were expressed as the number of cells per milliliter of fluid recovered. Cytocentrifuge slides prepared for immunocytochemistry were air dried for five minutes, fixed in PLP for 10 minutes at 4°C, placed in a 15% sucrose phosphate buffer solution for 10 minutes at 4°C and air dried overnight. Slides were then wrapped in pairs in foil and stored at -70°C.

### Tissue Eosinophils

Paraffin-embedded sections, prepared from the biopsy sample fixed in 4% paraformaldehyde, were stained with Chromotrope 2R (Sigma) to enumerate eosinophils in the tissue (Lendrum, 1944). Slides were coded prior to evaluation. For each section, the number of positively stained cells along the length of the basement membrane to a depth of 115  $\mu\text{m}$  (delineated by a squared eyepiece graticule) was counted. A computerized and calibrated graphics tablet (Apple IIe) was used to determine the area of the lamina propria in which counts were made. Results were expressed as the number of eosinophils per unit area of lamina propria ( $1 \text{ mm}^2$ ).

### Immunocytochemistry

Immunocytochemistry was performed on BAL cytopins and tissue sections. To remove remaining OCT compound and sucrose and to enhance permeability and block non-specific reactions, slides were incubated with Dulbecco's phosphate buffered saline (GIBCO), supplemented with 0.01 M HEPES buffer (Boehringer Mannheim Canada Ltd.) containing 0.1% saponin (Sigma) (DPBS H + S) for five minutes at room temperature, two times. To block non-specific binding further, slides were incubated at room temperature with DPBS H + S containing 75% heat inactivated human AB sera for one hour and then with DPBS H + S containing 25% heat inactivated normal rabbit serum (Zymed, San Francisco, Ca.) for one

hour. Slides were washed briefly with DPBS H + S before application of the monoclonal antibodies. The monoclonal antibodies used were EG2 (Pharmacia, Sweden) which recognizes the cleaved form of human ECP and is thought to represent eosinophil activation (Tai et al., 1984) and a mouse monoclonal antibody which reacts with human GM-CSF protein (Genzyme, Boston, Ma.). EG2 and GM-CSF antibodies were diluted in DPBS H + S containing 0.1% bovine serum albumin (Zymed) at final concentrations of  $1 \mu\text{g}\cdot\text{ml}^{-1}$  and  $3 \mu\text{g}\cdot\text{ml}^{-1}$ , respectively. Slides were incubated overnight at  $4^{\circ}\text{C}$  and then washed in DPBS H + S. Labelling of the monoclonal antibodies was detected by the alkaline phosphatase anti-alkaline phosphatase method (APAAP Kit, Dako, Santa Barbara, Ca.) according to the manufacturer's instructions. Slides were washed in distilled water, lightly counterstained with Mayer's hematoxylin (Sigma) and mounted. Appropriate system and specific controls were included in each assay.

#### Immunocytochemistry Quantification

All slides were coded before evaluation. For BAL cytospins, the cell pellet was divided into four equal sections, delineated with a squared eyepiece graticule. At least 100 cells were counted in each section. Results were expressed as the percentage of positively stained cells. The intra-class correlation coefficient, a stringent test of reproducibility, was 0.99 for repeated counts of EG2 and 0.99 for repeated counts of GM-CSF. Assessment of staining for the biopsy

specimens was performed with a semi-quantitative method, previously described for evaluating immunohistochemical staining (Leung et al., 1989). Briefly, two observers, blinded to the type of challenge, graded the extent and intensity of positive staining for each biopsy specimen on a scale from 1 (negative) to 5 (strongly positive). Intra-class correlation coefficients between observers were 0.81 for EG2 and 0.92 for GM-CSF.

#### ECP and GM-CSF Assays

Levels of ECP in serum and BAL fluid were determined using a double-antibody radioimmunoassay (Kabi Pharmacia Diagnostics AB, Sweden) with results expressed in micrograms per liter. Assay sensitivity for ECP was  $2 \mu\text{g}\cdot\text{L}^{-1}$ . Measurement of GM-CSF in serum and 20-fold concentrated (Amicon-10, Amicon Corp., Danvers, Ma) BAL fluid was performed using an enzyme-linked immunosorbent assay (Quantikine, Research and Diagnostics System, Minneapolis, Mn) with results expressed in picograms per milliliter. Assay sensitivity for GM-CSF was  $2.8 \text{ pg}\cdot\text{ml}^{-1}$  for serum and  $1.5 \text{ pg}\cdot\text{ml}^{-1}$  for BAL fluid. In all assays, samples were analyzed in duplicate.

#### Statistical Analysis

Statistical analyses were performed using the CSS Statistica computer software program. Data distributions were checked for normality using

Kolmogorov-Smirnoff and Chi-Square analyses. As most distributions were significantly different from normal, non-parametric statistics were used. Given the a priori hypothesis that airway eosinophils would be increased after allergen compared to diluent challenge, one-tailed Wilcoxon Signed-Rank tests for paired samples were used for airway eosinophil counts. Differences in indices of eosinophil activation (ECP levels and EG2 staining) and GM-CSF between allergen and diluent were examined using two-tailed Wilcoxon Signed-Rank tests. Spearman's rank correlation coefficient test ( $R_s$ ) was used to examine the association between inflammatory parameters and physiological measures of airway function. A p value of  $< 0.05$  was accepted as statistically significant.

## **Results**

In mild atopic asthmatics, allergen inhalation caused early and late asthmatic responses (Table 5. 1). The early asthmatic response after allergen, with a mean fall in  $FEV_1$  of 30% was significantly greater than that after diluent, with a mean fall in  $FEV_1$  of 4% ( $p < 0.000$ ). The late asthmatic response after allergen, with a mean fall in  $FEV_1$  of 30%, was also significantly greater than that after diluent, with a mean fall in  $FEV_1$  of 3% ( $p = 0.004$ ).



Allergen inhalation caused an increase in eosinophils in the bronchoalveolar lavage and biopsy tissue of mild asthmatic subjects. The percentage of BAL eosinophils was significantly greater after allergen, with a median value of 2.8% (range: 1.0 to 23%), than after diluent, with a median of 1.3% (range: 0.0 - 2.3%) ( $p = 0.021$ ) (Table 5. 2). The absolute number of eosinophils in the lavage was also significantly greater after allergen than after diluent ( $p = 0.032$ ). The proportionate increase in BAL eosinophils after allergen was balanced by a decrease in the proportion of BAL lymphocytes. Within bronchial biopsy tissue, allergen challenge caused a significant increase in eosinophils, with a median value of  $30.0\text{-mm}^2$  (range: 12.5 - 45.7) , compared to diluent challenge, with a median value of  $15.1\text{-mm}^2$  (range: 7.4 - 33.3) ( $p = 0.014$ ) (Figure 5. 1). The absolute number ( $\times 10^4\text{-ml}^{-1}$ ) of circulating eosinophils was not significantly increased after allergen, with a median of 47 (range: 22 - 84), compared to after diluent, with a median of 36 (range: 16 - 75) ( $p = 0.176$ ).

Table 5.2: Bronchoalveolar lavage cell counts after diluent and allergen challenges

Diluent						
Subject No.	Return (ml)	Total ( $\times 10^4 \cdot \text{ml}^{-1}$ )	M	N	L	E
			(%)			
1	55	5.59	92.0	1.8	4.8	1.5
2	66	8.35	95.0	0.0	3.5	1.3
3	67	4.85	95.5	2.5	2.0	0.0
4	55	9.09	95.3	1.0	2.5	1.3
5	57	1.39	94.5	2.5	0.8	2.3
6	69	2.87	93.5	4.3	1.5	0.8
7	67	7.99	85.3	1.0	12.3	1.5
Median	66	5.59	94.5	1.8	2.5	1.3
Allergen						
Subject No.	Return (ml)	Total ( $\times 10^4 \cdot \text{ml}^{-1}$ )	M	N	L	E
			(%)			
1	52	7.89	88.0	3.0	3.3	6.0
2	67	11.87	95.0	0.5	2.0	3.0
3	68	4.19	94.8	2.0	1.3	2.0
4	40	6.15	72.8	3.3	1.5	23.0
5	64	4.96	98.3	0.8	0.0	1.0
6	53	2.81	88.0	7.0	2.3	2.8
7	88	3.84	94.5	0.3	3.8	1.5
Median	64	4.96	94.5	2.0	2.0	2.8
p value*	1.00	1.00	0.46	0.45	0.04	0.02

M, macrophage; N, neutrophil; L, lymphocyte; E, eosinophil.

\* p value for comparison between diluent and allergen challenge.

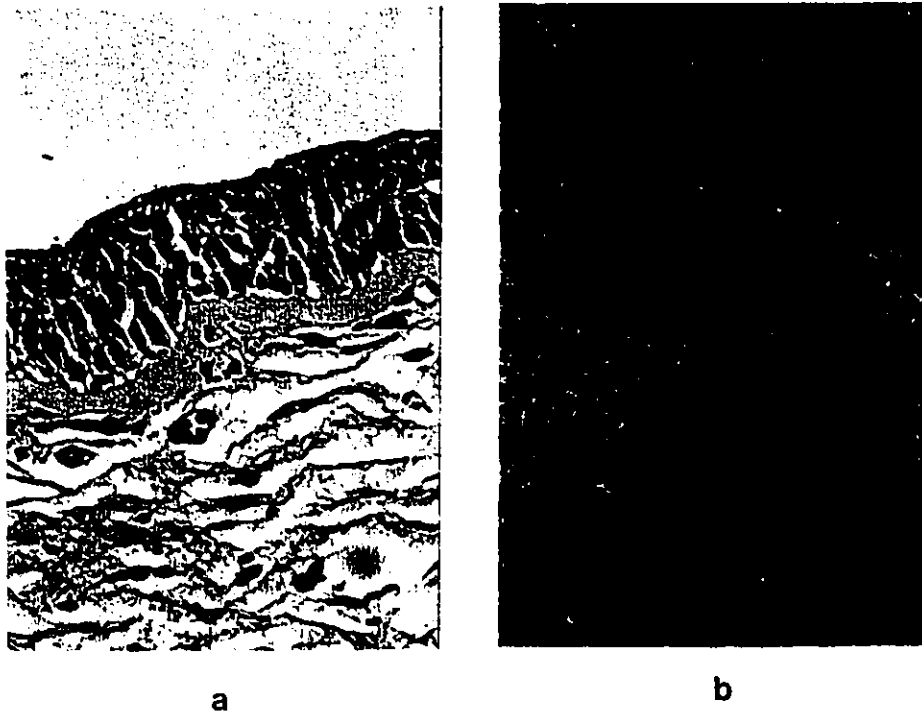


Figure 5.1. Chromotrope 2R stained eosinophils after (a) diluent and (b) allergen challenge in bronchial biopsy tissue from a mild asthmatic (internal scale = 50  $\mu\text{m}$ ). Allergen challenge caused a significant increase in the number of tissue eosinophils.

Immunocytochemistry on BAL cells demonstrated that allergen inhalation resulted in an increased number of cells positive for EG2 and GM-CSF (Figure 5. 2). The median value of EG2-positive cells increased from 2.3% after diluent to 5.0% after allergen ( $p = 0.008$ ). The median value of GM-CSF-positive cells increased from 18% after diluent to 53% after allergen ( $p = 0.028$ ). In BAL cells, staining for EG2 was present in the cytoplasm and was also seen extracellularly, suggestive of ECP secretion (Figure 5. 3). Staining for GM-CSF in BAL cells was evident in the cytoplasm of a variety of cells which, based on size and morphology, included eosinophils, alveolar macrophages and lymphocytes (Figure 5. 4). No staining was present in slides stained with IgG<sub>1</sub> (negative control).

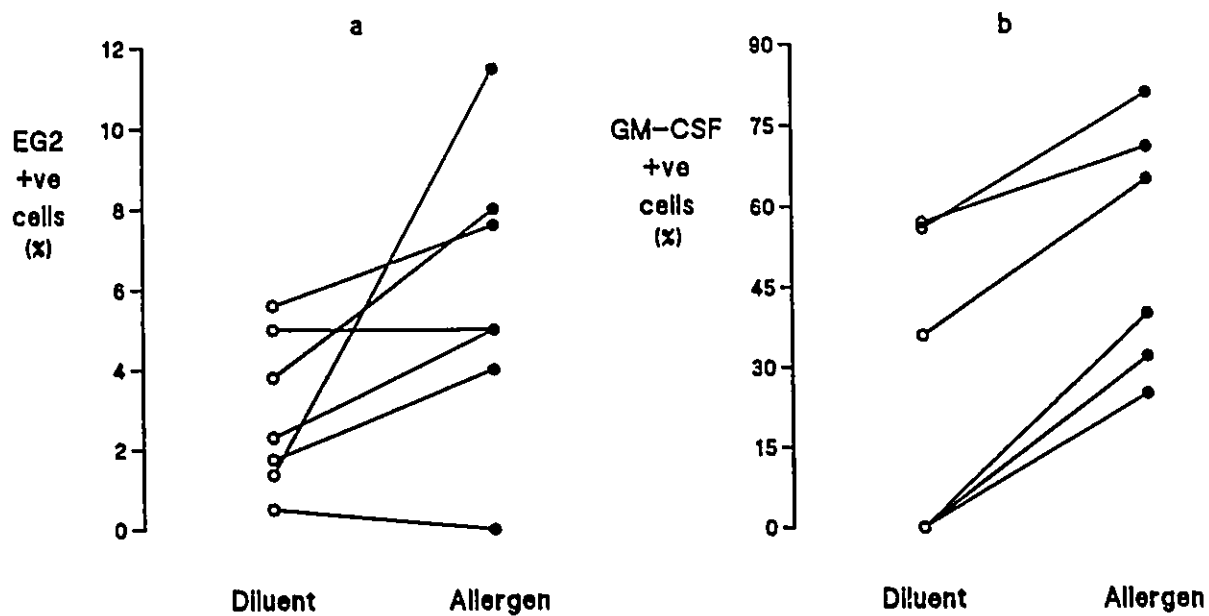
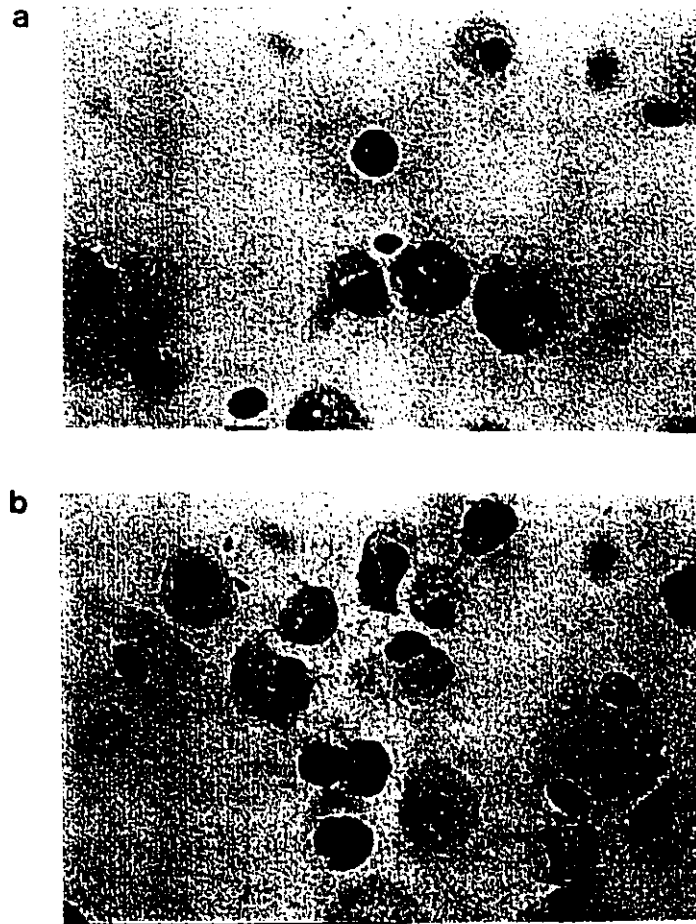


Figure 5.2. Percentage of BAL cells positive for (a) EG2 and (b) GM-CSF in asthmatic subjects after diluent or allergen challenge. Allergen challenge significantly increased the number of BAL cells positive for EG2 and GM-CSF.



**Figure 5.3.** Immunocytochemistry for EG2 in BAL cells from an asthmatic subject after (a) diluent and (b) allergen challenge (internal scale = 50  $\mu\text{m}$ ). Positive (red) staining with EG2 is seen in the cytoplasm, with faint staining also seen surrounding the eosinophils.

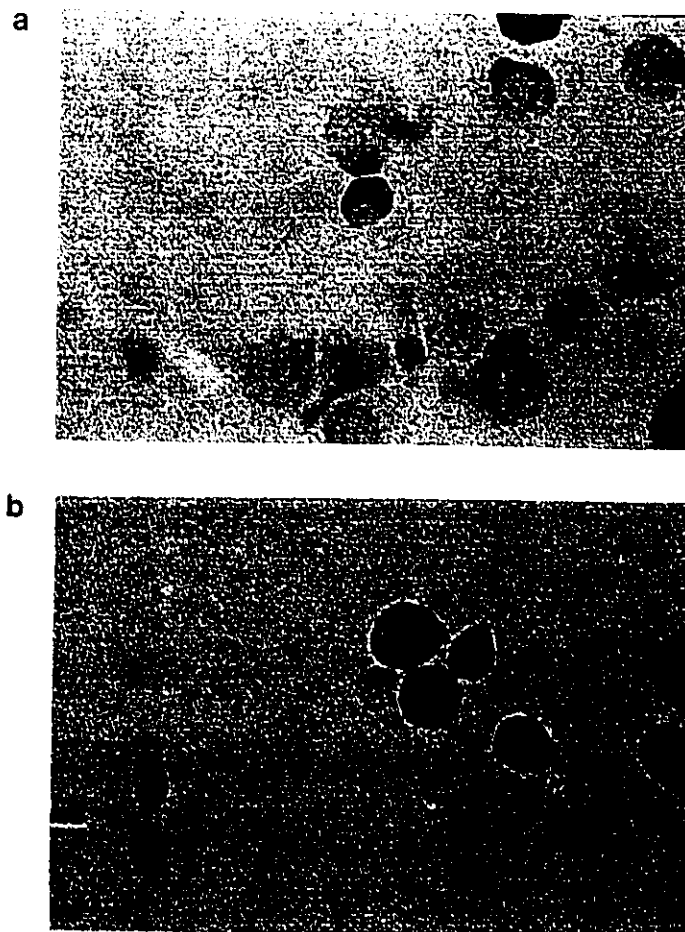


Figure 5.4. Immunocytochemistry for GM-CSF in BAL cells from an asthmatic subject after (a) diluent and (b) allergen challenge (internal scale = 50  $\mu\text{m}$ ). Positive (red) staining for GM-CSF is seen in a variety of cells including an eosinophil, based on size and morphology.

Immunohistochemistry on biopsy tissue demonstrated that allergen inhalation did not cause significantly increased staining for EG2 but did result in significantly decreased staining for GM-CSF (Figure 5. 5). The median value of EG2-staining was 1.75 after diluent and 2.63 after allergen ( $p = 0.208$ ). For GM-CSF staining, the median value decreased from 2.13 after diluent to 1.63 after allergen ( $p = 0.028$ ). Staining for EG2 after diluent was mainly intra-cellular and localized primarily beneath the basement membrane. In some subjects after allergen, however, diffuse extra-cellular staining was apparent and intra-epithelial EG2-positive cells, likely eosinophils, were observed (Figure 5. 6). Staining for GM-CSF in biopsies obtained after both diluent and allergen challenge was intra- and extra-cellular and was apparent both in the submucosa and epithelium (Figure 5. 7). No staining was present in the negative controls.



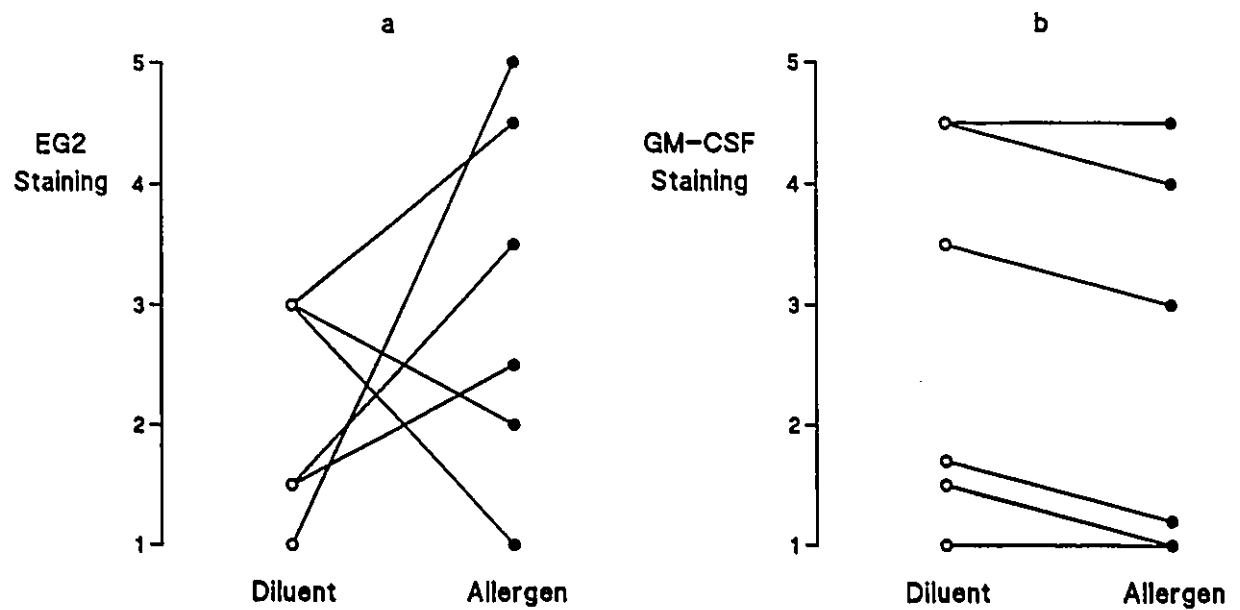


Figure 5.5. Staining of asthmatic biopsy tissue for (a) EG2 and (b) GM-CSF after diluent or allergen challenge. Allergen challenge did not significantly alter EG2 staining but did result in significantly decreased staining for GM-CSF.

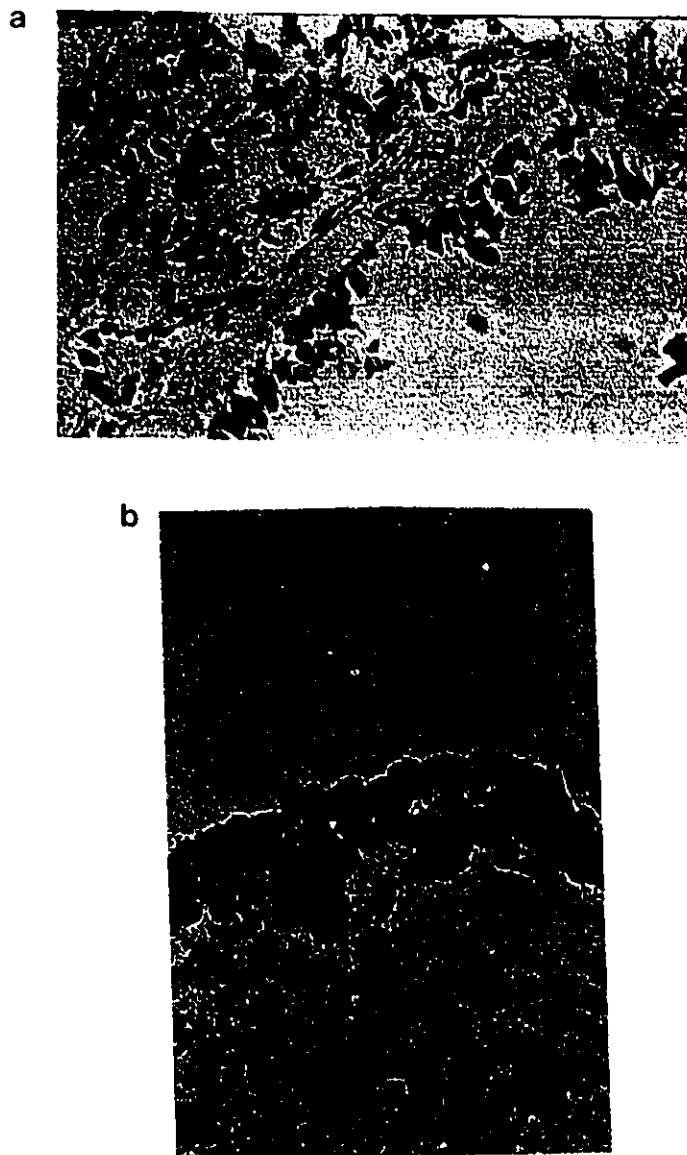


Figure 5.6. Immunohistochemistry for EG2 on bronchial biopsy tissue from an asthmatic subject after (a) diluent and (b) allergen challenge (internal scale = 50  $\mu$ m). Intra- and extra-cellular positive (red) staining for EG2 is seen. In this subject, more intense staining was evident after allergen.

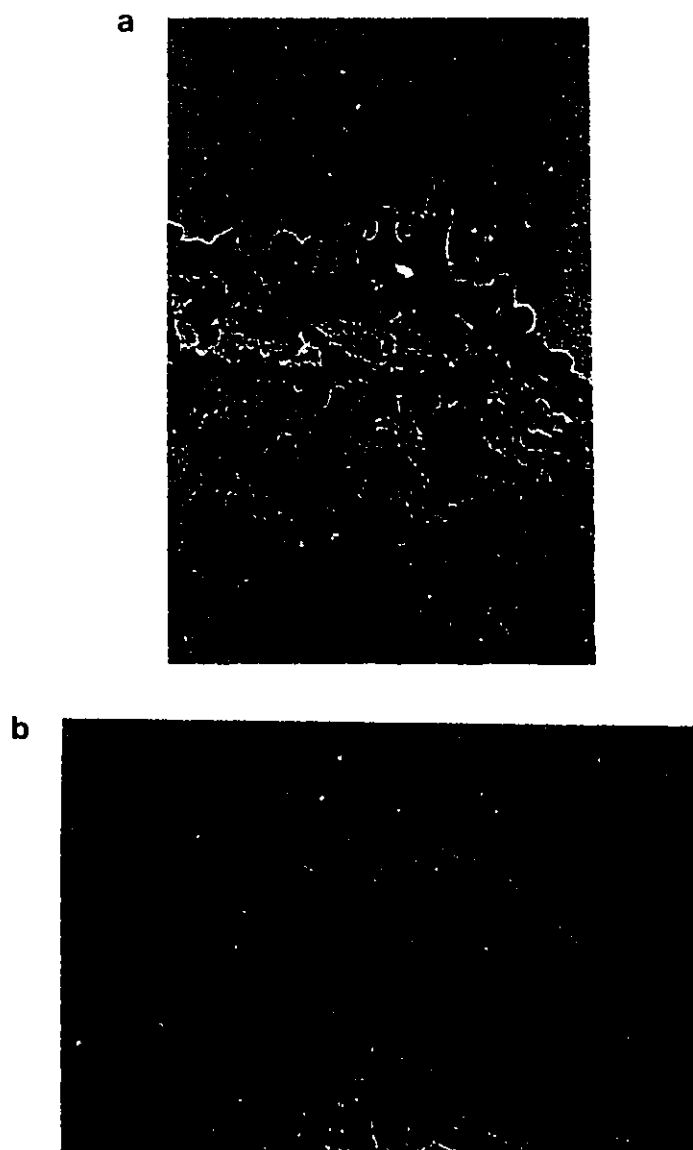


Figure 5.7. Immunohistochemistry for GM-CSF on bronchial biopsy tissue from an asthmatic subject after (a) diluent and (b) allergen challenge (internal scale = 50  $\mu\text{m}$ ). Positive (red) staining for GM-CSF is seen within the epithelium and in the lamina propria. In this subject, staining for GM-CSF was decreased after allergen.

In bronchoalveolar lavage fluid, allergen inhalation resulted in significant increases in ECP and GM-CSF (Figure 5. 8). The median concentration ( $\mu\text{g}\cdot\text{L}^{-1}$ ) of ECP increased from 3.4 after diluent to 9.0 after allergen ( $p = 0.018$ ). The median concentration of GM-CSF ( $\text{pg}\cdot\text{ml}^{-1}$ ) in concentrated lavage fluid increased from 13.9 after diluent to 19.8 after allergen ( $p = 0.018$ ). In the serum, median concentrations of ECP and GM-CSF were slightly, but not significantly, higher after allergen compared to diluent challenge.

The severity of the late asthmatic response was significantly correlated with the number of BAL eosinophils present after allergen ( $R_s = 0.82$ ;  $p = 0.02$ ). A trend was evident also between the severity of the late asthmatic response and the concentration of GM-CSF in the BAL supernatant after allergen ( $R_s = 0.71$ ;  $p = 0.07$ ). Relationships between GM-CSF and eosinophils after allergen were suggested by the correlation between the concentration of GM-CSF in the BAL supernatant and the percentage of BAL eosinophils ( $R_s = 0.75$ ;  $p = 0.05$ ).

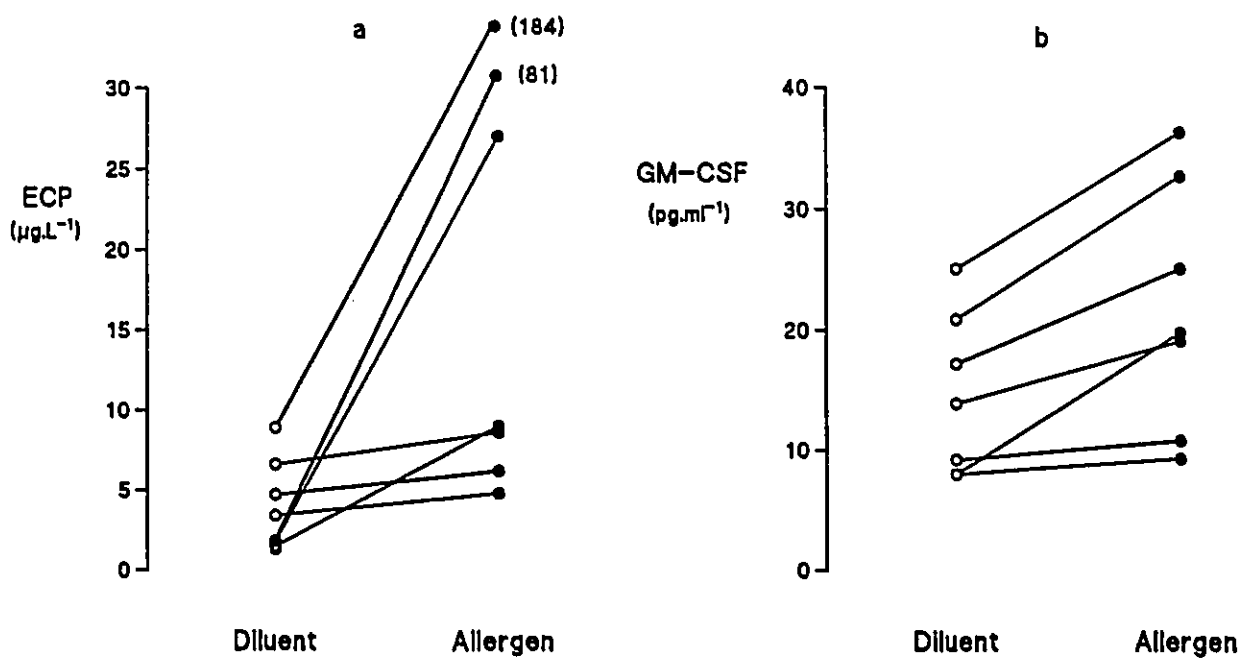


Figure 5.8. Concentrations of (a) ECP and (b) GM-CSF in BAL supernatant from asthmatic subjects after diluent or allergen challenge. Allergen challenge resulted in significant increases in ECP and GM-CSF.

## Discussion

This study demonstrated that allergen inhalation by mild, stable asthmatics, who developed both early and late asthmatic responses, resulted in significant changes in the number and activation of eosinophils and GM-CSF protein in the airways. Relationships existed between numbers of eosinophils and levels of GM-CSF. Also, allergen-induced changes in these inflammatory indices were related to the severity of the late asthmatic response. These findings suggest that the eosinophil and GM-CSF, an eosinophil regulatory cytokine, contribute to allergen-induced airway inflammation.

Eosinophil numbers were increased in BAL and biopsy tissue after allergen inhalation. The allergen-induced increase in BAL eosinophils confirms findings from previous studies (Brusasco et al., 1990; De Monchy et al., 1985; Metzger et al., 1986; Broide et al., 1992; Broide et al., 1991; Rossi et al., 1991). The magnitude of BAL eosinophilia reported in these studies has varied depending, in part, on whether allergen has been instilled locally or inhaled. We have used allergen inhalation rather than local instillation, and the increases in both the percentage and number of BAL eosinophils we observed are similar to results reported in previous studies using allergen inhalation (Brusasco et al., 1990; Metzger et al., 1986; Rossi et al., 1991).

Previous studies investigating eosinophil numbers in bronchial tissue have reported an increase after allergen instillation (Jarjour et al., 1993) but no significant changes after allergen inhalation (Beasley et al., 1989; Crimi et al., 1991). The discrepancy between our study and other studies using allergen inhalation (Beasley et al., 1989; Crimi et al., 1991) may be due to differences in study design and/or statistical power. In our study, comparisons were made between the allergen and diluent control day. In contrast, Crimi et al. (1991) made comparisons across groups and Beasley et al. (1989) compared pre- and post-allergen results. In addition, the sample sizes of these studies were smaller than ours and, while each study showed allergen-induced increases in tissue eosinophils, the increases were not statistically significant.

Allergen challenge may also cause eosinophil activation. In our study, BAL eosinophils appeared to be activated after allergen, based on the increases we found in BAL ECP concentration and EG2 staining in BAL cells. De Monchy et al. (1985) reported that in asthmatics with late responses, ECP levels were increased in BAL fluid collected 6-7 hours after allergen challenge. Our finding of increased BAL ECP levels extends this finding by implying that airway eosinophils continue to secrete ECP for at least 24 hours after allergen challenge in asthmatics. The increase in EG2 staining in BAL cells 24 hours after allergen inhalation is a new finding. Recently, Aalbers et al. (1993) reported a significant increase in EG2 positive BAL cells 3 hours after allergen inhalation challenge but

no significant increase 24 hours after challenge. As not all of the subjects challenged, however, demonstrated a late response, the lack of significant EG2 staining at 24 hours may have been due to the inclusion of data from isolated early responders. Our result suggests that eosinophils within the bronchial lumen retain the ability to actively secrete ECP many hours after allergen exposure. The in vivo consequences of ECP secretion are likely to be adverse given that this cationic protein has been shown in vitro to cause damage to pulmonary epithelium (Ayers et al., 1985). This prolonged activation of airway eosinophils may contribute to the long duration of reduced airway function often observed with late asthmatic responses.

While ECP was increased in BAL fluid and BAL cells after allergen, we did not find significant increases in biopsy tissue. This is in contrast to the other reports on EG2 staining in biopsy tissue after allergen inhalation challenge (Bentley et al., 1993; Aalbers et al., 1993). This discrepancy may be due to differences in sample size or kinetics of ECP synthesis and degradation. Bentley et al. (1993) studied thirteen patients and reported a significant increase in EG2 staining in biopsy tissue after allergen. However, approximately one third of the subjects in the study by Bentley et al. (1993) and in our study (Figure 5. 5a) demonstrated a decrease in biopsy EG2 staining after allergen. Kinetics may contribute to this variability, with differences occurring between asthmatics in the time of peak ECP secretion or the rate of ECP degradation. In a kinetic study, Aalbers et al. (1993)



reported increased EG2 staining in biopsies obtained 3 and 24 hours after challenge, compared to pre-challenge staining. Interestingly, however, a decline in EG2 staining was observed between 3 and 24 hours and a negative correlation noted between EG2 staining in biopsy tissue and EG2 positive cells in bronchial lavage at 24 hours. These results suggest that activated eosinophils move from the tissue to the lumen and it is probable that the kinetics of this movement differ between subject. Individual differences in markers of airway inflammation after allergen challenge are not surprising and probably account for the variability between asthmatics in the allergen-induced changes in lung function.

Similar to our findings with ECP, we found an increase in GM-CSF protein in BAL fluid and BAL cells after allergen inhalation. Broide and Firestein (1991) reported that GM-CSF in BAL fluid increased significantly after local allergen challenge. The GM-CSF values reported, however, are approximately ten fold greater than what we observed after allergen inhalation (Figure 5. 8b). The difference in GM-CSF levels between studies may be due to the method of allergen delivery. With local instillation (Broide et al., 1991), both GM-CSF levels and eosinophil numbers are approximately ten fold more than with allergen inhalation (Table 5. 2, Figure 5. 8b). This suggests that, although the magnitude may differ, similar mechanisms may be operating after either type of allergen challenge. The concentrations of GM-CSF that we observed likely exert biological effects in vivo as in vitro studies have demonstrated that GM-CSF can enhance

eosinophil survival and cytotoxicity at concentrations of less than 20 pM (Lopez et al., 1986; Owen et al., 1987).

Although no previous reports have documented GM-CSF protein in BAL cells after allergen inhalation, increases in GM-CSF positive BAL cells have been observed after local allergen challenge (Broide et al., 1992; Broide et al., 1991). Based on mRNA findings, Broide and Firestein attributed the cellular sources of GM-CSF to lymphocytes and alveolar macrophages (Broide et al., 1991). In a subsequent study, however, it was recognized that eosinophils were also capable of expressing GM-CSF mRNA and protein (Broide et al., 1992). Our results are in agreement with these findings, demonstrating that GM-CSF is increased in a variety of inflammatory cells, including eosinophils, after allergen challenge.

Less information is available concerning the effect of allergen challenge on GM-CSF in biopsy tissue. Bentley et al. (1993) reported an increase in GM-CSF mRNA positive cells in biopsy tissue from asthmatics after allergen inhalation. In contrast, we found a significant decrease in GM-CSF protein in asthmatic biopsy tissue. This apparent conflict may be explained by differences between the presence of mRNA and protein. Increased mRNA does not necessarily result in increased synthesis of protein (Wiesner et al., 1991). While allergen challenge may elicit an initial increase in the transcription and translation of GM-CSF within the bronchial tissue, it is possible that protein translation subsides by 24 hours even though mRNA for the protein is still present. Alternatively, cells may continue to

synthesize GM-CSF protein but migrate from the tissue to the lumen, retaining or secreting the protein. This would account for the increases in GM-CSF protein in the BAL cells and BAL fluid that we observed 24 hours after challenge.

In contrast to the allergen-induced changes in airway eosinophils and GM-CSF, no significant changes were observed in these indices in the circulation. This study is the first to directly investigate the effects of allergen inhalation on eosinophil numbers and GM-CSF in blood, BAL and biopsy. Previous studies, have reported an increase (Cookson et al., 1989; Durham et al., 1989) or no change (Frick et al., 1989) in circulating eosinophils 24 hours after allergen challenge in asthmatics. Cookson et al. (1989) also reported that eosinophils continued to increase for 48 hours after challenge. While we found a non-significant increase in circulating eosinophils at 24 hours, significant increases may have been detected at a later time point. Serum concentrations of ECP and GM-CSF were also non-significantly increased 24 hours after allergen challenge. Previous studies investigating serum ECP have reported decreases for up to 6 hours after allergen challenge (Venge et al., 1988) or a relative increase 6 to 12 hours after allergen, compared to methacholine, challenge (Durham et al., 1989). To our knowledge, the effect of allergen challenge in asthmatics on serum GM-CSF has not been previously reported. Findings from our study and the available literature suggest that allergen-induced changes are likely to be more evident in the airways than in the circulation.

The relationships we observed between the late asthmatic response and BAL eosinophils and GM-CSF suggest that the eosinophil and its regulatory cytokines contribute to allergen-induced decreases in lung function. The correlation we observed between BAL eosinophil numbers and the fall in FEV<sub>1</sub> during the late response complements the significant correlation reported by Brusasco et al. (1990) between allergen-induced increases in BAL eosinophils and airway responsiveness. We also observed a trend between the severity of the late response and GM-CSF levels in BAL fluid. This is the first observation relating allergen-induced changes in GM-CSF to changes in lung function. The relationship between BAL eosinophils and BAL GM-CSF suggests that the influence of these variables on allergen-induced changes in lung function may be inter-dependent. The involvement of other cytokines able to influence eosinophil function, including interleukin-3 and interleukin-5, may also affect allergen responses and studies are currently underway to examine this issue.

In summary, we found that allergen inhalation by stable, mild asthmatics with late responses caused significant changes in the number of eosinophils, ECP and GM-CSF protein in bronchoalveolar lavage and biopsy tissue. Relationships were apparent between eosinophils and GM-CSF and the severity of the late response was related to changes in these inflammatory indices. These findings highlight the involvement of the eosinophil and GM-CSF in allergen-induced airway inflammation.

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## CHAPTER 6: INCREASES IN AIRWAY EOSINOPHILS, EOSINOPHIL CATIONIC PROTEIN AND METACHROMATIC CELLS, BUT NOT INTERLEUKIN-3, IN MILD ASTHMATICS COMPARED TO NON-ASTHMATICS

### Abstract

The importance of airway inflammation in asthma, even in its mild form, is being increasingly recognized. Compared to non-asthmatics, mild asthmatics have been shown to have increases in the number and activation of airway eosinophils and metachromatic cells. Interleukin-3 (IL-3) has been shown in vitro to regulate the growth, differentiation and activation of eosinophils and metachromatic cells. The potential role of IL-3 in vivo in asthma, however, is not known. We compared the number of metachromatic cells in the bronchoalveolar lavage (BAL) and the number and activation of eosinophils and levels of IL-3 protein in the blood, BAL and biopsy tissue obtained from 8, mild, atopic asthmatics and 10 non-asthmatics. Metachromatic cells were significantly increased in the BAL of asthmatic subjects. The number and activation (based on measures of eosinophil cationic protein secretion) of eosinophils were significantly increased in the BAL fluid, BAL cells and biopsy tissue of asthmatics. IL-3 protein was present in both asthmatic and non-asthmatic subjects, with no difference

apparent between the two groups. No relationships were apparent between IL-3 and eosinophils or metachromatic cells. Airway responsiveness ( $PC_{20}$ ) was significantly correlated with the number of tissue eosinophils ( $R_s = -0.71$ ;  $p = 0.00$ ) and the activation of eosinophils in BAL ( $R_s = -0.48$ ;  $p = 0.046$ ) and biopsy tissue ( $R_s = -0.65$ ;  $p = 0.004$ ). These results suggest that airway eosinophils and metachromatic cells, but not IL-3, are involved in the airway inflammation associated with asthma. In addition, increased airway responsiveness, a characteristic of asthma, may be consequence of eosinophilic infiltration and activation.

## Introduction

The importance of airway inflammation in the pathogenesis of asthma, even in its mild form, is being increasingly recognized (O'Byrne, 1990). Studies using bronchoalveolar lavage (BAL) have shown that asthmatics have increased numbers of inflammatory cells in their airways, including eosinophils (Adelroth et al., 1990; Wardlaw et al., 1988; Metzger et al., 1986) and metachromatic cells (mast cells/basophils) (Kirby et al., 1987; Robinson et al., 1992; Tomioka et al., 1984), compared to non-asthmatics. In addition, several studies have reported that BAL eosinophils (Adelroth et al., 1990; Wardlaw et al., 1988) and mast cells (Wenzel et

al., 1988) are more activated in asthmatic subjects. Although fewer studies have examined bronchial tissue directly, increases in eosinophils and mast cells in asthmatic biopsy tissue have been reported (Azzawi et al., 1990; Bousquet et al., 1990; Jeffery et al., 1992). These findings, however, have not been universal (Djukanovic et al., 1990; Jeffery et al., 1989).

Recently, attention has been directed toward the potential role of cytokines in regulating the number or activation state of inflammatory cells in asthmatic airways (Calhoun et al., 1993). In vitro studies have demonstrated that the cytokine, interleukin-3 (IL-3), can enhance or induce eosinophil growth and differentiation (Sonoda et al., 1989; Saito et al., 1988), chemotaxis (Sehmi et al., 1992; Czech et al., 1993), survival (Kita et al., 1991; Warringa et al., 1993; Anwar et al., 1993) and degranulation (Fujisawa et al., 1990; Tai et al., 1990). IL-3 has also been shown to promote the growth, survival and activation of murine mast cells (Ihle et al., 1983) and to increase the number of mast cells in rodent airways (Du et al., 1993). Recent research, however, has questioned the ability of IL-3 to affect human lung mast cells (Bischoff et al., 1992; Valent et al., 1990). The potential role of IL-3 in regulating human eosinophils or mast cells in vivo remains to be determined.

Information on the involvement of IL-3 in asthma is limited. Several recently published abstracts (Sur et al., 1993; Restrickett et al., 1993; Tadokoro et al., 1991) and papers (Robinson et al., 1992; Walker et al., 1991) have investigated IL-

IL-3 levels in asthmatic subjects. Restrick et al., (1993) reported that concentrations of IL-3 were less than  $1 \text{ pg}\cdot\text{ml}^{-1}$  in BAL fluid from mild asthmatics and non-asthmatics, with no difference evident between groups. Tadokoro et al., (1991) measured IL-3 in sputum from seventeen asthmatics experiencing an attack of severe asthma and reported that IL-3 was detectable ( $>40 \text{ pg}\cdot\text{ml}^{-1}$ ) in only seven subjects. Walker et al., (1991) investigated the presence of IL-3 in the supernatant from peripheral blood CD4+ T lymphocytes cultured *in vitro* and in serum. Based on the use of neutralizing antibodies, the authors concluded that IL-3 was present in samples from one of four mild, atopic asthmatics. Conflicting results are apparent with respect to measurement of IL-3 mRNA. Sur et al., (1993) was unable to detect IL-3 mRNA in BAL cells from either asthmatics or non-asthmatics. In contrast, Robinson et al., (1992) reported that the number of BAL cells expressing IL-3 mRNA was increased in mild, atopic asthmatics compared to non-atopic, non-asthmatics. Currently, no studies have investigated whether IL-3 protein is present in bronchial tissue or differs between asthmatics and non-asthmatics.

The purpose of the present study was to compare the number of eosinophils and metachromatic cells (mast cells/basophils) and the levels of ECP (used as a marker of eosinophil activation) and IL-3 in the blood, BAL and bronchial tissue of mild asthmatics and non-asthmatics. We hypothesized that eosinophils and metachromatic cells would be increased in the asthmatic subjects.

We also sought to examine whether IL-3 was related to eosinophil number and function or metachromatic cells. To evaluate the physiological significance of these markers of inflammation, we examined the relationships between methacholine airway responsiveness and eosinophil numbers, ECP and IL-3.

## **Methods**

### **Subjects**

Eight patients with mild asthma and ten non-asthmatic subjects were selected for study (see Table 4. 1). The study was approved by the Ethics Committee of McMaster University Medical Center and all subjects provided written informed consent. All asthmatics and five non-asthmatics were atopic, as indicated by one or more positive weal and flare responses to a battery of 16 skin prick tests to common antigens. All subjects were non-smokers and none had experienced a respiratory infection during the four weeks prior to the study. The asthmatic subjects were stable at the time of study, requiring only intermittent use of inhaled  $\beta_2$ -agonists and having baseline FEV<sub>1</sub> values > 70% predicted.

### Study Design

Subjects attended the laboratory on two occasions. At the initial visit, subjects completed a respiratory questionnaire and a full medical history, physical examination, skin prick tests and an ECG was performed. A blood sample was taken to measure white blood cell count, platelet count, prothrombin time and partial thromboplastin time. Baseline spirometry and a methacholine inhalation test were then performed. Within one week, subjects returned to the laboratory to complete a short follow-up questionnaire and perform spirometry. Providing the FEV<sub>1</sub> was within 5-10% of the baseline value, fiberoptic bronchoscopy was performed. After bronchoscopy, subjects were observed until they had recovered from the procedure.

### Methacholine Inhalation Test

To determine the degree of airway responsiveness, a methacholine inhalation challenge was performed as described by Cockcroft et al., (1991). Subjects inhaled doubling concentrations of methacholine chloride from a Wright nebulizer, for two minutes by tidal breathing. The test was continued until a fall in FEV<sub>1</sub>  $\geq$  20% of the baseline value occurred. The results were expressed as the provocative concentration causing a 20% fall in FEV<sub>1</sub> (PC<sub>20</sub>).

### Fiberoptic Bronchoscopy

Fiberoptic bronchoscopy was performed according to the recommendations of the National Institutes of Health (Workshop Committee, 1985). Subjects arrived at the research bronchoscopy suite at McMaster University Medical Center at 8:00 am, having fasted since midnight. An intravenous line was secured and blood samples obtained. Premedication with atropine (0.6 mg subcutaneous) was given 30 minutes prior to bronchoscopy. If sedation was required, midazolam ( $0.07 \text{ mg} \cdot \text{kg}^{-1}$ ) was given intravenously. Asthmatic subjects inhaled 400  $\mu\text{g}$  of salbutamol, administered via a spacer device, before topical anesthesia with lidocaine (Xylocaine) was applied to the upper airways. The bronchoscope (Olympus BF-1T20D; Olympus Optical Co., Tokyo, Japan) was inserted via a mouthpiece under further anesthesia to suppress cough. During the entire procedure, oxygen at  $4 \text{ L} \cdot \text{min}^{-1}$  was administered via a nasal cannula and subjects were monitored with ECG and ear oximetry. No complications were observed in any subjects during or following bronchoscopy.

### Blood Samples

Two venous blood samples were obtained from each subject. Samples were collected in either EDTA-treated tubes, for total and differential white blood cell counts, or non-treated tubes, to obtain serum. Total cell counts were performed using a hemocytometer and differential cell counts were made using

blood smears stained with a modified Wright-Geimsa (Diff-Quik, Baxter, McGraw Park, IL). Duplicate cell counts (200 cells counted per slide) were performed by one investigator blinded to the subject tested. Cells were classified as neutrophils, eosinophils, lymphocytes, monocytes and basophils using standard morphological criteria. To obtain serum, venous blood was stored at room temperature for two hours, centrifuged twice (1350 g, 10 min) and then stored at -70°C.

#### Biopsy Samples

Following insertion of the bronchoscope into the left lung, four mucosal biopsies were obtained from the lobar carinae and carinae of the basal segments using a separate forceps (FB-20C) for each biopsy. The position of the biopsy site and the appearance of the mucosal surface were carefully recorded. Two biopsies were placed immediately in glutaraldehyde for electron microscopy analysis and one biopsy was fixed in 4% paraformaldehyde for future studies. The biopsy sample for immunohistochemistry was placed immediately in periodate-lysine-paraformaldehyde (PLP) fixative and stored at 4°C for four hours. The tissue was then transferred through four solutions (phosphate buffered saline, 10%, 20% and 30% sucrose phosphate buffer), each for 4-6 hours at 4°C. The tissue was then frozen in Tissue-Tec OCT compound (Miles, Elkhart, In.) in -70°C isopentane (Aldrich, Milwaukee, Wi.) and stored at -70°C. Serial cryostat sections (6 µm) were cut onto slides coated with 3-aminopropyltriethoxysilane (Sigma, St. Louis, Mo.).



### Bronchoalveolar Lavage

The bronchoscope was subsequently inserted into the middle lobe of the right lung and wedged into a segment or sub-segment. Five 20 ml aliquots of pre-warmed (37°C), sterile normal saline were infused and gently aspirated back into polypropylene tubes kept on ice. The fluid was then centrifuged at 200 g for 10 minutes at 4°C. The supernatant was decanted and stored at -70°C. The cell pellet was washed in PBS and a total cell count was performed using a hemocytometer. The cells were then diluted with PBS to a concentration of  $1 \times 10^6$  cells·ml<sup>-1</sup>. Cytocentrifuge slides were prepared and stained with a modified Wright-Geimsa (Diff-Quik, Baxter, McGraw Park, IL) for a differential cell count (400 cells counted) or toluidine blue (pH = 0.05) for a metachromatic cell count (10,000 cells counted). Duplicate cell counts were performed by one investigator blinded to the subject tested. Cells were classified as macrophages, neutrophils, eosinophils, lymphocytes and metachromatic cells using standard morphological criteria. Total and differential cell counts were expressed as the number of cells per milliliter of fluid recovered. Cytocentrifuge slides prepared for immunocytochemistry were air dried for five minutes, fixed in PLP for 10 minutes at 4°C, placed in a 15% sucrose phosphate buffer solution for 10 minutes at 4°C and air dried overnight. Slides were then wrapped in pairs in foil and stored at -70°C.

### Tissue Eosinophils

Paraffin-embedded sections, prepared from the biopsy sample fixed in 4% paraformaldehyde, were stained with Chromotrope 2R (Sigma) to enumerate eosinophils in the tissue (Lendrum, 1944). Slides were coded prior to evaluation. For each section, a count was made of the positively staining cells within intact epithelium and another count made of the positively staining cells in the lamina propria to a depth of 115  $\mu\text{m}$  (delineated by a squared eyepiece graticule). A computerized and calibrated graphics tablet (Apple IIe) was used to determine the length of the epithelium and the area of the lamina propria in which counts were made. Results were expressed as the number of eosinophils per unit length of epithelium (1 mm) and area of lamina propria (1  $\text{mm}^2$ ).

### Immunocytochemistry

Immunocytochemistry was performed on BAL cytopins and tissue sections. To remove remaining OCT compound and sucrose and to enhance permeability and block non-specific reactions, slides were incubated with Dulbecco's phosphate buffered saline (GIBCO), supplemented with 0.01 M HEPES buffer (Boehringer Mannheim Canada Ltd.) containing 0.1% saponin (Sigma) (DPBS H + S) for five minutes at room temperature, twice. To block non-specific binding further, slides were incubated at room temperature with DPBS H + S containing 75% heat inactivated human AB sera for one hour and then with DPBS H + S containing

25% heat inactivated normal rabbit serum (Zyomed, San Francisco, Ca.) for one hour. Slides were washed briefly with DPBS H + S before application of the monoclonal antibodies. The monoclonal antibodies used were EG2 (Pharmacia, Sweden) which recognizes the cleaved form of human ECP and is thought to represent eosinophil activation (Tai et al., 1984) and a rat monoclonal antibody which reacts with human IL-3 protein (generous gift from Dr J.S. Abrams, DNAX, Ca.). EG2 and IL-3 antibodies were diluted in DPBS H + S containing 0.1% bovine serum albumin (Zyomed) at final concentrations of  $1 \mu\text{g}\cdot\text{ml}^{-1}$  and  $5 \mu\text{g}\cdot\text{ml}^{-1}$ , respectively. Slides were incubated overnight at  $4^{\circ}\text{C}$  and then washed in DPBS H + S. Labelling of the monoclonal antibodies was detected by the alkaline phosphatase anti-alkaline phosphatase method (APAAP Kit, Dako, Santa Barbara, Ca.). Slides were washed in distilled water, lightly counterstained with Mayer's hematoxylin (Sigma) and mounted. Appropriate system and specific controls were included in each assay.

#### Immunocytochemistry Quantification

All slides were coded before evaluation. For BAL cytospins, the cell pellet was divided into four equal sections, delineated with a squared eyepiece graticule. At least 100 cells were counted in each section. Results were expressed as the percentage of positively stained cells. The intra-class correlation coefficient, a stringent test of reproducibility, for repeated counts was 0.99 for EG2.

Assessment of staining for the biopsy specimens was performed with a semi-quantitative method, previously described for evaluating immunohistochemical staining (Leung et al., 1989). Briefly, two observers graded the extent and intensity of positive staining for each biopsy specimen on a scale from 1 (negative) to 5. Intra-class correlation coefficients between observers were 0.81 for EG2 and 0.84 for IL-3.

#### ECP and IL-3 Assays

Levels of ECP in serum and BAL fluid were determined using a double-antibody radioimmunoassay (Kabi Pharmacia Diagnostics AB, Sweden), with results expressed in micrograms per liter. Assay sensitivity for ECP was  $2 \mu\text{g}\cdot\text{L}^{-1}$ . Measurement of IL-3 in serum and 20-fold concentrated (Amicon-10, Amicon Corp., Danvers, Ma) BAL fluid was performed using an enzyme-linked immunosorbent assay (Quantikine, Research and Diagnostics System, Minneapolis, Mn), with results expressed in picograms per milliliter. Assay sensitivity for IL-3 was  $7.4 \text{ pg}\cdot\text{ml}^{-1}$ . In all assays, samples were analyzed in duplicate.

#### Statistical Analysis

Statistical analyses were performed using the CSS Statistica computer software program. Data distributions were checked for normality using

Kolmogorov-Smirnoff and Chi-Square analyses. As most distributions were significantly different from normal, non-parametric statistics were used. Given the a priori hypothesis that eosinophils and metachromatic cells would be increased in asthmatic compared to non-asthmatic subjects, one-tailed Mann-Whitney U tests for unpaired samples were used for these cell counts. Differences in indices of eosinophil activation (ECP levels and EG2 staining) and IL-3 between asthmatics and non-asthmatics were examined using two-tailed Mann-Whitney U tests. Spearman's rank correlation coefficient test ( $R_s$ ) was used to examine the association between inflammatory parameters and the physiological measurement of airway responsiveness. A p value of  $< 0.05$  was accepted as statistically significant.

## Results

Eosinophils were increased in the blood, bronchoalveolar lavage and biopsy tissue from mild asthmatic subjects. The percentage of circulating eosinophils was significantly greater in the asthmatic subjects, with a median of 5% (range: 3 - 13%), than in non-asthmatic subjects, with a median of 3% (range: 1 - 9%) ( $p = 0.019$ ). The absolute number ( $\times 10^4 \cdot \text{ml}^{-1}$ ) of circulating eosinophils was also significantly greater in asthmatics, with a median of 36 (range: 16 - 100), than in non-asthmatics, with a median of 17 (range: 6 - 52) ( $p = 0.008$ ). The percentage of BAL eosinophils was also significantly increased in asthmatic subjects, with a median value of 1.4% (range: 0.0 to 5.0%), compared to non-asthmatics, with a median of 0.5% (range: 0.0 - 2.0%) ( $p = 0.034$ ) (Table 6. 1). Metachromatic cells in the BAL were significantly increased in asthmatic subjects, with a median value of 0.02% (range: 0.02 to 0.08%), compared to non-asthmatics, with a median of 0.00% (range: 0.00 - 0.03%) ( $p = 0.017$ ) (Table 6. 1).

Table 6.1: Bronchoalveolar lavage cell counts for subjects

Asthmatic Subjects							
Subject No.	Return (ml)	Total ( $\times 10^4 \cdot \text{ml}^{-1}$ )	M	N	L (%)	E	MC
1	55	5.59	92.0	1.8	4.8	1.5	0.02
2	66	8.35	95.0	0.0	3.5	1.3	ND
3	49	3.88	92.0	1.0	2.0	5.0	ND
4	67	4.85	95.5	2.5	2.0	0.0	0.02
5	55	9.09	95.3	1.0	2.5	1.3	0.08
6	57	1.39	94.5	2.5	0.8	2.3	0.04
7	69	2.87	93.5	4.3	1.5	0.8	0.02
8	67	7.99	85.3	1.0	12.3	1.5	0.02
Median	62	5.22	94.0	1.4	2.3	1.4	0.02
Non-Asthmatic Subjects							
Subject No.	Return (ml)	Total ( $\times 10^4 \cdot \text{ml}^{-1}$ )	M	N	L (%)	E	MC
1	65	4.23	89.0	10.3	0.3	0.5	0.03
2	47	3.15	89.0	6.5	3.5	1.0	ND
3	62	8.61	97.3	0.0	0.8	2.0	ND
4	65	4.42	93.3	2.3	4.5	0.0	0.00
5	62	9.64	93.0	3.3	2.3	1.5	0.00
6	68	13.16	87.0	7.8	5.0	0.3	ND
7	67	23.06	97.3	1.8	1.0	0.0	0.00
8	51	6.72	94.0	1.8	4.3	0.0	0.00
9	71	11.83	98.3	0.3	0.5	1.0	0.00
10	76	9.70	94.8	2.5	2.3	0.5	0.00
Median	65	9.13	93.7	2.4	2.3	0.5	0.00
p value*	0.59	0.08	0.86	0.27	0.59	0.03	0.02

M macrophage; N neutrophil; L lymphocyte; E eosinophil; MC metachromatic cell.  
 ND not determined

\* p value for comparison between asthmatics and non-asthmatics.

In bronchial biopsy tissue, the number of eosinophils was again significantly greater in asthmatic than non-asthmatic subjects (see Figure 4. 1). In areas of intact epithelium, intra-epithelial eosinophils could be seen in asthmatics, with a median of  $1.05\text{-mm}^{-1}$  (range: 0.67 - 3.18) but were never observed in non-asthmatic subjects ( $p = 0.002$ ). Within the lamina propria, eosinophils were increased in asthmatics, with a median of  $17.02\text{-mm}^{-2}$  (range: 7.41 - 78.95), compared to non-asthmatics, with a median of  $1.41\text{-mm}^{-2}$  (range: 0.00 - 10.81) ( $p = 0.003$ ).

Immunocytochemistry on BAL cells demonstrated that asthmatics had a significantly increased number of cells positive for EG2 (see Figure 4. 2). The median value of EG2-positive cells was 2.03% for asthmatics and 0.10% for non-asthmatics ( $p = 0.008$ ). In BAL cells from asthmatics, staining for EG2 was present in the cytoplasm and was also observed external to the cell surface, suggestive of ECP secretion (see Figure 4. 3). Occasionally, staining for EG2 was apparent in alveolar macrophages, suggesting engulfment of secreted ECP. In non-asthmatics, few BAL cells were positive for EG2 (see Figure 4. 3). No staining was present in the slides stained with IgG<sub>1</sub> (negative control).

Immunohistochemistry on biopsy tissue demonstrated that asthmatics had significantly increased staining for EG2 but not for IL-3 (Figure 6. 1). The median value of EG2 staining was 2.25 for asthmatics and 1.25 for non-asthmatics ( $p = 0.026$ ). The median value of IL-3 staining was 3.25 for asthmatics and 3.13



for non-asthmatics ( $p = 0.626$ ). In asthmatic tissue, staining for EG2 was mainly intra-cellular and, although localized primarily beneath the basement membrane, intra-epithelial EG2-positive cells were observed in some asthmatics (see Figure 4.6). In non-asthmatic tissue, minimal staining was observed for EG2 (see Figure 4.6). For both asthmatics and non-asthmatics, staining for IL-3 was primarily intra-cellular, with substantial staining localized to the epithelium (Figure 6.2). No staining was present in the negative control slides.

In the serum and BAL supernatant, the median concentration of ECP was not significantly higher in the asthmatic compared to the non-asthmatic subjects. In the serum, the median concentration ( $\mu\text{g}\cdot\text{L}^{-1}$ ) of ECP was 10.25 for asthmatics (range: 5.10 - 45.50) and 9.30 (range: 1.70 - 32.80) for non-asthmatics ( $p = 0.594$ ). In lavage fluid from asthmatics, the median concentration ( $\mu\text{g}\cdot\text{L}^{-1}$ ) of ECP was 4.05 (range: 1.30 - 16.20) and was 3.35 (range: 1.30 - 9.70) in lavage fluid from non-asthmatics ( $p = 0.790$ ). In both asthmatic and non-asthmatic subjects, no IL-3 was detected in serum and concentrated BAL supernatant samples (detection limit of assay =  $7.4 \text{ pg}\cdot\text{ml}^{-1}$ ).

No relationships were apparent between staining for IL-3 in biopsy tissue and eosinophil number or function, metachromatic cells or methacholine airway responsiveness. However, airway responsiveness was related to eosinophil number and activation. Significant correlations were found between MCh  $\text{PC}_{20}$  and the number of circulating eosinophils ( $R_s = -0.53$ ;  $p = 0.02$ ), the number of

eosinophils in the epithelium ( $R_s = -0.95$ ;  $p < 0.00$ ) and the number of eosinophils in the lamina propria ( $R_s = -0.71$ ;  $p < 0.00$ ). Significant correlations were also observed between MCh PC<sub>20</sub> and staining for EG2 in BAL cells ( $R_s = -0.48$ ;  $p = 0.046$ ) and biopsy tissue ( $R_s = -0.65$ ;  $p = 0.004$ ).

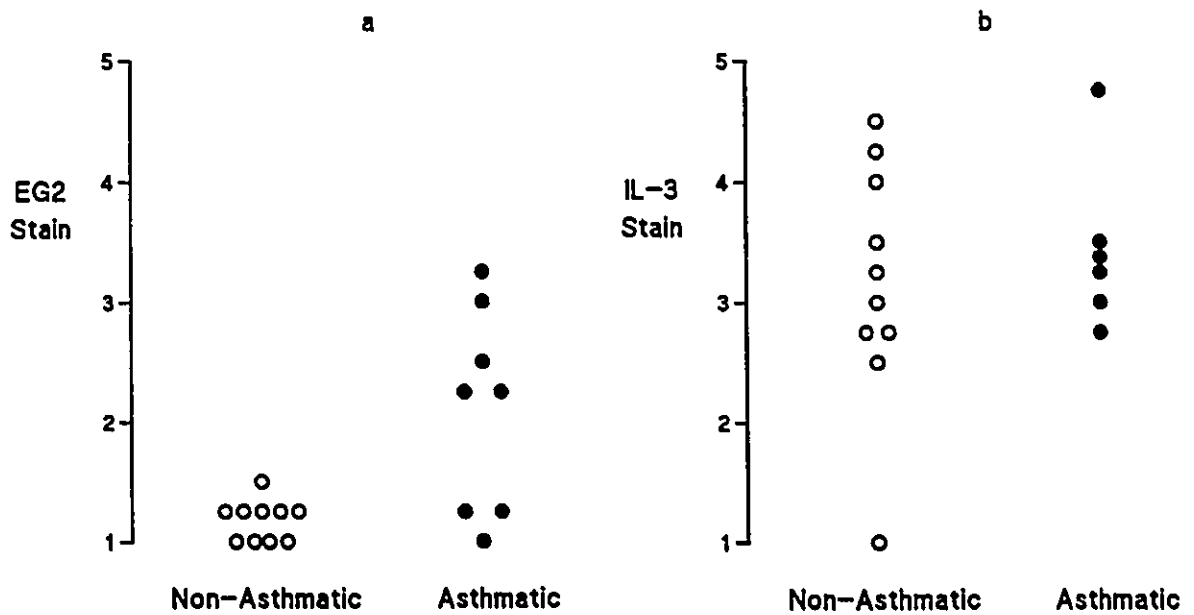
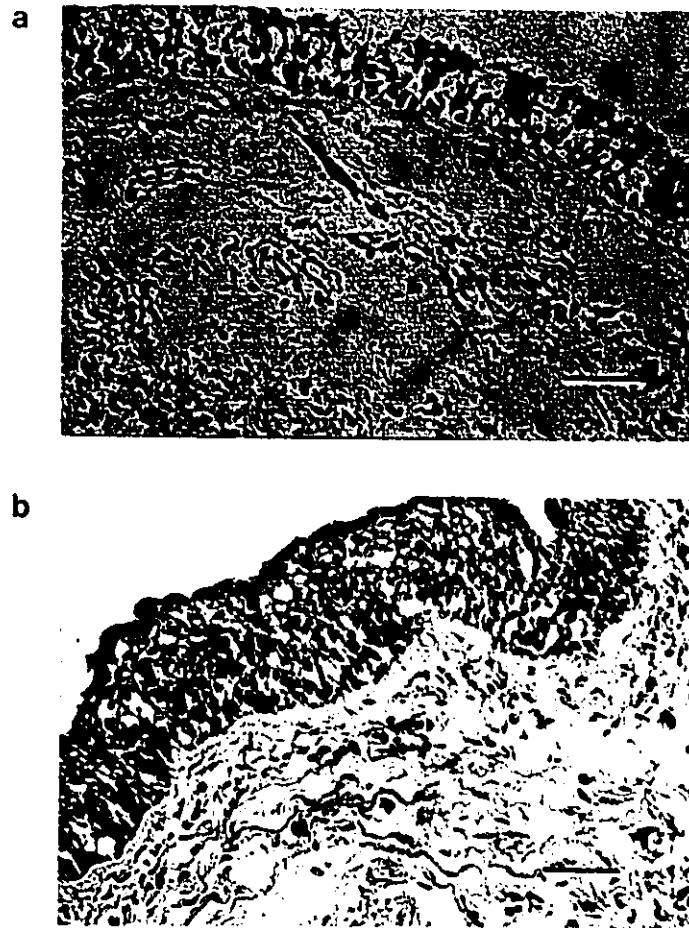


Figure 6.1. Staining of biopsy tissue for (a) EG2 and (b) IL-3 in non-asthmatic and asthmatic subjects. Biopsy tissue from asthmatics exhibited significantly greater staining for EG2, but not IL-3, compared to non-asthmatics.



**Figure 6.2.** Immunohistochemistry for IL-3 on bronchial biopsy tissue from a (a) non-asthmatic and (b) asthmatic subject (scale bar = 50  $\mu\text{m}$ ). Staining is evident in tissue from both the asthmatic and non-asthmatic subject. Positive (red) staining for IL-3 is primarily intra-cellular and mainly within the epithelium.

## Discussion

The results from the present study demonstrate that the number and activity of eosinophils in the blood, BAL and bronchial biopsy tissue from mild, stable asthmatics were increased compared to non-asthmatic controls. The number of metachromatic cells in the BAL was also increased in the asthmatic subjects. The cytokine, IL-3, was present in bronchial biopsy tissue from asthmatic and non-asthmatic subjects, with no difference evident between the two groups. IL-3 protein was not detected in serum or BAL fluid in either asthmatics or non-asthmatics. No relationships were apparent between IL-3 and eosinophils or metachromatic cells. Eosinophil number and function, however, were significantly correlated with methacholine airway responsiveness. The novel finding of constitutive intracellular localization of IL-3 protein within asthmatic and non-asthmatic tissue suggests that IL-3 may play a role in normal airway physiology. The results concerning eosinophils and metachromatic cells suggest that these cells contribute to the ongoing airway inflammation apparent even in mild asthma and that airway hyperresponsiveness may be a consequence of eosinophilic inflammation.

The increased number of eosinophils and metachromatic cells that we found in mild asthmatics, compared to non-asthmatics, is in agreement with

previous observations. In most studies, increased numbers of eosinophils in blood (Ellis, 1908; Durham et al., 1989; Bousquet et al., 1990) and BAL (Kirby et al., 1987; Adelroth et al., 1990; Wardlaw et al., 1988; Metzger et al., 1986) samples from asthmatics have been consistently reported. Similarly, an increase in metachromatic cells in BAL fluid from asthmatics has been frequently observed (Kirby et al., 1987; Adelroth et al., 1990; Wardlaw et al., 1988; Robinson et al., 1992; Tomioka et al., 1984). Studies on inflammatory cells in bronchial biopsy tissue, however, are more limited and have yielded conflicting results. While several studies have documented increased numbers of eosinophils (Jeffery et al., 1992; Azzawi et al., 1990; Bousquet et al., 1990; Beasley et al., 1989) and mast cells (Jeffery et al., 1992) in bronchial tissue from asthmatics, compared to non-asthmatics, other studies have reported no significant differences in eosinophils (Jeffery et al., 1989) and mast cells (Djukanovic et al., 1990; Beasley et al., 1989) between the two groups. These inconsistencies may reflect the mild state of disease of the subjects studied and highlight the variability that is often observed in studies of patients with asthma.

Our observations of increased eosinophil activity in BAL and biopsy samples from mild asthmatics, compared to non-asthmatics, confirms and extends previous findings. Earlier studies have inferred that activated eosinophils are present in the bronchial lumen of asthmatics, based on findings of increased ECP in BAL supernatant (Broide et al., 1992; Adelroth et al., 1990; Bousquet et al.,

1991). Our finding of an increased number of EG2-positive cells in the BAL from asthmatics is novel and provides direct evidence that eosinophils within the lumen retain the ability to secrete ECP. The consequences of ECP secretion in the lumen are likely to be adverse based on in vitro studies which have documented the ability of ECP to damage pulmonary epithelium (Ayers et al., 1985; Young et al., 1986). Similar to recent studies (Beasley et al., 1989; Djukanovic et al., 1990; Bousquet et al., 1990), we observed increased staining for the secreted form of ECP in bronchial tissue from asthmatic, compared to non-asthmatic, subjects. Our study demonstrates that eosinophils in both the lumen and the mucosa are activated in subjects with mild asthma. As biopsy and BAL samples reflect central and more peripheral airways, respectively, these findings imply that activated eosinophils are present in much of the asthmatic airway.

The mechanism(s) underlying the eosinophilia associated with asthma are currently under investigation. Cytokines, such as GM-CSF and IL-3, have been shown in vitro to enhance or induce the growth and differentiation (Sonoda et al., 1989; Saito et al., 1988), chemotaxis (Sehmi et al., 1992; Czech et al., 1993), survival (Kita et al., 1991; Warringa et al., 1993; Anwar et al., 1993) and degranulation (Fujisawa et al., 1990; Tai et al., 1990) of eosinophils. These cytokines have been postulated to regulate eosinophils in vivo and to contribute to the eosinophilic airway inflammation associated with asthma (Arm et al., 1992; Barnes, 1993; Wardlaw et al., 1993; Calhoun et al., 1993). We have recently

shown that GM-CSF protein is increased in BAL cells and biopsy tissue of mild asthmatics compared to non-asthmatics (Woolley K.L. et al., submitted). We also found that GM-CSF was significantly correlated with airway function, based on the measurement of airway responsiveness. These findings support the hypotheses that GM-CSF regulates eosinophils in vivo and that GM-CSF contributes to the altered airway function evident with asthma.

In contrast, our observations that IL-3 protein was present in both asthmatic and non-asthmatic airways and that IL-3 was not correlated with eosinophils, metachromatic cells or airway function imply that IL-3 does not play a major role in asthmatic airway inflammation. Localization of IL-3 protein in bronchial tissue from both asthmatic and non-asthmatics is a novel finding. The biological significance of IL-3 in normal airways, however, remains to be determined. As inflammation is a homeostatic process (Gauldie et al., 1992; Weissman, 1988), IL-3 may play a protective role in the ongoing defense of normal airway mucosa. For example, IL-3 may be involved in regulating recruitment of inflammatory cells in response to potentially harmful agents that gain access to the airways. In this regard, IL-3 has been shown in vitro to upregulate the expression of ICAM-1 (Czech et al., 1993), a cell adhesion molecule believed to play a pivotal role in inflammatory cell migration (Wegner et al., 1990; Leff et al., 1991; Wegner et al., 1992). Furthermore, recent studies have shown that, similar to our findings with IL-3, ICAM-1 is constitutively expressed in both asthmatic and non-asthmatic



bronchial tissue (Montefort et al., 1992; Bentley et al., 1993; Gosset et al., 1993).

Our inability to detect IL-3 in the serum or lavage fluid from asthmatics and non-asthmatics suggests that secretion of IL-3 is limited and not associated with mild asthma. Findings from previous studies support these observations. Restrick et al. (1993) reported that the concentration of IL-3 in BAL fluid from asthmatics and non-asthmatics was less than  $1 \text{ pg}\cdot\text{ml}^{-1}$ , with no differences evident between the groups. The detection of IL-3 in sputum from seven of seventeen asthmatics with a severe attack of asthma (Tadokoro et al., 1991), raises the possibility that secretion of IL-3 may occur with more severe disease. However, IL-3 in sputum may reflect leakage, rather than secretion, of IL-3 from epithelial cells sloughed off and damaged during an asthma attack. Consistent with this are our observations that IL-3 was predominantly localized to epithelial cells in bronchial tissue and not secreted into the lumen. In addition, recent in vitro studies in our laboratory have shown that human epithelial cell lines contain, but do not spontaneously secrete, IL-3 protein (Sallenave, J.M. et al., submitted). Limited secretion of IL-3 would presumably restrict the actions of IL-3 to the local milieu, which may be particularly important in controlling inflammation within normal airways.

In our study, IL-3 was not significantly correlated with metachromatic cell number in the BAL. This finding provides indirect support for studies which have shown that IL-3 does not affect human lung mast cells in vitro (Valent et al., 1990).

However, as the toluidine blue stain we used identifies metachromatic cells, namely mast cells and basophils, a potential role for IL-3 in regulating basophils in vivo cannot be discounted. In vitro studies have shown that IL-3 enhances the growth and differentiation (Saito et al., 1988) and activation (MacDonald et al., 1989) of human basophils. The apparent inability of IL-3 to affect human lung mast cells indicates that other factors may mediate increases in the number and activation of airway mast cell in asthma. One such potential factor may be c-kit ligand, a stem cell growth factor which has been shown recently to enhance pre-formed and newly synthesized mediator release from human lung mast cells in vitro (Bischoff et al., 1992).

In conclusion, we have found that eosinophils and metachromatic cells are increased in the airways of mild asthmatics, compared to non-asthmatics. Furthermore, the number and function of eosinophils was correlated with airway responsiveness. We also found that IL-3 protein was present, but not significantly different, in bronchial tissue from asthmatic and non-asthmatic subjects. No relationships were evident between IL-3 and eosinophils, metachromatic cells or airway responsiveness. These results suggest that eosinophils and metachromatic cells, but not IL-3, are involved in the airway inflammation associated with asthma.

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**CHAPTER 7: ALLERGEN CHALLENGE INCREASES AIRWAY EOSINOPHILS, EOSINOPHIL CATIONIC PROTEIN AND METACHROMATIC CELLS, BUT NOT INTERLEUKIN-3, IN MILD ASTHMATICS.**

**Abstract**

Interleukin-3 (IL-3) has been shown in vitro to regulate the growth and differentiation and activation of eosinophils and metachromatic cells. The role that IL-3 may play in vivo in the increases in eosinophils and metachromatic cells that can occur after allergen inhalation in asthmatic subjects remains to be clarified. We investigated the effects of allergen inhalation on eosinophil number and activation (based on secretion of eosinophil cationic protein), metachromatic cells and IL-3 protein in blood, bronchoalveolar lavage (BAL) and biopsy tissue. Seven, mild, atopic asthmatics, with late asthmatic responses, completed diluent and allergen inhalation challenges. Bronchoscopy was performed 24 hours after challenge and samples collected. Allergen inhalation caused a significant increase in the number of BAL metachromatic cells and BAL and biopsy eosinophils. Allergen inhalation also significantly increased ECP in the BAL fluid and BAL cells. IL-3 was present in biopsy tissue but not detected in serum or BAL fluid. Allergen inhalation did not affect the level of IL-3 in biopsy tissue and no relationships were

evident between IL-3 and eosinophils or metachromatic cells. A significant correlation was demonstrated between BAL eosinophil number and the severity of the late asthmatic response ( $R_s = 0.82$ ,  $p = 0.02$ ). These results suggest that the increases in airway eosinophils and metachromatic cells observed after allergen challenge are not mediated by IL-3. In addition, eosinophils, in particular, appear to contribute to the late asthmatic response.

## Introduction

The allergen challenge model has been widely utilized for investigating airway inflammation in asthma. In asthmatics with allergen-induced late asthmatic responses, increases in peripheral blood eosinophils (Booij-Noord et al., 1972; Lam et al., 1987) and bronchoalveolar lavage (BAL) eosinophils (De Monchy et al., 1985; Metzger et al., 1986; Lam et al., 1987; Brusasco et al., 1990; Broide et al., 1991) have been reported. In addition, several studies have demonstrated allergen-induced increases in the activation of BAL eosinophils (De Monchy et al., 1985; Metzger et al., 1986) and metachromatic cells (mast cells/basophils) (Metzger et al., 1986; Wenzel et al., 1988). More recently, researchers have investigated the effects of allergen challenge on eosinophils and metachromatic cells within bronchial biopsy tissue. Currently, however, results are conflicting, with

increases (Jarjour et al., 1993; Bentley et al., 1993; Aalbers et al., 1993) or no changes (Beasley et al., 1989; Crimi et al., 1991) reported for the number or activation state of eosinophils within biopsy tissue after allergen challenge. Similarly, increases (Crimi et al., 1991) or non-significant changes (Beasley et al., 1989) have been reported for metachromatic cells in bronchial tissue after allergen challenge.

The mechanisms which may regulate allergen-induced inflammatory events are now under investigation. Of particular interest is the potential role played by cytokines such as granulocyte-colony stimulating factor (GM-CSF) and interleukin-3 (IL-3), in regulating the number and activation of eosinophils in the tissue. Interest in IL-3 has also been generated by in vitro studies demonstrating the effects of IL-3 on murine mast cell growth, development and activation (Ihle et al., 1983) and in vivo evidence of increased numbers of mast cells and eosinophils in rodent airways following pre-treatment with human recombinant IL-3 (Du et al., 1993).

We have recently shown that significant changes occur in the level of GM-CSF protein in BAL cells, BAL fluid and bronchial tissue obtained from mild asthmatics 24 hours after allergen challenge (Woolley K.L. et al., submitted). Currently, however, limited information is available concerning the effects of allergen challenge on IL-3 in asthmatic airways. Investigation of IL-3 protein in BAL fluid obtained after allergen challenge from subjects with allergic rhinitis has

produced conflicting results. Ohnishi et al., (1991) reported that IL-3 activity was present in BAL fluid, based on partial inhibition of eosinophil survival with IL-3 neutralizing antibodies. In contrast, Kato et al., (1992), using a bioassay for IL-3, reported that allergen challenge did not result in detectable increases in IL-3. Studies examining the late phase response in the skin (Kay et al., 1991) or nose (Durham et al., 1992) with in situ hybridization reported that the number of cells expressing IL-3 mRNA was increased at allergen, compared to diluent, challenged sites. Most recently, however, the same group investigated the effects of allergen challenge on IL-3 mRNA in BAL cells (Hamid et al., 1993) and bronchial tissue (Bentley et al., 1993) from asthmatics and reported no changes after allergen challenge. To date, no studies have investigated levels of IL-3 protein in BAL fluid or biopsy tissue from mild asthmatics after allergen challenge.

The purpose of the present study, therefore, was to determine the effects of allergen inhalation challenge on eosinophil number, eosinophil cationic protein (ECP), used as a marker of eosinophil activation, metachromatic cells and IL-3 protein in the blood, BAL and bronchial tissue of mild asthmatics. We hypothesized that allergen inhalation would induce increases in eosinophils and metachromatic cells. We also sought to investigate whether IL-3 protein was related in vivo to allergen-induced changes in eosinophils or metachromatic cells. The physiological significance of inflammatory changes was examined by relating the severity of the late asthmatic response to eosinophils, metachromatic cells,

ECP and IL-3.

## **Methods**

### Subjects

Seven patients with mild asthma were selected for study (see Table 5. 1). The study was approved by the Ethics Committee of the McMaster University Medical Center and all subjects provided their written informed consent prior to starting the study. Subjects were atopic, as indicated by one or more positive weal and flare responses to skin prick tests. Subjects were non-smokers and none had experienced a respiratory infection during the four weeks prior to the study. Asthmatics were stable at the time of study, only requiring intermittent use of inhaled  $\beta_2$ -agonists and had baseline FEV<sub>1</sub> values > 70% predicted.

### Study Design

Subjects attended the laboratory on six occasions. At the initial visit, subjects completed a respiratory questionnaire and a full medical history, physical examination, skin prick test, ECG and chest x-ray were performed. A blood sample was taken to determine white blood cell count, platelet count, prothrombin time and partial thromboplastin time. Baseline spirometry and a methacholine inhalation test were performed. Within one week, subjects returned to the

laboratory to complete a short follow-up questionnaire, perform spirometry and undertake a diluent inhalation challenge. Fiberoptic bronchoscopy was performed 24 hours after the diluent challenge. After a period of three to four weeks, subjects returned to the laboratory to perform spirometry and complete a methacholine inhalation test and skin prick test titration test with the relevant allergen. Within one week, subjects returned to the laboratory to complete a short follow-up questionnaire, perform spirometry and undertake an allergen inhalation challenge. Fiberoptic bronchoscopy was again performed 24 hours after the allergen challenge. After each bronchoscopy, subjects were observed closely until the local anesthesia had worn off.

#### Methacholine Inhalation Challenge and Skin Test

Methacholine inhalation challenge was performed as described by Cockcroft et al., (1991). Subjects inhaled doubling concentrations of methacholine chloride from a Wright nebulizer, for two minutes by tidal breathing. Increasing concentrations of methacholine were administered until a fall in  $FEV_1 \geq 20\%$  of the baseline value occurred. Results were expressed as the provocative concentration causing a 20% fall in  $FEV_1$  ( $PC_{20}$ ). For the skin prick titration test, the allergen used in the allergen inhalation challenge was administered in doubling dilutions, in duplicate. The dilution giving a two millimeter weal response at 15 minutes was used in the calculation of the predicted  $PC_{15}$  of allergen.

### Diluent Inhalation Challenge

Three doses of normal saline were given at ten minute intervals. The aerosol was generated by a Wright nebulizer with an output of  $0.13 \text{ ml}\cdot\text{min}^{-1}$ . The aerosol was inhaled for two minutes by tidal breathing using a Hans-Rudolph valve box and mouthpiece with a filter attached to the expiratory route and the nose clipped. FEV<sub>1</sub> measurements were made after each inhalation. After the final inhalation, spirometry was performed at 10, 20, 30, 45, 60, 90 and 180 minutes and then every hour for seven hours after the final inhalation.

### Allergen Inhalation Challenge

Results from the methacholine inhalation challenge and the skin prick titration test were used to predict the allergen PC<sub>15</sub> according to the formula described by Cockcroft et al., (1987). Doubling concentrations of allergen were inhaled at ten minute intervals until a fall of 15% or more occurred in the subject's FEV<sub>1</sub>. Measurements of FEV<sub>1</sub> were made in the same manner as for the diluent challenge. From the predicted allergen PC<sub>15</sub>, the starting concentration of allergen was chosen to be three doubling doses below the predicted value.

### Fiberoptic Bronchoscopy

Fiberoptic bronchoscopy was performed according to the recommendations of the National Institutes of Health (Workshop Committee, 1985).

Subjects arrived at the research bronchoscopy suite at McMaster University Medical Center at 8:00 am, having fasted since midnight. An intravenous line was secured and blood samples obtained. Premedication with atropine (0.6 mg subcutaneous) was given 30 minutes prior to bronchoscopy. If sedation was required, midazolam ( $0.07 \text{ mg} \cdot \text{kg}^{-1}$ ) was given intravenously. Subjects inhaled 400  $\mu\text{g}$  of salbutamol administered via a spacer device before topical anesthesia with lidocaine (Xylocaine; Astra Pharma Inc., Mississauga, Canada) was applied to the airways. The bronchoscope (Olympus BF-1T20D; Olympus Optical Co., Tokyo, Japan) was inserted via a mouthpiece under further anesthesia to suppress cough. During the entire procedure, oxygen at  $4 \text{ L} \cdot \text{min}^{-1}$  was administered via a nasal cannula and subjects were monitored with ECG and ear oximetry. No complications were observed in any subjects during or following bronchoscopy.

### Blood Samples

Two venous blood samples were obtained from each subject. Samples were collected in either EDTA-treated tubes for total and differential white blood cell counts or untreated tubes to obtain serum. Total cell counts were performed using a hemocytometer and differential cell counts were made from blood smears stained with Diff-Quik. Cell counts were performed in duplicate by one investigator blinded to the type of challenge undertaken by the subject. Cells were classified as neutrophils, eosinophils, lymphocytes, monocytes and basophils



using standard morphological criteria. To obtain serum, venous blood was stored at room temperature for two hours, centrifuged twice (1350 g, 10 min) and then stored at -70°C.

### Biopsy Samples

Following insertion of the bronchoscope into the left lung, four mucosal biopsies were obtained from the lobar carinae and carinae of the basal segments using a separate forceps (FB-20C) for each biopsy. The position of the biopsy site and the appearance of the mucosal surface were carefully recorded. Two biopsies were placed immediately in glutaraldehyde for electron microscopy analysis and one biopsy was fixed in 4% paraformaldehyde for future studies. The biopsy sample for immunohistochemistry was placed immediately in periodate-lysine-paraformaldehyde (PLP) fixative and stored at 4°C for four hours. The tissue was then transferred through four solutions (phosphate buffered saline, 10%, 20% and 30% sucrose phosphate buffer), each for 4-6 hours at 4°C. The tissue was then frozen in Tissue-Tec OCT compound (Miles, Elkhart, In.) in -70°C isopentane (Aldrich, Milwaukee, Wi.) and stored at -70°C. Serial cryostat sections (6 µm) were cut and positioned on slides coated with 3-aminopropyltriethoxysilane (Sigma, St. Louis, Mo.).

### Bronchoalveolar Lavage

The bronchoscope was inserted into the middle lobe of the right lung and wedged into a segment or sub-segment. Five 20 ml aliquots of pre-warmed (37°C), sterile normal saline were infused and gently aspirated back into polypropylene tubes kept on ice. The fluid was then centrifuged at 200 g for 10 minutes at 4°C. The supernatant was decanted and stored at -70°C. The cell pellet was washed in PBS and a total cell count was performed using a hemocytometer. The cells were then diluted with PBS to a concentration of  $1 \times 10^6$  cells·ml<sup>-1</sup>. Cytocentrifuge slides were prepared and stained with a modified Wright-Geimsa (Diff-Quik, Baxter, McGraw Park, IL) for a differential cell count (400 cells counted) or toluidine blue (pH = 0.05) for a metachromatic cell count (10,000 cells counted). Duplicate cell counts were performed by one investigator blinded to the subject tested. Cells were classified as macrophages, neutrophils, eosinophils, lymphocytes and metachromatic cells using standard morphological criteria. Total and differential cell counts were expressed as the number of cells per milliliter of fluid recovered. Cytocentrifuge slides prepared for immunocytochemistry were air dried for five minutes, fixed in PLP for 10 minutes at 4°C, placed in a 15% sucrose phosphate buffer solution for 10 minutes at 4°C and air dried overnight. Slides were then wrapped in pairs in foil and stored at -70°C.

### Tissue Eosinophils

Paraffin-embedded sections, prepared from the biopsy sample fixed in 4% paraformaldehyde, were stained with Chromotrope 2R (Sigma) to enumerate eosinophils in the tissue (Lendrum, 1944). Slides were coded prior to evaluation. For each section, the number of positively staining cells along the length of the basement membrane to a depth of 115  $\mu\text{m}$  (delineated by a squared eyepiece graticule) was counted. A computerized and calibrated graphics tablet (Apple IIe) was used to determine the area of the lamina propria in which counts were made. Results were expressed as the number of eosinophils per unit area of lamina propria ( $1 \text{ mm}^2$ ).

### Immunocytochemistry

Immunocytochemistry was performed on BAL cytopins and tissue sections. To remove remaining OCT compound and sucrose and to enhance permeability and block non-specific reactions, slides were incubated with Dulbecco's phosphate buffered saline (GIBCO), supplemented with 0.01 M HEPES buffer (Boehringer Mannheim Canada Ltd.) containing 0.1% saponin (Sigma) (DPBS H + S) for five minutes at room temperature, twice. To block non-specific binding further, slides were incubated at room temperature with DPBS H + S containing 75% heat inactivated human AB sera for one hour and then with DPBS H + S containing 25% heat inactivated normal rabbit serum (Zymed, San Francisco, Ca.) for one

hour. Slides were washed briefly with DPBS H + S before application of the monoclonal antibodies. The monoclonal antibodies used were EG2 (Pharmacia, Sweden) which recognizes the cleaved form of human ECP and is thought to represent eosinophil activation (Tai et al., 1984) and a rat monoclonal antibody which reacts with human IL-3 protein (generous gift from Dr J.S. Abrams, DNAX, Ca.). EG2 and IL-3 antibodies were diluted in DPBS H + S containing 0.1% bovine serum albumin (Zymed) at final concentrations of  $1 \mu\text{g}\cdot\text{ml}^{-1}$  and  $5 \mu\text{g}\cdot\text{ml}^{-1}$ , respectively. Slides were incubated overnight at  $4^{\circ}\text{C}$  and then washed in DPBS H + S. Labelling of the monoclonal antibodies was detected by the alkaline phosphatase anti-alkaline phosphatase method (APAAP Kit, Dako, Santa Barbara, Ca.). Slides were washed in distilled water, lightly counterstained with Mayer's hematoxylin (Sigma) and mounted. Appropriate system and specific controls were included in each assay.

#### Immunocytochemistry Quantification

All slides were coded before evaluation. For BAL cytospins, the cell pellet was divided into four equal sections, delineated with a squared eyepiece graticule. At least 100 cells were counted in each section. Results were expressed as the percentage of positively stained cells. The intra-class correlation coefficient, a stringent test of reproducibility, for repeated counts was 0.99 for EG2. Assessment of staining for the biopsy specimens was performed with a semi-

quantitative method, previously described for evaluating immunohistochemical staining (Leung et al., 1989). Briefly, two observers graded the extent and intensity of positive staining for each biopsy specimen on a scale from 1 (negative) to 5. Intra-class correlation coefficients between observers were 0.81 for EG2 and 0.84 for IL-3.

#### ECP and IL-3 Assays

Levels of ECP in serum and BAL fluid were determined using a double-antibody radioimmunoassay (Kabi Pharmacia Diagnostics AB, Sweden), with results expressed in micrograms per liter. Assay sensitivity for ECP was  $2 \mu\text{g}\cdot\text{L}^{-1}$ . Measurement of IL-3 in serum and 20-fold concentrated (Amicon-10, Amicon Corp., Danvers, Ma) BAL fluid was performed using an enzyme-linked immunosorbent assay (Quantikine, Research and Diagnostics System, Minneapolis, Mn), with results expressed in picograms per milliliter. Assay sensitivity for IL-3 was  $7.4 \text{ pg}\cdot\text{ml}^{-1}$ . In all assays, samples were analyzed in duplicate.

#### Statistical Analysis

Statistical analyses were performed using the CSS Statistica computer software program. Data distributions were checked for normality using Kolmogorov-Smirnoff and Chi-Square analyses. As most distributions were

significantly different from normal, non-parametric statistics were used. Given the a priori hypothesis that airway eosinophils and metachromatic cells would be increased after allergen compared to diluent challenge, one-tailed Wilcoxon Signed-Rank tests for paired samples were used for airway eosinophil counts. Differences in indices of eosinophil activation (ECP levels and EG2 staining) and IL-3 between allergen and diluent were examined using two-tailed Wilcoxon Signed-Rank tests. Spearman's rank correlation coefficient test ( $R_s$ ) was used to examine the association between inflammatory parameters and physiological measures of airway function. A p value of  $< 0.05$  was accepted as statistically significant.

## Results

In mild atopic asthmatics, allergen inhalation caused early and late asthmatic responses (see Table 5. 1). The early asthmatic response after allergen, with a mean fall in FEV<sub>1</sub> of 30% was significantly greater than that after diluent, with a mean fall in FEV<sub>1</sub> of 4% ( $p < 0.000$ ). The late asthmatic response after allergen, with a mean fall in FEV<sub>1</sub> of 30%, was also significantly greater than that after diluent, with a mean fall in FEV<sub>1</sub> of 3% ( $p = 0.004$ ).

Allergen inhalation caused an increase in eosinophils in the bronchoalveolar lavage and biopsy tissue of mild asthmatic subjects. The percentage of BAL eosinophils was significantly higher after allergen, with a median value of 2.8% (range: 1.0 to 23%), than after diluent, with a median of 1.3% (range: 0.0 - 2.3%) ( $p = 0.021$ ) (Table 7. 1). The absolute number of eosinophils in the lavage was also significantly higher after allergen than after diluent ( $p = 0.032$ ). The proportionate increase in BAL eosinophils after allergen was balanced by a decrease in the proportion of BAL lymphocytes. Within bronchial biopsy tissue, allergen challenge caused a significant increase in eosinophils, with a median value of 30.0/mm<sup>2</sup> (range: 12.5 - 45.7) , compared to diluent challenge, with a median value of 15.1/mm<sup>2</sup> (range: 7.4 - 33.3) ( $p = 0.014$ ) (see Figure 5. 1). The absolute number ( $\times 10^4/\text{ml}^{-1}$ ) of circulating eosinophils was not significantly increased after allergen, with a median of 47 (range: 22 - 84), compared to after

diluent, with a median of 36 (range: 16 - 75) ( $p = 0.176$ ).

Allergen inhalation caused a significant increase in the percentage of metachromatic cells in the BAL. After allergen inhalation, the median percentage of metachromatic cells was 0.04% (range: 0.03 to 0.09%) and after diluent was 0.02% (range: 0.02 - 0.08%) ( $p = 0.022$ ) (Table 7. 1).



Table 7.1: Bronchoalveolar lavage cell counts after diluent and allergen challenges

Diluent							
Subject No.	Return (ml)	Total ( $\times 10^4 \cdot \text{ml}^{-1}$ )	M	N	L (%)	E	MC
1	55	5.59	92.0	1.8	4.8	1.5	0.02
2	66	8.35	95.0	0.0	3.5	1.3	ND
3	67	4.85	95.5	2.5	2.0	0.0	0.02
4	55	9.09	95.3	1.0	2.5	1.3	0.08
5	57	1.39	94.5	2.5	0.8	2.3	0.04
6	69	2.87	93.5	4.3	1.5	0.8	0.02
7	67	7.99	85.3	1.0	12.3	1.5	0.02
Median	66	5.59	94.5	1.8	2.5	1.3	0.02
Allergen							
Subject No.	Return (ml)	Total ( $\times 10^4 \cdot \text{ml}^{-1}$ )	M	N	L (%)	E	MC
1	52	7.89	88.0	3.0	3.3	6.0	0.03
2	67	11.87	95.0	0.5	2.0	3.0	ND
3	68	4.19	94.8	2.0	1.3	2.0	0.07
4	40	6.15	72.8	3.3	1.5	23.0	0.09
5	64	4.96	98.3	0.8	0.0	1.0	0.04
6	53	2.81	88.0	7.0	2.3	2.8	0.04
7	88	3.84	94.5	0.3	3.8	1.5	0.04
Median	64	4.96	94.5	2.0	2.0	2.8	
p value*	1.00	1.00	0.46	0.45	0.04	0.02	0.02

M macrophage; N neutrophil; L lymphocyte; E eosinophil; MC metachromatic cell.  
 ND not determined

\* p value for comparison between diluent and allergen challenge.

Immunocytochemistry on BAL cells demonstrated that allergen inhalation resulted in an increased number of cells positive for EG2 (see Figure 5. 2). The median value of EG2-positive cells increased from 2.3% after diluent to 5.0% after allergen ( $p = 0.008$ ). In BAL cells, staining for EG2 was present in the cytoplasm and was also seen extra-cellularly, suggestive of ECP secretion (see Figure 5. 3). No staining was present in slides stained with IgG, (negative control).

Immunohistochemistry on biopsy tissue demonstrated that allergen inhalation did not cause significantly increased staining for either EG2 or IL-3 (Figure 7. 1). The median value of EG2-staining was 1.75 after diluent and 2.63 after allergen ( $p = 0.208$ ). For IL-3 staining, the median value was 3.31 after diluent and 3.00 after allergen ( $p = 0.463$ ). Staining for EG2 after diluent was mainly intra-cellular and localized primarily beneath the basement membrane. In some subjects after allergen, however, diffuse extra-cellular staining was apparent and intra-epithelial EG2-positive cells, likely eosinophils, were observed (see Figure 5. 6). Staining for IL-3 in biopsies obtained after both diluent and allergen challenge was primarily intra-cellular and was localized mainly to the epithelium (Figure 7. 2). No staining was present in the negative controls.

In bronchoalveolar lavage fluid, allergen inhalation resulted in a significant increase in ECP (see Figure 5. 8). The median concentration ( $\mu\text{g}\cdot\text{L}^{-1}$ ) of ECP increased from 3.4 after diluent to 9.0 after allergen ( $p = 0.018$ ). In the

serum, the median concentration of ECP was slightly, but not significantly, higher after allergen compared to diluent challenge. After either diluent or allergen inhalation, no IL-3 was detected in serum or BAL samples (detection limit of assay = 7.4 pg·ml<sup>-1</sup>).

The severity of the late asthmatic response was significantly correlated with the number of BAL eosinophils present after allergen ( $R_s = 0.82$ ;  $p = 0.02$ ). No relationships were evident between IL-3 and eosinophil number or function, metachromatic cells or airway function.

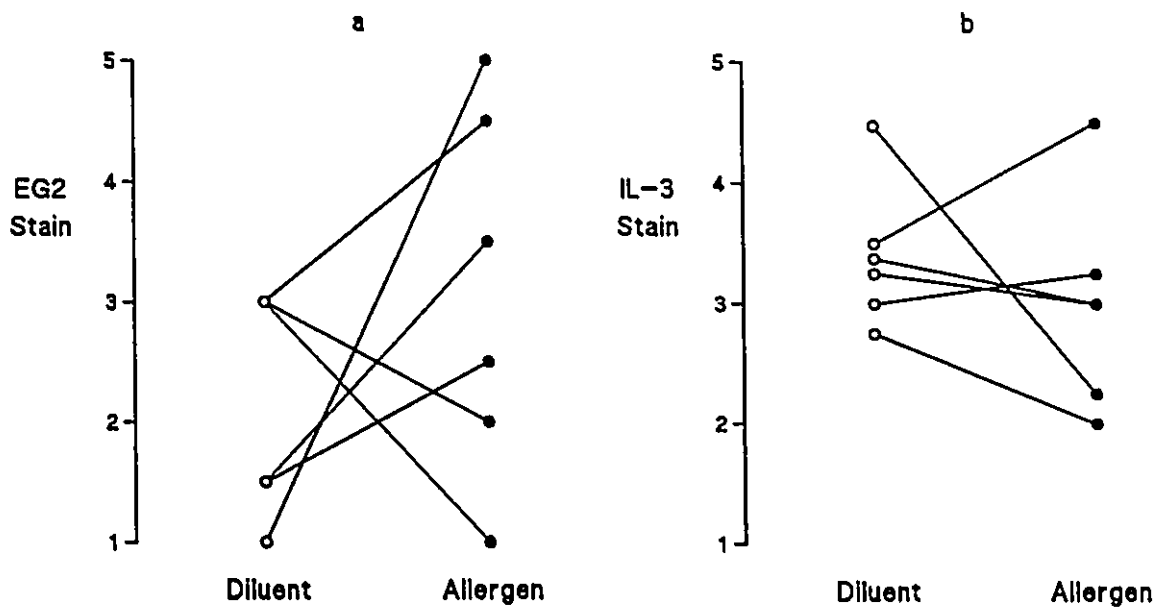


Figure 7.1. Staining of asthmatic biopsy tissue for (a) EG2 and (b) IL-3 after diluent or allergen challenge. Allergen challenge did not significantly alter staining for either EG2 or IL-3.

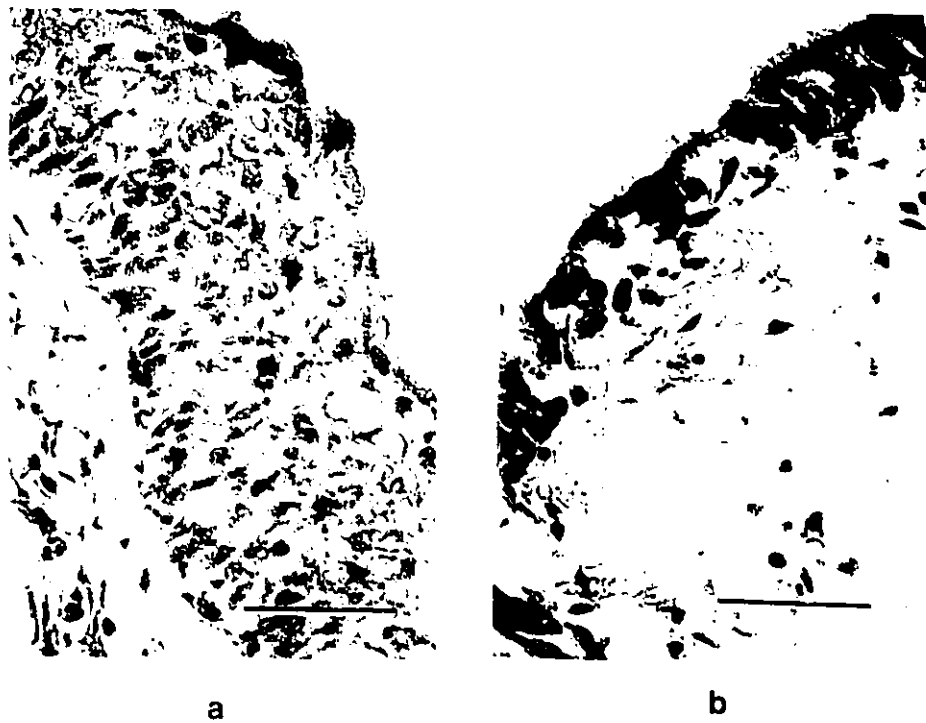


Figure 7.2. Immunohistochemistry for IL-3 in bronchial biopsy tissue from an asthmatic subject after (a) diluent and (b) allergen challenge (internal scale = 50  $\mu\text{m}$ ). Positive (red) staining for IL-3 is primarily intracellular and localized mainly within the epithelium.

## Discussion

In the present study, inhalation of allergen by mild asthmatics, with early and late asthmatic responses, was associated with significant increases in the number and activation of airway eosinophils. Allergen inhalation was also associated with an increase in the number of airway metachromatic cells. However, allergen inhalation did not change levels of the cytokine, IL-3, in bronchial biopsy tissue and IL-3 levels were not related to eosinophils or metachromatic cell number or airway function. A significant relationship was observed between the number of BAL eosinophils and the severity of the late asthmatic response. These results indicate that eosinophils and metachromatic cells, but not IL-3, are involved in allergen-induced airway inflammation and that eosinophils, in particular, contribute to the late asthmatic response.

Our findings of allergen-induced increases in the number of eosinophils in BAL and biopsy tissue are in agreement with previous studies (Metzger et al., 1986; De Monchy et al., 1985; Jarjour et al., 1993). The method of allergen challenge appears to influence the magnitude of allergen-induced BAL eosinophilia, with greater increases in eosinophils occurring after allergen is instilled locally (Broide et al., 1991; Broide et al., 1992), rather than inhaled (Rossi et al., 1991; Brusasco et al., 1990). Our findings are consistent with the

percentage and number of eosinophils reported in previous studies using the allergen inhalation method (Rossi et al., 1991; Brusasco et al., 1990). Eosinophil numbers in biopsy tissue have been reported to increase significantly after allergen instillation (Jarjour et al., 1993) but not after allergen inhalation (Crimi et al., 1991; Beasley et al., 1989). Differences in statistical power may explain the inconsistency between our study and the other allergen inhalation studies. The sample sizes in the studies by Crimi et al. (1991) and Beasley et al. (1989) were smaller than ours and while both groups reported increases in tissue eosinophils after allergen, these differences failed to reach statistical significance. Differences in study design may also contribute to the lack of agreement between the studies. We compared eosinophil numbers in the same subjects after they inhaled diluent on one occasion and allergen on another. In contrast, Crimi et al. (1991) compared eosinophil numbers between separate allergen and diluent groups and Beasley et al. (1989) compared eosinophil numbers in the same subjects before and after allergen inhalation.

The increase that we observed in metachromatic cells in the BAL 24 hours after allergen, compared to diluent, inhalation is a novel finding. Previous studies have investigated allergen-induced changes in BAL mast cells at earlier time points. Wenzel et al. (1988), who performed BAL in atopic asthmatics before and 5 minutes after local instillation of allergen, reported increased activation of mast cells after allergen. Using electron microscopy, Metzger et al. (1986)

reported that 2 hours after allergen inhalation by symptomatic asthmatics, mast cell degranulation had increased. Diaz et al. (1989), who performed BAL 6 hours after mild asthmatics inhaled either diluent or allergen, found a significant decrease in BAL mast cell number after allergen. The authors postulated that the decrease in mast cells after allergen may have been due to mast cell degranulation, conveying the difficulty in counting degranulated mast cells. These studies suggest that mast cell degranulation begins minutes after allergen exposure, with full degranulation potentially occurring several hours later.

At later time points, an increase in airway mast cells may occur. In studies where bronchial biopsies have been obtained from asthmatic subjects 18 to 24 hours after allergen inhalation, a trend toward (Beasley et al., 1989) or a significant (Crimi et al., 1991) increase in mast cell number has been reported. The significant increase in BAL mast cells that we observed may reflect migration of mast cells to the lumen in response to chemotactic factors. In addition, if in situ maturation of mast cell progenitors occurs in the lower airway, as has been proposed for the upper airway (Otsuka et al., 1987), allergen-induced enhancement of mast cell maturation may also contribute to an increase in mast cell number.

In our study, allergen inhalation was associated with an increase in eosinophil activation, based on increases in ECP in BAL fluid and increased staining for EG2 in BAL cells. These results are consistent with and extend



findings from previous studies. De Monchy et al. (1985) reported increased levels of ECP in BAL fluid collected 6-7 hours after allergen inhalation by asthmatics with late responses. We found increased ECP in BAL fluid collected 24 hours after allergen inhalation, implying that airway eosinophils can continue to secrete ECP for many hours after challenge. Increased EG2 staining in BAL cells 24 hours after allergen inhalation has not been reported previously. Aalbers et al. (1993) documented a significant increase in EG2-positive BAL cells at 3, but not 24, hours after allergen inhalation challenge. In contrast to our study, however, where all subjects were dual responders, some subjects in the study by Aalbers et al. did not exhibit a late asthmatic response. The lack of significance noted by Aalbers et al., in BAL EG2-positive cells at 24 hours, therefore, may reflect inclusion of data from isolated early responders. As ECP has been shown *in vitro* to damage pulmonary epithelial cells (Ayers et al., 1985), increased secretion of ECP 24 hours after challenge suggests that epithelial cell damage may occur for many hours after initial allergen exposure. This prolonged activation of airway eosinophils may contribute to the long duration associated with the late asthmatic response.

In contrast to our findings in the BAL, ECP secretion did not appear to increase in the biopsy tissue after allergen challenge. Previous studies, however, have reported increased staining for EG2 in biopsy tissue after allergen inhalation (Bentley et al., 1993; Aalbers et al., 1993). Inter-subject variability and kinetic differences may explain the discrepancy between our study and others. Bentley

et al. (1993) reported a significant increase in EG2 staining in biopsies from mild asthmatics after allergen, compared to diluent, inhalation. However, examination of individual values shows that, similar to our results, one third of the subjects had lower EG2 staining after allergen than after diluent. Aalbers et al. (1993), examined the kinetics of EG2 staining in bronchial biopsies from asthmatics bronchoscoped on separate occasions, before, 3 and 24 hours after allergen inhalation. EG2 staining was highest at 3 hours, suggesting that EG2-positive cells may migrate from the tissue to the lumen, with less staining evident in the tissue at 24 hours. The individual variability and differing kinetics in inflammatory indices after allergen challenge may account for the variability in the onset, severity and duration of late asthmatic responses.

Contrary to our findings with eosinophils and metachromatic cells, no difference in IL-3 protein localization in biopsy tissue was apparent between diluent and allergen challenge. Our study is the first to investigate the effect of allergen inhalation on IL-3 protein in bronchial tissue from asthmatics. Nevertheless, our results are in line with results from several other studies on IL-3. Hamid et al. (1993) and Bentley et al. (1993) collected BAL cells and bronchial biopsy tissue, respectively, from mild atopic asthmatics 24 hours after diluent or allergen inhalation. Using in situ hybridization to examine IL-3 at the mRNA level, no differences were evident in IL-3 mRNA in BAL cells (Hamid et al., 1993) or biopsy tissue (Bentley et al., 1993) between diluent and allergen challenges.

Although previous studies have not investigated the effect of allergen challenge on IL-3 protein in BAL fluid from asthmatics, results from other studies support our results. Kato et al. (1992) used an IL-3 bioassay (sensitivity 150 pg·ml<sup>-1</sup>) to measure IL-3 in BAL fluid collected from subjects with allergic rhinitis 19 hours after local allergen challenge. The authors concluded that minimal IL-3 activity was evident in the BAL fluid after allergen. Despite using an assay with greater sensitivity (7.4 pg·ml<sup>-1</sup>) than the bioassay used by Kato et al. (1992), we were unable to detect IL-3 protein in BAL fluid after allergen inhalation. To our knowledge, no studies have examined the effect of diluent and allergen inhalation on IL-3 levels in the serum. Our inability to detect IL-3 in the serum is consistent with previous studies unable to detect cytokines in the serum (Nakamura et al., 1993; Woolley et al., 1992).

While in vitro studies have suggested that IL-3 can influence eosinophils and mast cells, we did not find significant relationships between tissue localization of IL-3 and eosinophils in BAL or tissue or metachromatic cells in BAL. These findings suggest that IL-3 may not contribute to the increased number of eosinophils and mast cells in asthmatic airways. Consistent with our results are the findings by Hamid et al. (1993) and Bentley et al. (1993), where an increased number of eosinophils in BAL and an increased number of EG2-positive eosinophils in biopsy tissue were observed without significant increases evident in IL-3 mRNA.

The increase in eosinophils in BAL that we found after allergen was significantly correlated with the severity of the late asthmatic response. This finding complements the significant correlation reported by Brusasco et al. (1990) between allergen-induced increases in the number of BAL eosinophils and the level of airway responsiveness. Together, these findings suggest that airway eosinophils contribute to allergen-induced decreases in lung function.

In conclusion, we found that the number of metachromatic cells and the number and activation of eosinophils in the BAL was significantly increased in mild, stable asthmatics after allergen, compared to diluent, inhalation. Allergen challenge was also associated with a significant increase in eosinophils in the bronchial tissue. IL-3 protein was not detected in serum or BAL but was present in biopsy tissue. Allergen challenge, however, did not affect IL-3 levels in biopsy tissue and no relationships were evident between IL-3 and metachromatic cells or eosinophils. The severity of the late response was significantly correlated with the number of eosinophils in the BAL. These results suggest that airway eosinophils and metachromatic cells, but not IL-3, are involved in allergen-induced airway inflammation and that eosinophils, in particular, contribute to the late asthmatic response.

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## **CHAPTER 8: EOSINOPHILS AND CYTOKINES IN ASTHMA: SUMMARY OF FINDINGS, GENERAL DISCUSSION AND FUTURE DIRECTIONS**

### **Summary of findings**

The results in this thesis suggest that in mild asthmatics, airway inflammation contributes to chronic and acute changes in airway function. The specific components of inflammation that were investigated in this thesis were the number and activation of eosinophils, the number of metachromatic cells and the presence and levels of the cytokines, GM-CSF and IL-3. The chronic component of asthma was examined by comparing mild asthmatics with non-asthmatics, while the acute component of asthma was investigated using the allergen inhalation model. The major findings of the studies in the thesis are summarized below:

**Chapter 4: Granulocyte-macrophage colony-stimulating factor, eosinophils and eosinophil cationic protein in mild asthmatics and non-asthmatics.**

In this study, mild asthmatics were found to have an increased number of eosinophils in their blood, BAL and bronchial tissue. ECP, used as a marker of eosinophil activation, and GM-CSF were increased in BAL cells and bronchial tissue from mild asthmatics. Correlations existed between GM-CSF and

eosinophils; airway responsiveness was correlated with eosinophils, ECP and GM-CSF.

Chapter 5: Effects of allergen challenge on granulocyte-macrophage colony-stimulating factor, eosinophils and eosinophil cationic protein in mild asthmatics.

In the mild asthmatics studied, inhalation of allergen caused early and late asthmatic responses. Compared to diluent inhalation, allergen inhalation caused an increase in eosinophils in BAL and bronchial tissue. Eosinophil activation, assessed with ECP, and GM-CSF were increased in BAL fluid and BAL cells. Increases in eosinophils were correlated with increases in GM-CSF and the severity of the late asthmatic response was correlated with the number of eosinophils.

Chapter 6: Increases in airway eosinophils, eosinophil cationic protein and metachromatic cells, but not interleukin-3, in mild asthmatics compared to non-asthmatics.

In this study, we investigated IL-3, a cytokine shown to regulate eosinophils and metachromatic cells in vitro. IL-3 was detected in biopsy tissue from both asthmatics and non-asthmatics. However, no difference in IL-3 levels were apparent between the groups, despite an increased number of eosinophils and metachromatic cells in the asthmatic subjects. No relationships were found between IL-3 and eosinophil number, eosinophil activation or metachromatic cell number. Eosinophil activation was increased in the BAL cells and biopsy tissue

of asthmatics and airway responsiveness was correlated with eosinophil number and activation.

**Chapter 7: Allergen challenge increases airway eosinophils, eosinophil cationic protein and metachromatic cells, but not interleukin-3, in mild asthmatics.**

In the previous study (Chapter 6), IL-3 was not associated with mild, ongoing asthma. In this study we investigated whether an acute exacerbation of asthma, elicited by allergen inhalation, would be associated with an increase in IL-3. Although allergen inhalation caused early and late asthmatic responses and increases in BAL eosinophils and metachromatic cells, an increase in IL-3 in bronchial tissue was not apparent. In addition, IL-3 was not related to increases in metachromatic cell number or the number and activation of eosinophils or the late asthmatic response.

## **General Discussion**

The aim of this thesis was to investigate the role of eosinophils and cytokines in mild and allergen-induced asthma. The following discussion addresses the presence and involvement of inflammatory cells (eosinophils and metachromatic cells) and cytokines (GM-CSF and IL-3) in asthma. In addition, the limitations of the studies used in the thesis are considered.

### Eosinophils

The eosinophil has had a long historical association with asthma (Ellis, 1908). Until the advent of fiberoptic bronchoscopy, however, direct evidence of eosinophil involvement in mild asthma was limited. The studies of this thesis clearly documented that the number and activation of eosinophils were increased in the airways of mild asthmatics and that further increases occurred after allergen inhalation. The eosinophilia observed in asthma may be a consequence of either increased recruitment of eosinophils to the airways or increased survival of eosinophils within the airways. Either mechanism may be mediated by cytokine action. Eosinophil recruitment may occur due to (i) the ability of cytokines, such as  $\text{TNF}\alpha$ , IL-4 and GM-CSF, to increase the expression of adhesion molecules mediating eosinophil adhesion and transendothelial migration (eg. VLA<sub>4</sub>, CD11b) (Moser et al., 1992; Czech et al., 1993; Schleimer et al., 1991) and (ii) the effects of cytokines, including GM-CSF, IL-5 or RANTES, on eosinophil chemotaxis (Warringa et al., 1991; Calhoun et al., 1993). Also, the actions of cytokines, such as GM-CSF, IL-3 and IL-5, which are able to enhance the survival of eosinophils, presumably by preventing apoptosis (Yamaguchi et al., 1991; Stern et al., 1992), may contribute to tissue eosinophilia. In addition, increases in circulating eosinophil progenitors (Gibson et al., 1991), possibly mediated by cytokine-enhanced bone marrow progenitor production and their differentiation into mature cells, might also contribute to the eosinophilia associated with asthma.

The importance of the eosinophil in asthma was highlighted in this thesis by correlations between eosinophils and the disordered airway function evident in mild, chronic asthma (increased airway responsiveness) and allergen-induced exacerbations of asthma (late asthmatic response). Several mechanisms have been proposed to explain how eosinophils may contribute to the changes in airway function associated with asthma. Increased airway responsiveness, for example, may result from eosinophil-mediated damage to the epithelium (eg. MBP- or ECP-mediated detachment or lysis of respiratory epithelial cells), with the subsequent increased exposure of nerve endings to bronchoconstrictor agents (Wardlaw et al., 1988; Jeffery et al., 1992; Beasley et al., 1989). Eosinophils may also contribute to the late asthmatic response by releasing mediators (eg. leukotrienes) able to cause smooth muscle contraction, mucus secretion and vasodilation (Aalbers et al., 1993; De Monchy et al., 1985; Weller et al., 1983).

#### Metachromatic cells

In conjunction with eosinophils, metachromatic cells (mast cells and basophils) are believed to play a central role in allergic diseases (Marone et al., 1993). Mast cells, localized near epithelial surfaces and bearing high affinity IgE receptors, are strategically located to interact with inhaled allergens (Friedman et al., 1987). The release of bronchoconstrictor mediators from mast cells is believed to underlie the early asthmatic response to inhaled allergen (White et al., 1991).

Mast cells might also contribute indirectly to the late asthmatic response by releasing pro-inflammatory cytokines, such as  $\text{TNF}\alpha$  and GM-CSF (Busse et al., 1993). In contrast, basophils, migrating from the circulation, are thought to contribute directly to the late asthmatic response by releasing histamine and leukotrienes (Marone et al., 1993).

Although, the primary interest in this thesis was the role of the eosinophil in asthma and its potential regulation *in vivo* by GM-CSF and IL-3, metachromatic cells were also examined in the IL-3 studies as IL-3 had been shown to influence these cells *in vitro* (Saito et al., 1988; Ihle et al., 1983; MacDonald et al., 1989). An increase in the number of airway metachromatic cells in mild asthmatics, compared to non-asthmatics, has been reported previously (Kirby et al., 1987; Adelroth et al., 1990; Tomioka et al., 1984). The studies in this thesis confirm this finding and also demonstrate that a further increase in airway metachromatic cells occurs 24 hours after allergen inhalation. The mechanisms mediating this increased number of metachromatic cells in asthma have not been defined. The increases detected in BAL may be a consequence of epithelial shedding, with the subsequent release of intra-epithelial metachromatic cells into the lumen (Friedman et al., 1987). Alternatively, increases may occur due to the chemotactic or local growth and differentiating actions of cytokines (Otsuka et al., 1987; Litchfield et al., 1992).

The actions of cytokines in the regulation of eosinophils or metachromatic cells in vivo in asthma is a subject of much current interest. In particular, GM-CSF and IL-3 have been ascribed potentially important roles:

...GM-CSF may be involved in eosinophil recruitment, survival in tissues and priming...IL-3 may be important in persistence of mast cells and eosinophils in airway tissues...

Barnes, P.J. (1993)

### GM-CSF

The studies in this thesis have shown that GM-CSF is increased in mild asthmatics, compared to non-asthmatics, and that the levels of GM-CSF increase after allergen inhalation. The correlations that were observed between GM-CSF and eosinophils are in agreement with in vitro studies and suggest that GM-CSF likely regulates eosinophil number and activation in vivo. The synthesis and release of GM-CSF in asthmatic airways may be an important factor in asthma chronicity (ie. the ongoing, disordered airway function that appears to be associated with persistent airway inflammation).

Identification of the cells responsible for producing GM-CSF represents the next logical step in investigating GM-CSF involvement in asthma. Definitive evidence of the cellular sources of GM-CSF will require the use of double labelling techniques. It is possible to speculate, however, on the cellular sources of GM-CSF, based on the morphology of cells that were identified immunochemically as

GM-CSF-positive. Localization of GM-CSF was observed in cells resembling eosinophils, epithelial cells, macrophages and lymphocytes. Of these cells, localization within eosinophils and epithelial cells is particularly intriguing with respect to asthma. Autocrine regulation of eosinophils is a plausible hypothesis to explain the increased number and activation of eosinophils in asthmatic airways. By producing GM-CSF, eosinophils could enhance their survival and activation, which in turn, could enable further production of GM-CSF. Most likely, however, eosinophils are subject to paracrine, as well as autocrine, regulation. Consistent with this is the ability of other cells, such as epithelial cells, to produce GM-CSF (Mattoli et al., 1992). The ability of epithelial cells to produce GM-CSF may be particularly important in asthma. In our studies, epithelial hyperplasia was evident in biopsy samples obtained from mild asthmatics. Conceivably, an increased number of epithelial cells capable of producing GM-CSF would contribute to a milieu that was conducive to enhanced eosinophil survival.

### IL-3

The novel demonstration of IL-3 protein in non-asthmatic airways indicates that IL-3 may be involved in the ongoing, normal defence of the airway mucosa (Tovey, 1988). In support of this, we observed substantial localization of IL-3 within epithelial cells, indicating that this cytokine is strategically located at the interface between potentially harmful environmental agents and host tissue. The



apparent cell association, rather than release, of IL-3 might also be beneficial in normal defence of the airways. By necessitating intimate cell to cell contact to exert its effects, IL-3 may selectively sequester inflammatory cells to the epithelium, restricting the extent of tissue damage that may occur after mounting an inflammatory response.

In contrast to GM-CSF, IL-3 does not appear to be associated with mild or allergen-induced asthma. Levels of IL-3 did not appear to differ between asthmatics and non-asthmatics and were not significantly altered after allergen inhalation. In addition, no correlations were evident between IL-3 and eosinophils or metachromatic cells in mild or allergen-induced asthma. Although an absence of correlations does not by itself negate a role for IL-3 in regulating these cells in vivo, these results do suggest that IL-3 does not have a major, direct influence on these cells in mild or allergen-induced asthma. In this regard, it appears that not all of the classically described "eosinophil regulatory cytokines" exert effects in vivo in asthma:

All animals are created equal, but some animals are more equal than others.

G. Orwell (1946)

As IL-3 has been shown to influence mast cells in rodents (Du et al., 1993) and regulate human eosinophils in vitro (Warringa et al., 1991; Czech et al., 1993), these results also highlight species differences and the discrepancies that

can occur between in vitro and in vivo studies.

### Limitations

While the studies in this thesis indicate cellular and cytokine involvement in mild and allergen-induced asthma, limitations exist with respect to subject selection, study design and outcome variables.

Mild, atopic asthmatics were selected for the studies as we sought to determine inflammatory events that occurred with mild disease and how these events might be altered after an experimental exacerbation of asthma. In addition, the potential risks associated with fiberoptic bronchoscopy (Workshop Committee, 1985) favoured the selection of mild asthmatics. Given these limitations, the results from our studies may not be extrapolated to asthmatics with a non-atopic etiology or generalized to asthmatics with more severe disease.

The effect of an exacerbation of asthma on inflammatory events was examined by comparing the effects of diluent and allergen inhalation on samples obtained 24 hours after inhalation. Inhalation, rather than instillation, of allergen was chosen because the inhalation of allergen in the laboratory provides a useful model of the type of exposure that asthmatics are subjected to in real life. In support of this, the BAL eosinophilia that we observed was similar in magnitude to the BAL eosinophilia reported for asthmatics during seasonal allergen exposure (Metzger et al., 1986). In addition, our laboratory has considerable experience with

allergen inhalation, having used this method successfully and safely in previous studies. Safety concerns were particularly important given that fiberoptic bronchoscopy was to be used in our laboratory for the first time. Comparisons between diluent and allergen inhalations were made so that each subject served as their own control. Practical and ethical constraints precluded examination of changes that might have occurred at time points other than 24 hours. While previous studies have documented changes in inflammatory events 24 hours after allergen inhalation (Cookson et al., 1989; Bentley et al., 1993; Aalbers et al., 1993), this single time point is not presumed to reflect the complex and dynamic events that probably occur in asthmatic airways after allergen exposure.

The aim of this thesis directed the selection of the outcome variables. Of prime interest was eosinophil number and activation. Enumeration of eosinophils in blood, BAL and biopsy tissue was completed using standardized techniques. A variety of eosinophil-derived products can be used to investigate eosinophil activation. We selected the secretion of ECP as a marker of eosinophil activation as (i) ECP has been shown to be cytotoxic to respiratory epithelium in vitro (Young et al., 1986; Ayers et al., 1985), (ii) the availability of a monoclonal antibody to the secreted form of ECP (EG2) enabled examination of eosinophil activation in cells and tissues (Tai et al., 1984) and (iii) our findings could be compared to previous studies that had measured ECP in asthmatic subjects (De Monchy et al., 1985; Bousquet et al., 1991; Bentley et al., 1993). Our results with

ECP indicated increased activation of eosinophils in mild and allergen-induced asthma. The involvement and contribution of other eosinophil products (eg. major basic protein, leukotrienes), however, cannot be inferred from these results as differential release of eosinophil products can occur (Kita et al., 1991).

Selection of the cytokines examined was based on (i) information from in vitro studies which indicated that GM-CSF (Lopez et al., 1986; Anwar et al., 1993; Silverstein et al., 1986) and IL-3 (Tai et al., 1990; Warringa et al., 1991; Czech et al., 1993) regulate eosinophil function and (ii) the availability of monoclonal antibodies to GM-CSF and IL-3. Our results with IL-3 indicate that in vitro findings cannot be readily extrapolated in vivo. Therefore, the involvement of other cytokines, also shown to regulate eosinophils in vitro (eg. interleukin-5, RANTES), can only be ascertained by specific examination in vivo.

### **Future Directions**

This thesis documented cellular and cytokine involvement in mild and allergen-induced asthma. Specifically, increases in eosinophils, metachromatic cells and GM-CSF were associated with chronic and acute asthma. The following section will examine issues that need to be addressed in future studies, including (i) defining, with more precision, the "airway inflammation" associated with asthma,

(ii) determining the relative contributions of individual cells and cytokines to the clinical manifestations of asthma and (iii) expanding the current view of inflammation to include factors which may serve to limit, rather than induce, inflammation.

Although airway inflammation is increasingly recognized as important in the pathogenesis of asthma, details of the inflammatory components that underlie asthma, await clarification. Airway inflammation, per se, is a generic term and can only provide an overall impression of what asthmatic airways may look like (eg. edematous, inflammatory cell infiltrate). However, more specific information is required to understand the mechanisms by which airway inflammation contributes to the pathophysiology of asthma. Future studies will need to (i) identify the cells and cytokines present and active in asthmatic airways (ii) determine the kinetics of cell ingress and egress and cytokine production and degradation (iii) examine interactions between cells (eg. inflammatory cells and tissue structural cells) and between cytokines (eg. synergism or antagonism) and (iv) determine the relationships between inflammatory events and the pathophysiological changes associated with asthma. Essentially, the panoply of inflammatory cells and cytokines that have been proposed to play a role in asthma must be systematically investigated to determine their actual involvement in this disease.

One important caveat to be kept in mind in determining the inflammation underlying asthma, however, is the heterogeneity associated with this disease. Paradoxically, one of the most common findings in asthma research is the remarkable variability evident between asthmatics. Nevertheless, information on differences in inflammatory events between asthmatics may explain the variation that also occurs in airway physiology. For example, the difference between asthmatics with an isolated early asthmatic response and asthmatics with a dual response (ie. early and late asthmatic response) after allergen inhalation, may be due to qualitative or quantitative differences in components of inflammation.

With knowledge of the specific cells and cytokines involved in chronic and acute asthma, a more precise and accurate approach can be taken toward drug development. The use of specific drugs will enable the relative contributions of cells and cytokines to be determined. Results from this thesis suggest that chronic and acute changes in lung function may be affected by inhibiting the migration, survival or activation of eosinophils. One method of abating eosinophil actions, for example, may be to reduce the level of GM-CSF in the airways.

In addition, more detailed knowledge of the cytokines associated with airway inflammation in asthma could benefit studies utilizing "knock in" (cytokine augmentation) or "knock out" (cytokine absence) transgenic animal models. For example, information on which cytokines are most likely to be involved in the pathogenesis of asthma could aid the selection of which cytokines to target in

these transgenic animal models. Unlike findings based on correlations, this experimental approach can provide evidence of causal links between cytokines and biological effects.

The recent upsurge in interest in asthma as an inflammatory disease has proceeded with a certain bias. Considerable research has been driven by the search for an upregulation of pro-inflammatory cells and cytokines in asthmatics. Subsequent attention has focussed on determining the "culprits" responsible for the increased presence of pro-inflammatory cells or the increased production of pro-inflammatory mediators. The risk that is inherent in this research strategy is ignorance of the crucial role that may be played by anti-inflammatory mechanisms. For example, the cytokine interleukin-10 can inhibit the release of pro-inflammatory cytokines in vitro (Fiorentino et al., 1991), reduces the enhanced survival of, and cytokine production by, eosinophils mediated by LPS in vitro (Takanashi, S. et al., manuscript in preparation) and can also exert anti-inflammatory effects in animal models in vivo (Howard et al., 1993) (Woolley M.J. et al., manuscript in preparation). In addition, certain products, released from cells during an inflammatory response, may neutralize potentially harmful inflammatory mediators. For example, heparin, which bears a highly anionic charge, is capable of binding cationic proteins in vitro (Venge et al., 1989), and may reduce the tissue damage mediated by eosinophil cationic proteins (Page, 1993).

Valuable information may be gained from future studies on anti-inflammatory mechanisms in asthma. For example, the chronic, self-perpetuating inflammation characteristic of even mild asthma may reflect inadequate anti-inflammatory processes. Similarly, in allergen challenge studies, the lack of or deficiencies in anti-inflammatory mechanisms may destine an asthmatic to develop a late asthmatic response. To fully understand the role of inflammation in asthma, both pro- and anti-inflammatory events must be examined.

True science suppresses nothing, but goes on searching, and is undisturbed in looking straight at things that it does not yet understand.

C. Bernard (1927)



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