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PHARMACOLOGICAL MODULATION OF ALLERGEN-INDUCED AIRWAY INFLAMMATION

By

GAIL M. GAUVREAU, M.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

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ABSTRACT

Asthma is a chronic respiratory disease which is characterized by reversible bronchoconstriction, airway hyperresponsiveness, and airway inflammation. Allergen inhalation by sensitized atopic asthmatics enhances airway hyperresponsiveness and inflammation, providing a model to study mild asthma exacerbation. Airway inflammation can be measured non-invasively from airway secretions by sputum induction. Prior to starting this thesis, information on allergen-induced inflammation measured from sputum was limited. Furthermore, the pro- or anti-inflammatory effects of asthma therapies had not been investigated using this model of allergen-induced airway inflammation. The aim of this thesis was to first characterize the allergen-induced changes in sputum inflammatory cells and determine the repeatability of measurements of sputum inflammatory cells following allergen inhalation challenge. In addition, this thesis was aimed to investigate the pro- or anti-inflammatory effects of asthma therapies on allergen-induced airway inflammation.

Inflammatory cells considered to be important in asthma, such as eosinophils and mast cells, were measured from induced sputum following allergen inhalation challenge. These cells remained significantly elevated in sputum for 7d following inhalation of allergen, compared to diluent control. Cytokines associated with the activation and chemotaxis of eosinophils such as IL-5, eotaxin and RANTES, were also significantly elevated following allergen inhalation challenge. We examined the repeatability of allergen-induced airway inflammation assessed by induced sputum, and calculated the subject sample sizes required

to demonstrate significant attenuation of sputum eosinophilia in placebo-controlled crossover studies.

Measurement of sputum inflammatory cells, particularly eosinophils, following allergen inhalation challenge were found to reflect the pro- or anti-inflammatory effects of asthma therapies. Inhaled budesonide and PGE_2 are known to protect against allergen-induced early and late asthmatic responses and allergen-induced airway hyperresponsiveness. We demonstrated that regular treatment with inhaled budesonide, and that inhalation of PGE_2 immediately before allergen inhalation challenge attenuated the allergen-induced early and late asthmatic responses, and the allergen-induced increase in airway hyperresponsiveness and inflammatory cells. In contrast, regular treatment with an inhaled β_2-agonist, an asthma therapy known to enhance the allergen-induced late asthmatic response, also enhanced the allergen-induced increase in sputum eosinophils during the late asthmatic response. These findings indicate that the measurements of airway eosinophils from induced sputum following allergen inhalation challenge reflect the allergen-induced late asthmatic response and airway hyperresponsiveness. Furthermore, allergen-induced sputum eosinophilia is a sensitive indicator of the pro- or anti-inflammatory effects of asthma therapies in mild atopic asthma.

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I am indebted to Dr. O'Byrne and Dr. Killian for overseeing the clinical studies. These experiments could not have been completed without the clinical skills of Rick Watson, who challenged research subjects with methacholine, allergen, and also with his sense of humour. Thanks to Rick for meeting our nearly impossible deadlines, which may be responsible for the progressive change in the colour of his hair. Many thanks to Tracy Rerecich for all of the early mornings and long days of laboratory work, and especially for her contagious enthusiasm for research. I also thank Joceline Otis for her expertise with the preparation of this thesis, Lorna, Roma, Terry, George, Mark, Russ, Jennifer, Merrill and Erin for countless favours, and Tim and Amer for thier motivation during the "thesis race". I have been priviledged to work with a dedicated and skilled group of people who uphold the reputation of Dr. O'Byrne's laboratory.

Special thanks to those who celebrated with me during my achievements, and

comforted me during my setbacks. It was honour to work with my colleagues, Lorna Wood and Roma Sehmi, who were not only insightful laboratory partners, but also my partners in crime and my confidantes, providing an endless source of camaraderie, friendship and fun. I am truely thankful for their support and advise. Comic relief was supplied regularly by Kieran, George and Rick, with various pranks sometimes being the highlight of the day (right, Mark?), and not easily forgotton (cologne dripping from the ceiling... who would have imagined!).

Finally, I would like to thank my family and friends for their endurance during my Ph.D., patiently listening to my stories with interest as I described the smallest details of my project. I thank Tan for changing the subject occasionally. Weekends with my family provided an escape to reality, a place with completely different challenges. My Mom's weekend prescription, including outdoor exercise, horses, dogs and good food, was the most effective means of putting things in to perspective. These weekend retreats provided the balance necessary for me to fully appreciate my Ph.D. experience.

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LIST OF ABBREVIATIONS

ANCOVA:	Analysis of covariance
ANOVA:	Analysis of variance
AUC:	Area under the curve of the FEV_1-time response
BAL:	Bronchoalveolar lavage
cAMP:	Cyclic 3',5' adenosine monophosphate
COPD:	Chronic obstructive pulmonary disease
DNA:	Deoxyribonucleic acid
DPBS:	Dulbecco's phosphate buffered saline
EAR:	Early airway bronchoconstrictor response
ECP:	Eosinophil cationic protein
EG2:	Antibody to the cleaved (activated) form of ECP
ELAM:	Endothelial leukocyte adhesion molecule
ELISA:	Enzyme-linked immunosorbent assay
Eo/B CFU:	Eosinophil/basophil colony forming unit
FEV_1:	Forced expiratory volume in 1 second
FITC:	Fluorescein isothiocyanate
GM-CSF:	Granulocyte-macrophage colony stimulating factor
HIM:	Hemopoietic inductive microenvironment

HEPES:	N-[2-Hydroxyethyl]piperazine-N'-[4-butanesulfonic acid]
IC:	Intraclass correlation
ICAM:	Intracellular adhesion molecule
IL:	Interleukin
LAR:	Late airway bronchoconstrictor response
LT:	Leukotriene
MBP:	Major basic protein
MCC:	Metachromatic cells
mRNA:	Messenger ribonucleic acid
NAMC:	Non-adherent mononuclear cells
PAF:	Platelet-activating factor
PC_{20}:	Provocative concentration for 20% fall in FEV_1
PGE_2:	Prostaglandin E_2
RANTES:	Regulated upon activation in T cells, expressed and secreted
RT-PCR:	Reverse transcription-polymerase chain reaction
SCF:	Stem cell factor
TXA_2:	Thromboxane A_2
TNF:	Tumor necrosis factor
VCAM:	Vascular cell adhesion molecule

PREFACE

The following studies have been submitted for publication and form the basis of chapters two, three, four, five, and appendix 1 of this thesis:

KINETICS OF ALLERGEN-INDUCED AIRWAY RESPONSES AND AIRWAY INFLAMMATION. Gail M. Gauvreau, Rick M. Watson, and Paul M. O'Byrne.
Submitted to: American Journal of Respiratory Critical Care Medicine

REPEATABILITY OF ALLERGEN-INDUCED AIRWAY INFLAMMATION. Gail M. Gauvreau, Rick M. Watson, and Paul M. O'Byrne.
Submitted to: Journal of Allergy and Clinical Immunology

EFFECTS OF INHALED BUDESONIDE ON ALLERGEN-INDUCED AIRWAY RESPONSES AND AIRWAY INFLAMMATION. Gail M. Gauvreau, Joel Doctor, Rick M. Watson, Manel Jordana, and Paul M. O'Byrne.
Published : American Journal of Respiratory Critical Care Medicine 1996; 154:1267-1271.

EFFECT OF REGULAR INHALED ALBUTEROL ON ALLERGEN-INDUCED LATE RESPONSES AND SPUTUM EOSINOPHILS IN ASTHMATIC SUBJECTS. Gail M. Gauvreau, Manel Jordana , Rick M. Watson, Donald W. Cockcroft, and Paul M. O'Byrne.
Published: American Journal of Respiratory Critical Care Medicine 1997; 156:1738-1745.

PROTECTIVE EFFECTS OF INHALED PGE_2 ON ALLERGEN-INDUCED AIRWAY RESPONSES AND AIRWAY INFLAMMATION. Gail M. Gauvreau, Rick M. Watson, and Paul M. O'Byrne.
Accepted for publication: American Journal of Respiratory Critical Care Medicine

ENHANCED EXPRESSION OF GM-CSF IN DIFFERENTIATING EOSINOPHILS OF ATOPIC AND ATOPIC ASTHMATIC SUBJECTS. Gail M. Gauvreau, Paul M. O'Byrne, Redwan Moqbel, Juan Velazquez, Rick M. Watson, Karen J. Howie, and Judah A. Denburg.
Published: American Journal of Respiratory Cell Molecular Biology 1998; 18:1-8.

These studies are the first clinical studies, completed by the Asthma Research Group in Hamilton, Canada, which have been performed using induced sputum to assess airway inflammation following pharmacological modulation of allergen-induced airway responses. These allergen challenge studies required input from many people. Dr. Paul O'Byrne provided and organized the financial support, laboratory space, equipment, and technical support. Dr Paul O'Byrne and Dr. Kieran Killian supervised the clinical procedures, and provided expertise during screening and testing of research subjects. Rick Watson carried out the clinical procedures, including allergen and methacholine challenges. Dr. Judah Denburg together with Dr. Manel Jordana offered considerable expertise, and Tracy Rerecich provided technical support to analyze the collected samples.

I am first author on the papers which form the basis of this thesis. I was involved with the planning and design of the experiments and submitted proposals outlining the purpose, methods and analysis of the experiments. I was involved with the clinical procedures, and performed methacholine and allergen inhalation challenges, and sputum induction. I was responsible for all of the subsequent assays on the samples collected, including sputum processing, methylcellulose tissue culture, and cytochemical and immunocytochemical staining of cells. Pilot studies were required to modify the staining techniques specifically for the samples collected in these experiments. 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 it was my responsibility to write each paper.

CHAPTER 1:

INTRODUCTION

Asthma

Asthma is a disease of the airways characterized by symptoms caused by episodic bronchoconstriction and circadian variation in pulmonary function (Szefler et al, 1991). Airway inflammation is an important characteristic in patients with current symptomatic asthma (Bradley et al, 1991; Kirby et al, 1987; Pin et al, 1992), leading to changes in the airway such as edema, increased secretions and smooth muscle constriction (Boschetto et al, 1991; Fabbri et al, 1988; Hulbert et al, 1981; Persson et al, 1986), and hyperresponsiveness to non-specific stimuli such as methacholine (Hargreave et al, 1981), and specific stimuli such as allergen (Cockcroft et al, 1977).

Allergen Inhalation Challenge

Allergen inhalation by atopic asthmatics results in acute bronchoconstriction and airway secretions caused by the immediate release of direct-acting mediators, and in 50-60% of adult subjects, this is followed by a late bronchoconstrictor response (LAR) (Booij-Noord et al, 1972; Robertson et al, 1974). The late response to inhaled allergen is associated with the development of allergen-induced airway hyperresponsiveness (Cartier et al, 1982) and by an allergen-induced increase in the number of airway inflammatory cells, as measured in bronchial biopsies (Crimi et al, 1991), BAL (Aalbers et al, 1993) and induced sputum (Fahy

et al, 1994; Gauvreau et al, 1996).

Allergen inhalation challenge can be used as a stimulus to trigger airway inflammation in atopic asthmatics. Using this airway challenge, asthma therapies can be evaluated for their ability to protect the airway by measuring their effects on the allergen-induced airway bronchoconstrictor responses, hyperresponsiveness and inflammation.

Asthma Therapies

Asthma is a common chronic disease, with a prevalence of approximately 10-15% (Sears 1990) of the population. Asthma medications currently prescribed include bronchodilators, which target airway narrowing, and anti-inflammatory drugs, which target airway inflammation. Bronchodilators act predominantly by relaxing airway smooth muscle, whereas anti-inflammatory drugs suppress the inflammation in asthmatic airways.

Glucocorticoids

Glucocorticoids are the most potent anti-inflammatory agents available for the treatment of asthma. The actions of glucocorticoids can occur through binding of the steroid/receptor complex to the promoter site of target genes (Beato 1989). This binding can directly increase transcription, as in the case of β_2-receptor (Mak et al, 1995) and neutral endopeptidase (Borson and Gruenert, 1991), or directly suppress expression of genes coding for inflammatory mediators (Kern et al, 1988). In addition, glucocorticoids can exert gene repression indirectly by binding of the steroid/receptor complex to transcription factors (Adcock et al, 1995), and by inducing inhibitors of transcription factors.

Inhaled topical glucocorticoids with a high ratio of topical to systemic activity have been developed for the treatment of asthma in order to minimize systemic toxicity such as growth inhibition, cataracts and osteoporosis (Fahy et al, 1995). Inflammation is a complex interaction of cells and mediators, and glucocorticoids are thought to be effective topically in the airway because of their wide spectrum of activities, including cell chemotaxis, mediator synthesis and release, and vascular permeability. Inhaled glucocorticoids, such as budesonide, beclomethasone dipropionate and fluticasone, have been shown to decrease the numbers and activation status of airway inflammatory cells such as eosinophils, mast cells and T lymphocytes (Djukanovic et al, 1990; Wilson et al, 1994; Laitinen et al, 1992; Burke et al, 1992; Trigg et al, 1994; Whang et al, 1994). With respect to the airway eosinophilia, which is characteristic of allergic asthma, glucocorticoids have been shown to inhibit chemotaxis of eosinophils (Warringa et al, 1993; Altman et al, 1981; Taborda-Barata et al, 1996), inhibit the survival of eosinophils (Lamas et al, 1991; Wallen et al, 1991; Cox et al, 1991), and inhibit the production of chemokines by the epithelium (Lilly et al, 1997; Kwon et al, 1995). Furthermore, inhaled glucocorticoids appear to maintain some systemic effect by decreasing peripheral blood eosinophil counts (Evans et al, 1992), and inhibiting the release of eosinophils from the bone marrow (Gibson et al, 1991). The net result of the above actions is effective inhibition of eosinophil localization in the airways of asthmatics. Although use of inhaled steroids is a first line therapy for the treatment of moderate to severe asthma, the long term effects of inhaled steroids must be considered in sub-populations of

asthmatics, such as postmenopausal women where bone loss may be accelerated (Ip et al, 1994).

β_2-agonists

β_2-adrenoceptor agonists, on the other hand, are well established for relief of acute asthma bronchospasm (Barnes 1995), and their main beneficial pharmacological effects include bronchodilation and functional antagonism (protection from bronchoconstrictor agents). β_2-agonists produce bronchodilation predominantly by activating β_2-receptors on airway smooth muscle cells (Nijkamp et al, 1992). Activation of β_2-receptors results in activation of adenylyl cyclase, and through stimulatory G proteins results in an increase in intracellular cyclic 3',5' adenosine monophosphate (cAMP). Increased cAMP leads to phosphorylation of several proteins including large conductance calcium-activated potassium channels, resulting in hyperpolarization of the cell membrane and relaxation of the airway smooth muscle cell (Goldie et al, 1991). In airway smooth muscle, β_2-receptors may be directly coupled to large conductance calcium-activated potassium channels, suggesting that elevation of cAMP is not necessary for the bronchodilator response to β_2-agonists (Kume et al, 1994). β-receptors are also localized to many other cell types in the airways, such as mast cells and cholinergic nerves. Activation of these receptors by β_2-agonists may inhibit release of bronchoconstrictor mediators, such as histamine, arachidonic acid metabolites and acetylcholine, contributing further to the bronchodilator effects.

With widespread use of β_2-agonists in the management of asthma, issues regarding

deleterious effects (increased morbidity and mortality) of frequent use of these agents in asthma have emerged (Taylor et al, 1996), suggesting the development of tolerance to the beneficial effects of inhaled β_2-agonists. The development of tolerance to β_2-agonists has been demonstrated with reduced functional antagonism to non-specific bronchoconstrictor stimuli such as exercise (Gibson et al, 1978), histamine (Vathenen et al, 1988; Wahedna et al, 1993) and methacholine (O'Connor et al, 1992; Cockcroft et al, 1993) and to specific bronchoconstrictors such as allergen (Cockcroft et al, 1993). The clinical relevance of these findings, however, remains uncertain, as there remains a considerable functional antagonist effect even after tolerance develops.

Although the β_2-agonists and glucocorticoids are widely prescribed therapies for the treatment of asthma, they are not always effective alone or in combination with each other. Inhaled glucocorticoids are not effective in rare patients whose asthma is resistant to steroid treatment (Cypcar and Busse 1993). Masking of clinical symptoms with regular β_2-agonist use may allow progression of the underlying airway inflammation, leading to exacerbation. Combination therapy of β_2-agonist plus glucocorticoid may not be complimentary, as results from recent studies have formed a basis for the hypothesis that simultaneous use of β_2-agonists and glucocorticoids may impair the anti-inflammatory effects of both endogenous and exogenous steroids. β_2-agonists exert their bronchodilator properties through stimulating the production of cAMP, which occurs in a dose-dependent manner (Adcock et al, 1995b). Preliminary studies have demonstrated that binding of glucocorticoid complexes to specific

DNA sequences may be inhibited by increased intracellular concentrations of cAMP (Peters et al, 1995). Interactions of these therapies must be considered in the treatment of asthma, as there are clinical data to confirm this hypothesis (Cockcroft et al, 1995; Wong et al, 1994) and in vitro evidence that β_2-agonists block corticosteroid inhibition in eosinophils (Nielson et al, 1998).

Prostaglandin E_2

There is a need for novel asthma therapies which can suppress airway inflammation, and provide relief of acute bronchospasm in asthma without untoward interactions. New strategies, such as the antileukotriene drugs, have been developed to target specific mediators and receptors of the arachidonic acid pathway, which are thought to play an important role in the pathogenesis of airway bronchoconstriction and inflammation. The inhibitory prostanoids, which have a broader spectrum of action, may provide another alternative.

Prostaglandin E_2 (PGE_2) has demonstrated bronchodilatory properties (Pasargiklian et al, 1976), and plays a regulatory role in the inflammatory process. Prostaglandin E_2 is present in human airways as a cyclo-oxygenase product of airway epithelium (Churchill et al, 1989) and airway smooth muscle (Delamere et al, 1994), and similar to β_2-agonists, receptor binding leads to increased intracellular cAMP. PGE_2 demonstrates bronchoprotective effects in patients with bronchial asthma by attenuating exercise-induced (Melillo et al, 1994), allergen-induced (Pavord et al, 1993; Pasargiklian et al, 1976), and aspirin-induced bronchoconstriction (Sestini et al, 1996), as well as bronchoconstrictor

agents such as metabisulfite, methacholine and histamine (Pavord et al, 1991; Manning et al, 1989; Walters et al, 1982).

There are many other events involved in the development of allergic inflammation, which PGE_2 may modulate. PGE_2 has been shown to regulate the production of peripheral blood mononuclear cell-derived cytokines such as interleukin (IL)-2, IL-4 and IL-5, by elevating intracellular levels of cAMP (Kaminuma et al, 1996), and has been shown to induce shift in the functional profile of cytokine mRNA in T lymphocytes (Gold et al, 1994), possibly by the same mechanism. These results suggest that PGE_2 may be an important endogenous mediator for regulation of inflammation in asthmatic airways.

Assessment of Asthma Therapies

Given the current consensus that airway inflammation plays a central role in asthma, therapies are now being evaluated for their anti-inflammatory effects on airway inflammation. New novel therapies include treatment with anti-IgE antibody, which has been shown to attenuate the late response to inhaled allergen (Fahy et al, 1997), and anti-IL-5 antibody which is currently being evaluated for use in humans. Assessment of the anti-inflammatory effects of these therapies is essential to evaluate their potential role in the treatment of allergic asthma. Airway measurements of inflammatory cells traditionally have been obtained with invasive procedures, which limit the use of these methods. Measuring changes in compartments other than the airway, such as peripheral blood, has advanced our understanding of the anti-inflammatory properties of glucocorticoids (Butterfield et al, 1986;

Evans et al, 1993). ECP levels in peripheral blood are higher in asthmatics compared to non-asthmatics, but peripheral blood ECP is not an accurate marker of asthmatic airway inflammation (Pizzichini et al, 1997b), likely because mediators secreted in the airway must move from the tissue back into the vasculature in order to be detected in serum. The preferred compartment to study airway inflammation is by direct measurements obtained from the airway itself.

Measurement of Airway Inflammation

Airway inflammation has, in the past, been measured by changes in bronchial biopsies and bronchoalveolar lavage. The bronchoscopy procedures required to collect these samples from the lower airways is invasive, expensive and difficult to obtain repeated samples over short periods of time from the same subject. Hypoxia, bronchoconstriction and side effects to lidocaine are possible during bronchoscopy (Van Vyve et al, 1992; Smith et al, 1993).

More recently a technique has been developed to collect airway secretions non-invasively and repeatedly by sputum induction. Using comparisons with BAL and bronchial washings, the criterion validity of indices measured from induced sputum have been investigated. Samples from induced sputum have been shown to yield cells with a similar morphological appearance as those in BAL (Fahy et al, 1995b). Induced sputum from asthmatic subjects contains higher total cell counts and higher percentages of neutrophils and eosinophils than BAL (Fahy et al, 1995b; Maestrelli et al, 1995; Keatings et al, 1995; Kidney

et al, 1995), which may reflect the relative proportion of inflammatory cells in different compartments of the airway. BAL primarily samples the alveolar compartment, whereas sputum is derived mainly from the bronchioles and bronchi that are affected in the asthmatic process, and contain granulocytes, the terminal cells involved in the inflammatory process. Despite these differences, the percentage of eosinophils in induced sputum and bronchoalveolar lavage and bronchial biopsies are significantly correlated with one another (Fahy et al, 1995b; Maestrelli et al, 1995).

Sputum is induced by inhalation of nebulized hypertonic saline which enhances clearance of secretions from the lung (Pavia et al, 1978; Robinson et al, 1994). Cell differentials from sputum samples are not affected by the induction process alone (Pizzichini et al, 1996; Fahy et al, 1995b) choice of nebulizer (Popov et al, 1995), concentration of hypertonic saline (Bacci et al, 1996; Popov et al, 1995; Iredale et al, 1994), pretreatment with salbutamol (Popov et al, 1995) or processing technique (plugs vs whole sample method) (Pizzichini et al, 1996b; Peleman et al, 1995; Gershman et al, 1996) permitting valid comparisons to be made between laboratories. Furthermore, homogenization with dithiothreitol to break apart mucus plugs does not alter the proportion of cells or significantly interfere with measurements of cell surface markers or intracytoplasmic markers of inflammation or the measurement of proteins in fluid phase supernatants detected by immunocytochemistry (Efthimiadis et al, 1997; Kidney et al, 1994; Girgis-Gabardo et al, 1994; Hoshi et al, 1995).

Sputum induction with hypertonic saline has the advantage of being a safe (Wong et al, 1997) non-invasive procedure, and samples may be obtained on multiple occasions from the same subject (Gibson et al, 1989; Pin et al, 1992) with a success rate >80% (Pin et al, 1992; Fahy et al, 1993; Popov et al, 1995). Cell counts and fluid phase measurements from stable asthmatic subjects are reproducible (In't Veen et al, 1996; Pizzichini et al, 1996c; Spanavello et al, 1996), with good intra- and inter-observer consistency (Iredale et al, 1994; Pizzichini et al, 1996c), which however decreases with increasing contamination of the sample with squamous cells (Efthimiadis et al, 1995). Successive sputum inductions, however, appear to elevate the proportion of neutrophils in subsequent samples (Kips et al, 1995; Holtz et al, 1997), and increase bronchial hyperresponsiveness (Bacci et al, 1996), which implies release of inflammatory mediators. This must be considered when evaluating cell counts and soluble markers following repeated sputum induction, and controlled for in the study design.

Sputum sampling is a valid measure of underlying airway inflammation. Using comparisons between asthmatics, COPD and healthy subjects, the content validity has been investigated. Content validity (comparisons between disease states) is more valuable than criterion validation (comparisons with other sampling techniques), as it allows for inference all based on measurements of sputum (O'Byrne and Inman, 1996). Induced sputum has been useful to define airway disease by clearly differentiating between asthmatics, COPD and healthy subjects (Fahy et al, 1993; Pin et al, 1992; Keatings et al, 1996; Pizzichini et al,

1996c; Gibson et al, 1989; Ronchi et al, 1996). Sputum composition has been shown to reflect asthma severity (Pizzichini et al, 1996c; Baigelman et al, 1983; Ronchi et al, 1997; Kips et al, 1996; Foresi et al, 1997), responds to factors known to affect the degree of airway inflammation, such as anti-inflammatory drugs (Claman et al, 1994; Brown et al, 1958; Gibson et al, 1995; Keatings et al, 1997; Gauvreau et al, 1996; Pizzichini et al, 1997) and allergen inhalation challenge (Fahy et al, 1994; Pin et al, 1992b; Keatings et al, 1997b), and demonstrates the effects of asthma therapies on allergen-induced airway inflammation (Wong et al, 1992; Pizzichini et al, 1996d; Gauvreau et al, 1996; Gauvreau et al, 1997; Kidney et al, 1997). Disadvantages of using induced sputum include the lag time between clinical symptoms and expectoration of sputum, which may explain why changes in cellularity do not always correlate with symptom severity. Furthermore, measurements of inflammatory cells from induced sputum do not accurately reflect non-granulocytic inflammatory cells involved in airway inflammation, such as lymphocytes and mast cells which are typically tissue-associated effector cells and appear only in low levels in the sputum.

Sputum is being used to measure cytokines and cell mediators as markers of inflammation to improve the current understanding of the inflammatory mechanisms of asthma (In't Veen et al, 1996; Pizzichini et al, 1996c; Fahy et al, 1993; Louis et al, 1997; Keatings et al, 1996; Keatings et al, 1997b; Adachi et al, 1996; Pizzichini et al, 1997b; Kurashima et al, 1996; Konno et al, 1996; Hoshi et al, 1995; Gelder et al, 1995). Phagocyte

ingestion of apoptotic cells and proteins from activated granulocytes as observed in bronchial biopsies (Adelroth et al, 1994; Cox et al, 1995; Stern et al, 1992) may be exaggerated in sputum containing apoptotic cells being cleared from the airways, and interfere with the evaluation of intracellular cytokines and proteins. Most proteins are present in higher concentrations in sputum supernatant than BAL fluid, permitting measurements which would otherwise be below the detection limits of the assays.

Measurements of airway inflammation can be made repeatedly from sputum samples following allergen inhalation challenge, permitting examination of the kinetics of allergen-induced airway inflammation. Furthermore, allergen-induced airway inflammation can be compared following treatment with asthma therapies. We have employed this model to investigate the pro- or anti-inflammatory properties of asthma therapies known to result in enhancement or protection of allergen-induced asthmatic early and late responses and airway hyperresponsiveness.

Overall Hypothesis:

Allergen-induced late responses and airway hyperresponsiveness are caused by the presence of an increased number of activated airway eosinophils and mast cells after allergen inhalation.

Specific Hypotheses:

Drugs known to inhibit the physiological responses to allergen inhalation will attenuate airway eosinophil and mast cell inflammation, while drugs known to enhance allergen-

induced physiological responses will enhance airway eosinophil and mast cell inflammation. That:

a) regular treatment of inhaled corticosteroid or inhalation of prostaglandin E_2 immediately before allergen inhalation will inhibit the allergen-induced late response and increased airway hyperresponsiveness, and also, will inhibit allergen-induced increase in the number of eosinophils, activated eosinophils in induced sputum.

b) regular treatment with β_2-agonist will enhance the allergen-induced airway responses, and increase the number of eosinophils and activated eosinophils appearing in sputum after allergen inhalation.

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CHAPTER 2:

KINETICS OF AND REPEATABILITY OF ALLERGEN-INDUCED AIRWAY RESPONSES AND AIRWAY INFLAMMATION

Abstract

Allergen inhalation challenge has been shown to be a sensitive clinical model to investigate the effects of asthma therapies on allergen-induced airway eosinophilia. Several cytokines, such as IL-5, eotaxin and RANTES are implicated in tissue eosinophilia, coordination of these cytokines seems to be necessary to support the different stages of eosinophil extravasation including adhesion, chemotaxis and activation. We investigated the reproducibility of allergen-induced asthmatic and airway inflammatory responses, and predicted the subject sample size required to demonstrate protection by asthma therapies. Each of 16 subjects completed two allergen inhalation challenges using the same dose of allergen on both occasions. Eight atopic asthmatics demonstrating a dual response to inhaled allergen completed a diluent-controlled cross-over study to characterize the kinetics of allergen-induced inflammatory cell recruitment to the airways, with particular attention to the involvement of cytokines selective for eosinophil chemotaxis and activation. Baseline measurements of FEV_1, the provocative concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}), and differential cell counts from induced sputum were determined the day

before and the day after the inhalation challenges. The FEV_1 was measured for 7 hours following challenges, and sputum samples were obtained at 7 hours. To investigate the kinetics of the response, PC_{20} was measured and sputum collected until 7 days following challenge. Subjects demonstrated significant early and late airway responses to allergen ($p<0.05$), and an allergen-induced increase in the number of eosinophils and metachromatic cells in sputum ($p<0.05$). The number of eosinophils immunopositive for IL-5, eotaxin and RANTES increased at 7 hours following allergen inhalation ($p<0.05$), coincident with a peak in the number of activated (EG2-positive) eosinophils. The intraclass correlation (IC) for maximal late fall in FEV_1 was 0.32, and for the area under the curve of the late response was 0.61. The sample size predicted to be necessary to observe 50% attenuation of both the maximal late fall in FEV_1 and the late AUC with a power of 0.9, is 8 subjects. The IC for % allergen-induced sputum eosinophils was 0.60 and 0.53 for 7 hours and 24 hours, respectively. The sample size predicted to be necessary to observe a 50% attenuation of % allergen-induced eosinophils using a randomized cross-over study design, is 4 subjects. These results confirm the presence of cytokines specific for the activation and chemotaxis of eosinophils, and the data suggest that co-operation of these cytokines may be important for the accumulation of activated eosinophils at the site of allergic inflammation. Understanding regulation of tissue eosinophilia may be important to develop new therapies for the control of allergic asthma. Allergen inhalation challenge with measurements of eosinophils in sputum is a non-invasive and sensitive method for assessing the effectiveness

of anti-inflammatory asthma therapies.

Introduction

Eosinophilic infiltration into affected tissue is one of the hallmarks of allergic inflammation. Airway eosinophilia has been demonstrated in asthmatics when compared to normal controls, (1, 2), and may be further enhanced in the airways of atopic asthmatics following allergen inhalation challenge (3,4,5) in association with development of a late airway bronchoconstrictor response to allergen and airway hyperresponsiveness to methacholine (6,7,8).

Allergen inhalation challenge in atopic, mild asthmatics is commonly used as a model of allergic asthma. This model has provided a reproducible measurement of early and late responses as measured by maximal fall in FEV_1 (9,10), and has proven to be a useful model to assess bronchoprotective effects of asthma drugs (11,12,13) and the relative potency of inhaled corticosteroids (14). Furthermore, allergen inhalation challenge has been shown to be a sensitive clinical model to investigate the effects of asthma therapies on allergen-induced sputum eosinophilia in asthmatics (6,7,15), however, the repeatability of allergen-induced inflammation in sputum has not been investigated. As sputum eosinophilia is currently being utilized as a primary outcome variable in studies investigating the potential anti-inflammatory role of asthma drugs on allergen-induced airway inflammation, it is necessary to determine the repeatability of these measurements, and the predicted sample

size necessary to observe statistically significant attenuation of sputum eosinophils following allergen challenge.

The kinetics of allergen-induced airway eosinophilia has been investigated in BAL fluid (3), however the invasive nature of the procedure restricts the number of samples obtainable. Advantages of sputum examination include the ability to obtain repeated, non-invasive samples of airway secretions, which is more appropriate than a single time point measurement for examination of inflammatory cell kinetics. Furthermore, we have demonstrated by immunocytochemistry, increased inflammatory markers in sputum following allergen (6, 7), in particular, those associated with eosinophil activation and chemotaxis (16).

Regulation of eosinophil recruitment to allergic tissues is an area of study that has not been clarified. Tissue eosinophilia is likely due to a combination of several rather specific and co-ordinated cellular processes appearing at the different stages of eosinophil extravasation including adhesion, chemotaxis and activation (17). Several cytokines are implicated in eosinophilia. IL-5 was first described as a factor important for proliferation and differentiation of bone marrow eosinophils (18), and is known to prime eosinophils (19) and prolong their survival (20). Chemokines selective for eosinophils include eotaxin and regulated upon activation in normal T-cells, expressed and secreted (RANTES), which induce chemotaxis as well as specifically activate eosinophils (21,22). Studies in vitro have demonstrated specificity of these eosinophil-selective cytokines in the attraction and

activation of eosinophils, and furthermore, support their participation for the specific recruitment of eosinophils to the airways of asthmatics in vivo.

Understanding the mechanisms of eosinophil recruitment to the airways and targeting pathways critical for regulation of eosinophil chemotaxis and activation will aid in the development of new anti-inflammatory agents for the treatment of asthma. Using high dose allergen inhalation challenge as a model of asthma exacerbation, we have characterized the kinetics of inflammatory cell recruitment to the airways, with particular attention to the involvement of cytokines selective for eosinophil chemotaxis and activation, and cytokines known to regulate the TH2-type inflammatory response.

Methods

Subjects

Eight subjects were selected to examine the kinetics of allergen-induced airway inflammation. The sample size to examine the kinetics of airway inflammation was considered sufficient, as previous studies have shown that 8 or more subjects can demonstrate allergen-induced airway inflammation assessed with induced sputum using the same methodology employed in this study (6,7). In addition, the data from 16 subjects who had completed 2 allergen inhalation challenges using the same dose of allergen were used to examine the repeatability of allergen-induced airway responses and inflammatory cells measured from induced sputum. All subjects were non-smoking with mild atopic asthma,

selected because they demonstrated an allergen-induced early and late airway response of at least 15% reduction in the forced expiratory volume in 1 second (FEV_1). Signed informed consent was given to participate in the studies, which were approved by the Ethics Committee of McMaster University Health Sciences Center. Subjects were not exposed to sensitizing allergens and did not have asthma exacerbations or respiratory tract infections for at least four weeks prior to allergen challenges. All subjects had stable asthma with FEV_1 greater than 70% of predicted normal on all study days before challenge to the airways, and used no regular medication other than infrequent (<twice weekly) inhaled β_2-agonist as required to treat their symptoms. All medications were withheld for at least 8 hours before each visit, and subjects were instructed to refrain from rigorous exercise, tea or coffee in the morning before visits to the laboratory.

Study Design

Inflammatory Cell Kinetics. Eight subjects completed an allergen and a diluent (0.9% saline) inhalation challenge. Each challenge study period consisted of 6 visits to the laboratory. Baseline measurements of FEV_1, the provocative concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}), and inflammatory cell counts from induced sputum were determined the day before the inhalation challenge. Inhalation challenge was carried out the following morning, and the FEV_1 was measured for 7 hours following challenge. Sputum samples were obtained at 7 hours, during the late response. Sputum could not be induced earlier than 7 hours, because of the requirement of pretreatment with inhaled β_2-agonists

before sputum induction, which would interfere with subsequent measures of FEV_1. Methacholine PC_{20} and sputum samples were obtained again at 24 hours, 2 days, 4 days, and 7 days following challenge.

Repeatability of Airway Responses Sixteen subjects completed 2 allergen inhalation challenges using the same dose of allergen on both occasions. Allergen challenges were separated by at least 3 weeks, and not more than 11 months. Each allergen challenge consisted of 3 visits to the laboratory (baseline, allergen inhalation and 24 hours post-allergen) and measurements of FEV_1, PC_{20} and sputum were determined as described earlier.

Laboratory Procedures

Methacholine Inhalation Test Methacholine inhalation challenge was performed as described by Cockcroft (23). Subjects inhaled normal saline, then doubling concentrations of methacholine phosphate from a Wright nebulizer for 2 minutes. FEV_1 was measured at 30, 90, 180 and 300 seconds after each inhalation. Spirometry was measured with a Collins water sealed spirometer and kymograph. The test was terminated when a fall in FEV_1 of 20% of the baseline value occurred, and the methacholine PC_{20} was calculated.

Allergen and Diluent Inhalation Challenge Allergen challenge was performed as described by O'Byrne and colleagues (8). The allergen producing the largest skin wheal diameter was diluted in 0.9% saline for inhalation. The concentration of allergen extract for inhalation predicted to cause a 20% early fall in FEV_1 was determined from a formula described by Cockcroft (24) using the results from the skin test and the methacholine PC_{20}.

The starting concentration of allergen extract for inhalation was two doubling concentrations below that predicted to cause a 20% early fall in FEV_1, and doubling concentrations of allergen were inhaled until a 20% early fall in FEV_1 was reached. The FEV_1 was measured at 10 minutes, then again at 20, 30, 40, 50, 60, 90 and 120 minutes post allergen inhalation, then each hour until 7 hours. The early airway response was taken to be the largest fall in FEV_1 within 2 hours after allergen inhalation, and the late airway response was taken to be the largest fall in FEV_1 between 3 hours and 7 hours after allergen inhalation. Only subjects who developed a late fall in FEV_1 of at least 15% were used in the study. The area of the response was determined during the early (0-2 hours) and late (3-7 hours) response by plotting the response using graphics software (Fig P., Fig P Software Corporation, Durham, NC), which calculated the area of the FEV_1-time response (AUC). The diluent challenge was similar to allergen challenge, with 0.9% saline inhaled rather than allergen.

Sputum Analysis Sputum was induced and processed using the method described by Pizzichini and co-workers (25). Subjects inhaled 3%, 4% then 5% saline for 7 minutes each. The induction was stopped when an adequate sample was obtained, or if the FEV_1 dropped 20% from baseline. Cell plugs with little or no squamous epithelial cells were selected from the sample, separated from saliva, and weighed. Samples were aspirated in 4 times their volume of 0.1% dithiothreitol (Sputolysin, Calbiochem Corp. San Diego, CA) and 4 times their volume of Dulbecco's phosphate buffered saline (DPBS) (Life Technologies Inc., Grand Island, NY). The cell suspension was filtered through a 52 μm nylon gauze (BNSH

Thompson, Scarborough, Ont., Canada) to remove debris, centrifuged at 1500 rpm for 10 minutes. Supernatant was removed and stored at -70°C. The total cell count was determined using a Neubauer hemocytometer chamber (Hausser Scientific, Blue Bell, PA) and expressed as the number of cells per ml sputum. Cells were resuspended in DPBS at 0.75-1.0x10^6/ml and cytospins were prepared on glass slides using 50 µl of cell suspension and a Shandon III cytocentrifuge (Shandon Southern Instruments, Sewickly, PA), at 300 rpm for 5 minutes. Differential cell counts were obtained from the mean of two slides with 400 cells counted per slide stained with Diff Quik (American Scientific Products, McGaw Park, IL). The same observer counted all study slides, with a high reproducibility of the cell counts using these methods (25). Metachromatic cell (MCC) (mast cells and basophils) counts on slides stained with toluidine blue, were obtained from the mean of two slides with 1500 cells observed on each slide.

Cytospins were also prepared on aptex coated slides and fixed for 10 minutes in periodate-lysine-paraformaldehyde for immunocytochemical staining. Slides were stained with murine monoclonal antibodies to human ECP at 1.0 ug/ml (EG2) (Kabi Pharmacia, Uppsala, Sweden), IL-5 and RANTES at 30.0 ug/ml, eotaxin at 100 ug/ml (R&D Systems, Minneapolis, MN). All antibodies were diluted in 1.0% bovine serum albumin (Sigma Chemical Co, St. Louis, MO) in wash buffer made up of DPBS, 0.01M HEPES buffer (Life Technologies Inc., Grand Island, NY) and 0.01% saponin (Sigma Chemical Co, St. Louis, MO). Briefly, slides were incubated overnight with anti-human monoclonal primary

antibodies and protein was detected the following day by the alkaline phosphatase anti-alkaline phosphatase (APAAP) method (26) using rabbit anti-murine or rabbit anti-rat secondary antibodies, and murine APAAP tertiary antibodies (DAKO, Denmark). Non-specific staining of the primary and secondary antibodies were blocked by incubation with 75% human AB serum and 25% normal rabbit serum (Sigma Chemical Co, St. Louis, MO) diluted in wash buffer. Negative isotype controls (murine IgG_1, Sigma Chemical Co, St. Louis, MO) were included using the same concentration as the primary antibody. Calcium ionophore-stimulated human peripheral blood eosinophils and lipo-polysaccharide-stimulated human peripheral blood mononuclear cells were included as positive controls. The percentage of positive immunoreactive cells was determined from a count of 400 cells under light microscopy. To demonstrate specific staining of eosinophils, all slides were double stained with fluorescein isothiocyanate (FITC) (Sigma Chemical Co, St. Louis, MO), a specific stain for eosinophils (27,28,29), for 10 minutes at a concentration of 10 ug/ml. Immunopositive eosinophils were enumerated using a florescence microscope, from a count of 400 cells.

Eotaxin was also measured in duplicate in sputum supernatant using a quantitative sandwich enzyme immunoassay technique and a monoclonal antibody specific for eotaxin (R&D Systems, Minneapolis, MN). The sensitivity of the assay was 5 pg/ml. Concentrations of eotaxin were determined by constructing a standard curve with a range 12.5 pg/ml to 1000 pg/ml.

Statistical Analysis

Summary statistics are expressed as mean and standard error of the mean (SEM), with the exception of methacholine PC_{20} measurements which are expressed as geometric mean and geometric standard error of the mean (GSEM). Methacholine PC_{20} measurements were made by linear interpolation of log dose response curves resulting in logarithmic values for PC_{20}, which were then subjected to statistical analysis. The allergen-induced shift (change from baseline) in PC_{20} and sputum inflammatory cells were analyzed using repeated measures ANOVA (30). Data not normally distributed (MCC) were log-transformed prior to analysis. The allergen-induced EAR and LAR (maximum % fall and AUC) were compared to diluent challenge using Student's paired t-test. The repeatability of allergen-induced changes were assessed by the reliability coefficient (intraclass correlation coefficient) as the proportion in the variance between the two measurements compared to the variance between subjects (31). The relationships between variables were compared using the correlation coefficient. Sample size estimates for non-repeated measurements (early and late responses) were predicted using the variance of the difference between challenge 1 and challenge 2 (32). Sample size estimates for repeated measurements (sputum inflammatory cells) were predicted using the time/challenge interaction error term from ANOVA tables as an estimate of variance.

Results

Inflammatory Cell Kinetics

Airway All 8 subjects demonstrated a dual response to allergen, with a maximal fall in FEV_1 of 36.1% ± 3.2 % during the early response ($p<0.001$), and 26.3% ± 2.0 % during the late response ($p<0.001$) (Figure 2.1). The allergen-induced change in FEV_1 was significantly lower than diluent control for 2 days ($p<0.05$) (Figure 2.2), and there was significant increase in non-specific airway responsiveness measured by methacholine PC_{20} following allergen compared to diluent for 7 days ($p<0.003$) (Figure 2.2).

Sputum The total sputum cell count did not increase following allergen challenge compared to diluent ($p=0.76$). There was an allergen-induced change from baseline of the percent sputum inflammatory cells including % eosinophils ($p=0.02$), % metachromatic cells ($p=0.01$), % neutrophils ($p=0.004$) and % macrophages ($p=0.01$) (Table 2.1). There was a significant allergen-induced increase in the number of eosinophils for 7 days ($p<0.02$) and EG2-positive eosinophils for 2 days ($p=0.02$) (Figure 2.2) in sputum compared to diluent challenge. Metachromatic cells in sputum increased significantly following allergen ($p=0.01$), being maximal at 7 hours (range 0.4 $x10^4$/ml - 62.8 $x10^4$/ml) and remained higher than diluent for 7 days (Figure 2.2). There was no difference in the number of neutrophils ($p=0.11$) or mononuclear cells ($p=0.59$) following diluent or allergen challenge. There were no significant correlations between the numbers of inflammatory cells and airway physiology ($p>0.05$) measured before or after allergen challenge.

Slides stained by immunocytochemical methods demonstrated a specific positive signal, as we did not observe a positive signal on the isotype-matched negative control slides. We observed eosinophils and neutrophils to be immunopositive for eotaxin, IL-5 and RANTES, and mononuclear cells (macrophage/monocytes and lymphocytes) to be immunopositive for eotaxin and IL-5. There were no significant increases in the number of sputum cells immunopositive for RANTES, eotaxin or IL-5 following allergen challenge (Figure 2.3). By ELISA, we were not able to detect eotaxin in baseline samples of sputum supernatant. At 7 hours, however, we detected eotaxin in sputum supernatant from 2 subjects (103 pg/ml and 173 pg/ml), but not at any other time point.

There was a significant allergen-induced increase in the number of sputum eosinophils immunopositive for eotaxin at 7 hours ($p=0.0002$), for IL-5 from 7 to 24 hours ($p<0.01$), and for RANTES at 7h ($p=0.0002$). Peak immunopositivity coincided with the maximal number of EG2-positive eosinophils, which occurred 7 hours following allergen challenge (Figure 2.3).

Repeatability of Airway Responses

Sixteen subjects completed 2 allergen inhalation challenges using the same dose of allergen on both occasions. Challenges were separated by at least 3 weeks and no more than 11 months. Most subjects completed both challenges within 2 months (n=9), with others 2 to 6 months (n=6) and 6 to 11 months (n=2)

Airway The maximal early fall in FEV_1 was 27.2% ± 2.3% and 28.9% ± 2.4% for

challenge 1 and 2, respectively, with an intraclass correlation (IC) of 0.40. The maximal late fall in FEV_1 was 24.8% ± 1.6% and 23.9% ± 2.5% for challenge 1 and 2, respectively, with an IC of 0.32 (Figure 2.4). The area of the late response was also repeatable, being 51.6 ± 5.0 and 48.7 ± 7.3, with IC=0.61. The sample size predicted to be necessary to observe 50% attenuation of both the maximal late fall in FEV_1 and the late AUC with a power of 0.9, is n=8 (Figure 2.4).

The methacholine PC_{20} decreased from baselines of 1.6 mg/ml (GSEM 1.3 mg/ml) and 2.1 mg/ml (GSEM 1.3 mg/ml) IC=0.70, to 0.5 mg/ml (GSEM 1.3 mg/ml) ($p<0.001$), and to 1.1 mg/ml (GSEM 1.3 mg/ml) ($p<0.002$) (IC=0.51) following challenge 1 and 2, respectively (Figure 2.5).

Sputum The number of sputum eosinophils increased significantly from baseline following both the 1st and 2nd allergen challenges ($p<0.001$), increasing from 11.4 ± $2.3x10^4$/ml and 12.5 ± $4.3x10^4$/ml (IC=0.36), to 114.1 ± $36.2x10^4$/ml and 78.9 ± $18.4x10^4$/ml at 7 hours (IC=-0.02) and to 192.1 ± $45.8x10^4$/ml and 91.4 ± $16.8x10^4$/ml at 24 hours (IC=0.34). The mean percent sputum eosinophils and IC are shown in Figure 2.5. The percent eosinophils increased significantly following allergen ($p<0.001$) with higher reproducibility than the absolute number of eosinophils (Figures 2.6 and 2.7). The sample sizes predicted to be necessary to observe a 50% attenuation in the sputum eosinophils following allergen inhalation, using a power level of 0.9, are n=4 and n=17, for eosinophils expressed as percentages and absolute numbers, respectively (Figures 2.6 and 2.7).

The number of metachromatic cells increased significantly following allergen ($p=0.004$), from $1.9 \pm 0.8 \times 10^3$/ml and $2.0 \pm 0.8 \times 10^3$/ml at baseline (IC=0.16), to $15.2 \pm 6.8 \times 10^3$/ml and $12.5 \pm 4.5 \times 10^3$/ml at 7 hours following challenge (IC=0.17), and to $26.4 \pm 5.6 \times 10^3$/ml and $20.7 \pm 8.4 \times 10^3$/ml at 24 hours following challenge (IC=0.12). The mean percent metachromatic cells and IC are shown in Figure 2.5. The percent metachromatic cells increased significantly following allergen ($p<0.001$) with higher post-allergen reproducibility than the absolute metachromatic cell counts.

The absolute number of neutrophils did not change following allergen ($p=0.30$), however the percent neutrophils decreased significantly ($p=0.03$) from $47.0 \pm 1.3\%$ and $46.0 \pm 1.5\%$ before, to $37.7 \pm 1.3\%$ and $35.1 \pm 1.4\%$ at 24 hours following the 1st and 2nd allergen challenges, respectively.

There was a significant correlation between the maximal % fall in FEV_1 during the late response and the allergen-induced increase in the percentage of sputum eosinophils 7 and 24 hours following allergen ($p=0.03$, $p<0.001$, respectively) (Figure 2.8). There was a significant correlation between the baseline methacholine PC_{20} and sputum eosinophils ($p=0.006$), however this relationship was not present following allergen inhalation.

Discussion

This study describes, for the first time, the allergen-induced kinetics of inflammatory cells, IL-5, RANTES and eotaxin in the airways of asthmatics. We have demonstrated

maximal numbers of activated eosinophils, metachromatic cells, and eosinophils immunopositive for eotaxin and RANTES and IL-5 occur at 7 hours following allergen challenge. Furthermore, we have demonstrated that allergen-induced sputum eosinophilia is a reproducible measurement which corresponds significantly to the magnitude of the allergen-induced late airway response.

In our investigation of allergen-induced inflammatory cell kinetics, inhalation of allergen resulted in a maximal fall in FEV_1 of 36.1% ± 3.2 % during the early response, and 26.3% ± 2.0 % during the late response. These airway asthmatic responses are similar to those observed in previous studies from this laboratory (6,7). Allergen challenge had prolonged effects on measurements of airway physiology, such as FEV_1 and methacholine PC_{20}. Prolonged allergen-induced airway hyperresponsiveness has been demonstrated previously (33,34). Methacholine PC_{20} has been shown to correlate with sputum eosinophils in asthmatics (35), possibly as a result of damage to the airway epithelium by eosinophils (36,37).

The number of eosinophils remained elevated for 7 days following allergen inhalation, demonstrating prolonged effects of the allergen challenge on eosinophil recruitment to the airways. The number of EG2-positive eosinophils, however, remained elevated for only 24 hours following challenge, suggesting that eosinophil recruitment alone does not necessarily lead to their activation. We have demonstrated increased numbers of eosinophils immunopositive for IL-5 for 24 hours following allergen challenge, suggesting

that participation of activating cytokines, such as IL-5, may be necessary for recruited eosinophils to become activated. In asthmatics, EG2-positive eosinophils have been shown to express IL-5 receptor α-chain, further supporting this notion (38).

Influx of eosinophils into the airway is thought to be partly a result of release of eosinophil-attracting/activating cytokines. The number of activated eosinophils peaked 7 hours following allergen challenge, coincident with maximal immunopositivity of eosinophils for eotaxin, IL-5 and RANTES. The number of eosinophils immunopositive for eotaxin and RANTES were only significantly increased 7 hours following allergen, demonstrating participation of airway eosinophilia by eotaxin and RANTES no longer contribute significantly 24 hours after challenge. High circulating levels of eosinophils may be necessary for chemoattraction by chemokines. IL-5 demonstrated a longer association with allergen-induced airway eosinophilia, maintaining increased immunopositivity by eosinophils until 24 hourss after challenge.

We did not detect a significant overall increase of IL-5 immunopositive cells in sputum following allergen challenge. This is most likely due to lack of statistical power, but may be interpreted in several other ways. Immunocytochemistry is unable to distinguish between intracellular protein (synthesized, stored or phagocytosed), and extracellular protein (receptor-bound). The majority of post-allergen eosinophils are immunopositive for IL-5, which may be due to expression of IL-5 by eosinophils themselves, receptor-bound IL-5 released from other cells in sputum, or receptor-bound IL-5 expressed by cells distal to the

airway. IL-5 has been reported to be synthesized by cells in the sputum of asthmatics (39) and is elevated in sputum supernatant of atopic asthmatics 24 hours following allergen challenge (40), supporting allergen-induced release if IL-5 by cells in the airway, such as T lymphocytes and eosinophils (41,42). Recent work in mice, however, has proposed a role of IL-5 distal to the airway. Systemic IL-5 may contribute to local eosinophil accumulation by releasing the eosinophil from the bone marrow, priming, and/or facilitating migration through the microvascular endothelium (43,44), and may be more relevant than the IL-5 measured downstream in the sputum. That we did not measure an overall increase in intracellular IL-5 following allergen suggests IL-5 in sputum is best measured in fluid phase supernatant.

The number of eosinophils immunopositive for eotaxin and RANTES were significantly elevated 7 hours following allergen challenge. We did not, however, detect a significant overall increase in the number of sputum cells immunopositive for these chemokines, most likely due to a lack of statistical power. Known producers of chemokines include airway epithelial and endothelial cells. As these structural cells do not commonly appear in samples of sputum, increased expression of eotaxin and/or RANTES by these cells by immunocytochemistry would not be reflected in sputum. We did not observe RANTES immunopositive mononuclear cells, suggesting either it is not chemotactic for mononuclear cells at this concentration, or that the mononuclear cells require a priming signal rendering them responsive to RANTES, as IL-5 priming of eosinophils increases chemotactic

properties of RANTES on eosinophils (45). RANTES is found in higher levels in sputum supernatant of asthmatics compared to normal controls (46), suggesting fluid phase measurements may better reflect the levels of RANTES and eotaxin in the allergen-challenged airway.

We were able to detect eotaxin in sputum supernatant at 7 hours following challenge, however, we may be grossly underestimating the level of eotaxin in our samples. Processing of the sputum sample requires a dilution of the sample (9 X) allowing only detection of eotaxin levels greater than 45 pg/ml (sensitivity 5 pg/ml). Detection of eotaxin in the 100-200 pg/ml level only 7 hourss following challenge suggests that there is indeed an allergen-induced increase in the level of eotaxin in the human airway.

Eotaxin is associated with early tissue recruitment and activation of eosinophils (47), where allergen-induced expression of eotaxin by lumen-associated cells determine a tissue gradient in the airway for recruitment through the tissues and into the airway lumen. In the guinea pig, mRNA is rapidly induced in airway epithelium and alveolar macrophages 3 hours following allergen (47), suggesting our measurements in sputum at 7 hours following challenge may be too late to measure allergen-induced increases of eotaxin intracellularly.

The number of metachromatic cells increased significantly following allergen, however, with a much higher allergen-induced number than previously reported using the same method for sputum processing and staining (6,7). Detection of metachromatic cells using the toluidine blue stain, is dependent upon retention of granule proteins within the mast

cells/basophils. Degranulation of metachromatic cells is possible following allergen challenge, thus staining of metachromatic granules within cells may underestimate the actual number of mast cells (48).

There is no consistent evidence for allergen-induced neutrophilia in the airways of asthmatics (5,6,40), and we did not observe an effect of allergen on the number of neutrophils in sputum. If the neutrophil is involved in the airway response to inhaled allergen, it may reflect an early, non-specific response which occurs with acute tissue injury (49), unlike the prolonged eosinophilia which occurs in response to specific allergic stimuli. The percent of sputum neutrophils (the ratio of neutrophils to other cells) was significantly elevated above baseline following diluent challenge, which may reflect the effects of repeated sputum induction (50,51). However, the absolute number of neutrophils (the load of neutrophils in the airway) remains unaffected by diluent challenge, and may be more relevant since it is not dependent upon flux of other cells, as does the percentage.

Although we observed neutrophils immunopositive for RANTES, this chemokine does not appear to be recruiting neutrophils to the airways. Allergen-inhalation has also been shown to increase the level of IL-8 in sputum without increasing the number of sputum neutrophils (16). A neutrophilic response to these potent neutrophils chemoattractants may require a co-stimulus, such as adhesion molecules or cytokines which prime neutrophils, but are not present in the airway following allergen challenge.

High reproducibility of the allergen-induced late response requires careful

administration of a suitable dose of relevant allergen, and airway stability of the subjects studied. Subjects enrolled in allergen challenge studies in our laboratory are characterized as stable, mild asthmatics with FEV_1 greater than 70% of predicted. It is important to carry out allergen inhalation studies when subjects are not affected by seasonal allergies, and to minimize the washout time between repeated allergen challenges to eliminate the possibility of seasonal shifts in airway reactivity. In the investigation of repeatability of the allergen-induced airway responses, the average washout time between challenges was 3.4 months, yet the allergen-induced airway responses were remarkably consistent, indicating these subjects had stable asthma.

The late airway response to allergen is measured by recording the maximal fall in FEV_1 occurring between 3 and 7 hours following allergen, or by constructing a FEV_1-time curve and measuring the area under the curve from 3 to 7 hours following allergen. We compared the reproducibility of the late response measured by maximal fall in FEV_1 and AUC to determine if AUC is a more sensitive indicator of the magnitude of the late response than a single time-point maximal fall in FEV_1. The higher IC for the AUC demonstrated this is indeed a more reproducible measurement than the maximal fall in FEV_1, however, the sample sizes predicted to be necessary to achieve a significant attenuation of the late response by either of these measurements are identical; the sample size predicted to be necessary for a 50% attenuation at a power level of 0.90, in late maximal fall in FEV_1 and for AUC is 8.

We have demonstrated that there is an inverse relationship between the magnitude of the late response to allergen, and the magnitude of airway eosinophilia (21). Eosinophils and ECP levels have also been inversely correlated with impairment of FEV_1 (25,53,54). Eosinophils may release cytotoxic mediators, such as ECP and MBP, which are shown to be elevated in the airways of asthmatics (36) and may lead to airway damage (53).

Eosinophils and MCC have been implicated as major participants in IgE-driven allergic disease. Both of these cell types increase in the airway following allergen inhalation, and bear receptors for IgE. We demonstrate an allergen-induced increase in the number of sputum eosinophils and metachromatic cells, suggesting these cells have a defined role during the allergic asthmatic response.

The allergen-induced eosinophilia is an important measurement when assessing the anti-inflammatory properties of asthma drugs, and it is necessary to determine the clinical feasibility of including this measurement in clinical trials. Based on estimates of variance using repeated measures ANOVA, the sample size necessary to achieve a 50% attenuation in the number of sputum eosinophils following allergen with a power of 0.90, is 17 subjects, and with a power level of 0.70 is 10 subjects (Figure 2.7). This sample size estimation is consistent with previous work from our laboratory where we demonstrated with 7 subjects that 1 wk treatment with inhaled steroid can reduce allergen-induced airway eosinophilia by approximately 50% (6), and with 10 subjects that 1 wk treatment with inhaled albuterol can increase allergen-induced airway eosinophilia at 7 hours by over 50% (7).

Repeated measures ANOVA is a powerful statistical tool in a randomized cross-over study design. When applied to measurements of eosinophils in sputum, repeated measures ANOVA allows for practical sample sizes to demonstrate attenuation in the number of sputum eosinophils. If, for example, the number of allergen-induced eosinophils were measured at only one time point (ie. 7 hours or 24 hours) following challenge, the predicted sample size required to statistically demonstrate a 50% reduction in the number of eosinophils, using a paired t-test, would be approximately 30-70 subjects. A great advantage of sputum induction is that it does allow for repeated sampling of airway inflammatory cells, yielding a more sensitive measure of variance.

The reproducibility of sputum eosinophils and metachromatic cells reflect the variability in airway asthmatic responses to the same dose of allergen, as well as the variability in the time-course of the inflammatory response to allergen. Furthermore, reproducibility of the sputum counts reflect the quality of sputum sample and consistency of processing and quantification. The poor reproducibility of the total cell count in sputum (25) adds a considerable amount of variability in a repeated measures analysis, elevating the sample size required to demonstrate changes in the absolute eosinophil number when compared to percent changes in eosinophils (Figures 2.6 and 2.7). Despite the variability in absolute eosinophil counts, we have demonstrated drug effects on the allergen-induced number of eosinophils corresponding to the drug effects on the allergen-induced late response, with a reasonable sample size not more than 10 subjects (6,7).

This study has demonstrated that inhalation of allergen by atopic asthmatics induces an inflammatory response in the airways consisting of eosinophils and metachromatic cells. Metachromatic cells are associated with initiation and possible prolongation of the allergic response and eosinophils are associated with the resulting damage to the airways and subsequent reduction in pulmonary function. These data confirm the presence of several chemokines specific for the activation and chemotaxis of eosinophils following allergen inhalation challenge, and suggests these cytokines participate in the acute allergen-induced eosinophilia observed within the first 24 hours following challenge. Furthermore, these data support the notion that cooperation between chemokines may be necessary to induce eosinophil accumulation in vivo, as suggested in the guinea pig model of intradermal injection (43). Measurement of sputum eosinophilia as an index of airway inflammation is shown to be reproducible, proving to be a valuable clinical tool for assessing the anti-inflammatory properties of asthma therapies. Understanding the regulation of airways eosinophilia and the interaction between cytokines in the development of airways eosinophilia may be a useful strategy in the control of allergic asthma.

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Table 1: Total sputum cell count and percent sputum inflammatory cells at baseline and following allergen and diluent challenges.

	Baseline		7 hours		24 hours		2 days		4 days		7 days	
	Ag	Dil	Ag	Dil	Ag	Dil	Ag	Dil	Ag	Dil	Ag	Dil
TCC (10^6/ml)	4.2±1.9	1.6±0.3	8.2±2.7	1.8±0.6	7.2±3.5	1.9±0.3	4.4±1.3	3.2±0.4	2.4±0.7	2.0±0.2	4.0±1.6	1.8±0.2
Eos (%)	4.9±1.7	4.0±1.3	* 21.6±4.0	4.2±1.5	* 25.4±2.9	8.1±2.8	* 21.3±3.9	6.4±3.0	* 18.5±6.3	6.9±2.3	* 8.2±2.0	3.7±1.3
MCC (%)	.05±.03	.04±.02	* 1.11±.43	.09±.06	.40±.09	.28±.02	0.23±.04	.18±.14	.15±.04	.13±.07	.14±.06	.06±.02
Neut (%)	62.0±5.5	40.7±8.5	* 61.1±6.2	66.2±7.4	* 59.5±8.6	53.8±8.3	* 36.8±9.7	46.6±7.8	* 41.1±8.6	61.1±4.2	* 55.6±9.0	58.4±5.3
Macro (%)	32.7±4.5	54.3±7.9	* 19.1±3.7	29.3±7.4	* 23.3±5.9	29.3±8.0	* 40.3±8.0	46.4±8.4	* 40.0±4.8	32.7±3.9	* 35.5±7.6	37.0±5.1

Ag; allergen challenge, Dil; diluent challenge, TCC; total sputum cell count, Eos; eosinophils, MCC; metachromatic cells, Neut; neutrophils, Macro; macrophages; *$p<0.05$ Ag vs Dil.

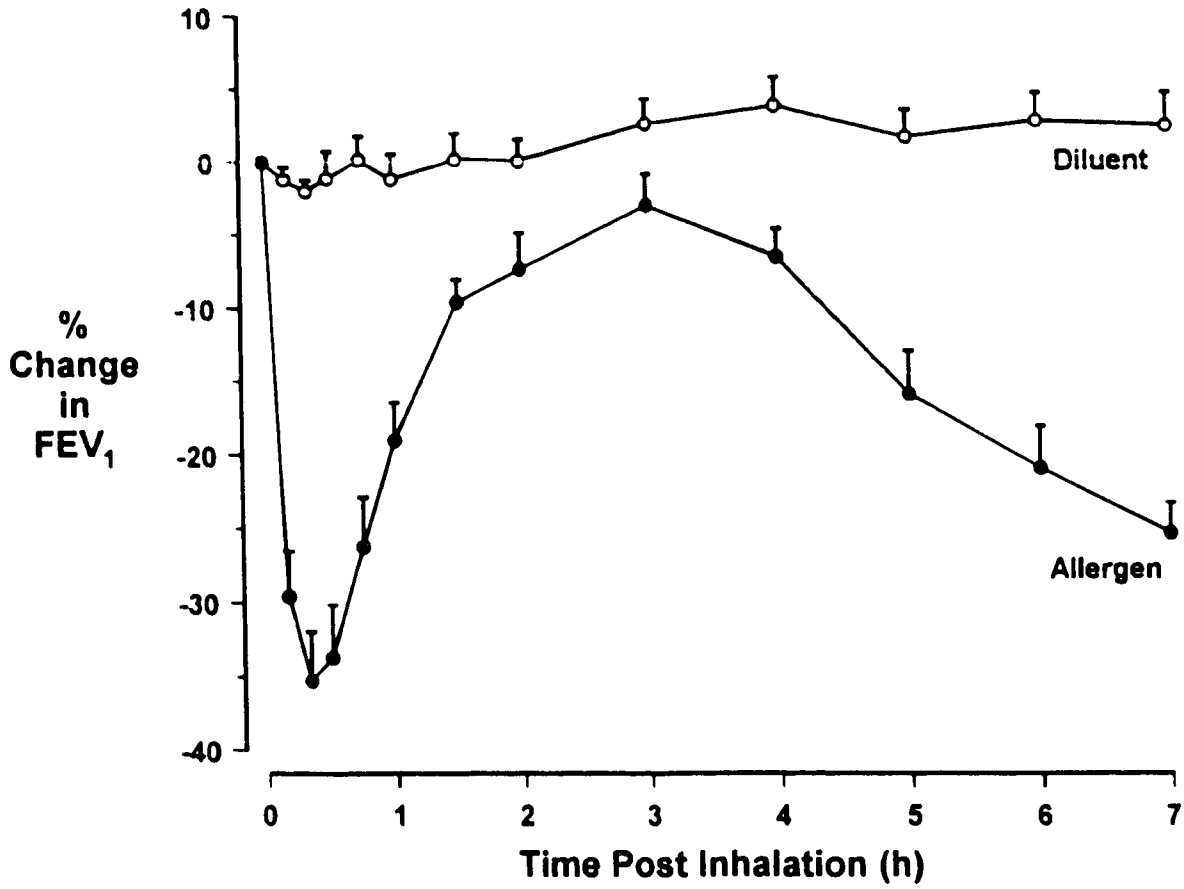

Figure 2.1: The early and late airway responses after allergen inhalation (solid circles) and diluent inhalation (open circles) challenge. Allergen challenge resulted in significant early and late airway responses ($p<0.05$) compared to diluent.

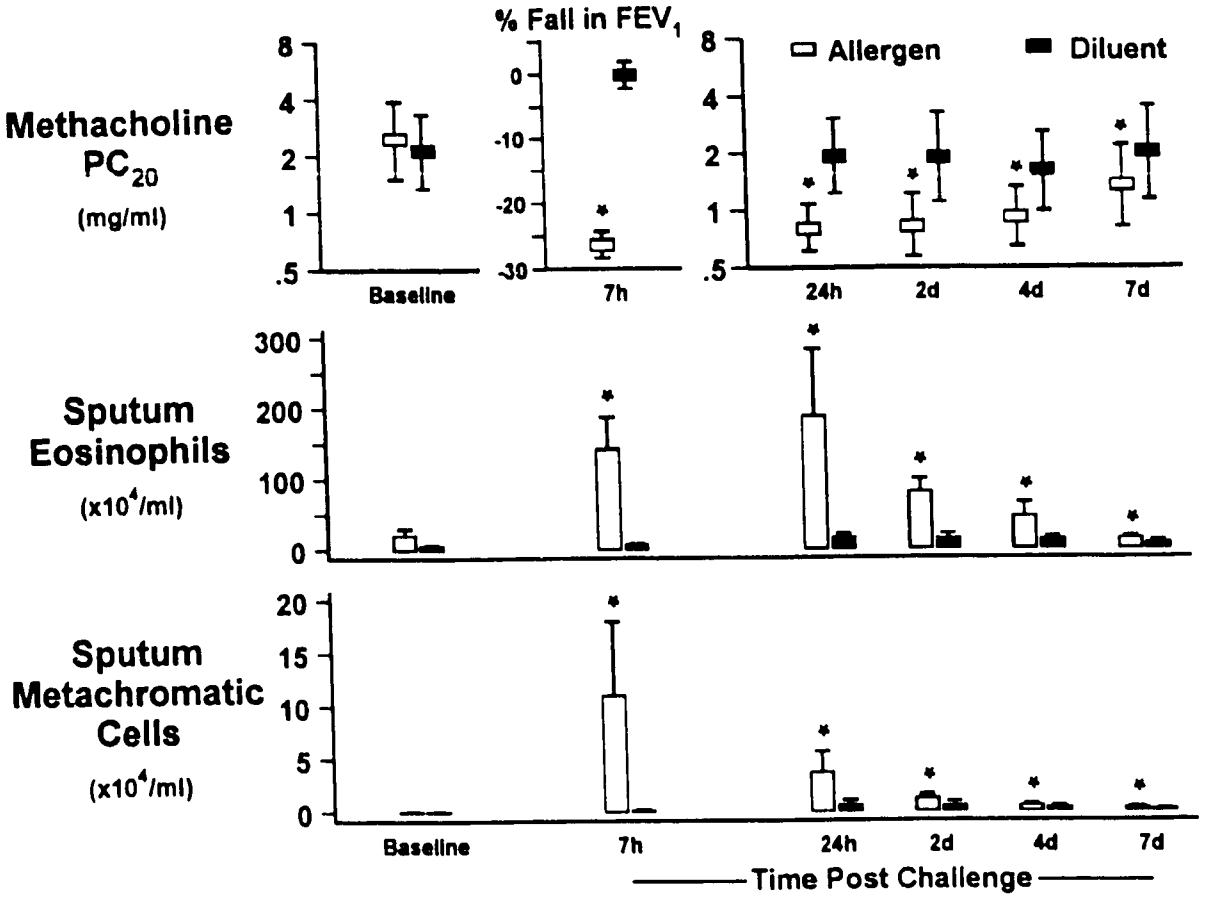

Figure 2.2: Methacholine PC_{20} and maximal late fall in FEV_1 (top panel), sputum eosinophils (middle panel) and sputum metachromatic cells (bottom panel) following allergen inhalation (open bars) and diluent inhalation (solid bars) challenge.

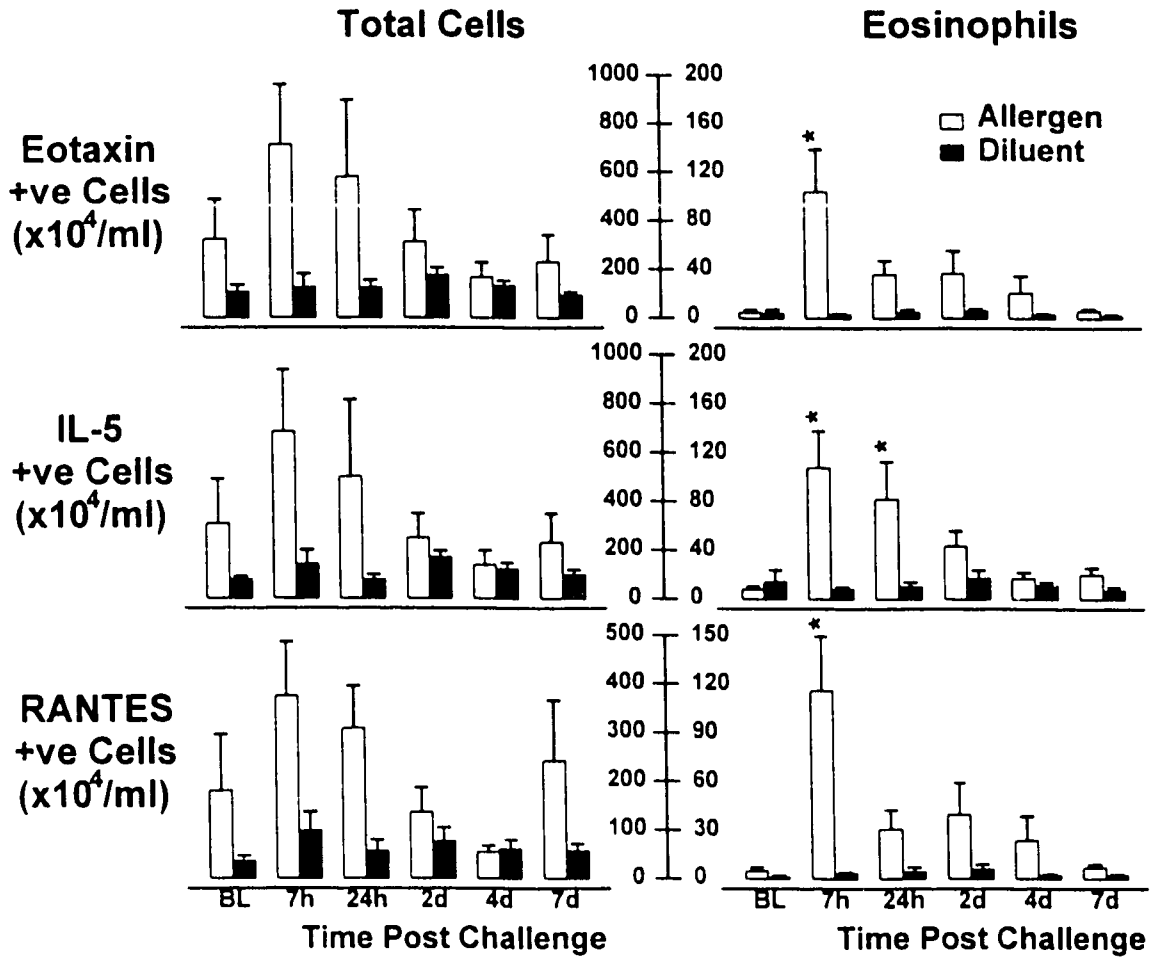

Figure 2.3: Number of sputum cells (left panel) and sputum eosinophils (right panel) immunopositive for eotaxin (top panel), IL-5 (middle panel) and RANTES (bottom panel) following allergen inhalation (open bars) and diluent inhalation (solid bars) challenge.

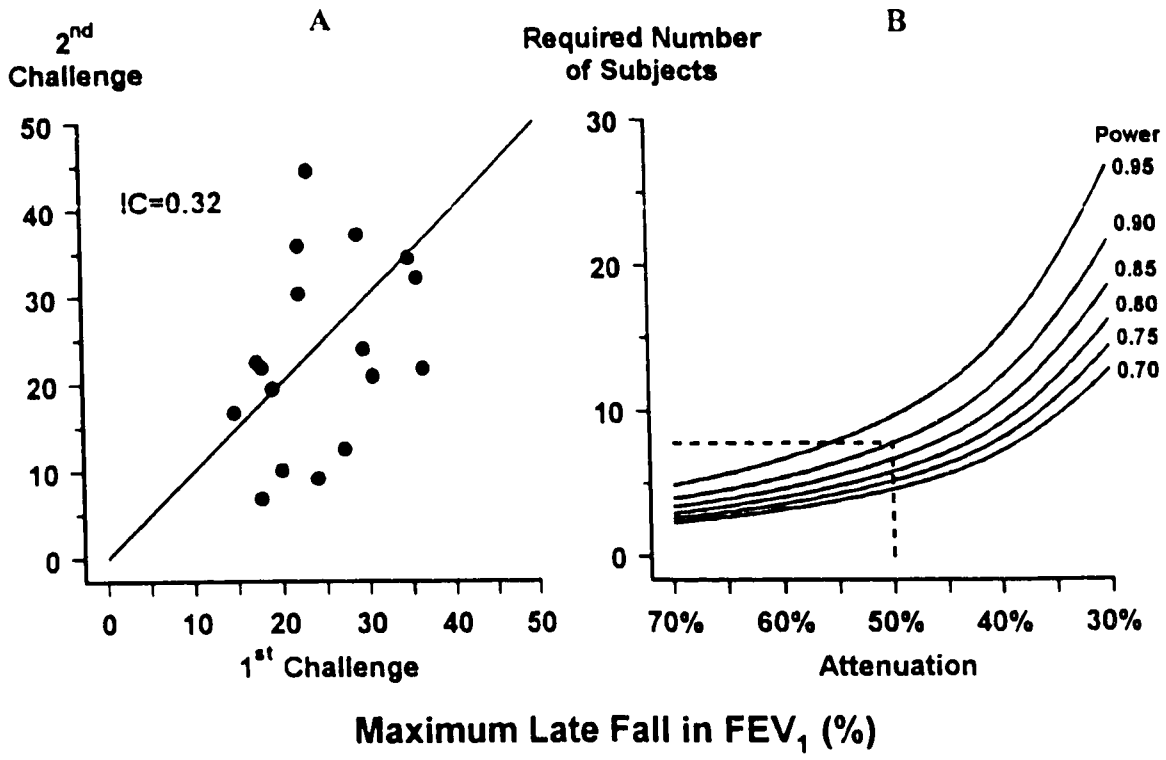

Figure 2.4: A) Maximum allergen-induced late fall in FEV_1 (%) demonstrating the reproducibility between the first and the second allergen inhalation challenges. The intraclass correlation between subsequent challenges is 0.32. B) Estimated sample sizes for attenuation of the maximum % late fall in FEV_1. The number of subjects required to demonstrate a 50% attenuation of the maximum late % fall in FEV_1 at a power level of 0.90 is 8 subjects.

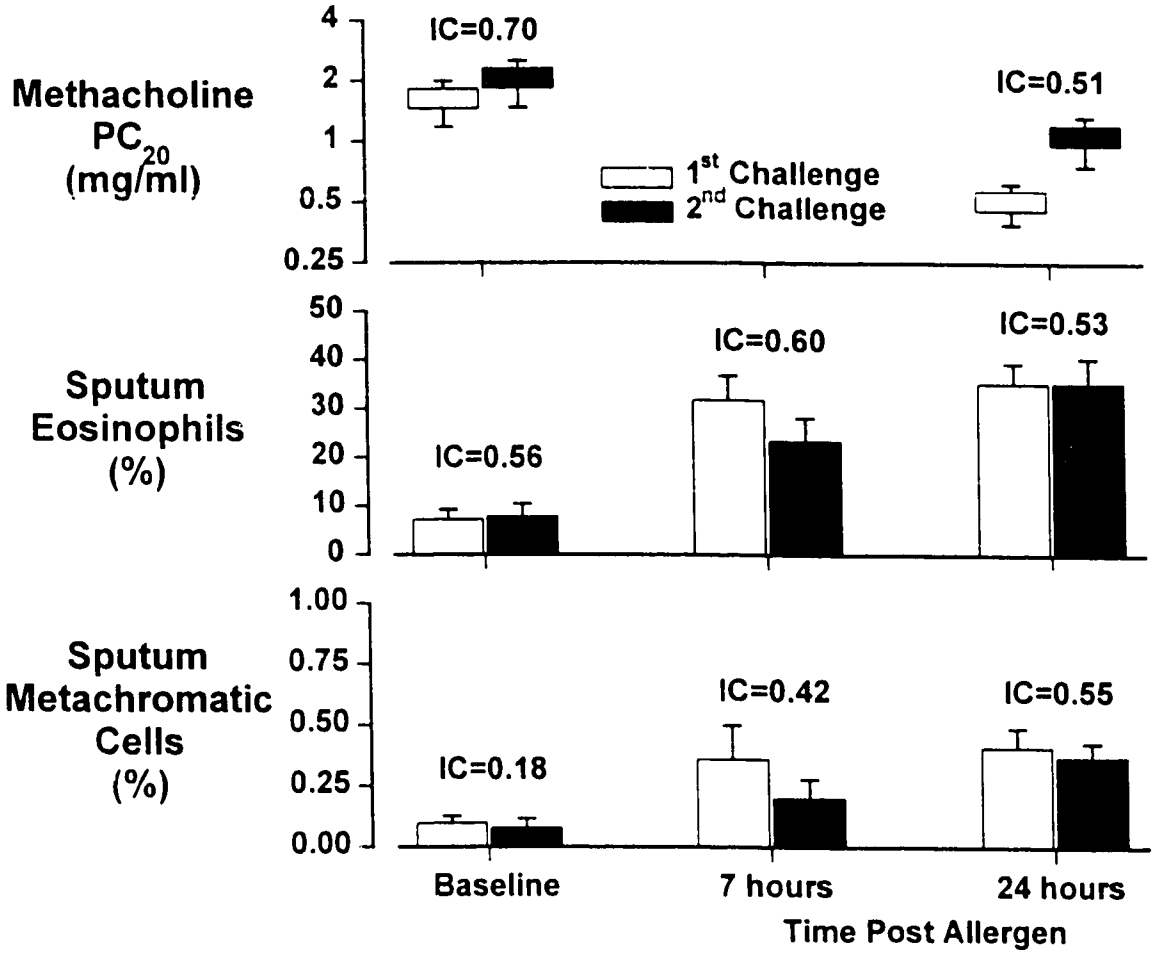

Figure 2.5: Comparison of methacholine PC_{20} (top panel), sputum eosinophils (middle panel) and sputum metachromatic cells (bottom panel) measured at baseline and following the first (open bars) and the second (solid bars) allergen inhalation challenges. Bars represent mean ± SEM (mean ± GSEM for methacholine PC_{20}); IC values represent the intraclass correlation between repeated measurements of individual subjects.

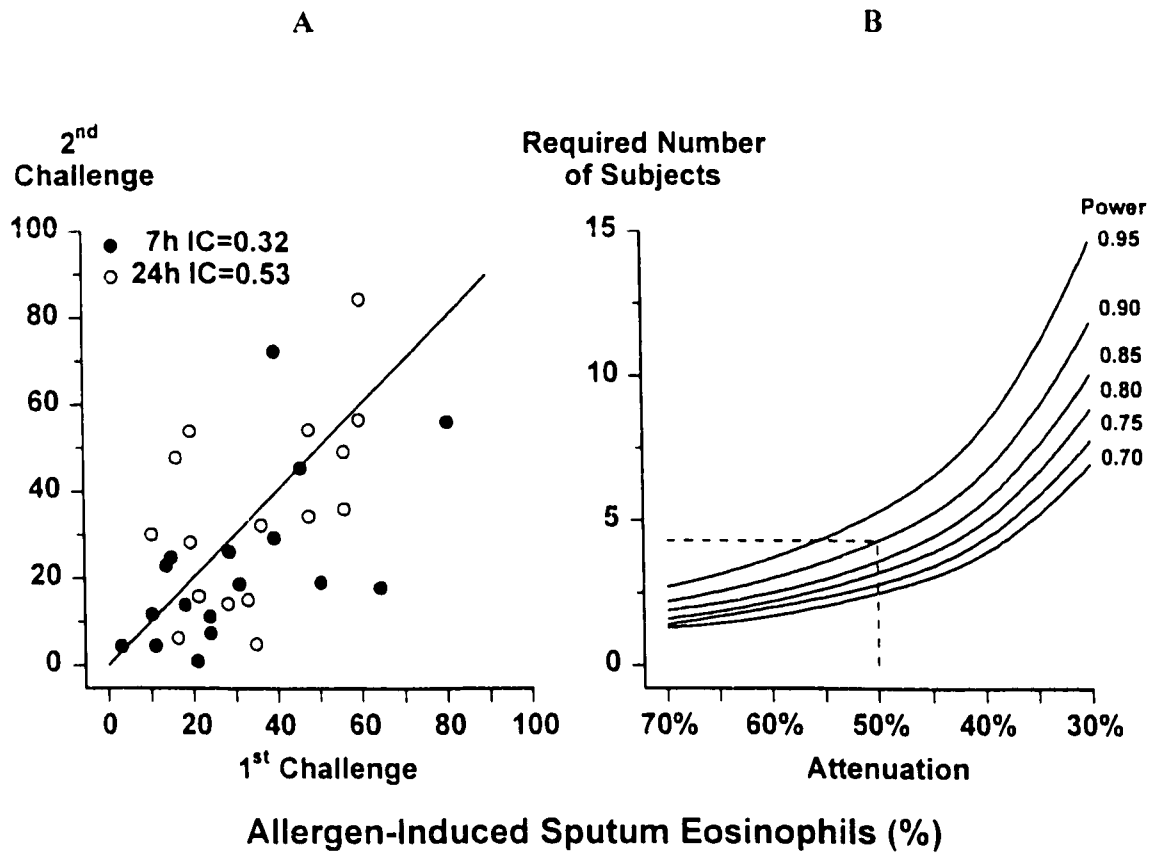

Figure 2.6: A) Percent allergen-induced sputum eosinophils at 7h (solid circles) and 24h (open circles) demonstrating the reproducibility between the first and the second allergen inhalation challenges. The intraclass correlations between subsequent challenges are 0.02 and 0.32 for 7h and 24h, respectively. B) Estimated sample sizes for attenuation of the allergen-induced percent sputum eosinophils. The number of subjects required to demonstrate a 50% attenuation of the allergen-induced percent eosinophils at a power level of 0.90 is 4 subjects.

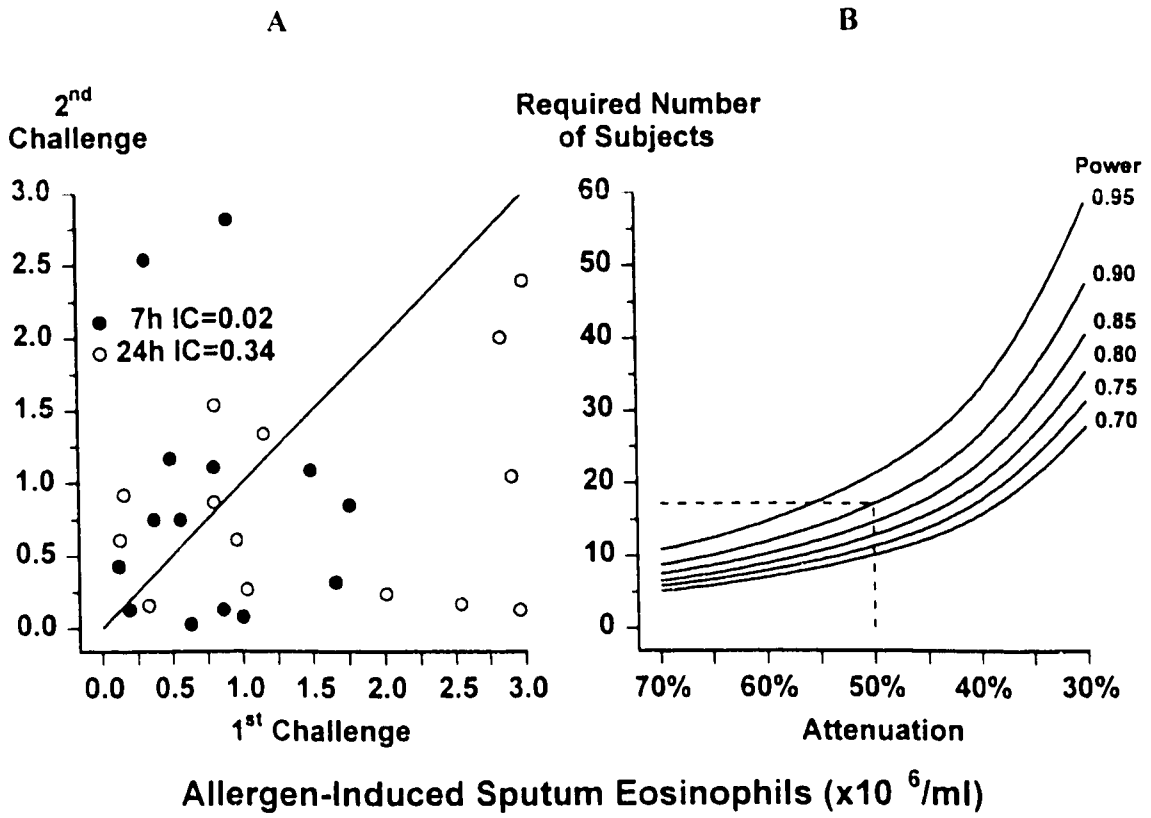

Figure 2.7: A) Number of allergen-induced eosinophils at 7h (solid circles) and 24h (open circles) demonstrating the reproducibility between the first and second allergen inhalation challenges. The intraclass correlations between subsequent challengs are 0.02 and 0.34 for 7h and 24h, respectively. B) Estimated sample sizes for attenuation of the allergen-induced number of sputum eosinophils. The number of subjects required to demonstrate a 50% attenuation of the allergen-induced number of sputum eosinophils at a power level of 0.90 is 17 subjects.

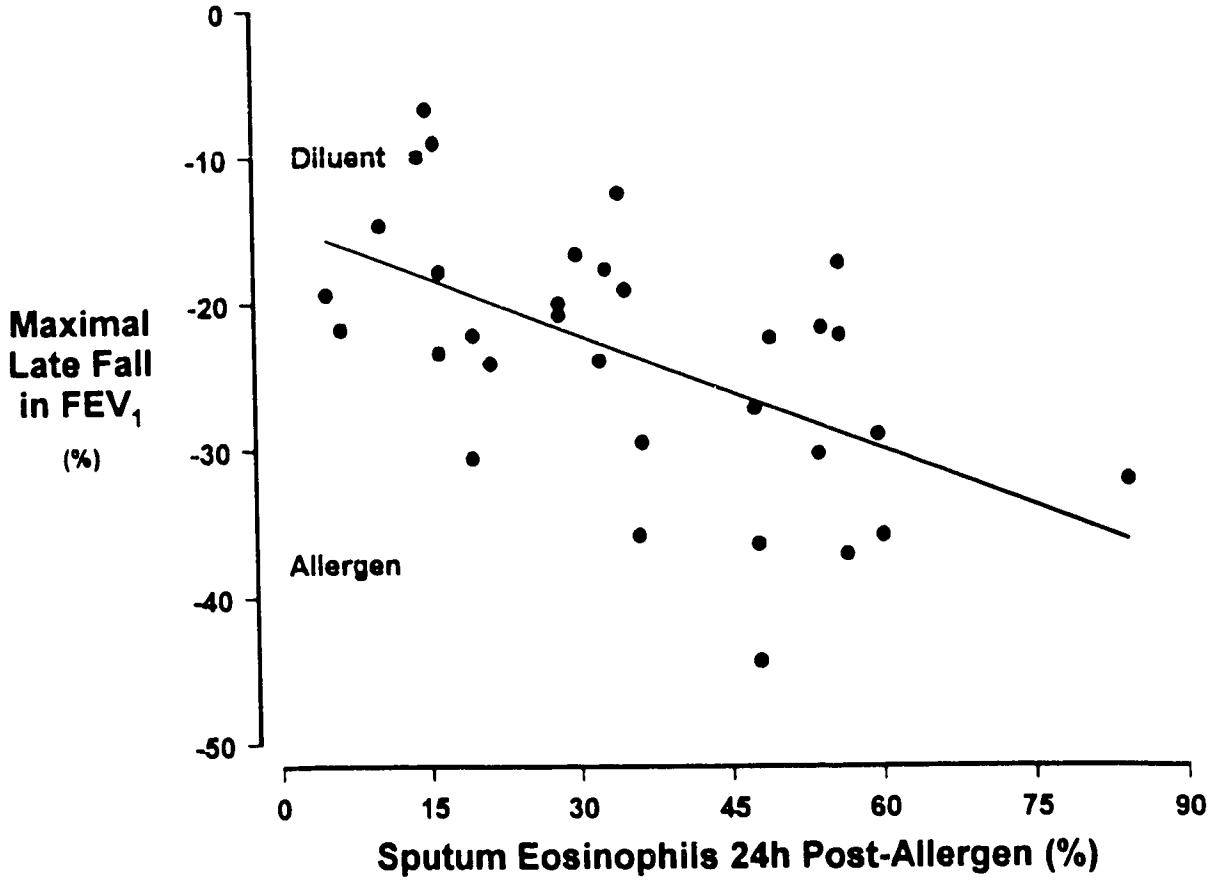

Figure 2.8: Relationship between the maximal late % fall in FEV_1 and the allergen-induced increase in the percentage sputum eosinophils 24h following allergen challenge (R=-0.56, $p<0.001$).

CHAPTER 3:

EFFECTS OF INHALED BUDESONIDE ON ALLERGEN-INDUCED AIRWAY RESPONSES AND AIRWAY INFLAMMATION

Published in The American Journal of Respiratory and Critical Care Medicine in 1996

Gail Gauvreau's contribution:

Processing of laboratory samples
Quantification of cells
Analysis of data
Preparation of manuscript

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Effects of Inhaled Budesonide on Allergen-induced Airway Responses and Airway Inflammation

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Allergen inhalation by sensitized subjects results in acute bronchoconstriction, which can be followed by a later bronchoconstrictor response, allergen-induced airway hyperresponsiveness, and increases in airway inflammatory cells. Treatment with inhaled glucocorticosteroids attenuates allergen-induced asthmatic airway responses. The purpose of this study was to determine whether a 1-wk pretreatment with inhaled budesonide influences allergen-induced changes in inflammatory cells in blood and induced sputum. Seven subjects with mild atopic asthma were treated in a double-blind, placebo-controlled, randomized, crossover fashion with either inhaled budesonide 400 μg/d, or placebo for 7 d. Allergen challenges were carried out the morning after treatment was discontinued and sputum samples were obtained 7 h after allergen inhalation. Methacholine airway responsiveness was measured, and blood and sputum samples were obtained 24 h post-allergen. Budesonide treatment attenuated the magnitude of both the early and the late asthmatic response, reduced allergen-induced methacholine airway hyperresponsiveness, and attenuated allergen-induced increases in total eosinophils and activated eosinophils. These results suggest that the effects of inhaled glucocorticosteroids on allergen-induced airway responses may be mediated through their inhibition of allergen-induced eosinophil migration and activation. **Gauvreau GM, Doctor J, Watson RM, Jordana M, O'Byrne PM. Effects of inhaled budesonide on allergen-induced airway responses and airway inflammation.**

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Airway inflammation is an important characteristic in patients with current symptomatic asthma. This has been demonstrated by high numbers of inflammatory cells present in bronchial biopsies (1), bronchoalveolar lavage (BAL) fluid (2), and sputum (3) from asthmatics when compared with nonasthmatics. Airway hyperresponsiveness to a variety of bronchoconstrictor mediators is another characteristic finding in patients with asthma (4), the severity of which reflects the severity of asthma (5). Inhaled glucocorticosteroids are known to improve airway hyperresponsiveness in asthmatics (6, 7), an effect that is thought to occur by suppressing the degree of inflammation in the airways (8, 9).

Allergen challenge is a valuable laboratory model for the study of pathogenesis of airway inflammation and allergen-induced asthma. Allergen inhalation results in acute bronchoconstriction in sensitized subjects, and in 50 to 60% of adult subjects, this is followed by a late bronchoconstrictor response that is associated with the development of allergen-induced airway hyperresponsiveness (10). The allergen-induced late asthmatic response has been associated with increases in the number of airway inflammatory cells, particularly eosinophils and metachromatic cells (MCC), in bronchial biopsies (11), BAL (12), and induced sputum (13, 14). Treatment with inhaled corticosteroids has been shown to abolish allergen-induced late responses (15, 16), and to inhibit allergen-induced airway hyperresponsiveness (16, 17). More recently, oral treatment with prednisone was associated with a decrease in inflammatory markers in induced sputum (18). The mechanisms of action of glucocorticosteroids on late responses and airway hyperresponsiveness have been suggested to occur through the inhibition of the allergen-induced airway inflammatory response. However, this has not been previously directly studied. The purpose of this study was to determine whether pretreatment with the inhaled corticosteroid budesonide, administered for 1 wk, a duration of treatment known to inhibit allergen-induced airway responses, influences allergen-induced changes in eosinophils and MCC in blood and induced sputum, as well as the physiologic changes after inhaled allergen.

METHODS

Subjects

Nine nonsmoking subjects (Table 1) with mild asthma (five female, four male) were selected for the study because of a previously documented allergen-induced early and late asthmatic response, and they gave signed consent to participate in the study. The study was approved by the Ethics Committee of the McMaster University Health Sciences Center. All subjects had stable asthma with a FEV, greater than 70% of predicted normal on all study days. Subjects were not exposed to sensitizing allergens and had not had asthma exacerbations or respiratory tract infections for at least 6 wk prior to entering the study. Subjects used only inhaled

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TABLE 1

SUBJECT CHARACTERISTICS UPON SCREENING VISIT

Subject No (n = 7)	Age (yr)	Treatment	FEV_1 (% pred)	PC_{20} (mg/ml)	Allergen (dilution)
102	20	Salbutamol	83 1	0 357	HDM (1 2,048)
104	23	Bricanyl	77 6	0 500	HDM (1 256)
105	24	Salbutamol	96 3	1 669	Cat (1 128)
106	20	Salbutamol	70 6	0 145	HDM (1 1,024)
107	21	Salbutamol	72 5	1 083	HDM (1 1,024)
108	22	Salbutamol	80 0	2 462	RW (1 128)
109	19	None	95 5	19 924	HDM (1 16)

Definition of abbreviations: PC_{20} = provocative concentration of methacholine causing a 20% fall in FEV_1; HDM = house dust mite; RW = ragweed

beta-2-agonist as required. Medication was withheld for at least 8 h before each visit, and subjects were instructed to refrain from rigorous exercise and tea or coffee in the morning before visits to the laboratory. Two of the subjects dropped out of the study because of protocol violations. These were asthma deterioration and the randomization criteria not fulfilled.

Study Design

The study was carried out with a double-blind, placebo-controlled, randomized, crossover design. The subjects completed two treatment periods where they were treated with either inhaled budesonide 400 μg/day or an identical placebo for 7 d. Each treatment period consisted of four visits to the laboratory. Baseline measurements of FEV_1, the provocative concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}), blood and induced sputum differential, and total cell counts were determined before treatment and on the seventh day of treatment with budesonide or placebo. Allergen challenges were carried out in the morning and 12 h after treatment was discontinued, and sputum samples were obtained 7 h after allergen inhalation. Methacholine PC_{20}, blood and sputum samples were obtained 24 h postallergen. Each treatment period was separated by a washout period of at least 3 wk.

Methacholine Inhalation Test

Methacholine inhalation challenge was performed as described by Cockcroft (19). Subjects inhaled normal saline, then doubling concentrations of methacholine phosphate from a Wright nebulizer for 2 min. FEV_1 was measured at 30, 90, 180, and 300 s after each inhalation. Spirometry was measured with a Collins water-sealed spirometer and kymograph (Warren E. Collins, Braintree, MA). The test was terminated when a fall in FEV_1 of 20% of the baseline value occurred, and the PC_{20} was calculated.

Allergen Inhalation Test

Allergen challenge was performed as described by O'Byrne and colleagues (20). The allergen producing the largest skin wheal diameter was diluted in normal saline. The concentration of allergen extract for inhalation was determined from a formula described by Cockcroft and colleagues (16) using the results from the skin test and the methacholine PC_{20}. The starting concentration of allergen extract for inhalation was two doubling concentrations below that predicted to cause a 20% fall in FEV_1. The same three doses of allergen were administered during each treatment period, and the FEV_1 was measured at 10, 20, 30, 40, 50, 60, 90, and 120 min postallergen inhalation, then each hour until 7 h after allergen inhalation. The early bronchoconstrictor response was taken to be the largest fall in FEV_1 within 2 hr after allergen inhalation, and the late response was taken to be the largest fall in FEV_1 between 3 and 7 h after allergen inhalation.

Differential Blood Counts

Blood was collected into heparinized tubes by direct venipuncture, and blood smears were made for differential staining (Diff Quik; American Scientific Products, McGaw Park, IL). Differential cell counts were obtained from the mean of two slides with 300 cells counted per slide. Total leukocyte count was determined using a hemocytometer, and cell populations were expressed as the number per milliliter of blood.

Sputum Analysis

Sputum was induced and processed using the method described by Popov and colleagues (21). Subjects inhaled 3, 4, then 5% saline for 10 min each. The induction was stopped when an adequate sample was obtained, or if the FEV_1 dropped 20% from baseline. Cell plugs were selected from the sample and processed using 0.1% dithiothreitol (Sputolysin; Calbiochem-Behring, San Diego, CA) and Dulbecco's phosphate buffered saline (Gibco Diagnostics, Tucson, AZ). Cytospins were prepared on glass slides for staining. Differential cell counts were obtained from the mean of two slides with 400 cells counted per slide stained with Diff Quik. MCC counts (mast cells and basophils) on slides stained with toluidine blue were obtained from the mean of two slides with 5,000 cells observed on each slide. If possible, cytospins were also prepared on aptex-coated slides and fixed in periodate-lysine-paraformaldehyde for immunocytochemical staining for eosinophil cationic protein (ECP), using a monoclonal antibody directed against cleaved ECP (EG2) (Kabi Pharmacia, Upsala, Sweden) (22). The percentage of EG2-positive cells was determined from a count of 500 cells under light microscopy, and it was expressed as the number of EG2-positive cells per milliliter of sputum plugs.

Statistical Analysis

Methacholine PC_{20} measurements are made by linear interpolation of log dose-response curves resulting in logarithmic values for PC_{20}, which are then subjected to statistical analysis. Summary statistics for methacholine PC_{20} are expressed as geometric mean and range. All other summary statistics are expressed as mean and SEM. Student's *t* test for paired and unpaired observations was used to compare the airway responses to allergen and to compare the effects of placebo and budesonide treatment on airway and blood cellular changes from pretreatment baseline, respectively. As not all subjects had an adequate number of slides for EG2 staining, and therefore unequal numbers of slides were available from each treatment arm, Student's *t* test for unpaired observations were used to analyze the effects of placebo and budesonide on the number of EG2-positive cells in sputum. All other allergen-induced changes in sputum were analyzed, using a two-factor repeated-measures ANCOVA, for the effects of time and treatment on log-transformed data (23).

RESULTS

The maximal mean early asthmatic response was attenuated by budesonide treatment, being 33.0% (SEM, 4.5%) after placebo treatment and 19.5% (SEM, 5.1%) after budesonide treatment ($p = 0.01$) (Figure 1). The maximal mean late asthmatic response was also attenuated by budesonide treatment, being 23.7% (SEM, 4.6%) after placebo treatment and 6.6% (SEM, 2.2%) after

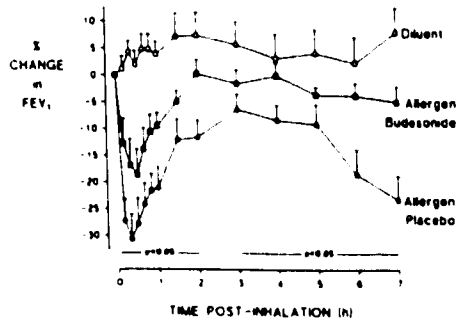

Figure 1. Allergen-induced airway responses. Percent change in FEV_1 (mean and SEM) after inhalation of diluent (*open circles*), allergen with budesonide treatment (*closed squares*), and allergen with placebo treatment (*closed circles*). Both the early (0–2 h) and the late (3–7 h) asthmatic responses to allergen were significantly reduced with budesonide treatment ($p < 0.05$).

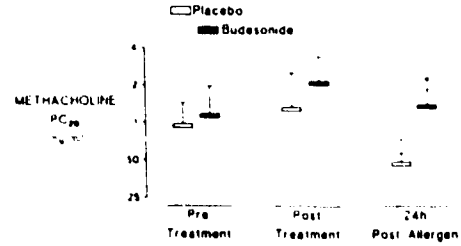

Figure 2. Allergen-induced methacholine hyperresponsiveness. Methacholine PC_{20} (mean and geometric SEM) before treatment, after 7 d of 400 μg/d budesonide (*closed bars*) or placebo (*open bars*), and 24 h after allergen inhalation. Allergen-induced methacholine airway hyperresponsiveness was significantly different. (*$p < 0.05$ budesonide significantly reduced the allergen-induced increase from pretreatment baseline.) (▼$p < 0.05$ significant allergen-induced increase from pretreatment baseline).

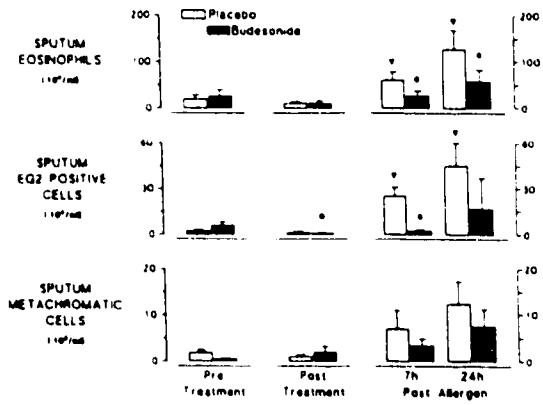

Figure 3. Allergen-induced increases of inflammatory cells in induced sputum. Number of sputum eosinophils (*top panel*), EG2-positive cells (*middle panel*), and metachromatic cells (*bottom panel*) before treatment, after 7 day of 400 μg/d budesonide (*closed bars*) or placebo (*open bars*), and at 7 and 24 h after allergen inhalation. (*$p < 0.05$ budesonide significantly reduced the allergen-induced increase from pretreatment baseline). (▼$p < 0.05$ significant allergen-induced increase from pretreatment baseline)

budesonide treatment ($p = 0.02$) (Figure 1). Budesonide treatment also significantly reduced the area under the curve of both the early ($p = 0.03$) and the late ($p = 0.02$) asthmatic response (Figure 1).

Budesonide treatment slightly increased the baseline measurements of methacholine PC_{20} from 1.15 (0.21 to 19.9) mg/ml before budesonide to 2.14 (0.37 to 25.1) mg/ml after budesonide, but this increase was not statistically significant when compared with placebo ($p = 0.25$) (Figure 2). The baseline measurements of methacholine PC_{20} were not significantly different during placebo treatment, being 0.95 (0.15 to 14.9) mg/ml before and 1.32 (0.16 to 30.3) mg/ml after placebo ($p = 0.22$). There was no significant treatment effect of budesonide on baseline FEV_1 or VC.

Allergen inhalation significantly decreased the methacholine PC_{20} during placebo treatment from 1.32 (0.16 to 30.3) mg/ml before to 0.46 (0.09 to 14.35) mg/ml 24 h after allergen ($p = 0.01$) (Figure 2). The methacholine PC_{20} did not change significantly after allergen during budesonide treatment, being 2.14 (0.37 to 25.1) mg/ml before and 1.38 (0.40 to 5.85) mg/ml 24 h after allergen ($p = 0.10$) (Figure 2). The decrease in methacholine PC_{20} from preallergen to postallergen inhalation during placebo treatment was 2.86-fold. The decrease in methacholine PC_{20} from preallergen to postallergen inhalation during budesonide treatment was 1.55-fold ($p = 0.05$).

Treatment with budesonide did not reduce the number of sputum eosinophils before allergen inhalation, but it did significantly reduce the number of sputum EG2-positive eosinophils from 5.7 (SEM, 1.9) to 0.6 (SEM, 0.5) × 10^4/ml ($p = 0.03$) (Figure 3). Treatment with placebo had no significant effect on the numbers of sputum eosinophils or EG2-positive eosinophils before allergen.

Allergen inhalation increased the number of sputum eosinophils and EG2-positive cells after allergen inhalation (Table 2 and Figure 3). On placebo treatment, the sputum eosinophils increased from 10 (SEM, 4) to 60 (SEM, 18) × 10^4/ml at 7 h, and to 127 (SEM, 41) × 10^4/ml at 24 h after allergen ($p < 0.05$), and the sputum EG2-positive cells increased from 1.10 (SEM, 0.60) to 25.3 (SEM, 5.6) × 10^4/ml at 7 h, and to 44.6 (SEM, 15.4) × 10^4/ml at 24 h after allergen ($p < 0.05$). Budesonide treatment significantly attenuated the allergen-induced increases in sputum eosinophils and EG2-positive cells after allergen inhalation ($p < 0.05$) (Table 2 and Figure 3). The sputum eosinophils were 27 (SEM, 10) × 10^4/ml at 7 h, and 58 (SEM, 25) × 10^4/ml at 24 h after allergen, and the sputum EG2-positive cells were 2.4 (SEM, 1.1) × 10^4/ml at 7 h, and 16.7 (SEM, 7.5) × 10^4/ml at 24 h after allergen.

Allergen inhalation also increased the number of sputum MCC slightly, but there was no significant effect of time ($p = 0.20$) (Table 2 and Figure 3). The number of MCC were reduced approximately 2-fold with budesonide treatment, but this effect was not statistically significant. There were no significant effects of time or treatment in sputum neutrophils after allergen (Table 2).

Lastly, allergen inhalation during placebo treatment increased the numbers of blood eosinophils 24 h after allergen from 33 (SEM, 6) × 10^4/ml to 57 (SEM, 9) × 10^4/ml ($p < 0.001$). Budesonide treatment significantly prevented this increase ($p < 0.01$), being 39 (SEM, 9) × 10^4/ml before and 36 (SEM, 6) × 10^4/ml after allergen (Table 2).

DISCUSSION

This study confirms previous studies, which have demonstrated that inhaled corticosteroids attenuate the early and the late bronchoconstrictor responses after inhaled allergen (15) and protect against allergen-induced methacholine airway hyperresponsiveness (16). In addition, the study demonstrated that treatment with inhaled budesonide also attenuates allergen-induced increase of inflammatory cells in blood and in the airways, as assessed by increases in cells in induced sputum. This suggests that the effects of inhaled glucocorticosteroids on allergen-induced airway responses is mediated through their effects on inflammatory cell migration and activation.

We have previously demonstrated that the numbers of sputum eosinophils and MCC increased by 7 h after allergen inhalation, and they remained elevated at 24 h (24). The number of MCC and eosinophils likely began to increase in airways sooner than 7 h; however, sputum was not collected for analysis before 7 h because sputum induction may cause bronchoconstriction, thus interfering with subsequent spirometry measurements.

TABLE 2

COMPARISON OF BUDESONIDE TREATMENT (7 DAYS, 400 μg/DAY) VERSUS PLACEBO TREATMENT ON INFLAMMATORY CELLS IN BLOOD AND INDUCED SPUTUM BEFORE AND AFTER ALLERGEN INHALATION*

	Pretreatment		Post-treatment		Seven Hours After		Twenty four Hours After	
	Placebo	Budesonide	Placebo	Budesonide	Placebo	Budesonide	Placebo	Budesonide
Sputum								
Eosinophils	19	26	10	10	60‡	27†	127‡	58†
$\times 10^4$/ml	(9)	(14)	(4)	(6)	(18)	(10)	(41)	(25)
Neutrophils	173	326	140	230	211	477	163	176
$\times 10^4$/ml	(55)	(150)	(43)	(67)	(80)	(188)	(27)	(66)
Metachromatic cells	1 8	0 49	0 92	1 98	6 96	3 39	12 40	7 60
$\times 10^3$/ml	(0 62)	(0 18)	(0 47)	(1 23)	(4 02)	(1 47)	(4 88)	(3 72)
EG2 + ve cells	2 6	5 7	1 1	0 6†	25 3‡	2 4†	44 6‡	16 7
$\times 10^4$/ml	(1 0)	(1 9)	(0 6)	(0 5)	(5 6)	(1 1)	(15 4)	(7 5)
Total cell count	2 7	5 1	2 3	3 7	3 8	4 3	4 7	3 0
$\times 10^6$/ml	(0 9)	(1 7)	(0 6)	(1 2)	(1 1)	(2 0)	(1 5)	(0 8)
Blood								
Eosinophils	36	42	33	39			57‡	36†
$\times 10^4$/ml	(8)	(6)	(6)	(9)			(9)	(6)

* Values are means with SEM shown in parentheses.
† $p < 0.05$ budesonide significantly reduced the allergen-induced increase
‡ $p < 0.05$ significant allergen-induced increase from pretreatment baseline

Unlike the previous findings (7, 8) inhaled steroid treatment did not reduce the number of circulating eosinophils before allergen inhalation. The dose of budesonide in the present study, however, was considerably lower and administered for a shorter period of time. In sputum, budesonide treatment for 7 d reduced the number of EG2-positive cells before allergen inhalation, and it appears to have done this by decreasing the percentage of activated eosinophils recruited into airways, as the number of eosinophils was not significantly reduced. This result suggests that the number of activated eosinophils may not be related to the degree of baseline airway hyperresponsiveness.

Eosinophilia is the hallmark of asthma exacerbation. Budesonide treatment blunted the allergen-induced increases in the number of circulating eosinophils, and in the number of eosinophils in the airways. The number of eosinophils calculated per milliliter sputum, in part, depends on the density of cells present in the sputum. When statistical analyses were performed on the percentages of eosinophils the results were similar, showing that the increase in the number of eosinophils was not only due to a higher number of cells present per milliliter of induced sputum.

The allergen-induced increase of EG2-positive cells follows the same pattern as the number of total eosinophils measured after allergen-inhalation; however, the data are not directly comparable because not all of the prepared slides were available for immuno-cytochemical staining. The increase in the number of EG2-positive cells after allergen inhalation is likely due to the increase in eosinophil numbers rather than to increased activation of eosinophils, as the percentage of EG2-positive cells remained similar after allergen inhalation. However, the reduction in EG2-positive cells by budesonide treatment appears to be due to a reduction in eosinophil activation, as there was no significant reduction in the numbers of eosinophils after 7 d of treatment with budesonide.

The final dose of budesonide was given 12 h before allergen inhalation. The pharmacokinetics of budesonide suggest there would be very low plasma levels at this time (25); however, budesonide was still observed to reduce airway inflammation. This suggests that budesonide may be preventing initiation of a cascade of events caused by allergen inhalation that are important in causing eosinophil migration and activation and the late response and airway hyperresponsiveness.

Budesonide may have reduced the numbers of MCC in sputum after allergen inhalation. This has not been shown statistically in the present study because a high between-subject variability may have masked previously documented allergen-induced increases in MCC as well as the 2-fold reduction of MCC by budesonide. This reduction in MCC may be physiologically relevant, as the mast cell is involved in the immediate response to allergen, and through release of mediators, may help to drive the late response, which is associated with airway inflammation.

In conclusion, this study confirms previous observations that inhaled corticosteroids inhibit the early and the late asthmatic responses and the airway hyperresponsiveness after inhalation of allergen. In addition, the study has demonstrated that this is associated with attenuation of increases in blood and airway eosinophils and in the state of activation of the airway eosinophils. These results suggest that the mechanism of action of inhaled corticosteroids may be through their ability to inhibit the influx and activation of airway eosinophils.

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CHAPTER 4:

EFFECT OF REGULAR INHALED ALBUTEROL ON ALLERGEN-INDUCED LATE RESPONSES AND SPUTUM EOSINOPHILS IN ASTHMATIC SUBJECTS

Published in The American Journal of Respiratory and Critical Care Medicine in 1997

Gail Gauvreau's contribution:

Experimental design
Collection of clinical data
Processing of laboratory samples
Staining and quantification of cells
Analysis of data
Preparation of manuscript

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Gauvreau, G.M., M. Jordana, R.M. Watson, D.W. Cockcroft, P.M. O'Byrne. Effects of Inhaled Albuterol on Allergen-induced Late Responses and Sputum Eosinophils in Asthmatic Subjects. *Am. J. Respir. Crit. Care Med.* 1997; 156:1738-1745. Please note that I am co-author of this work.

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Effect of Regular Inhaled Albuterol on Allergen-Induced Late Responses and Sputum Eosinophils in Asthmatic Subjects

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Treatment with inhaled β_2-agonists immediately before allergen inhalation inhibits allergen-induced early, but not late asthmatic responses (LAR). By contrast, 2 wk treatment with inhaled albuterol increases airway responses to inhaled allergen. We examined the effects of regular albuterol treatment on allergen-induced increases in inflammatory cells in blood and induced sputum. Ten mild, stable allergic asthmatics inhaled albuterol (800 μg/day) or placebo for 7 d in a controlled, randomized, double-blind, crossover study. Allergen inhalation was performed 12 h after the final dose. Methacholine airway responsiveness and blood samples were analyzed before and 24 h after, and induced sputum was obtained before, 7 h and 24 h after allergen. Allergen significantly reduced methacholine PC_{20}, increased blood eosinophil numbers, and numbers of sputum neutrophils, EG2 positive and metachromatic cells ($p < 0.05$), without significant differences between treatments. Albuterol treatment significantly increased the LAR compared to placebo treatment ($p = 0.003$) and significantly enhanced the number of sputum eosinophils ($p = 0.009$) and sputum ECP ($p = 0.04$) at 7 h but not 24 h post-allergen ($p > 0.05$). We conclude that regular use of inhaled albuterol significantly increases the LAR to inhaled allergen, in association with an increase in the number of sputum eosinophils and the release of ECP, suggesting albuterol increases the late response by increasing eosinophil influx into the airways. **Gauvreau GM, Jordana M, Watson RM, Cockcroft DW, O'Byrne PM. Effect of regular inhaled albuterol on allergen-induced late responses and sputum eosinophils in asthmatic subjects.**

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Airway inflammation is an important characteristic in patients with current symptomatic asthma. This has been demonstrated by increased numbers of inflammatory cells, particularly eosinophils, mast cells and lymphocytes present in bronchial biopsies (1) bronchoalveolar lavage fluid (BAL) (2) and sputum (3) from asthmatics when compared to nonasthamtics. Allergen challenge is a valuable laboratory model for the study of the pathogenesis of airway inflammation in asthma. Allergen inhalation results in acute bronchoconstriction in sensitized subjects, and in 50–60% of adult subjects, this is followed by a late bronchoconstrictor response (LAR), which is associated with the development of allergen-induced airway hyperresponsiveness (4), and increases in the number of airway inflammatory cells, particularly eosinophils and metachromatic cells (MCC), in bronchial biopsies (5), BAL (6), and induced sputum (7, 8). Sputum induction is a safe, noninvasive method of directly obtaining repeated samples of airway secretions (3). Measurements of inflammatory cells from sputum are repeatable (9), demonstrate changes associated with airway responses after inhaled allergen (8), and has been shown to demonstrate the effects of a therapeutic intervention with inhaled corticosteroids (10).

Inhaled β_2-adrenoceptor agonists are the most effective bronchodilator agents for the symptomatic treatment of asthma. β_2-agonists reverse airway obstruction primarily by relaxing airway smooth muscle (11). In addition, β_2-agonists are potent inhibitors of the release of histamine and newly synthesized mediators from activated mast cells (12) *in vitro*, and β_2-adrenoceptors have been found on other immune cells including macrophages, eosinophils, neutrophils and lymphocytes, and may modulate adhesion of inflammatory cells through a cAMP-dependant kinase (13). Thus, these *in vitro* studies suggest that β_2-agonists may have some anti-inflammatory properties for the treatment of asthma.

However, recent studies have demonstrated that regular treatment with β_2-agonists increase airway responsiveness to nonallergic stimuli (14, 15), and enhance allergen-induced late bronchoconstrictor responses (16, 17). Regular treatment with albuterol is also associated with increased levels of activated eosinophils in bronchial biopsies (18). Given the association between allergen-induced airway responses and airway eosinophilia (3, 6, 7, 10), we hypothesized that regular treatment with inhaled β_2-agonist might also enhance the allergen-induced

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TABLE 1

SUBJECT CHARACTERISTICS, ALLERGEN DOSE, AND TREATMENT RANDOMIZATION

Subject Number	Age (yr)	Gender	FEV_1 (% predicted)	Methacholine PC_{20} (mg/ml)	Inhaled Allergen	Allergen Dilution	Order of Treatment	Rescue Medication
1	20	F	91.9	6.71	HDM	1:16	A/P	0/0
2	24	M	93.8	2.58	Cat	1:128	A/P	0/1
3	21	F	93.4	1.91	HDM	1:1,024	P/A	1/0
4	21	M	77.6	3.06	Ragweed	1:256	P/A	1/2
5	21	F	93.6	0.37	HDM	1:1,024	P/A	0/0
6	21	F	82.7	3.41	Ragweed	1:128	A/P	0/0
7	20	M	81.1	1.30	HDM	1:512	P/A	4/0
8	21	F	81.9	0.87	HDM	1:512	A/P	1/0
9	21	M	70.5	5.76	Grass	1:1,024	A/P	0/0
10	22	M	96.1	1.59	Grass	1:16	P/A	0/0

Definition of abbreviations: FEV_1 = forced expiratory volume in 1 second; methacholine PC_{20} = provocative concentration of methacholine for 20% reduction in FEV_1; F = female, M = male; HDM = house dust mite; P = placebo treatment; A = albuterol treatment; rescue medication = number of occasions during A or P treatment when two puffs ipratropium bromide was inhaled to relieve bronchoconstriction.

airway eosinophilia. Therefore, the purpose of this study was to determine whether regular treatment with the inhaled β_2-agonist, albuterol, administered for 1 wk, a duration of treatment known to enhance the allergen-induced late bronchoconstrictor responses (17), influences allergen-induced changes in inflammatory cells, particularly eosinophils, in blood and in the airways as assessed by changes in induced sputum.

METHODS

Subjects

Fourteen nonsmoking subjects (Table 1) with mild atopic asthma (eight female, six male), were selected for the study because of a previously documented allergen-induced early and late bronchoconstrictor response of at least 15% reduction in the forced expiratory volume in 1 s (FEV_1) during a screening period, and gave signed consent to participate in the study. Inhalation challenge with the allergen diluent, 0.9% saline, was completed during the screening period in order to correct the allergen-induced fall in FEV_1 for normal airflow variability during the allergen challenge day. Four of the subjects dropped out of the study due to protocol violations. Two of these subjects were unable to inhale the same three doses of allergen at all three allergen inhalation challenges, because of marked bronchoconstriction at the lowest inhaled dose of allergen during the albuterol treatment period. Two subjects were excluded from the study because their diary cards indicated they had used albuterol instead of the medication provided as a rescue medication during the study. The statistical analysis was performed on results from the remaining 10 subjects. This sample size was considered sufficient, as a previous study has shown that eight or more subjects can demonstrate a 50% change in the LAR with a power of > 90%, using the same methodology employed in this study (19). The study was approved by the Ethics Committee of McMaster University Health Sciences Center. Subjects were not exposed to sensitizing allergens and did not have asthma exacerbations or respiratory tract infections for at least four weeks prior to entering the study. All subjects had stable asthma with FEV_1 greater than 70% of predicted normal on all study days before allergen inhalation. Subjects used no regular medication other than infrequent (< twice weekly) inhaled β_2-agonist as required to treat their symptoms. Ipratropium bromide replaced β_2-agonists as a rescue medication during the study period. All medications were withheld for at least 8 h before each visit, and subjects were instructed to refrain from rigorous exercise, tea, or coffee in the morning before visits to the laboratory.

Study Design

The study was carried out with a double blind, placebo-controlled, randomized, two-period cross-over design (Figure 1). During each period, subjects were treated with either inhaled albuterol 200 μg (2 puffs) four times daily (Ventolin; Glaxo Canada, Toronto, Ontario), or an identical placebo 2 puffs four times daily for 1 wk. Each treatment period consisted of four visits to the laboratory. Baseline measurements of FEV_1, the provocative concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}), blood and induced sputum differential and total cell counts were determined before treatment and on the 7th day of treatment with albuterol or placebo. Allergen challenges were carried out the following morning, 12 h after treatment was discontinued, and FEV_1 was measured for the next 7 h. Sputum samples were obtained during the late response, 7 h after allergen inhalation, 19 h after treatment was discontinued. Sputum could not be induced earlier than 7 h, because of the requirement of pretreatment with inhaled β_2-agonists before sputum induction, which would interfere with subsequent measures of allergen-induced airway responses. Blood was not obtained 7 h after allergen because allergen-induced eosinophilia has not been shown to occur at this time after allergen inhalation (20). Methacholine PC_{20}, blood and sputum samples were obtained 24 h post-allergen, 36 h after treatment was discontinued. Each treatment period was separated by a washout period of at least 3 wk.

Laboratory Procedures

Methacholine inhalation test. Methacholine inhalation challenge was performed as described by Cockcroft (21). Subjects inhaled normal saline, then doubling concentrations of methacholine phosphate from a Wright nebulizer for 2 min. FEV_1 was measured at 30, 90, 180, and 300 s after each inhalation. Spirometry was measured with a Collins water-sealed spirometer and kymograph. The test was terminated when a fall in FEV_1 of 20% of the baseline value occurred, and the methacholine PC_{20} was calculated.

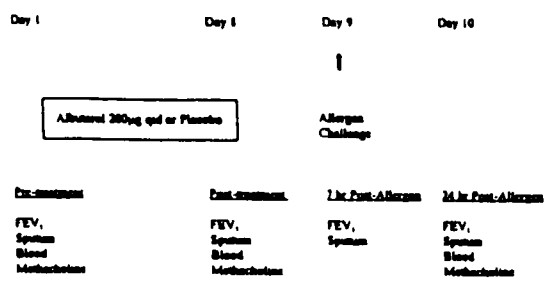

Figure 1. Study design.

Allergen Inhalation Test

Allergen challenge was performed as described by O'Byrne and coworkers (22). The allergen producing the largest skin wheal diameter was diluted in normal saline. The concentration of allergen extract for inhalation was determined from a formula described by Cockcroft and coworkers (23) using the results from the skin test and the methacholine PC_{20}. The starting concentration of allergen extract for inhalation was two doubling concentrations below that predicted to cause a 20% fall in FEV_1. The same doses of allergen were administered during each treatment period, and the FEV_1 was measured at 10, 20, 30, 40, 50, 60, 90, and 120 min post allergen inhalation, then each hour until 7 h after allergen inhalation. The early bronchoconstrictor response was taken to be the largest fall in FEV_1 within 2 h after allergen inhalation, and the late response was taken to be the largest fall in FEV_1 between 3 h and 7 h after allergen inhalation. The area under the curve was determined during the early (0–2 h) and late (3–7 h) response by plotting the response using graphics software (Fig P., Fig P Software Corporation, Durham, NC), which calculated the area of the FEV_1-time response. All allergen challenges were performed with the same allergen dose utilized during the screening challenge.

Differential blood counts. Blood was collected into heparinized tubes by direct venipuncture, and blood smears were made for differential staining (Diff Quik; American Scientific Products, McGaw Park, IL). Differential cell counts were obtained from the mean of two slides with 300 cells counted per slide. Total leukocyte count was determined using a hemocytometer (Neubauer Chamber; Hausser Scientific, Blue Bell, PA), and cell populations were expressed as the number per ml blood by dividing by the total number of cells counted, and multiplying by the total leukocyte count.

Sputum analysis. Sputum was induced and processed using the method described by Popov and coworkers (24). Subjects inhaled 3%, 4%, then 5% saline for 7 min each. The induction was stopped when an adequate sample was obtained, or if the FEV_1 dropped 20% from baseline. Cell plugs with little or no squamous epithelial cells were selected from the sample using an inverted microscope, separated from saliva, and weighed. Samples were aspirated in two times their volume of 0.1% dithiothreitol (Sputolysin, Calbiochem Corp., San Diego, CA) and two times their volume of Dulbecco's phosphate buffered saline (Gibco, Grand Island, NY). The cell suspension was filtered through a 52 μm nylon gauze (BNSH Thompson, Scarborough, ON, Canada) to remove debris, then centrifuged at 1,500 rpm for 10 minutes. Supernatants were collected and stored at −70° C for fluid phase measurements of eosinophil cationic protein (ECP) by radioimmunoassay (Pharmacia, Uppsala, Sweden). The total cell count was determined using a hemocytometer (Neubauer Chamber) and expressed as the number of cells per ml sputum. Cells were resuspended in Dulbecco's phosphate buffered saline (DPBS) at 0.75–1.0 × 10^6/ml. Cytospins were prepared on glass slides using 50 μl of cell suspension and a Shandon III cytocentrifuge (Shandon Southern Instruments, Sewickly, PA), at 300 rpm for 5 min. Differential cell counts were obtained from the mean of two slides with 400 cells counted per slide stained with Diff Quik. The same observer counted all study slides, and the reproducibility of the cell counts using these methods is excellent (9). For example, the interclass correlation coefficient is 0.97 for sputum eosinophils. Metachromatic cell (MCC) (mast cells and basophils) counts on slides stained with toluidine blue, were obtained from the mean of two slides with 5,000 cells observed on each slide. Cell types were enumerated by dividing by the total number of cells counted, and multiplying by the total cell count per milliliter of sputum. If possible, cytospins were also prepared on apex coated slides and fixed for 10 min in periodate-lysine-paraformaldehyde for immunocytochemical staining for ECP. Slides were stained with a mouse monoclonal antihuman antibody directed against cleaved ECP (EG2) (Kabi Pharmacia, Uppsala, Sweden) which was diluted in 1.0% BSA (Sigma Chemical Co.) and wash buffer made up of DPBS, 0.01 M HEPES buffer (Boehringer Mannheim Canada Ltd., Burlington, ON) and 0.01% saponin (Sigma Chemical Co.), and were incubated overnight at a concentration of 1 μg/ml. Labeling of EG2 was detected by the alkaline phospatase anti-alkaline phosphatase method (25). Mouse IgG1 (Sigma Chemical Co.) was used as a negative control. The percentage of EG2 positive cells was determined from a count of 500 cells under light microscopy, and were expressed as the number of EG2 positive cells per ml of sputum. These slides were also double stained with 10 μg/ml FITC for 10 min,

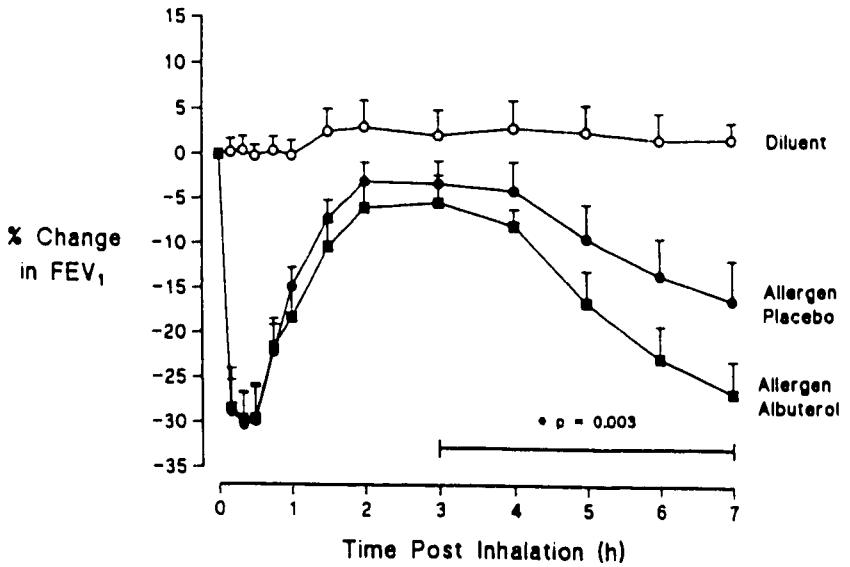

Figure 2. Allergen-induced airway responses. Percent change in FEV_1 (mean and SEM) after inhalation of diluent (*open circles*), allergen with albuterol treatment (*closed squares*), and allergen with placebo treatment (*closed circles*). The late (3–7 h) asthmatic response to allergen was significantly enhanced with albuterol ($p = 0.003$).

TABLE 2

ALLERGEN-INDUCED EARLY AND LATE AIRWAY RESPONSES, AND METHACHOLINE RESPONSIVENESS AFTER 7 d REGULAR ALBUTEROL TREATMENT VERSUS PLACEBO

	Placebo	Albuterol	p Value
Early response, maximum fall in FEV_1, %	−34.3 ± 3.7	−32.2 ± 3.8	0.53
Early response, area under the curve	63.2 ± 10.4	79.3 ± 9.7	0.11
Late response, maximum fall in FEV_1, %	−18.2 ± 3.6	−28.3 ± 3.1	0.003
Late response, area under the curve	46.1 ± 15.9	72.8 ± 9.3	0.04
PC_{20}, log difference pre-post allergen, mg/ml	0.50	0.54	0.73

Definition of abbreviations: FEV_1 = forced expiratory volume in 1 second, PC_{20} = provocative concentration of methacholine for 20% reduction in FEV_1.

Early and late responses are shown as the mean ± SEM and methacholine PC_{20} is shown as the geometric mean ± GSEM.

which is a specific stain for eosinophils (26–28). The level of eosinophil activation was determined by examination of 100 fluorescent cells for appearance of immunolocalization of the EG2 antibody, and expressed as the number of EG2 positive/100 eosinophils.

Statistical Analysis

Methacholine PC_{20} measurements are made by linear interpolation of log dose response curves resulting in logarithmic values for PC_{20}, which are then subjected to statistical analysis. Summary statistics for methacholine PC_{20} are expressed as geometric mean and geometric standard error of the mean (GSEM). All other summary statistics are expressed as mean and SEM. Two-tailed Students *t* test for paired observations was used to compare the early and late airway responses to allergen. The effects of 7 d treatment with placebo and albuterol treatment on airway and blood cellular and log transformed sputum cellular changes were analyzed, using a one-way repeated measured ANOVA, for the effects of treatment. The effects of placebo and albuterol treatment on allergen-induced airway and blood cellular and log transformed sputum cellular changes from post treatment baseline, were analyzed using a two-factor repeated measures ANOVA, for the effects of time and treatment (29). As not all subjects had an adequate number of sputum slides for EG2 staining, complete sets of slides were only available from six subjects to compare the effects of 7 d albuterol treatment on baseline activated eosinophils, and from three subjects to compare the effects of albuterol in allergen-induced increases in activated eosinophils. All sputum cellular data were log transformed prior to analysis.

RESULTS

The allergen-induced late bronchoconstrictor response was significantly enhanced after albuterol treatment when com-

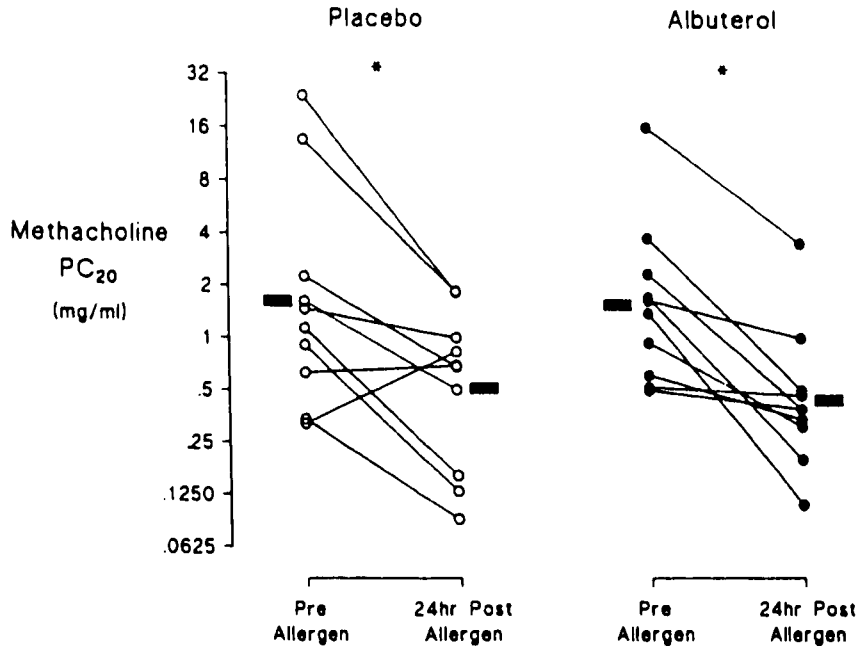

Figure 3. Allergen-induced methacholine hyperresponsiveness. Individual and geometric mean methacholine PC_{20} (*solid bars*) values before treatment, after 7 d of 800 μg/day albuterol (*solid circles*) or placebo (*open circles*), and 24 h after allergen inhalation. Allergen-induced methacholine airway hyperresponsiveness was not significantly different between albuterol and placebo treatments. *Significant allergen-induced change from post-treatment baseline.

TABLE 3

THE EFFECT OF ALBUTEROL ON ALLERGEN-INDUCED CHANGES IN SPUTUM MEASURED 7 AND 24 HOURS AFTER ALLERGEN INHALATION

	Pre-treatment		Post-treatment		7 h Post-allergen		24 h Post-allergen	
	Placebo	Albuterol	Placebo	Albuterol	Placebo	Albuterol	Placebo	Albuterol
Sputum								
Eosinophils, × 10^4/ml sputum	12 ± 5	14 ± 5	12 ± 3	8 ± 2	91 ± 62*	220 ± 156*†	166 ± 78*	75 ± 22*
Neutrophils, × 10^4/ml sputum	88 ± 21	167 ± 105	214 ± 77	124 ± 50	264 ± 66*	373 ± 106*	146 ± 32*	242 ± 105*
MCC, × 10^3/ml sputum	1.1 ± 0.5	1.1 ± 0.5	4.7 ± 2.3	1.4 ± 0.6	9.0 ± 5.0*	6.8 ± 2.0*	17.5 ± 7.3	4.8 ± 1.9*
ECP, μg/ml supernatant	0.4 ± 0.1	0.8 ± 0.2	1.1 ± 0.6	0.8 ± 0.4	1.8 ± 0.6*	4.3 ± 1.8*†	8.1 ± 3.5*	5.1 ± 2.3*
EG2 positive, eosinophils/100	86.7 ± 7.9	64.6 ± 17.9	81.1 ± 13.4	57.2 ± 13.6	60.0 ± 17.0	48.2 ± 10.8	67.7 ± 14.1	57.5 ± 18.5
Blood								
Eosinophils, × 10^4/ml	36 ± 6	37 ± 8	39 ± 7	35 ± 7			58 ± 10*	56 ± 6*
Neutrophils, × 10^4/ml	452 ± 47	417 ± 48	412 ± 35	394 ± 36			421 ± 46	474 ± 51

Values shown are the mean ± SEM

* $p < 0.05$ significant difference between pre and post-allergen inhalation

† $p < 0.05$ significant difference between placebo and albuterol

pared to placebo (Figure 2). The maximal % fall in FEV_1 during the late response was 28.3 ± 3.1% after albuterol treatment and 18.2 ± 3.6% after placebo treatment ($p = 0.003$) and the area under the curve was also significantly increased ($p = 0.04$) (Table 2). The early bronchoconstrictor response was not significantly changed after albuterol treatment versus placebo (Figure 2). The maximal % fall in FEV_1 during the early response was 32.2 ± 3.8% after albuterol treatment and 34.2 ± 3.7% after placebo treatment (Table 2). Rescue medication with ipratropium bromide was rarely required during either of the treatment periods (Table 1).

The methacholine PC_{20} decreased significantly 24 h after allergen inhalation after albuterol treatment from 1.55 mg/ml (GSEM 1.37) to 0.44 mg/ml (GSEM 1.32) ($p = 0.002$) and after placebo treatment from 1.62 mg/ml (GSEM 1.54) to 0.51 mg/ml (GSEM 1.38) ($p = 0.002$) (Figure 3), however, there

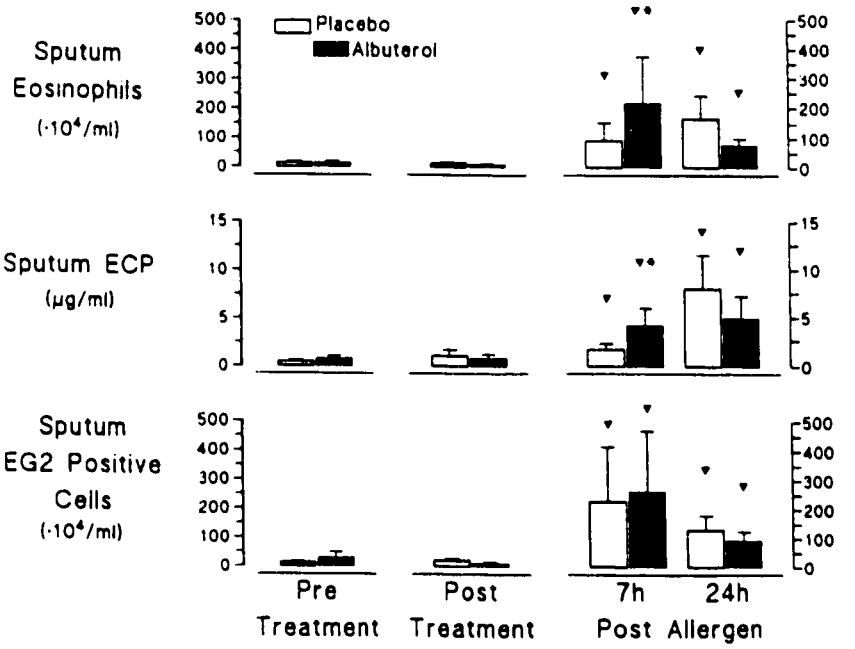

Figure 4. Allergen-induced increase of eosinophils, activated eosinophils, and eosinophil cationic protein in induced sputum. Number of sputum eosinophils (*top panel*), sputum fluid phase ECP (*middle panel*), and activated eosinophils (*bottom panel*) before treatment, after 7 d of 800 μg/day albuterol (*solid bars*) or placebo (*open bars*), and at 7 and 24 h after allergen inhalation. †Significant allergen-induced change from post-treatment baseline. *Significant allergen-induced difference between albuterol and placebo treatments.

was no difference in the magnitude of the allergen-induced increase in airway responsiveness between albuterol and placebo treatments (Table 2).

Allergen inhalation increased the number of eosinophils per milliliter in sputum both at 7 h after allergen, during the late bronchoconstrictor response, and 24 h after allergen when methacholine airways responsiveness was increased ($p = 0.01$). Albuterol treatment, however, significantly enhanced the numbers of eosinophils per milliliter in sputum 7 h after allergen to $220 \pm 156 \times 10^4$/ml when compared to $91 \pm 62 \times 10^4$/ml after placebo treatment ($p = 0.009$) (Figure 4). This difference was no longer present in sputum 24 h after allergen, the number of eosinophils being $75 \pm 22 \times 10^4$/ml after albuterol treatment and $166 \pm 78 \times 10^4$/ml after placebo treatment ($p = 0.31$) (Table 3, Figure 4). The absolute change in the number of allergen-induced eosinophils at 7 h was greater after albuterol treatment in nine subjects, as compared with the change after placebo treatment, and decreased in one subject (Figure 5).

The concentration of ECP increased significantly after allergen inhalation from 1.1 ± 0.6 μg/ml during placebo to 1.8 ± 0.6 μg/ml at 7 h and 8.1 ± 3.5 μg/ml at 24 h after allergen ($p = 0.00001$). During albuterol treatment, the concentration of fluid phase sputum ECP increased from 0.8 ± 0.4 μg/ml to 4.3 ± 1.8 μg/ml at 7 h and 5.1 ± 2.3 μg/ml at 24 h after allergen (Table 3, Figure 4). As with the number of sputum eosinophils, the allergen-induced increase in the concentration of sputum ECP was significantly enhanced by albuterol at 7 h after allergen inhalation ($p = 0.045$). The allergen-induced changes in the number of eosinophils was correlated with the concentration of fluid phase ECP ($p = 0.00004$, $r = 0.62$). The number of EG2 positive eosinophils significantly increased after allergen inhalation during placebo, from $18 \pm 7 \times 10^4$/ml to $223 \pm 188 \times 10^4$/ml at 7 h and $127 \pm 50 \times 10^4$/ml at 24 h after allergen, and during albuterol treatment, from $10 \pm 5 \times 10^4$/ml to $258 \pm 207 \times 10^4$/ml at 7 h and $91 \pm 32 \times 10^4$/ml at 24 h after allergen ($p = 0.004$) (Table 3, Figure 4). However, we did not observe a significant effect of albuterol treatment on allergen-induced increases in EG2 positive cells ($p = 0.29$).

There was a significant correlation between the magnitude of the LAR (expressed as area under the curve) and the increase in sputum fluid phase ECP ($r = 0.67$, $p = 0.002$), and between the maximal fall in FEV_1 and the increase in sputum ECP ($r = 0.56$, $p = 0.015$). There was no significant correlation between the allergen-induced increase in the number of sputum eosinophils and the magnitude of the LAR ($r = 0.18$), or maximal fall in FEV_1 ($r = 0.18$).

Allergen inhalation also increased the numbers of sputum neutrophils ($p = 0.04$) and the numbers of MCC ($p = 0.02$) measured 7 and 24 h after allergen inhalation, however there was no significant difference between albuterol and placebo treatments either at 7 or 24 h (Table 3).

The number of blood eosinophils increased significantly 24 h after allergen inhalation after albuterol treatment from $35.1 \pm 7.5 \times 10^4$/ml to $56.1 \pm 6.4 \times 10^4$/ml ($p = 0.001$) and after placebo treatment from $38.9 \pm 7.4 \times 10^4$/ml to $57.7 \pm 9.5 \times 10^4$/ml ($p = 0.001$). This increase was the same magnitude for placebo and albuterol treatment periods (Table 3). The number of blood neutrophils measured 24 h after allergen inhalation did not change significantly after 7 d of albuterol treatment, and there was no difference in allergen-induced changes between albuterol and placebo (Table 3).

Regular use of albuterol for 7 d did not significantly alter the baseline FEV_1, being 3.3 ± 0.2 L after albuterol treatment and 3.5 ± 0.2 L after placebo treatment, or the baseline methacholine PC_{20}, being 1.55 mg/ml (GSEM 1.37) after albuterol treatment, and 1.62 mg/ml (GSEM 1.54) after placebo. Also, regular albuterol treatment for 7 d did not significantly change the numbers of baseline blood eosinophils or neutrophils, nor

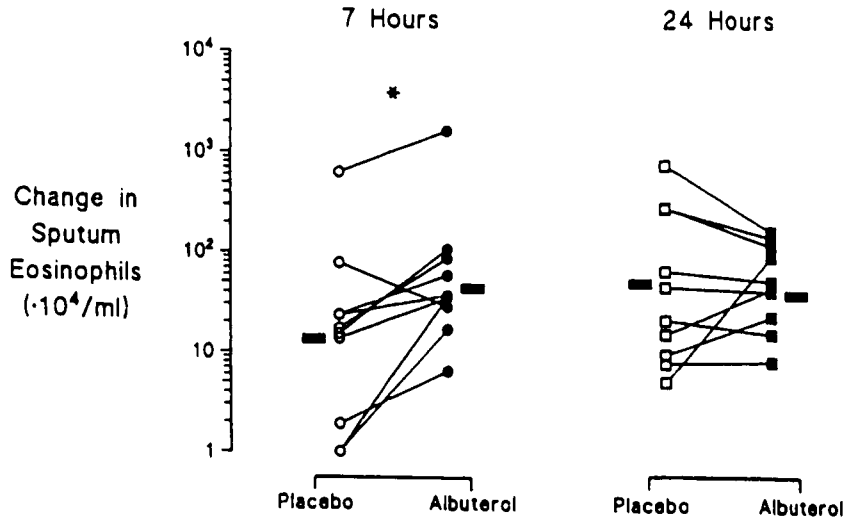

Figure 5. Allergen-induced change in sputum eosinophil numbers. The allergen-induced changes (post-allergen minus pre-allergen) in the number of sputum eosinophils (*circles*) after regular albuterol treatment for 7 d (*closed symbols*) or placebo (*open symbols*) and 7 h (*circles*) and 24 h (*squares*) after allergen inhalation. The geometric means are also shown (*closed rectangles*). As the difference from baseline was negative in two subjects on placebo treatment at 7 h after allergen inhalation, zero was used to complete the figure. *Significant allergen-induced difference between albuterol and placebo treatments.

the number of sputum inflammatory cells; eosinophils or activated eosinophils, MCC or neutrophils, nor the concentration of ECP measured in sputum (Table 3).

DISCUSSION

This study confirms previous reports which have demonstrated that regular treatment with inhaled albuterol increases allergen-induced late responses (16, 17). Use of ipratropium bromide as a rescue medication was infrequent and similar during both treatment periods, and should not bias the results. The allergen-induced late response is associated with airway inflammation, particularly increases in airway eosinophils and MCC (8). We have previously demonstrated that the number of eosinophils and the number of EG2 positive cells in sputum increase by 7 h after allergen inhalation, and remain elevated at 24 h (10). We therefore hypothesised that the increase in allergen-induced late responses may be caused by albuterol-induced increases in allergen-induced airway inflammation, as measured by changes in induced sputum.

Consistent with this hypothesis, albuterol enhanced allergen-induced sputum eosinophils and ECP at 7 h after allergen inhalation, during the late response, but there were no significant increases in the number of sputum neutrophils or MCC. This suggests the albuterol-enhanced late response may be associated with increased numbers of eosinophils rather than neutrophils or MCC, and is supported by the observation that sputum ECP levels in the present study, and sputum eosinophils in other studies of patients with asthma (30, 31) correlate with parameters of airflow obstruction.

We did not confirm the observations of Maestrelli and coworkers (31), who demonstrated a significant correlation between the magnitude of bronchoconstriction after isocyanate inhalation and the increase in sputum eosinophil counts. It is possible that this lack of correlation is because of different mechanisms involved. The lack of correlation is more likely due to large intersubject variability in the magnitude of allergen-induced eosinophilia, which has previously been described (8), and suggests the need for a larger subject sample size to further investigate the relationship between airway responses and airway eosinophilia. However, the level of ECP in sputum correlated well with airway responses, supporting the relationship between eosinophil activation and airflow obstruction.

The allergen inhalation was started 12 h after the last dose of albuterol, which is longer than its duration of pharmacological action. This suggests that the increases in allergen-induced late responses and eosinophil influx during the late response are a consequence of events in the airways which persist for 8–12 h after the pharmacological effects of albuterol are over. In addition, there was no difference in allergen-induced airway hyperresponsiveness measured at 24 h, between albuterol and placebo treatments, which was 36 h after the last dose of albuterol. This suggests that the untoward effects of albuterol on allergen-induced responses are short-lived. We did not observe a difference in blood or sputum eosinophils measured 24 h after allergen inhalation, suggesting albuterol may locally enhance allergen-induced eosinophilia in the airways, 7 h after allergen-inhalation, by altering the kinetics of eosinophil influx across the airway. The time course of the changes in allergen-induced airway eosinophils after albuterol treatment also suggests that the rate of trafficking of eosinophils through the airway may be increased. Thus, regular treatment with albuterol may lead to a faster onset of the appearance of eosinophils in the airways, as well as a faster resolution, as sputum eosinophils were slightly but not statistically lower 24 h following allergen, without altering the trafficking of other inflammatory cells. However, there is no direct evidence, as yet, to support any of these hypotheses.

Measurement of induced sputum is a sensitive indicator of eosinophilic airway inflammation, and it can be used to distinguish between different types of airway inflammation (32). Using induced sputum, we have recently demonstrated that treatment with inhaled steroid therapy for 7 d caused a significant reduction of sputum eosinophils and EG2 positive cells (activated eosinophils) (10) associated with complete attenuation of the late response and significant reduction of allergen-induced airway hyperresponsiveness. In the present study, we also found an allergen-induced increase in both the number of eosinophils and the number of activated eosinophils. Quantification of eosinophils dual stained with FITC and EG2 demonstrated that the ratio of eosinophils localizing EG2 did not change after allergen inhalation (Table 3). This indicates that the allergen-induced increase in activated eosinophils into sputum is due to an influx of eosinophils into the airways rather than an increased level of activation of airway eosinophils. These results support our earlier observations with allergen-induced eosinophilia (10). Furthermore, albuterol may be enhancing the influx of eosinophils into the airway without affecting their level of activation.

The mechanism of the albuterol-induced increases in allergen-induced airway eosinophils was not investigated in this study. Short term treatment with albuterol has been reported to down-regulate pulmonary β_2-receptors *in vivo* in humans (33). Down-regulation of β_2-receptors on immune cells may render these cells more easily activated to participate in airways inflammation due to overexpression of adhesion molecules.

Finally, it is also possible that the enhanced late bronchoconstrictor response with albuterol treatment is not only associated with increased influx of eosinophils into the airways, but also may be associated with tolerance to the broncho-protective effect of the inhaled β_2-agonist (34). Desensitization of β_2-adrenoceptor-induced cAMP formation has been observed in cultured airway smooth muscle cells (36), and this response *in vivo* may render subjects less able to respond to endogenous catecholamines.

In this study, one week regular treatment with albuterol did not have an effect on baseline FEV_1, methacholine PC_{20}, allergen-induced early responses, or baseline numbers of inflammatory cells measured in peripheral blood or sputum. Studies with longer term regular treatment of β_2-agonist have shown increased airway hyperresponsiveness to nonallergic stimuli (14, 15), and increased EG2 positive cells in bronchial biopsies (18). It may require a similar length of albuterol treatment to demonstrate increased indices of inflammation in peripheral blood or sputum in this group of mild asthmatics.

The clinical consequences of the effect of regular albuterol use on allergen-induced airway responses and inflammation are not known. However, it is a concern that one reason for asthmatics to increase their usage of inhaled β_2-agonists is during allergen exposure, when asthma symptoms increase. Whether or not this repeated use of an inhaled β_2-agonist during regular and repeated allergen inhalation, rather than the single, high dose allergen inhalation used in this study to elicit late responses, further enhances allergen-induced responses and eosinophil influx requires further study.

In conclusion, this study confirms previous observations that regular treatment with inhaled β_2-agonist enhances the allergen-induced late bronchoconstrictor response. In addition, this study demonstrates an enhancement of allergen-induced eosinophil influx into the airways with regular albuterol treat-

ment, suggesting that albuterol enhances the late bronchoconstrictor response to inhaled allergen by increasing the rate of influx of airway eosinophils.

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CHAPTER 5:

PROTECTIVE EFFECTS OF INHALED PGE_2 ON ALLERGEN-INDUCED AIRWAY RESPONSES AND AIRWAY INFLAMMATION

Abstract

Inhalation of prostaglandin E_2 (PGE_2) has been reported to prevent allergen-induced bronchoconstrictor responses, however, alterations of PGE_2 levels in the lung may have beneficial or deleterious effects on airway function. This study compared the effects of inhaled PGE_2, administered at different times, on allergen-induced bronchoconstrictor responses and airway inflammation. Twenty mild asthmatics with a documented dual airway response to inhaled allergen were recruited into two separate double-blind randomized cross-over studies which differed only in the time between the drug inhalation (100µg) delivered 7 minutes (immediate) and 19 minutes (delayed) before allergen challenge. FEV_1 was measured for 7 hours. Sputum was sampled at baseline, 7 and 24 hours following challenge. Following the immediate challenge, PGE_2 attenuated the early (10.3±2.5% vs 24.4±3.6%; p.002) and late (12.6±3.6% vs 21.2±2.7%; p=0.03) maximal fall in FEV_1, airway hyperresponsiveness, (1.5mg/ml (GSEM 1.4) vs 1.0mg/ml (GSEM 1.4) p=0.03) and sputum eosinophils (21.0±7.3% vs 36.3±8.8%; p=0.01). Following the delayed challenge, PGE_2 enhanced the maximal fall in FEV_1 (28.1±2.9% vs 20.7±3.9%; p=0.01) and sputum

neutrophils (47.4±6.1% vs 30.6±5.1%; p=0.01) during the late airway response with no difference in airway hyperresponsiveness or sputum eosinophils (p>0.05). These results indicate that there is a therapeutic window for administration of PGE_2, which may provide anti-inflammatory protection from allergen challenge. In contrast, inhalation of PGE_2 outside this window may, in fact, worsen the late airway response to allergen.

Introduction

Prostaglandin E_2 (PGE_2) is present in human airways as a cyclo-oxygenase product of airway epithelium (1) and airway smooth muscle (2), and demonstrates bronchoprotective effects in patients with bronchial asthma. PGE_2 has been shown to protect against exercise-induced (3), allergen-induced (4,5), and aspirin-induced bronchoconstriction (6), as well as bronchoconstrictor agents such as metabisulfite, methacholine and histamine (7, 8, 9). PGE_2 may represent an endogenous protective mechanism for airways, however, alterations of PGE_2 levels in the lung may have beneficial or deleterious effects on airway function (10).

Airway inflammation is an important characteristic in patients with current symptomatic asthma. This has been demonstrated by increased numbers of inflammatory cells in bronchial biopsies and lavage, and in sputum (11) from asthmatics when compared to non-asthmatics. PGE_2 is thought to have inhibitory effects on most inflammatory cells, including antigen-induced mediator release from mast cells (12), and eosinophil degranulation (13) chemotaxis, and cytokine-stimulated survival (14).

Allergen challenge is a valuable laboratory model for the study of the pathogenesis of airway inflammation in asthma. Allergen inhalation results in acute bronchoconstriction in sensitized subjects, and in 50-60% of adult subjects, this is followed by a late bronchoconstrictor response (LAR). The LAR is associated with the development of allergen-induced airway hyperresponsiveness (15) and increases in the number of airway inflammatory cells, particularly eosinophils and metachromatic cells (MCC), in induced sputum (16,17). Sputum induction is a safe, non-invasive method of directly obtaining repeated samples of airway secretions (11). Measurement of inflammatory cells from sputum is repeatable (18,19), reflects airway responses to inhaled allergen (17), and has been shown to demonstrate the effects of therapeutic intervention (20, 21).

Inhaled PGE_2 has been demonstrated to prevent early and late allergen-induced bronchoconstrictor responses when given immediately before allergen inhalation challenge (4, 5). We postulated that PGE_2 may exert bronchoprotective effects on allergen-induced bronchoconstriction by inhibiting airway inflammation, which may be assessed by measurements of inflammatory cells in induced sputum. This study examines the anti-inflammatory role of PGE_2 inhaled before allergen challenge, and the therapeutic window for the anti-inflammatory effects of PGE_2.

Methods

Subjects

Twenty non-smoking subjects (Table 5.1) with mild atopic asthma (8 female, 12 male), were selected for the study because of a previously documented allergen-induced early and late bronchoconstrictor response, and gave signed consent to participate in the study. The study was approved by the Ethics Committee of the McMaster University Health Sciences Center. Subjects were not exposed to sensitizing allergens and did not have asthma exacerbations or respiratory tract infections for at least four weeks prior to entering the study. All subjects had stable asthma with a forced expiratory volume in one second (FEV_1) greater than 70% of predicted normal on all study days before allergen inhalation. Subjects used no medication other than inhaled β_2-agonist as required to treat their symptoms. Medication was withheld for at least 8 hours before each visit, and subjects were instructed to refrain from rigorous exercise, tea or coffee in the morning before visits to the laboratory. The dose of allergen that elicited a late response during the screening challenge was used on subsequent allergen challenges. Four of the subjects dropped out of the delayed allergen challenge due to protocol violations. Of these subjects, 3 did not complete the second treatment period, and 1 did not develop a late response during either treatment period.

Study Design

The study was carried out with a double-blind, placebo-controlled, randomized, cross-over design. Each subject completed two treatment periods with inhalation of either

100 μg PGE_2 (Sigma Chemical Co, St. Louis, MO) diluted in 0.05 ml 100% ethanol and 4.95 ml physiological saline, or placebo consisting of 0.05 ml 100% ethanol and 4.95 ml physiological saline. Each treatment period consisted of 3 visits to the laboratory. Baseline measurements of FEV_1, the provocative concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}), blood and induced sputum differential and total cell counts were determined one day before allergen challenge. Drug or placebo treatment and allergen challenges were carried out the following morning. Spirometry was measured after allergen inhalation until 7 hours, and sputum samples were obtained at this time. Sputum could not be induced earlier than 7 hours because inhalation of hypertonic saline can cause bronchoconstriction which would interfere with measures of allergen-induced airway responses. Methacholine PC_{20}, blood and sputum samples were obtained 24 hours post-allergen. Each treatment period was separated by a washout period of at least 3 weeks.

PGE_2 and Placebo Inhalation

Stock solution of PGE_2 was prepared at a concentration of 2 mg/ml by dissolving 10 mg PGE_2 (Sigma Chemical Co, St. Louis, MO) in 5.0 ml of 100% ethanol. This solution was stored at -70°C. Immediately before use, 0.50 ml of stock solution (equivalent to 100 μg PGE_2) was added to 4.95 ml physiological saline. Subjects inhaled all of this solution by tidal breathing from a Fisoneb ultrasonic nebuliser (Canadian Medical Products, Ltd., Markham, Ontario, output 1 ml/min, aerodynamic mass median diameter 5.6 μm). FEV_1 was measured before and after inhalation of PGE_2 or placebo, and side effects were recorded.

The allergen inhalation challenge was started within 5 minutes, and was completed within 7 minutes following PGE_2 or placebo inhalation. This dose and timing of administration of PGE_2 has been previously described to significantly attenuate allergen-induced bronchoconstrictor responses (4).

In normal subjects, inhalation of PGE_2 causes initial bronchoconstriction followed by bronchodilation, which can be accompanied by transient cough, retrosternal soreness and airway secretions. To avoid unblinding of investigators, PGE_2 and placebo were inhaled in a closed room, with only one investigator present. This unblinded investigator was responsible only for preparation and administration of drug, and recording side effects, if any. Allergen challenges, measures of methacholine hyperresponsiveness and sputum inflammatory cells were carried out by investigators who remained blinded to the treatment.

Methacholine Inhalation Challenge

Methacholine inhalation challenge was performed as described by Cockcroft (22). Subjects inhaled normal saline, then doubling concentrations of methacholine phosphate from a Wright nebulizer for 2 minutes. FEV_1 was measured at 30, 90, 180 and 300 seconds after each inhalation. Spirometry was measured with a Collins water sealed spirometer and kymograph. The test was terminated when a fall in FEV_1 of 20% of the baseline value occurred, and the PC_{20} was calculated.

Allergen Inhalation Challenge

Immediate Challenge: Allergen inhalation challenge, with 8 subjects was performed

as described for the delayed challenge, using only the highest concentration of allergen utilized during the screening allergen challenge.

Delayed Challenge: Allergen challenge, with 8 subjects, was performed as described by O'Byrne et al (23). The allergen producing the largest skin wheal diameter was diluted in normal saline. The concentration of allergen extract for inhalation was determined from a formula described by Cockcroft et al (24) using the results from the skin test and the methacholine PC_{20}. The first concentration of allergen extract for inhalation was one doubling concentration below that causing at least a 15% late fall in FEV_1 during the screening allergen challenge. The first concentration of allergen was inhaled from a Wright nebuliser for 2 minutes, and the FEV_1 was measured at 10 minutes following allergen inhalation. If the FEV_1 dropped >40% from baseline, the challenge was discontinued. If the FEV_1 was similar to the screening value, the allergen challenge was continued. The second concentration of allergen was then inhaled for 2 minutes (Figure 5.1), and post-allergen FEV_1 was measured at 10, 20, 30, 40, 50, 60, 90 and 120 minutes following the second allergen inhalation, then each hour until 7 hours after allergen inhalation. The same inhalation protocol and allergen concentrations were administered for the placebo and PGE_2 treatment study period.

Spirometry

The early bronchoconstrictor response was taken to be the largest fall in FEV_1 within 2 hours after allergen inhalation, and the late response was taken to be the largest fall in FEV_1 between 3 and 7 hours after allergen inhalation. The area under the curve (AUC) was determined during the early and late response by plotting the response using graphics software (Fig P., Fig P Software Corporation, Durham, NC, USA), which calculated the area of the FEV_1 response.

Differential Blood Counts

Blood was collected into heparinized tubes by direct venipuncture, and blood smears were made for differential staining (Diff Quick; American Scientific Products, McGaw Park, IL). Differential cell counts were obtained from the mean of two slides with 400 cells counted per slide. Total cell count was determined using a haemocytometer (Neubauer Chamber, Hausser Scientific, Blue Bell, Pa), and cell populations were expressed as the number per ml blood by dividing by the total number of cells counted, and multiplying by the total cell count.

Sputum Analysis

Sputum was induced and processed using the method described by Popov et al (18). Subjects inhaled 3%, 4% then 5% saline for 10 minutes each. The induction was stopped when an adequate sample was obtained, or if the FEV_1 dropped 20% from baseline. Cell plugs with little or no squamous epithelial cells were selected from the sample using an

inverted microscope, separated from saliva, and weighed. Samples were aspirated in 4 times their volume of 0.1% dithiothreitol (Sputolysin, Calbiochem Corp. San Diego, CA, USA) and 4 times their volume of Dulbecco's phosphate buffered saline (DPBS, Gibco BRL, Life Technologies, Grand Island, NY). The cell suspension was filtered through a 52 µm nylon gauze (BNSH Thompson, Scarborough, Ont., Canada) to remove debris, then centrifuged at 1500 rpm for 10 minutes. The total cell count was determined using a haemocytometer (Neubauer Chamber, Hausser Scientific, Blue Bell, Pa) and expressed as the number of cells per ml sputum. Cells were resuspended in DPBS at 0.75-1.0x10^6/ml. Cytospins were prepared on glass slides using 50 µl of cell suspension and a Shandon III Cytocentrifuge (Shandon Southern Instruments, Sewickly, PA, USA), at 300 rpm for 5 min. Differential cell counts were obtained from the mean of two slides with 400 cells counted per slide stained with Diff Quik. Metachromatic cell (mast cell and basophil) counts were obtained from slides stained with toluidine blue, from the mean of two slides with 5000 cells observed on each slide. Cytospins were also prepared on aptex coated slides and fixed in periodate-lysine-paraformaldehyde for immunocytochemical staining. Slides were stained for activated eosinophils using a mouse monoclonal anti-human antibody directed against cleaved eosinophil cationic protein (EG2) at 1 ug/ml (Kabi Pharmacia, Uppsula, Sweden). The EG2 antibody was diluted in 1.0% BSA (Sigma Chemical Co.) and wash buffer made up of DPBS, 0.01M HEPES buffer (Beohringer Manheim Canada Ltd) and 0.01% saponin (Sigma Chemical Co.), and slides were incubated with EG2 antibody overnight. Specific

binding of antibody was detected by the alkaline phosphatase anti-alkaline phosphatase method (25). Mouse IgG_1 (Sigma Chemical Co.) was used as a negative control. The percentage of cells positive for EG2 antibody was determined from a count of 500 cells under light microscopy.

Statistical Analysis

Measurements of FEV_1 are reported at body temperature and pressure saturated. All summary statistics are expressed as mean and standard error of the mean (SEM) with the exception of methacholine PC_{20} measurements which are measured by linear interpolation of log dose response curves resulting in logarithmic values for PC_{20}, and expressed as geometric mean and geometric standard error of the mean (GSEM) . Two-tailed Students t-test for paired observations was used to compare the early and late airway responses to allergen. The effects of placebo and PGE_2 treatment on baseline FEV_1, and on the allergen-induced change in methacholine PC_{20}, and blood and sputum inflammatory cells were analyzed using a 2 factor repeated measures ANOVA (26). Statistical analyses were performed using computer software (Statistica 4.5, Stat Soft Inc 1993).

Results

Airway Physiology

Immediate Challenge: There was no change in FEV_1 measured 5 minutes after inhaled placebo or PGE_2 ($p=0.41$), with the FEV_1 being 3.8 ± 0.1 L/sec before and 3.8 ± 0.2

L/sec after placebo, and 3.7 ± 0.1 L/sec before and 3.7 ± 0.2 L/sec after PGE_2. Inhaled PGE_2 significantly reduced the allergen-induced early response, measured as either the maximal fall in FEV_1, being 24.4 ± 3.6% after inhaled placebo and 10.3 ± 2.5% after inhaled PGE_2 (p=0.002) (Figure 2), or the AUC (p=0.008). Inhaled PGE_2 also significantly reduced the allergen-induced late response, measured as the maximal fall in FEV_1, being 21.2 ± 2.7% after inhaled placebo and 12.6 ± 3.6% after inhaled PGE_2. (p=0.03) (Figure 2). The methacholine PC_{20} decreased from 2.6 mg/ml (1.5 GSEM) before, to 1.0 mg/ml (1.4 GSEM) after allergen with placebo treatment (p=0.02), and this allergen-induced decrease of methacholine PC_{20} was significantly attenuated with PGE_2 treatment (p=0.03), being 1.6 mg/ml (1.3 GSEM) before, and 1.5mg/ml (1.4 GSEM) following challenge.

Delayed challenge: There was no change in FEV_1 measured 5 minutes after placebo or PGE_2 inhalation (p=0.44), with the FEV_1 being 3.4 ± 0.2 L/sec before and after placebo, and 3.5 ± 0.2 L/sec before and after PGE_2. Following the delayed allergen challenge, the allergen-induced early response was not different between treatments, as measured either by the maximal fall in FEV_1 being 26.9 ± 4.1% after inhaled placebo and 26.8 ± 3.4% after inhaled PGE_2 (p=0.98), or the AUC (p=0.30). However, the allergen-induced late response was significantly enhanced by inhaled PGE_2, as measured either by the maximal fall in FEV_1 being 20.7 ± 3.9% after inhaled placebo and 28.1 ± 2.9% after inhaled PGE_2 (p=0.01) (Figure 5.2), or the AUC (p=0.04). The allergen-induced change in methacholine PC_{20} was not different between treatment with placebo or drug, changing from 1.4 mg/ml (1.4 GSEM)

to 1.3 mg/ml (1.6 GSEM) after placebo and from 1.5 mg/ml (1.4 GSEM) to 0.9 mg/ml (1.5 GSEM) after PGE_2 (p=0.22).

Blood

Immediate Challenge: There was a significant allergen-induced increase in the number of circulating eosinophils from 27.8 ± 3.5 $x10^4$/ml to 42.5 ± 6.1 $x10^4$/ml (p=0.04), however, there was no effect of PGE_2 on the number of circulating eosinophils, being 37.8 ± 6.6 $x10^4$/ml before and 49.4 ± 7.8 $x10^4$/ml (p=0.67) 24 hours following allergen. There was no change in the number of circulating neutrophils following allergen with placebo or PGE_2 treatment (p=0.31).

Delayed Challenge: There was a significant increase in the number of circulating eosinophils from 36.4 ± 5.6 $x10^4$/ml to 53.3 ± 10.9 $x10^4$/ml 24 hours following allergen (p=0.05), however there was no significant effect of PGE_2 on this allergen-induced blood eosinophilia being 46.9 ± 10.6 $x10^4$/ml before and 87.5 ± 20.6 $x10^4$/ml following allergen (p=0.15). The number of allergen-induced circulating neutrophils increased from 364 ± 44 $x10^4$/ml to 444 ± 50 $x10^4$/ml with PGE_2 treatment, compared to a fall from 395 ± 36 $x10^4$/ml to 385 ± 43 $x10^4$/ml with placebo treatment 24 hours following allergen, however this difference did not reach statistical significance (p=0.07).

Sputum

Immediate Challenge: The number of cells retrieved per ml sputum following allergen was not statistically altered compared to baseline (p=0.07), nor was there an effect

of PGE_2 (p=0.38) (Table 5.2). There was an allergen-induced increase in the % eosinophils (p=0.045), % EG2-positive cells (p=0.03), and % neutrophils (p=0.02). The % MCC did not increase significantly following allergen (p=0.18). Treatment with PGE_2 significantly attenuated the allergen-induced increase in % eosinophils (p=0.01), % EG2-positive cells (p=0.02), and significantly reduced the % MCC following allergen (p=0.02) (Table 5.2, Figure 5.3).

Delayed Challenge: There was no significant effect of allergen or treatment on the total number of retrieved cells/ml sputum (p>0.06) (Table 5.2). There was a significant allergen-induced increase in the % sputum eosinophils (p<0.001) and % EG2-positive cells (p=0.02) which peaked at 24 hours following allergen (Table 5.2). There was no significant change in the % MCC (p=0.06) or % neutrophils (p=0.08) following allergen. Treatment with inhaled PGE_2 did not significantly change the allergen-induced increases in sputum % eosinophils (p=0.31), or % EG2-positive cells (p=0.31). There was, however, a significantly higher % sputum neutrophils with PGE_2 compared to placebo treatment at 7 hours following allergen (p=0.03) (Table 5.2).

Discussion

These results demonstrate that inhalation of PGE_2 immediately before allergen challenge suppresses allergen-induced airway inflammation in sputum, as indicated by a significant attenuation of allergen-induced increases in sputum eosinophils, EG2-positive

cells, and MCC up to 24 hours following challenge. This is the first time a non-steroidal agent has been shown to have anti-inflammatory properties in humans using inhaled allergen to induce airway inflammation.

Allergen inhalation by atopic asthmatics results in a sputum eosinophilia which develops during the late response, within 7 hours following challenge. Inhaled corticosteroids and β_2-agonists, which have been shown to alter the severity of the late response, also alter the increases in sputum eosinophils measured during the late response (20,27), suggesting the eosinophil is, at least in part, associated with the development of the late response after inhaled allergen. The results from the present study confirm these previous observations, this time with a non-steroidal agent, suggesting that inhibition of the late response by PGE_2 occurs as a result of attenuating airway eosinophilia.

There was a major difference in the effects of PGE_2 treatment in the airway when allergen was inhaled immediately following PGE_2 compared to when the challenge was delayed. Inhalation of allergen within 7 minutes following PGE_2 treatment resulted in attenuation of the allergen-induced early and late responses, airway hyperresponsiveness to methacholine, and sputum eosinophilia. In contrast, when inhalation of allergen was completed 19 minutes following PGE_2, there was a significant enhancement of the allergen-induced late response and no protection against airway hyperresponsiveness to methacholine or sputum eosinophilia. Inhalation of PGE_2 has been reported to cause transient bronchoconstriction, measured 5 minutes after inhalation in asthmatics, followed by slight

bronchodilation, lasting up to 1 hour (10), as well as decreased histamine airway responsiveness, when PGE_2 preceded histamine challenge by 5 minutes, and increased histamine airway responsiveness when PGE_2 preceded histamine challenge by 35 minutes (9). In the present study, bronchoconstriction did not occur after inhaled PGE_2. Bronchodilation following inhaled PGE_2 may account, in part, for the attenuation of the early responses observed following the immediate challenge. However, this could not account for the attenuation of the late response by PGE_2 following the immediate challenge or the absence of attenuation of the early and late airway responses by PGE_2 following the delayed challenge. Furthermore, attenuation of the early and late responses to allergen was accompanied by reduced allergen-induced airway inflammation, and protection against allergen-induced airway hyperresponsiveness. Taken together, the results of the present study indicate that inhaled PGE_2 has anti-inflammatory effects in asthmatic airways, and that its effects on allergen-induced asthmatic responses are not as a functional antagonist, as are the effects of inhaled β_2-agonists (27). These results also suggest that, as PGE_2 can attenuate allergen-induced airway responses long after its direct pharmacological activity has resolved (7 and 24 hours after inhaled allergen), its action is through inhibiting very early allergen-induced airway events, such as mast cell degranulation and mediator release.

The mechanisms by which PGE_2 may regulate airway hyperresponsiveness and airway inflammation following allergen challenge are speculative. The release of arachidonic acid from cell membrane phospholipids following challenge to the airways can

result in the production of a wide variety of mediators, including prostanoids, thromboxane, and leukotrienes, which may be relevant in the pathogenesis of asthma. Substances such as thromboxane (Tx)A_2 and the cysteinyl leukotrienes (LT)C_4, D_4 and E_4 are potent bronchoconstrictors in asthmatic airways (28,29), and can be modulated by PGE_2. TxA_2 is released following allergen challenge (30). Pre-incubation with PGE_2 has been shown to reduce arachidonic-induced release of thromboxane A_2 from human bronchial biopsies (31). There is more substantial evidence to support the role of cysteinyl leukotrienes in the pathogenesis of asthma. The cysteinyl leukotrienes are released following allergen challenge (32,33) and recently LTD_4 inhalation has been shown to cause bronchoconstriction and increased sputum eosinophils in asthmatics (34). Administration of leukotriene antagonists before challenge are effective in attenuating the allergen-induced early and late responses (35). Eosinophils and mast cells, which are present in greater numbers in the asthmatic lung (11), are the sources of the cysteinyl leukotrienes. Also, eosinophils are known to have several feedback mechanisms to promote their proliferation, activation and survival by synthesizing eosinophilopoietic cytokines. There are several ways in which PGE_2 may modulate this feedback. Endogenous PGE_2 inhibits PAF-induced LTC_4 synthesis by eosinophils (36) and PGE_2 has been shown to inhibit eosinophil degranulation (13). Increased intracellular levels of cAMP by PGE_2 have been shown to suppress leukotriene and prostaglandin production by neutrophils and eosinophils, and adenosine 3',5'-cyclic monophosphate (cAMP) inhibition of release of substrate arachidonic acid from

phospholipid pools occurs at levels consistent with interaction with the PGE receptor (37). The findings of the current study, that inhaled PGE_2 attenuates the LAR and sputum eosinophilia, are consistent with the hypothesis that PGE_2 may inhibit allergen-induced LTC_4 synthesis, and thereby eosinophil accumulation in the lung. Unfortunately, however, urinary levels of leukotriene were not measured in this study, which would demonstrate whether allergen-induced LTC_4 production was indeed inhibited by PGE_2.

There are many other events leading to the development of allergic inflammation, which PGE_2 may modulate. PGE_2 has been shown to regulate the production of PBMC-derived cytokines such as interleukin (IL)-2, IL-4 and IL-5 by elevating intracellular levels of cAMP (38), and has been shown to induce a shift in the functional profile of cytokine message in T lymphocytes (39), possibly by the same mechanism. The adherence of inflammatory cells to endothelium is one of the initial events necessary for migration of these cells through the vascular wall. PGE_2 has been shown to inhibit transendothelial migration of human lymphocytes (40), and inhibit tumour necrosis factor (TNF)-alpha-induced intercellular adhesion molecule (ICAM)-1, endothelial leukocyte adhesion molecule (ELAM)-1, and vascular cell adhesion molecule (VCAM)-1 expression, as well as lymphocyte adhesion to human airway smooth muscle (41). This suggests that PGE_2 can modulate cell recruitment indirectly through inhibition of TNF-alpha, and is consistent with experiments demonstrating that PGE_2 blocks lipopolysaccharide-induced TNF-alpha production and neutrophil recruitment to the lung (42).

Following the delayed challenge, PGE_2 significantly enhanced the allergen-induced LAR, increased sputum neutrophils during the late response compared to placebo, and there was a trend for blood neutrophils to be higher with PGE_2 treatment 24 hours following allergen. Administration of PGE_2 outside its therapeutic window may interfere with the regulation of TNF-alpha by endogenous PGE_2, leading to an enhanced neutrophilia rather than suppression of inflammation as in the immediate challenge. One mechanism may be through the prostanoid receptor refractoriness, as the prostanoid FP-receptor recognizing $PGF_{2\alpha}$, D_2 and E_2 is shown to become refractory when pretreated with a maximally effective concentration of prostaglandin (43). That PGE_2 did not protect the airways from allergen challenge during the delayed challenge is not likely due to the short half life of PGE_2, as 95% of infused PGE_2 is inactivated during one passage through the pulmonary circulation (44), and would no longer be present during either of the challenges. The amount of PGE_2 bound to receptor at the time of allergen inhalation may be more relevant, however, this is not currently known in vivo. Furthermore, regular treatment with β_2-agonist, which also mediates its actions by increasing intracellular levels of cAMP, and like PGE_2, has been shown to inhibit LTC_4 synthesis by platelet activating factor-stimulated eosinophils (36), and has been shown to enhance the LAR and sputum eosinophils during the late response (27), suggesting that agents which elevate cAMP may have the capability to enhance rather than inhibit cell activation following allergen challenge pending timing or duration of administration.

We have demonstrated that PGE_2 does indeed mediate anti-inflammatory effects in allergen-challenged airways. However, beneficial actions of PGE_2 are only demonstrated when it is inhaled within the therapeutic window, which we suggest is less than 19 minutes. PGE_2 is a complex mediator, whose effects, we have confirmed, are dependent on timing (9) of administration. Although PGE_2 formulation and administration for the treatment of airway inflammation in asthma may present many challenges, the therapeutic implications of regulating the immune response in the lung, by a mediator known to be synthesized by airway cells, remains an attractive alternative to the present anti-asthma therapies.

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Table 5.1: Subject lung function characteristics, allergen dose, and treatment randomization.

Subject Number	Age (years)	Gender	FEV_1 (% predicted)	Methacholine PC_{20} (mg/ml)	Inhaled Allergen	Allergen Dilution	Order of Treatment
1-I	19	F	97.3	3.14	HDM	1:256	PGE_2 Plac
2-I	24	M	78.8	1.89	Grass	1:1024	Plac PGE_2
3-I	19	M	93.1	3.84	Grass	1:1024	Plac PGE_2
4-I	21	M	90.7	2.59	Ragweed	1:256	PGE_2 Plac
5-I	24	M	83.6	1.66	HDM	1:512	PGE_2 Plac
6-I	21	F	95.0	1.51	HDM	1:256	PGE_2 Plac
7-I	23	M	81.1	1.51	Cat	1:16	Plac PGE_2
8-I	23	M	90.7	0.40	HDM	1:1024	Plac PGE_2
1-D	21	M	88.3	1.22	Grass	1:2048	Plac PGE_2
2-D	21	F	79.7	1.00	HDM	1:2048	PGE_2 Plac
3-D	23	F	85.3	0.54	HDM	1:256	Plac PGE_2
4-D	21	F	95.0	9.74	HDM	1:8	Plac PGE_2
5-D	45	M	36.5	1.92	Cat	1:64	Plac PGE_2
6-D	24	M	84.5	1.49	Ragweed	1:2048	Plac PGE_2
7-D	22	F	79.3	1.00	Ragweed	1:2048	PGE_2 Plac
8-D	21	M	74.5	1.17	Ragweed	1:4	Plac PGE_2

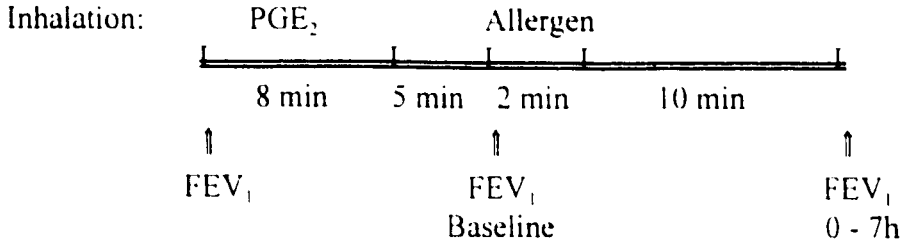
FEV_1: forced expiratory volume in 1 second, Methacholine PC_{20}: provocative concentration of methacholine for 20% reduction in FEV_1, I: immediate challenge, D: delayed challenge, F: female, M: male, HDM: house dust mite, Plac: placebo treatment, PGE_2: PGE_2 treatment

Table 5.2: Comparison of allergen-induced changes in sputum inflammatory cells with allergen challenge completed 7 minutes (immediate challenge) or 19 minutes (delayed challenge) following pretreatment with 100 ug PGE_2 or placebo

	BASELINE		7 HOURS POST ALLERGEN		24 HOURS POST ALLERGEN	
	Placebo	PGE_2	Placebo	PGE_2	Placebo	PGE_2
Immediate Challenge						
Eosinophils (%)	7.9 ± 4.7	11.1 ± 4.8	26.4 ± 8.3 ▲	17.6 ± 4.7 ▲•	36.3 ± 8.8 ▲	20.9 ± 7.3 ▲•
EG2 positive (%)	6.9 ± 4.3	8.7 ± 3.8	19.8 ± 8.7 ▲	10.5 ± 3.4 •	27.4 ± 7.1 ▲	13.1 ± 3.8 •
Neutrophils (%)	48.0 ± 9.8	34.7 ± 4.8	62.9 ± 8.1 ▲	58.0 ± 10.0 ▲	40.0 ± 8.7 ▲	38.5 ± 6.6 ▲
MCC (%)	0.08 ± 0.06	0.16 ± 0.08	0.37 ± 0.15	0.29 ± 0.11 •	0.66 ± 0.29	0.21 ± 0.06 •
Total cells (10^6/ml)	2.03 ± 0.47	2.43 ± 0.56	4.95 ± 1.78	3.58 ± 0.85	3.63 ± 0.96	4.15 ± 0.68
Delayed Challenge						
Eosinophils (%)	6.7 ± 1.5	5.2 ± 1.1	31.4 ± 6.4 ▲	26.6 ± 5.8 ▲	33.1 ± 5.7 ▲	35.9 ± 4.2 ▲
EG2 positive (%)	4.0 ± 1.7	5.6 ± 2.6	10.5 ± 4.0 ▲	11.7 ± 4.9 ▲	19.2 ± 6.0 ▲	24.3 ± 7.0 ▲
Neutrophils (%)	39.0 ± 7.2	32.9 ± 7.0	30.6 ± 5.1	47.4 ± 6.1 •	25.5 ± 4.8	27.7 ± 5.0
MCC (%)	0.14 ± 0.05	0.16 ± 0.03	0.27 ± 0.10	0.22 ± 0.04	0.23 ± 0.03	0.39 ± 0.13
Total cells (10^6/ml)	1.98 ± 0.23	1.63 ± 0.37	2.17 ± 0.54	3.45 ± 0.95	2.96 ± 0.69	3.51 ± 0.81

Data are presented as means ± SEM. MCC, metachromatic cells. ▲, $p<0.05$ allergen-induced change from baseline. •; $p<0.05$ difference from baseline, placebo versus PGE_2.

Immediate Challenge



Delayed Challenge

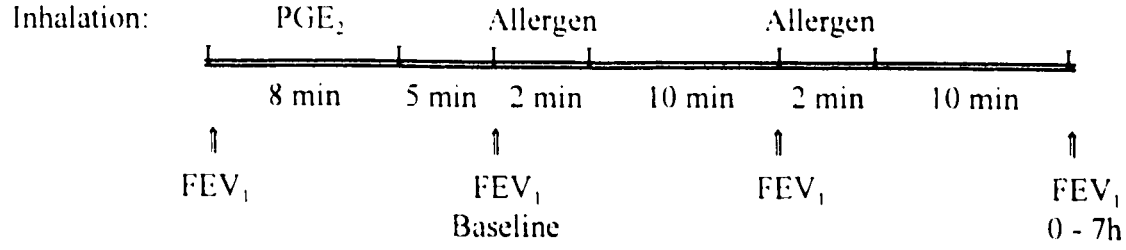


Figure 5.1: Schematic demonstrating the sequence and duration of PGE_2 and allergen inhalation during the immediate and delayed challenges. FEV_1; forced expiratory volume in 1 second, PGE_2; prostaglandin E_2.

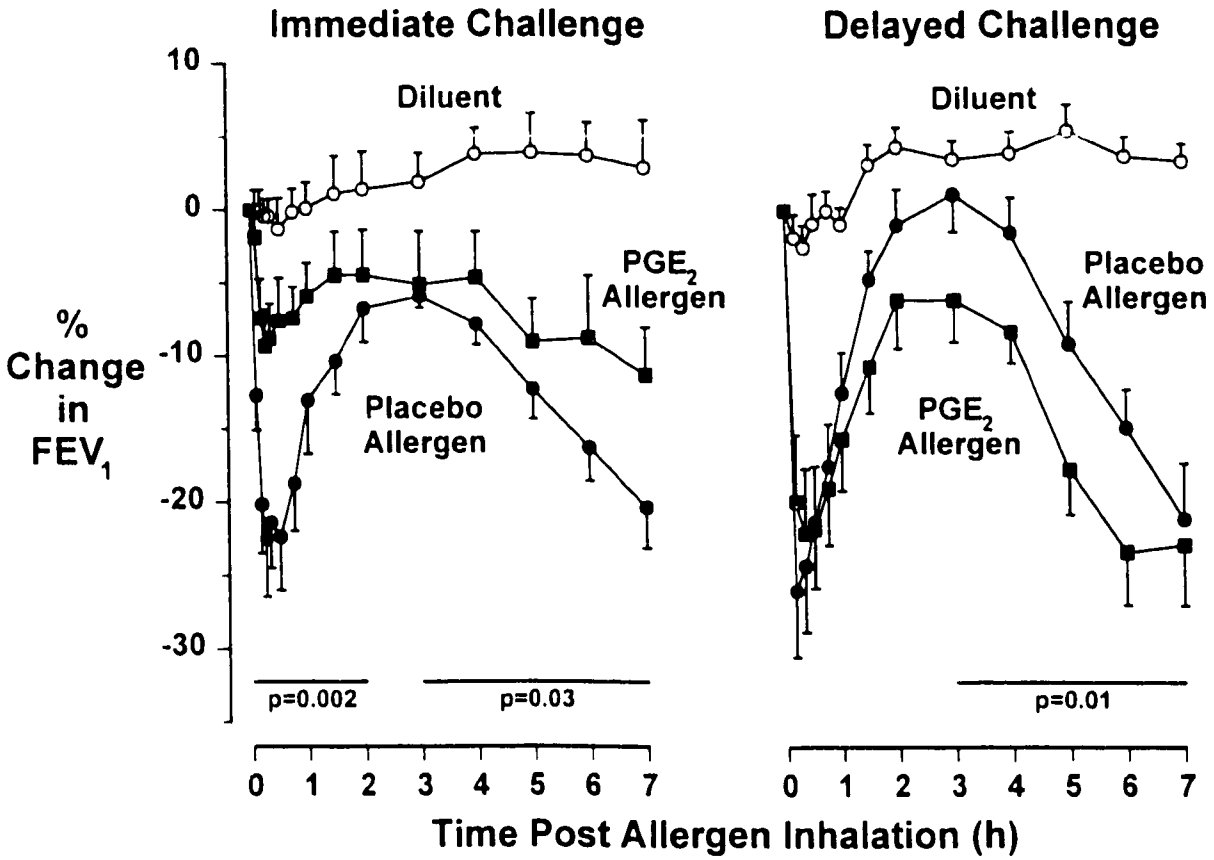


Figure 5.2: The allergen-induced early and late airway responses following the immediate challenge (left panel) and the delayed challenge (right panel) with allergen (solid symbols) or diluent (open circles), following treatment with placebo (solid circles) or PGE_2 (solid squares).

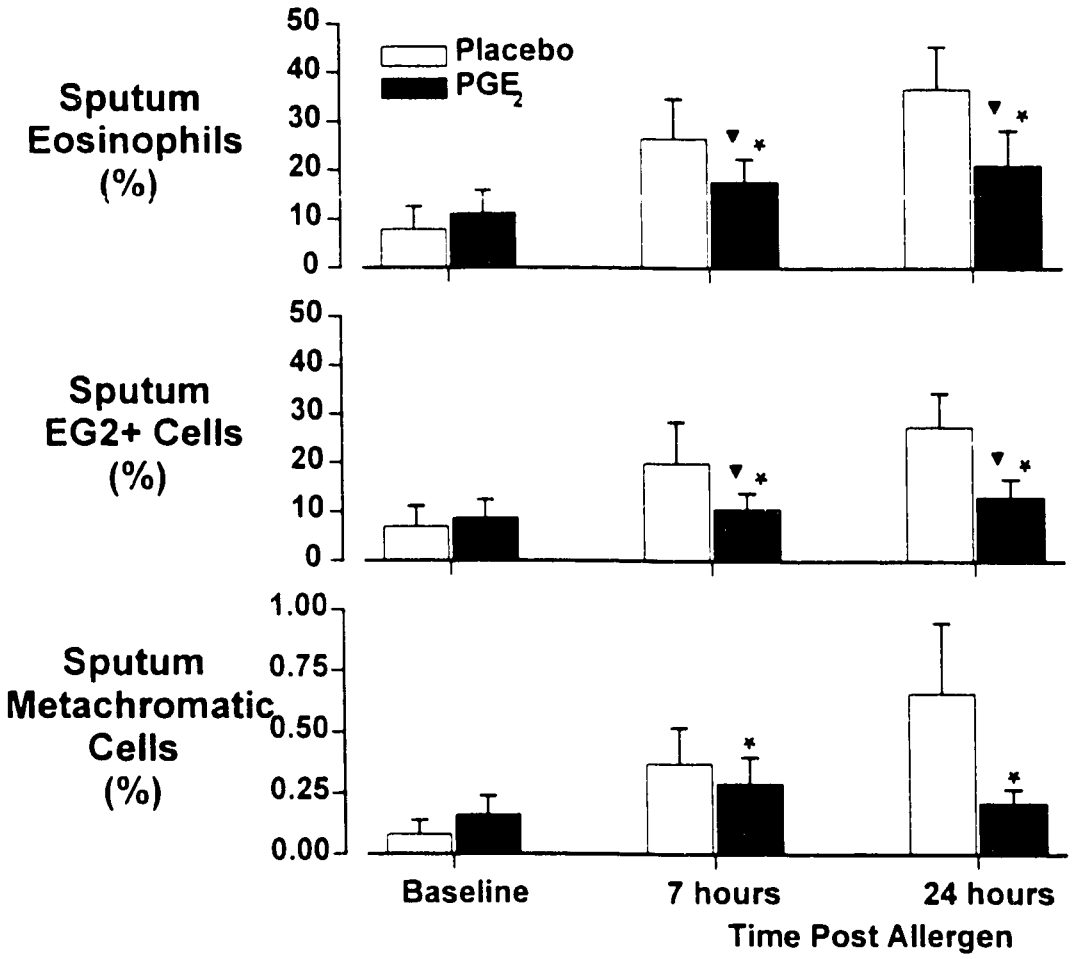


Figure 5.3: The percent sputum eosinophils (top panel), EG2+ cells (middle panel) and metachromatic cells (bottom panel) before, 7h and 24h following the immediate allergen inhalation challenge, following treatment with placebo (open bars) or PGE_2 (solid bars). ▼; $p<0.05$ allergen-induced increase, *; $p<0.05$ PGE_2 versus placebo difference

CHAPTER 6:

ALLERGEN-INDUCED AIRWAY INFLAMMATION: SUMMARY OF FINDINGS, GENERAL DISCUSSION AND FUTURE DIRECTIONS

Summary of Findings

The results in this thesis indicate that allergen inhalation challenge, together with measurements of allergen-induced sputum inflammatory cells, provides a repeatable and sensitive model for assessing the anti- and pro-inflammatory properties of established or new asthma therapies.

We have characterized the kinetics of allergen-induced airway inflammation for 7 days, demonstrating the degree and duration of eosinophilia and metachromacia in sputum. The optimal times for measuring changes in allergen-induced sputum eosinophils and metachromatic cells have been suggested to be 7 and 24 hours following challenge. We have also demonstrated that maximal numbers of activated eosinophils (EG2-positive) are coincident with maximal number of eosinophils immunoreactive for IL-5, eotaxin and RANTES at 7 hours, confirming their participation in allergen-induced airway inflammation. Measurements of cytokine levels in induced sputum taken before 7 hours could clarify the interaction that exists between these cytokines, leading to airway eosinophilia. The development of methods to measure inflammatory markers in sputum, such as

immunocytochemistry for IL-5, eotaxin and RANTES, will contribute to our understanding of recruitment of eosinophils to the airway after allergen challenge. For a comprehensive investigation of eosinophil recruitment, one must consider the kinetics of expression of adhesion molecules, eosinophil activators and chemotaxins, and cytokine receptor expression. These staining methods could be developed for future studies to evaluate the expression of these other markers of inflammation in serial sputum samples following allergen challenge.

We have demonstrated that the allergen-induced increase in sputum eosinophils is a reproducible measurement. Power calculations using data from repeated allergen challenges were used to predict sample sizes required to show allergen-induced differences in sputum eosinophils between placebo or active treatment. We have predicted that the sample size required to demonstrate a 50% attenuation of allergen-induced increases in % sputum eosinophils in a placebo-controlled cross-over study with repeated measurements of sputum eosinophils, is only 4 subjects. Repeated sputum sampling, which is much more difficult with sampling of BAL or bronchial biopsies, allows a sensitive statistical analysis and a practical sample size. The allergen-induced increase in sputum eosinophils may also be expressed as the absolute cell number per ml sputum, however, the sample size required to demonstrate a significant reduction in allergen-induced sputum eosinophils is considerably larger. This is because the total cell count is not a highly reproducible measurement and adds a substantial amount of variability to the eosinophil number per ml sputum.

Measurement of sputum eosinophils involves a non-invasive technique, can be measured repeatedly, and is becoming a widely accepted method for assessing airway inflammation. As sputum eosinophilia may be measured as a primary outcome variable for the evaluation of asthma therapies, we have provided the necessary power calculations to predict the sample sizes required to demonstrate treatment effects following allergen challenge in placebo-controlled cross-over studies.

We examined the effects of the inhaled steroid, budesonide, on allergen-induced airway inflammation. Budesonide is known to have anti-inflammatory properties, and is known to attenuate the allergen-induced late airway response and allergen-induced airway hyperresponsiveness. However, the effects of an inhaled steroid on allergen-induced airway inflammation has not been previously studied in asthmatics. This inhaled steroid was used as our "gold standard" anti-inflammatory therapy to investigate whether or not attenuation of allergen-induced inflammation can be measured in induced sputum. Following budesonide treatment, we observed a significant reduction in the allergen-induced late airway response, airway hyperresponsiveness, and eosinophils and metachromatic cells in the sputum. We also demonstrated that PGE_2, which has also been shown to attenuate the late airway response to allergen, significantly reduced allergen-induced airway inflammation.

We have provided data to demonstrate that the protection afforded by anti-inflammatory effects of an asthma therapy, budesonide, can indeed be quantified in induced sputum following allergen inhalation challenge. Furthermore, these studies provide

information regarding the actions of budesonide and PGE_2, which appear to attenuate the late airway response through their anti-inflammatory effects in the airway.

In contrast, we have demonstrated that regular use of an inhaled β_2-agonist, shown to enhance the allergen-induced late airway response, also enhanced the number of eosinophils in sputum,and the level of ECP in sputum at 7 hours, during the late airway response. These observations, taken together with the observations that the allergen-induced late airway response and airway eosinophils were both attenuated by budesonide and PGE_2, demonstrate that the late airway response is reflected by airway eosinophilia measured in induced sputum.

General Discussion

The aim of this thesis was to investigate the validity of induced sputum to examine pharmacological modulation of allergen-induced airway responses and airway inflammation. The following discussion will first address the suitability of the allergen challenge model, the limitations and assumptions of the techniques utilized to measure allergen-induced airway inflammation, and the interpretation of these measurements in sputum. The relationship between inflammatory indices from induced sputum and airway physiology will be addressed, as well as the importance of optimizing the design of studies for investigation of asthma therapies. The discussion will also include new insights into the mechanisms of the asthma therapies studied, and suggest future directions to better understand allergic

asthma and its pharmacologic control.

Allergen Inhalation Challenge Model

Allergen is inhaled from a nebulizer, which ensures that a constant, reproducible amount of allergen is delivered to the airways in a controlled and safe fashion. Reproducibility of allergen delivery is important for cross-over studies where it is necessary to give the same airway challenge on a number of occasions and compare the airway responses. A high dose allergen inhalation challenge represents a more physiological method to challenge areas of the lung which would normally encounter allergen,when compared to instillation of allergen using a bronchoscope which only challenges a local area of the lung.

High dose allergen inhalation challenge provides a model to assess the overall impact of an acute allergen exposure on the airway, and is associated with a measureable degree of airway inflammation for up to 1 week following inhalation. Furthermore, this model can be used to assess the overall effects of asthma therapies on acute exposure to sentitizing allergens. Repeated low dose allergen challenges may be more physiologically relevant to allergen exposure during an allergy season or long term exposure to a sensitizing allergen. This low dose allergen challenge model has not been well characterized, and is currently being investigated (Sulakvelidze et al, 1998). We have used the high dose allergen challenge model for all studies included in this thesis, as this model has been well characterized to assess therapeutic benefits of asthma therapy as measured by airway physiology, and to induce a measureable degree of airway inflammation. We have, in

Chapters 3, 4 and 5, demonstrated assessment of sputum to be sensitive enough to reflect, pharmacological effects of several asthma therapies; budesonide, albuterol and PGE_2, following high dose allergen challenge. Studies using low dose challenge are justified and will provide valuable information necessary to assess the pharmacological effects of asthma therapies with long term exposure to sensitizing allergens.

This model of allergen challenge, albeit sensitive, is not without limitations. One of the assumptions of allergen inhalation challenge in therapeutic trials is that the delivery of allergen is not altered by the status of the airway, when in fact, it may be. For example, asthmatic airways under the effect of a bronchodilator may allow allergen to be delivered more peripherally where the airway response to allergen may not be as great, highly inflamed and constricted airways may trap the allergen particles centrally where smooth muscle contraction occurs, thus altered allergen delivery to the airway may make it difficult to distinguish between the protection afforded by the drug, and the effects of altered allergen deposition.

The subjects recruited for these high dose allergen challenge studies represent a subpopulation of asthmatics with mild, well controlled asthma. Results from these studies may not be extrapolated to non-atopic asthmatics, or generalized to asthmatics with more severe disease. This group of mildly asthmatic subjects, however, are necessary to recruit for allergen challenge studies in order to safely induce a controlled level of airway inflammation, particularly in cross-over studies where the subjects will serve as their own

control.

Allergen inhalation has been used, in this thesis, as a model to induce airway inflammation, and can also been used to examine the systemic effects of acute airway challenge. In atopic, mild asthmatics, inhalation of allergen results in elevated circulating levels of eosinophils and their progenitors, possibly through increased expression of hemopoietic growth factors synthesized by these progenitors, leading to a more rapid maturation (Appendix 1). These observations provide a framework to investigate the pharmacological effects of asthma therapies in the blood and bone marrow. Achieving low systemic levels of drugs, such as steroids, may be important to regulate trafficking of inflammatory cells (Wood et al, 1998), therefore, measurements of the therapeutic effects on blood and bone marrow progenitor cells may provide additional clues to understand the mechanisms of anti-inflammatory therapies.

Laboratory Techniques

The methods for inducing and processing sputum samples have been carefully evaluated and optimized to minimize any effects on the final proportion of sputum cells measured on cyto-preparations of the sample. Identification of sputum cells can be difficult depending on the quality and viability of the sample (Efthimiadis et al, 1995), and furthermore, the morphology and staining characteristics can be altered by apoptosis and degranulation of the cells (Fokkens et al, 1992). There remain a number of other factors which may alter the cell counts in sputum. Repeated sputum induction has been shown to

elevate the percentage of neutrophils over a number of days (Kips et al, 1995; Holtz et al, 1997), and inhalation of methacholine to determine the PC_{20} immediately preceding sputum induction, as we have performed in these studies, may alter airway secretions leading to changes in total cell counts. We have controlled for these potential sources of variability by designing placebo-controlled cross-over studies, which take into account the effects of all procedures by comparing samples obtained at the same time points. It would have been interesting to examine airway inflammation also during the early response, however, we were unable to induce a sputum sample earlier than 7 hours following allergen inhalation because inhalation of a bronchodilator is necessary prior to sputum induction. As we were interested in documenting the late airway response to inhaled allergen, sputum could not be collected until after the last measurement of FEV_1 at 7 hours following allergen challenge.

Cells from induced sputum may undergo apoptosis and phagocytosis. In addition, as cells are recruited to the airway following allergen challenge, there is a lag time between the allergen-induced appearance of airway inflammatory cells in sputum and changes in airway physiology. Therefore, there are a number of assumptions made when one compares sputum samples from the same time points following allergen challenge. When comparing the allergen-induced airway inflammatory cell differential counts following drug and placebo treatments, the assumption is that the rate of apoptosis, phagocytosis or clearance of secretions from the airway remain unaltered by the active treatment. Alterations to any one of these events could alter the number of inflammatory cells present in the sputum at a given

point in time. Sputum sampling at two time points following allergen inhalation allows one to determine if there is a change in the kinetics of allergen-induced inflammatory cells, as was observed following regular treatment with albuterol. One can not readily distinguish whether this change in kinetics represents a change in recruitment of cells to, or clearance of cells from the airway.

Pharmacological modulation of the allergen-induced increase in sputum eosinophils could occur by several mechanisms, including alterations in production and delivery to the airway, survival in the airway, and clearance by pulmonary macrophages. Recruitment of cells to the airway following allergen challenge depends on the development of an allergen-induced response (Chapter 2), and is reflected by increased numbers of eosinophils and their precursors in the peripheral blood (Gibson et al, 1991, Appendix 1) and bone marrow (Wood et al, 1998a). This may be a site of regulation by asthma therapies, as inhaled budesonide has been shown to reduce the number of eosinophil colony-forming units in bone marrow (Wood et al, 1998b). Recruitment of eosinophils to the airway also depends on expression of adhesion molecules to enable binding to the vascular endothelium and migration into the tissue. Adhesion molecules are present on sputum eosinophils (Hansel et al, 1991). Pharmacological alteration of the kinetics of their expression following allergen challenge would dictate the eosinophil kinetics into the tissue. This could be examined in more detail with repeated sputum sampling following allergen challenge. Apoptosis of inflammatory cells and ingestion by macrophages is a mechanism for cell clearance from the airway, and

defective clearance by this mechanism could contribute to eosinophil accumulation in the airway following allergen challenge. The presence of IL-5 and GM-CSF inhibits apoptosis of eosinophils and the subsequent phagocytosis by macrophages (Stern et al, 1992), prolonging survival of eosinophils (Adachi et al, 1995). Apoptosis of cells is accompanied by cell shrinkage and DNA fragmentation, and can be identified using cytochemical staining and light microscopy (Woolley et al, 1996). Measurements of eosinophils and their precursors in blood, adhesion molecules on sputum eosinophils and apoptotic eosinophils in sputum may indicate the mechanism by which an asthma therapy alters the allergen-induced sputum eosinophilia.

We have used immunocytochemical methods in induced sputum to enumerate activated eosinophils, and measure the level of cytokine expression in the airway. Immunocytochemistry involves the specific binding of an antibody to the protein of interest, then colour development of this bound antibody. Of particular importance is the specificity of the antibody for the protein of interest. We have utilized monoclonal antibodies to cytokines which have been selected for their ability to neutralize the biological activity of the protein, and based on ELISA and western blot results, do not show any cross-reactivity with proteins of similar structure. Antibodies used for the identification of specific cell types should not be expressed by other cells. The antibody we have used to demonstrate the presence of activated eosinophils, EG2, is specific in that it distinguishes between the stored and secreted forms of eosinophil cationic protein (Tai et al, 1984). Furthermore, ECP has

been shown to be cytotoxic to respiratory epithelium in vitro (Young et al, 1986), and is commonly measured in sputum supernatant as a marker of eosinophil activation. ECP, however, can also be expressed by activated neutrophils, although they possess much less than eosinophils (Sur et al, 1991). For this reason, we have developed double-staining of sputum samples with FITC, which is specific for eosinophils at the pH utilized (Filley et al, 1982; Johnston et al, 1974), to confirm the identification of the cells staining positive for EG2.

A positive signal by immunocytochemistry demonstrates localization of the protein of interest. This protein may be intracellular, being synthesized and stored by cells known to express the protein, or phagocytosed by the phagocytic cells, such as macrophages and neutrophils. One would expect cells in sputum to be activated to not only express, but also secrete mediators. A positive signal by immunocytochemistry may also represent secreted extracellular protein, being bound to a receptor, or trapped by glycoprotein of the cell membrane. Either way, immunopositivity by a cell represents a specific binding of antibody to the protein. Although increased immunolocalization of protein in sputum may not be interpreted as increased protein synthesis by the cell localizing the protein, it may instead, be interpreted as a reflection of increased levels (expressed, secreted and receptor-bound) of protein in the airway.

Interpretation of Sputum Inflammatory Cell Measurements

Measurements of inflammatory cells from induced sputum have been reported in

published articles as the absolute number of cells per volume of sputum, or as the proportion of the total cell population, with no apparent preference for one or the other. One must be cautious when interpreting cell data presented as percentages of the total population, as the ratio of one cell type may change secondary to a change in another. For example, in a dynamic environment such as the airway, it is not possible to draw conclusions about an increase in the percentage of eosinophils without also considering if there has been a decrease in the percentage of another cell type. For this reason, it may be more appropriate to express the cells as absolute number per mg sputum to estimate the cell load in the airway. The absolute number of each cell type is dependent upon the total cell count per mg sputum as well as the ratio of cells. The total cell count, alone, is subject to variability introduced by airway secretions, and is not highly reproducible in sputum (Pizzichini et al, 1996), and therefore requires a higher sample size than percentages, as demonstrated in Chapter 2. The absolute number of eosinophils per mg sputum, however, may be easier to interpret than the percentage of eosinophils because it is not completely dependent on the ratio of other cell types.

Traditionally, inflammatory cells in the airway have been associated with activation of other inflammatory cells, and release of inflammatory mediators. In the preceding chapters, we have demonstrated that allergen-induced airway inflammation is associated with increased levels of activated eosinophils and metachromatic cells (mast cells and basophils), and cytokines associated with eosinophil chemotaxis and activation (eotaxin, IL-5 and

RANTES) (Elsner et al, 1996; Tai et al, 1991; Sehmi et al, 1992; Kameyoshi et al, 1992). Currently, airway inflammatory cells tend to be considered in terms of their pro-inflammatory contribution to airway inflammation. For example, activated eosinophils and mast cells are sources of many pro-inflammatory cytokines, including IL-5 and GM-CSF, which may participate in eosinophil recruitment in allergic inflammation (Broide et al, 1992; Gordon et al, 1990). Regulation of inflammation, however, is much more complex. Inflammatory cells may also synthesize mediators which suppress inflammation. Neutralization proteases which degrade harmful mediators (Caughey et al, 1989), heparin which can bind cytotoxic cationic proteins (Venge et al, 1989), and immunosuppressive interleukins such as IL-10 which can supress cellular activation (Fiorentino et al, 1991) and cytokine production by cells associated with allergic inflammation (Takanashi et al, 1994; de Vries et al, 1995). The presence of inflammatory cells, therefore, may also represent an important source of anti-inflammatory mediators.

Airway Physiology and Airway Inflammation

The allergen-induced changes in airway physiology are reflected by measurements in induced sputum. As compared to BAL, sputum cells mostly originate from the small central airways which respond to allergen, rather than the alveolar compartment which is more peripheral. In this respect, sputum cells are perhaps more relevant to allergen-induced airway inflammation than BAL.

We measured FEV_1, methacholine PC_{20}, and airway inflammation for 7 days

following allergen inhalation challenge to examine the kinetics and the relationship between these airway responses (Chapter 2). The maximal numbers of activated (EG2-positive) eosinophils, metachromatic cells, and eosinophils immunopositive for eotaxin and RANTES and IL-5 occured at 7 hours following allergen challenge, during the late response, demonstrating synchronization of airway responses, airway eosinophil responses, and levels of cytokines known to contribute to eosinophilic inflammation. In addition, the airway responses (FEV_1 and methacholine PC_{20}) were shifted from baseline for up to 7 days, as were the airway inflammatory cells. Therefore, in this investigation of the effects of allergen inhalation on airway physiology and airway inflammation, we have demonstrated that measurements of increased sputum inflammatory cells and increased immunostaining for pro-inflammatory cytokines coincide with the allergen-induced airway physiological responses.

We have also demonstrated that bronchoprotection by asthma therapies is reflected by sputum inflammatory cells. When the allergen-induced late airway response was attenuated following regular treatment with inhaled budesonide or inhalation of PGE_2, the allergen-induced increase in eosinophils and metachromatic cells in the sputum were also suppressed (Chapters 3 and 5). Conversely, when the allergen-induced late airway response was enhanced following regular treatment with inhaled albuterol, the allergen-induced increase in sputum eosinophils was also enhanced (Chapter 4). Therefore, despite the limitations of using induced sputum, including the lag time of cells appearing in the sputum,

phagocytosis and apoptosis of cells, and poor reproducibility of total cell counts, comparisons of sputum inflammatory cells from the same subjects in a randomized cross-over study have been shown to be sensitive enough to measure pro- and anti-inflammatory properties of asthma therapies following allergen challenge.

Although the allergen challenges in these studies produced a similar fall in FEV_1 among all subjects, the allergen-induced airway inflammatory cell responses of individual subjects varied considerably, as demonstrated in Chapter 2, Figures 4 and 6. As a result, there was no significant relationship between allergen-induced late airway response and allergen-induced airway eosinophils. We were unable to demonstrate a significant correlation between the allergen-induced late maximal fall in FEV_1 and sputum eosinophils with sample sizes of 8 or less (Chapters 3-5); however, there was a weak negative correlation between the late airway response and the percent sputum eosinophils when the sample size was increased to n=16. We did not observe a significant correlation between airway physiology and airway inflammation when the cells were expressed as absolute numbers per ml sputum, most likely because of the large between-subject variability in the total cell count. That we did observe significant changes in sputum eosinophils which coincided with changes in late airway responses in the randomized cross-over studies, suggests that these variables are in fact related, and lack of a significant correlation between measurements of airway inflammation and airway physiology most likely represents a power problem.

There are several mechanisms that have been proposed to explain the contribution

of the eosinophil to changes in airway function, such as eosinophil-mediated damage to the epithelium and consequent exposure of nerve endings to bronchoconstrictor agents (Jeffrey et al, 1992; Beasley et al, 1989). Eosinophils may directly contribute to the allergen-induced late airway response by releasing mediators, such as cysteinyl leukotrienes, which can cause smooth muscle contraction, mucus secretion and vasodilation (Aalbers et al, 1993; Weller et al, 1983). However, it may not be appropriate to attribute a physiological phenomenon such as allergen-induced late airway response, to a single inflammatory cell type. It is most likely that several inflammatory cell types, including mast cells, contribute to the measured changes in airway physiology following allergen challenge (Kaliner et al, 1989).

Statistics

The clinical investigations contained in this thesis were designed as placebo-controlled, randomized cross-over studies, in which each subject represented their own control. Repeated sputum samples were obtained from each subject during each arm of the study (placebo or active agent), allowing the use of repeated measures ANOVA for statistical analysis of the data. Repeated measures ANOVA is a powerful statistical tool in a randomized cross-over study design, as the error term is based only on the variability within subjects. As observed in Chapter 2, when applied to measurements of eosinophils in sputum, repeated measures ANOVA allows for practical sample sizes to demonstrate attenuation in the number of sputum eosinophils. Therefore, repeated sampling of airway inflammatory cells not only provides a means of examining the kinetics of the response to allergen, but also

allows for utilization of a statistical analysis yielding a very sensitive measure of variance.

Future Directions

We have demonstrated that measurements of eosinophils and metachromatic cells from induced sputum reflect the effects of allergen inhalation challenge as well as therapeutic modulation of the allergen-induced airway responses. With a growing interest in the development of a non-invasive method of assessing airway inflammation and the anti-inflammatory effects of asthma therapies, the model of high dose allergen provocation with measurements of airway inflammation from induced sputum tested in this thesis, is now being utilized in other laboratories (Fahy et al, 1997; Wong et al, 1992). Our own laboratory has recently demonstrated that repeated low dose allergen challenge results in increased sputum eosinophils, metachromatic cells, and levels of IL-5 (Sulakvelidze et al, 1998). It will be important to determine if pharmacological modulation of airway inflammation is measurable following this low dose allergen challenge, which represents exposure to allergen which is physiologically more similar to that of atopic asthmatics during seasonal allergen exposure.

Measurements of cell mediators provide additional information regarding the inflammatory status of the airway. We have developed immunocytochemical staining for many of the pro-inflammatory cytokines, including eotaxin, IL-5 and RANTES (Chapter 2), as well as GM-CSF and IL-8 (Gauvreau et al, 1997). Immunocytochemistry is a sensitive

method to demonstrate the presence of a mediator of interest, however, it can not be used to demonstrate which cells are synthesizing the mediator. To be certain where mediators are synthesized, one must demonstrate the presence of the specific mRNA in situ. Techniques such as in situ hybridization or in situ PCR can be used to confirm protein expression by specific cell types. We have been able to apply in situ PCR to differentiating eosinophils grown from peripheral blood, demonstrating these methods are possible to develop, and they provide essential information regarding protein synthesis (Appendix 1). Development of these techniques for use in sputum are not yet established, and the methods for sputum processing and fixation would need to be altered to enable detection of mRNA.

The studies in this thesis have investigated the pharmacological modulation of allergen-induced airway responses by examination of inflammatory cells in induced sputum. The inflammatory cells appearing in induced sputum are the granulocytes, as tissue-associated cells such as lymphocytes are present in very low numbers in sputum. The airway, however, is unquestionably the most appropriate site to measure the effects of inhaled allergen and protection by inhaled asthma therapies, as the allergen inhalation model induces a local challenge to the airway. This is not the only compartment, however, which responds to allergen challenge and asthma therapies. We and others have demonstrated circulating eosinophils and their progenitors increase following allergen challenge (Dahl et al, 1978; Gibson et al, 1991; Appendix 1), and this increase is accompanied by enhanced expression of GM-CSF by the colony cells themselves. We propose that this mobilization

and proliferation of eosinophils may be a necessary component for the development of allergen-induced airway eosinophilia. Furthermore, circulating eosinophils and eosinophil/basophil colony-forming units respond to steroid treatment (Butterfield et al, 1986; Gibson et al, 1990). This model of allergen-induced eosinophil progenitors may be promising as another objective measure to study the effects of allergen inhalation and mechanisms by which asthma therapy may be effective.

At a time when many new therapies are being evaluated, re-evaluated, and developed for the treatment of asthma, it is important to have a model that is sensitive and reliable, but also a model with which one can rapidly and thoroughly investigate the effects of asthma therapies. Sputum induction is well tolerated by asthmatic subjects, can be performed repeatedly, and provides a non-invasive technique to measure airway inflammation. Measurements of sputum, together with allergen inhalation challenge using a placebo-controlled cross-over study design should provide valuable information on the effects of asthma drugs. With the development of new molecular techniques for use with sputum cells, including in situ hybridization and PCR, these allergen challenge studies may begin to investigate the mechanisms of therapies for the treatment of asthma.

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APPENDIX 1:

ENHANCED EXPRESSION OF GM-CSF IN DIFFERENTIATING EOSINOPHILS OF ATOPIC AND ATOPIC ASTHMATIC SUBJECTS

Published in The American Journal of Respiratory Cell and Molecular Biology in 1998

Gail Gauvreau's contribution:

Experimental design
Collection of clinical data
Cell culture experiments
Staining and quantification of cells by immunohistochemistry
Staining of cells by in situ PCR
Analysis of data
Preparation of manuscript

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Enhanced Expression of GM-CSF in Differentiating Eosinophils of Atopic and Atopic Asthmatic Subjects

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Higher numbers of eosinophil/basophil colony-forming units (Eo/B CFU) are observed in blood of atopic individuals, and can be enhanced in atopic asthmatics by allergen-inhalation challenge. It is known that mature basophils and eosinophils synthesize cytokines relevant to allergic inflammation. To investigate the potential role of growth factors in allergic disease we examined the expression of the hemopoietic cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-5, in differentiating Eo/B colony cells from normal and atopic individuals, and from atopic asthmatics before and after allergen-inhalation challenge. Peripheral blood was collected from two normal and 12 atopic individuals, and also from 25 atopic asthmatics before and 24 h after allergen challenge. Nonadherent mononuclear cells were isolated and grown in semisolid growth medium. Eo/B colonies were selected and cytospins were prepared for immunocytochemical analysis of colony cells. Eo/B colonies, especially carbol chromotrope 2R+ cells, selected at Days 10, 14, and 18 from atopic donors contained messenger RNA for GM-CSF by combined *in situ* reverse transcription-polymerase chain reaction and cytochemistry, and demonstrated time-dependent expression of GM-CSF by immunocytochemistry ($P = 0.007$). Atopic individuals demonstrated a higher percentage of cells expressing GM-CSF than did normal subjects under all growth conditions when examined at Day 14 ($P = 0.04$). Atopic asthmatics challenged with inhaled allergen who demonstrated a dual airway response, an increase in the number of blood eosinophils ($P = 0.0001$), and an increase in the number of Eo/B CFU ($P = 0.02$) also demonstrated a significant increase in the percentage of colony cells expressing immunostainable GM-CSF ($P = 0.0009$), but only a variable effect on those expressing IL-5, 24 h after allergen. These results suggest that GM-CSF expression by differentiating Eo/Bs may provide an additional stimulus *in vivo* to enhance Eo/B progenitor differentiation in atopic and asthmatic individuals, especially after allergen challenge. The concept of microenvironmental differentiation, where blood progenitor cells may aid in their own differentiation, is supported by these *ex vivo* findings.
Gauvreau, G. M., P. M. O'Byrne, R. Moqbel, J. Velazquez, R. M. Watson, K. J. Howie, and J. A. Denburg. 1998. Enhanced expression of GM-CSF in differentiating eosinophils of atopic and atopic asthmatic subjects. Am. J. Respir. Cell Mol. Biol. 19:55–62.

Eosinophils are mature inflammatory cells present in increased numbers in tissue and blood in a variety of allergic conditions (1). Eosinophils and basophils arise from a common progenitor cell (2), the eosinophil/basophil colony-forming unit (Eo/B CFU), which circulates in increased levels in atopic individuals depending on symptomatology (3). Fluctuations in the levels of Eo/B CFU occur on natural allergen exposure (4) and during asthma exacerbation (5, 6).

Growth factors that promote Eo/B CFU proliferation and differentiation include granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3, and IL-5 (7). Mature eosinophils contain messenger RNA (mRNA) transcripts for GM-CSF, IL-3, and IL-5 (8–10), and eosinophils in bronchoalveolar lavage from asthmatics express GM-CSF and IL-5 mRNA before and after allergen challenge (11). While expression of these cytokines by eosinophils may play a role in acute and chronic inflammatory responses, it could also provide an autocrine source of growth factors for differentiating eosinophils. Indeed, the microenvironment of nasal polyp tissue, which includes

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Abbreviations: colony-forming units, CFU; Dulbecco's phosphate-buffered saline, DPBS; eosinophil/basophil, Eo/B; forced expiratory volume in 1 s, FEV_1; granulocyte-macrophage colony-stimulating factor, GM-CSF; interleukin, IL; the provocative concentration of methacholine causing a 20% fall in FEV_1, methacholine PC_{20}; messenger RNA, mRNA; nonadherent mononuclear cells, NAMC; reverse transcription-polymerase chain reaction, RT-PCR; stem cell factor, SCF.

abundant eosinophils capable of expressing GM-CSF (12) and IL-5 (13), as well as other cytokines (14), has been shown to promote differentiation of Eo/B CFU (15). However, it is not known whether autocrine, eosinophil-initiated hemopoietic effects can occur either *in vitro* or *in vivo* Previous experiments from our laboratory have demonstrated an increased number of Eo/B CFU in atopic versus nonatopic individuals (3), and an increased number of circulating Eo/B CFU in atopic asthmatics after allergen challenge versus before challenge (6). We hypothesized that, in addition to tissue-derived cytokines from fibroblasts and other structural cells (16), circulating Eo/B progenitors may be promoted to differentiate by cytokines derived from developing eosinophils themselves. In the current study, we have examined the expression of the eosinophilopoietins IL-5 and GM-CSF by differentiating eosinophils in semisolid cultures *in vitro*. We also compared the expression of cytokines by differentiating eosinophils in nonatopics and atopics, and in atopic asthmatics both before and after allergen challenge.

Materials and Methods

Studies were approved by the Ethics Committee of McMaster University Health Sciences Center. An initial set of experiments compared the expression of GM-CSF in peripheral blood Eo/B colony cells of atopic and normal individuals (Study 1), while a second set of experiments examined expression of GM-CSF and its message in a time-course study (Study 2). Finally, we compared the expression of GM-CSF and IL-5 in Eo/B colony cells before and after allergen inhalation in atopic asthmatics (Study 3). All subjects gave informed consent to participate in the studies.

Subjects

Studies 1 and 2. A total of 12 unchallenged atopic and two nonatopic subjects were studied. Atopy was confirmed with a positive skin test for one of 21 common allergens. Atopic subjects were studied out of season and thus not exposed to sensitizing allergens for at least 4 wk, since colony formation is depressed during prolonged allergen exposure (4). Subjects demonstrating a negative skin test served as nonatopic controls. Five of the atopics were used to examine time-dependent expression of GM-CSF and GM-CSF mRNA, and seven of the atopics were compared with normal subjects for expression of GM-CSF in Eo/B colony cells.

Study 3. Twenty-five nonsmoking subjects with mild atopic asthma (13 female/12 male) were selected for the allergen-inhalation experiments because of a previously documented allergen-induced early and late bronchoconstrictor response of at least 15% reduction in the forced expiratory volume in 1 s (FEV_1). Subjects were not exposed to sensitizing allergens and did not have asthma exacerbations or respiratory tract infections for at least 4 wk prior to entering the study. All subjects had stable asthma with FEV_1 greater than 70% of predicted normal on all study days before allergen inhalation, and used no regular medication other than infrequent (< twice weekly) inhaled β_2-agonist as required to treat their symptoms. All medications were withheld for at least 8 h before each visit, and subjects were instructed to refrain from rigorous exercise, tea, or coffee in the morning before visits to the laboratory.

Study Design

Each normal and each atopic subject attended the laboratory once. A skin test was performed and blood was obtained by direct venipuncture for assay of Eo/B CFU.

Each atopic asthmatic attended the laboratory on three occasions. Baseline measurements of FEV_1, the provocative concentration of methacholine causing a 20% fall in FEV_1 (methacholine PC_{20}), blood differentials, and CFU were determined the morning before allergen challenge. Allergen challenges were carried out the following morning, and FEV_1 was measured during the following 7 h. Measurements of FEV_1, methacholine PC_{20}, and blood were repeated 24 h after allergen challenge.

Laboratory Procedures

Methacholine-inhalation test. Methacholine-inhalation challenge was performed as described by Cockcroft (17). Subjects inhaled normal saline, then doubling concentrations of methacholine phosphate from a Wright nebulizer for 2 min. FEV_1 was measured at 30, 90, 180, and 300 s after each inhalation. Spirometry was measured with a Collins water-sealed spirometer and kymograph. The test was terminated when a fall in FEV_1 of 20% of the baseline value occurred, and the methacholine PC_{20} was calculated.

Allergen-inhalation test. Allergen challenge was performed as described by O'Byrne and colleagues (18). The allergen producing the largest skin-wheal diameter was diluted in normal saline. The concentration of allergen extract for inhalation was determined from a formula described by Cockcroft and associates (19) using the results from the skin test and the methacholine PC_{20}. The starting concentration of allergen extract for inhalation was two doubling concentrations below that predicted to cause a 20% fall in FEV_1. The same doses of allergen were administered during each treatment period, and the FEV_1 was measured at 10, 20, 30, 40, 50, 60, 90, and 120 min after allergen inhalation, then each hour until 7 h after allergen inhalation. The early bronchoconstrictor response was taken to be the largest fall in FEV_1 within 2 h after allergen inhalation, and the late response was taken to be the largest fall in FEV_1 between 3 and 7 h after allergen inhalation.

Differential blood counts. Blood was collected into heparinized tubes by direct venipuncture, and blood smears were made for differential staining (Diff Quik; American Scientific Products, McGaw Park, IL). Differential cell counts were obtained from the mean of two slides with 300 cells counted per slide. Total leukocyte count was determined using a hemocytometer (Neubauer Chamber; Hausser Scientific, Blue Bell, PA), and cell populations were expressed as the number per milliliter of blood by dividing by the total number of cells counted and multiplying by the total leukocyte count.

Methylcellulose assay. Methylcellulose assays for CFU were performed as previously described (4). Mononuclear cells were separated from whole peripheral blood using Percoll density-gradient (Pharmacia, Uppsala, Sweden),

then adherent cells were removed by a 2-h incubation at 37°C in plastic flasks. Nonadherent mononuclear cells (NAMC) were cultured in 0.9% methylcellulose (Sigma Chemical Co., St. Louis, MO) at 1×10^5 per 35 × 10 mm tissue culture dish (Falcon Plastics, Oxnard, CA) in Iscove's modified Dulbecco's medium and 20% fetal calf serum (GIBCO, Burlington, ON, Canada) supplemented with 1% penicillin-streptomycin and 5×10^{-5} mol/liter of 2-mercaptoethanol.

To compare Eo/B colony cells from atopic and normal subjects, cells were grown *in vitro* under each of three different stimulatory conditions: 100 U/ml rhGM-CSF (Pharmingen, Markham, ON, Canada), 10 ng/ml rhIL-5 (Pharmingen), or 10 ng/ml rhIL-3 (Genzyme, San Diego, CA); and Eo/B colonies were enumerated. Ten to 20 Eo/B colonies were randomly picked after 14 d of culture from plates stimulated with each cytokine. To examine the time dependency of GM-CSF expression, cells were grown *in vitro* in 10 ng/ml rhIL-3 and picked as above after 10, 14, and 18 d of culture. To evaluate the effect of allergen inhalation by atopic asthmatics, cells were grown *in vitro* in the presence of either 100 U/ml rhGM-CSF, or 0.5 ng/ml stem cell factor (SCF) (Amgen, Thousand Oaks, CA) plus 10 ng/ml rhIL-3. Day 14 Eo/B-type granulocyte colonies from either GM-CSF- or SCF plus IL-3-stimulated cells were enumerated in two replicate methylcellulose plates under inverted microscopy. The Eo/B colonies picked for cytokine and mRNA expression were identified by morphology, being tight, granulated, compact, round refractile cell aggregations (2, 3).

Immunocytochemical staining. Cells collected from all conditions were washed in 0.5 ml Dulbecco's phosphate buffered saline (DPBS; GIBCO) and resuspended in DPBS at 0.75–1.0 × 10^6/ml, and cytospins were prepared on aptex-coated glass slides using 50 μl of cell suspension and a Shandon III cytocentrifuge at 300 rpm for 5 min (Shandon Southern Instruments, Sewickly, PA), fixed for 10 min in periodate-lysine-paraformaldehyde, then 10 min in 15% sucrose; slides were stored at −70°C. Cells were stained with mouse monoclonal anti-human GM-CSF antibody (Genzyme, Cambridge, MA) (atopic versus normal subjects) or mouse monoclonal anti-human IL-5 antibody (R&D Systems, Minneapolis, MN) (allergen inhalation). Antibodies were diluted in 1.0% bovine serum albumin (Sigma) and wash buffer made up of DPBS, 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid buffer and 0.01% saponin (Sigma). Labeling of these antibodies was detected by the alkaline-phosphatase antialkaline-phosphatase method (20). Slides were incubated for 60 min with 75% human AB serum (Sigma) and for 30 min with 25% normal rabbit serum (Sigma) to block nonspecific binding of the first and second antibodies, respectively. Slides were incubated overnight at a concentration of 10 μg/ml anti-GM-CSF and 30 μg/ml anti-IL-5. Mouse IgG_1 (Sigma) was used as a negative control. A positive control for GM-CSF immunostaining consisted of peripheral blood monocytes stimulated with lipopolysaccharide; for IL-5, peripheral blood eosinophils stimulated with calcium ionophore. The percentage of cells immunolocalizing GM-CSF and IL-5 was determined from a count of 400 cells under light microscopy, based on a scale from 0 to 5. All cells demonstrating an intensity of stain > 1 were counted as positive, and those < 1 were counted as negative.

In situ *reverse transcription-polymerase chain reaction.* Cells were picked from colonies and washed once with diethyl pyrocarbonate-treated saline, then resuspended at a concentration of 1.2 × 10^6/ml. Droplets of this cell suspension (20 μl) were placed at three sites on *in situ* polymerase chain reaction (PCR) glass slides coated with an aminoalkylsilane (Perkin Elmer, Mississauga, ON, Canada), then fixed for 20 min in 4% PBS paraformaldehyde and stored at −20°C. Two representative slides were randomly chosen from each of the three time points. These cells were permeabilized with 2 μg/ml proteinase K digestion (Sigma) and treated overnight with DNase (Boehringer Mannheim, Laval, PQ, Canada) at 37°C to digest nuclear material. Reverse transcription (RT) by incubation with the 3' downstream primer and reverse transcriptase (Superscript™ RNase H⁻; GIBCO) for 3 h at 37°C converted the mRNA to complementary DNA, and this product underwent 35 cycles of PCR (GeneAmp 1000 System, Perkin Elmer) using the optimal annealing temperature for human GM-CSF of 60°C, in buffer including the 5' upstream (ATG TGG CTG CAG AGC CTG CTG C) and 3' downstream (CTG GCT CCC AGC AGT CAA AGG G) primers for human GM-CSF (Applied Biosystems, University of Alberta, Edmonton, AB, Canada), and 115 U/ml TAQ polymerase enzyme (GIBCO) and digoxigenin-11 deoxyuridine triphosphate (dUTP) (Boehringer Mannheim). Slides were incubated with anti-digoxigenin (Boehringer Mannheim) and color-developed with a solution of 4-nitro blue tetrazolium chloride (Boehringer Mannheim) and X-phosphate/5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim). Slides were counterstained 10 min in 1% carbol chromotrope 2R (Sigma), which is specific for eosinophils. The slides were enumerated by examining 500 cells per slide with conventional light microscopy. Positive and negative controls were processed in parallel with the test site, on the remaining two sites on the slide. The positive control was not treated with DNAse or reverse transcriptase, and the negative control was not treated with reverse transcriptase. To eliminate the possibility of false positivity due to high background on the test site, one slide was treated with an RNase solution (Amersham Life Science, Cleveland, OH); another was treated with an irrelevant primer sequence. Both tests showed a high background that remained high even when mRNA was not amplified. Human β2 microglobulin was used as a system control.

Statistical Analysis

All summary statistics are expressed as mean and SEM, except for methacholine PC_{20}, which is expressed as geometric mean and geometric standard error of the mean (GSEM). Methacholine PC_{20} was measured by linear interpolation of log dose-response curves resulting in logarithmic values for PC_{20}, which were then subjected to statistical analysis. Student's paired *t* test was used to compare the allergen-induced changes in Eo/B CFU, blood eosinophils, percentage of Eo/B cells immunolocalizing GM-CSF and IL-5, and methacholine airway responsiveness. Repeated-measures analysis of variance was used to compare

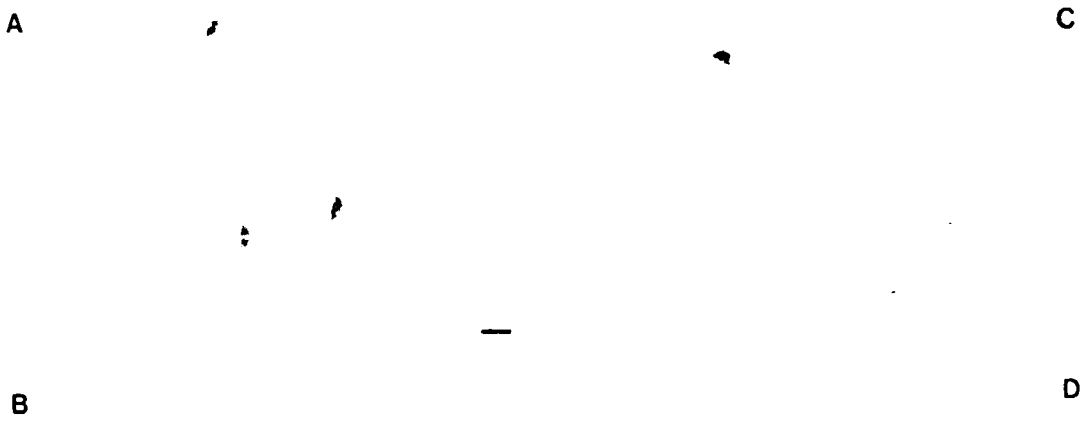

Figure 1. Immunoreactivity for GM-CSF (*A*, *B*) and IL-5 (*C*) with isotype control (*D*) in Eo/B colony cells. *Bar* = 5.5 μm (*A*, *C*, *D*); *bar* = 2.8 μm (*B*).

atopic and normal GM-CSF expression in colony cells grown under three different conditions, and to examine immunoreactive GM-CSF in Eo/B colony cells over time.

Results

GM-CSF Expression in Colony Cells Increases Temporally

Eo/B colony cells from atopic subjects grown with rhSCF plus rhIL-3 demonstrated immunoreactivity to anti-GM-CSF and anti-IL-5 (Figures 1A and 1C). Although only cells staining homogeneously with intensity > 1 were counted as positive, many of the cells demonstrated a faint positive signal localized next to the nucleus (Figure 1B). The percentage of Eo/B colony cells with immunoreactive GM-CSF after 14 and 18 d of culture was significantly higher than cells grown for 10 d ($P < 0.007$), being 8.1 ± 2.2%, 21.3 ± 4.6%, and 18.0 ± 4.0% after 10, 14, and 18 d, respectively. In addition, these cells were demonstrated by *in situ* RT-PCR to contain mRNA for GM-CSF at these times, being 2.7 ± 0.4%, 3.5 ± 0.2%, and 5.5 ± 0.8% after 10, 14, and 18 d, respectively (Figure 2).

Expression of GM-CSF in Colony Cells Is Greater in Atopic versus Normal Individuals

The number of Eo/B CFU grown with rhGM-CSF in atopics was 36.5 ± 21.5 per 10^6 NAMC versus 25.5 ± 11.5 per 10^6 NAMC in normal individuals. When the percentage of GM-CSF-immunoreactive Eo/B colony cells grown under three different conditions was compared, they were significantly increased in atopic subjects versus normal controls: under conditions of stimulation with rhGM-CSF, 53.4 ± 14.9% versus 14.5 ± 1.4%; rhIL-3, 73.2 ± 10.4% versus 24.0 ± 7.5%; and rhIL-5, 56.0 ± 11.3% versus 6.0 ± 1.2%, respectively ($P = 0.04$) (Figure 3). There was no significant difference in the percentage of GM-CSF-positive colony cells between the three growth conditions ($P = 0.26$). Cultures from two atopic subjects were excluded from analysis because of technical problems with the assay.

Allergen Inhalation

Twenty-five subjects completed the allergen-inhalation challenge and developed a dual airway response. The maximal percentage fall in FEV_1 during the early response was 32.4 ± 2.5% and during the late response was a 22.3 ± 2.7% maximal fall from pre-allergen baseline FEV_1 (Figure 4). The subjects also developed allergen-induced airway hyperresponsiveness 24 h following allergen inhalation, with a significant reduction in the methacholine PC_{20} from 1.60 mg/ml (1.31 GSEM) to 0.62 mg/ml (1.34 GSEM) ($P < 0.001$).

The number of peripheral blood eosinophils increased significantly 24 h after allergen inhalation, from pre-allergen values of 38.0 ± 3.8 × 10^4/ml to 57.3 ± 5.6 × 10^4/ml ($P = 0.005$) (Figure 5). Individual allergen-induced changes

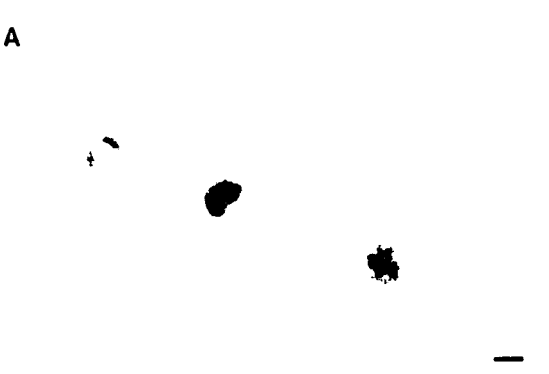

Figure 2. GM-CSF mRNA expression in Eo/B colony cells. Positive control (*A*), negative control (*B*), and test positive (*C*) for GM-CSF mRNA. *Bar* = 2.8 μm

in blood eosinophils ranged from a decrease of 28.5% to an increase of 614.6% from pre-allergen baseline, with a mean increase of 50.8%. The number of *in vitro* Eo/B CFU responsive to GM-CSF stimulation was also significantly elevated 24 h after allergen inhalation, from 10.2 ± 1.5 per 10^5 NAMC to 13.9 ± 1.8 per 10^5 NAMC ($P = 0.02$) (Figure 5). Individual changes in blood Eo/B CFU ranged from a 58.3% decrease to an increase of 433.3%, with an average increase of 36.3%. There was, however, no correlation between the individual changes in blood eosinophils and the individual changes in blood Eo/B CFU ($r = 0.07$, $P = 0.76$).

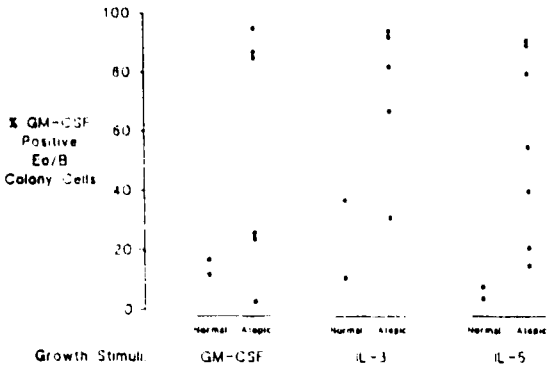

Figure 3. Immunoreactive GM-CSF in Eo/B colony cells from atopics and normal individuals. A comparison of the percentage of GM-CSF-positive Eo/B colony cells after 14 d culture, stimulated with GM-CSF, IL-3, and IL-5 in atopics versus normal controls ($P = 0.04$).

Colony cells grown *in vitro* under conditions of SCF and IL-3 stimulation contained immunoreactive GM-CSF and IL-5. The percent of colony cells expressing GM-CSF increased significantly from baseline values of 15.4 ± 1.3% before allergen to 29.9 ± 2.9% at 24 h after allergen inhalation ($P < 0.001$). There was a trend for IL-5 expression by colony cells to increase after allergen from 3.9 ± 0.8% to 5.7 ± 0.8%, however, this was not statistically significant ($P = 0.12$) (Figure 6).

Discussion

Eosinophils and their progenitors are present at elevated levels in atopic individuals, and can be further induced in atopic asthmatics by allergen-inhalation challenge. However, very little is understood about the mechanisms un-

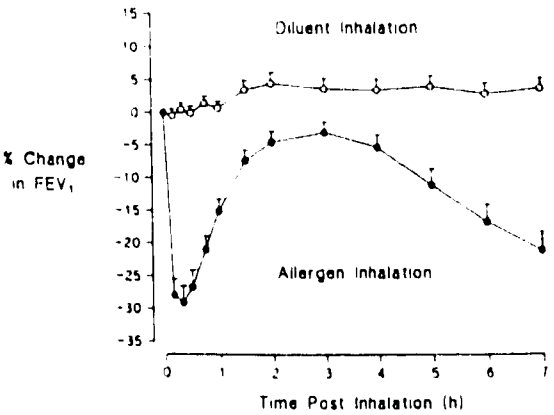

Figure 4. Allergen-induced airway responses. Percent change in FEV_1 (mean and SEM) in 25 atopic asthmatics after inhalation of diluent (*open circles*), and after inhalation of allergen (*closed circles*).

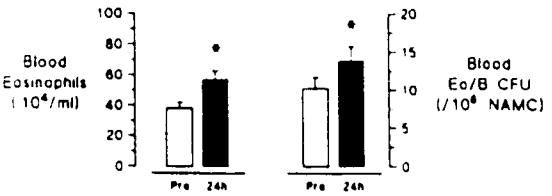

Figure 5. Peripheral blood eosinophils and Eo/B CFU before and after allergen-inhalation challenge. Circulating eosinophils (*left panel*) and Eo/B CFU (*right panel*) before allergen (*open bars*) and 24 h after allergen (*solid bars*). There was a significant increase in the number of eosinophils ($P = 0.0001$) and Eo/B CFU ($P = 0.02$) after allergen inhalation.

derlying this eosinophilia. There is evidence supporting the role of the tissue microenvironment in the accumulation of inflammatory cells at the site of inflammation (15). This is likely to be one mechanism contributing to elevation of eosinophils and their progenitors in the circulation of atopic individuals. This study examined the role of the constitutive expression of growth factors in differentiating eosinophils, which may represent a separate, autocrine mechanism enhancing the process of eosinophil differentiation shown to be upregulated in atopic individuals.

We have demonstrated by combined *in situ* RT-PCR and cytochemistry (particularly, carbol chromotrope 2R+ cells, i.e., eosinophilic) that Eo/B colony cells grown in culture express mRNA for GM-CSF. Carbol chromotrope is known to stain the granules of eosinophils that are cationic in nature (1). We have also demonstrated by immunocytochemistry that a significantly higher percentage of maturing, but not yet fully mature, progeny of eosinophil progenitors (Eo/B CFU) from the blood of atopic individuals express GM-CSF, when compared with controls. For technical reasons, the baseline colony growth and expression of GM-CSF in Eo/B colony cells was higher in these experiments (Study 1) than in the subsequent experiments (Studies 2 and 3). The latter demonstrated increased expression of GM-CSF in cells that have undergone further

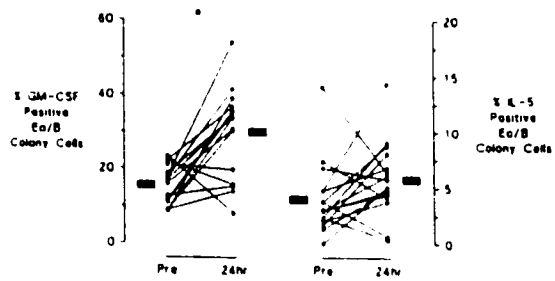

Figure 6. Immunoreactivity for GM-CSF and IL-5 in Eo/B colony cells before and after allergen-inhalation challenge. The percentage of GM-CSF-positive (*left panel*) and IL-5-positive (*right panel*) Eo/B colony cells before and 24 h after allergen. There was a significant increase in the percentage of Eo/B colony cells expressing GM-CSF ($P = 0.0009$), but not IL-5 ($P = 0.12$).

differentiation and a higher percentage of GM-CSF-positive colony cells 24 h after allergen inhalation in atopic asthmatics when compared with cells grown in culture before the allergen challenge. The higher GM-CSF expression observed in atopics and allergen-challenged asthmatics suggests that the atopic state per se, and/or allergen provocation, can directly or indirectly induce changes in Eo/B CFU or other nonadherent mononuclear cells, which confer an upregulated cytokine profile upon Eo/B CFU progeny *in vivo*. One implication of this activation state is that it may initiate another autocrine mechanism of Eo/B differentiation, further increasing the number of progenitors committed to this lineage. Similarly, atopy and/or allergen provocation may provide a "priming" effect for these cells such that they mature more quickly and develop the cytokine profile of a more mature cell earlier than nonatopic/unchallenged individuals.

Immunocytochemistry is a sensitive method to demonstrate the presence of intracellular protein, but it does not distinguish between protein that has been synthesized by the cell and extracellular protein that may be receptor-bound or bound to the interior of the cell. It is possible that GM-CSF may have been detected because of the binding of exogenous cytokine to receptors on maturing colony cells. We did, however, find differences in colony-cell immunostaining for GM-CSF even when other cytokines (SCF and IL-3) were used to stimulate the culture. This suggested that exogenous cytokine does not account for the immunostaining. However, in these cultures, which also contained GM colonies, it was possible that non-Eo/B cells generated GM-CSF, which then bound to GM-CSF receptors on the Eo/B colony cells. The staining pattern of GM-CSF and IL-5 expression, however, was typical of that observed for intracellular protein, being homogeneous throughout the cell rather than in a halo distribution typical of cells that have protein bound on their surface. In addition, gene transcription of GM-CSF by Eo/B colony cells was demonstrated with the presence of specific mRNA, confirming that these cells were capable of synthesizing this cytokine. Furthermore, the localized immunoreactivity adjacent to the nucleus may support the view that synthesis and storage of GM-CSF can be associated with intracellular organelles of the cell.

Mature eosinophils have the ability to synthesize IL-5 (8), IL-3 (9), and GM-CSF (10). This study demonstrates that immature and nascent eosinophils synthesize at least one of these growth factors. Increased constitutive expression of cytokines in maturing Eo/B CFU from atopic individuals may represent an autocrine mechanism for enhanced proliferation, differentiation, and activation of eosinophils in allergic responses. In support of this, there is also evidence for GM-CSF as an autocrine differentiating factor in an eosinophilic leukemia cell line, EoL-1 cells (21). GM-CSF is not only an important growth factor for eosinophils, but it also plays a role in cell viability by prolonging eosinophil survival *in vitro* (22). IL-5 is involved in terminal differentiation, stimulates function, and prolongs survival of the eosinophil (23, 24), whereas IL-3 prolongs survival and enhances functional properties of the eosinophil (25). IL-5 and GM-CSF are upregulated in diseases associated with blood and tissue eosinophilia (26), sup-

porting the hypothesis that cytokine inhibition or delay of apoptosis could be a mechanism for the development of blood and tissue eosinophilia in diseases such as asthma (27, 28). Tyrosine phosphorylation of cytokine receptors regulates the activation and inhibition of apoptosis in human eosinophils (29). Activation of IL-5 and GM-CSF receptors by common tyrosine kinases through the β receptor subunit may be essential for anti-apoptotic effects of IL-5 and GM-CSF (30). In our experiments, we did not compare expression of IL-5 in colony cells between atopic and normal individuals, but expression of IL-5 in eosinophil colony cells of atopic asthmatics was increased in some individuals after allergen challenge. The level of expression of IL-5 compared with GM-CSF in the colony cells from atopic asthmatics was considerably lower. This may reflect the relative immaturity of the colony cells, since IL-5 has shown to be expressed only by a limited number of fully differentiated cells (31).

Although immediate progeny (i.e., colony cells) of Eo/B progenitors derived from atopic individuals constitutively express higher levels of GM-CSF, the hemopoietic inductive microenvironment (HIM) itself may play a significant role in the development of eosinophilia during allergic inflammatory reactions. There are several mechanisms by which bone-marrow stroma can provide signals to control the process of hemopoiesis (32). Signals may be directed by cell-cell interactions, secreted soluble bioactive factors, and cell–matrix interactions. Atopic individuals may differ from normals in the set of signals provided for progenitors in the bone marrow. Thus, eosinophilic inflammation in atopic asthmatics may represent the effects of a cascade of cytokines, including growth and differentiation factors derived from resident inflammatory cells (33); airway structural cells such as fibroblasts and endothelial and epithelial cells (15, 34–37); and an "activated" HIM. Furthermore, subcutaneous injection of GM-CSF has been associated with increases in the numbers of circulating, colony-forming progenitors in human peripheral blood (38). This suggests that elevations of circulating hemopoietic cytokines in chronic tissue inflammation can potentially mobilize specific hemopoietic progenitor cells. Indeed, specific responsiveness to cytokines of progenitor cells can be induced in atopic individuals and allergic asthmatics through a process involving IL-5 receptor modulation on bone marrow and blood progenitors (39, 40). We are currently exploring the mechanism for this. Similarly, allergen challenge in airway hyperresponsive dogs elicits serum hemopoietic activity that can upregulate bone-marrow myeloid progenitors (41).

Previous work from this laboratory has demonstrated the importance of the microenvironment on the development of progenitor cells. The current study adds another dimension to the regulation of progenitor differentiation, and proliferation, suggesting that in atopic individuals, developing eosinophils may be altered by signals released after allergen inhalation to ultimately produce more hemopoietic cytokines. These cytokines in "activated" progenitors of eosinophils could contribute to the mobilization, differentiation, and activation of both maturing and mature eosinophils. Further experiments are required to determine how much of this cytokine expression by maturing eosinophils is secondary to signals from inflamed tissue and how much it reflects a primary (constitutive) upregulation of programs for cytokine expression in developing eosinophils in atopic and normal individuals.

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