Proliferative Response of Airway Smooth Muscle Cells to Macrophage-Derived Products

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Dedications

To Tante Mara whose light was an unwavering beacon in the darkness and whose brilliant shine was extinguished much too early for those of us left behind without our angel to guide us. Mara, you started all of this and for that I will be forever grateful.

To my beloved family for their unending support throughout the years. Mami and Papi, you encouraged my scientific tendencies from when I used to collect worms and observe them in my little bowl, and when I used to catch grasshoppers in glass jars. When I was young, you held my hand through the nights when my asthma attacks were so fierce that I thought each breath would be my last, and you consoled me when I couldn't play outside with my sisters because I wasn't well enough. (How appropriate that the disease that plagued my childhood should be the topic of my research.) Your unrelenting support gave me the strength to make it this far, and I could never have done it without you.

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Abstract

Brown Norway rats show increased airway smooth muscle content following repeated allergen challenges. Macrophages synthesize many growth factors *in vitro* which potentially stimulate proliferation of airway smooth muscle cells. Leukotriene C_4, an important mediator of allergic airway responses, can stimulate macrophages to release platelet-derived growth factor (PDGF) which is a potent stimulator of proliferation. The purpose of this study was to investigate the role of the macrophage in the proliferative response of airway smooth muscle cells leading to airway remodelling. Macrophages were harvested from 7- to 9-week-old male Brown Norway rats, allowed to adhere to plastic 25cm^2 culture flasks for 25 minutes, rinsed with sterile PBS at 37°C to wash off cells other than macrophages and maintained in short-term culture in serum-free medium. Airway smooth muscle cells were also harvested and cultured. Macrophage medium was collected after 12 hours and frozen. Smooth muscle cells were grown to confluence, subcultured at a density of 10^4 cells per well, allowed to grow for 72 hours in medium supplemented with 10% FBS, and growth arrested for 72 hours in medium supplemented with 1% FBS. For 6 hours following growth arrest, smooth muscle cells were treated with only the collected macrophage medium, and then [^{3}H]-thymidine was added to be incorporated into the cells to measure stimulation for proliferation. Supernatant from macrophages significantly stimulated airway smooth muscle cell proliferation 5-fold as compared to controls ($p<0.05$). This stimulation was affected neither by the addition of the cyclooxygenase inhibitor ASA, nor by the addition of the LTD_4 inhibitor MK-571. However, stimulation was decreased by the addition of the PDGF inhibitor suramin, as

well as by an anti-PDGF polyclonal antibody. Maximal inhibition observed with antibody was 34% ($p<0.01$). We conclude that macrophages have the ability to stimulate airway smooth muscle cell proliferation by releasing growth factors, that one of these growth factors is PDGF, and that PDGF contributes 34% of the total airway smooth muscle cell proliferation. Therefore, it is likely that macrophages play an important role in airway remodelling and that this airway remodelling may be an important component in the pathology of asthma.

Résumé

Chez des rats bruns de Norvège, les voies respiratoires présentent une plus forte teneur en muscles lisses après plusieurs provocations allergéniques. Les macrophages synthétisent de nombreux facteurs de croissance *in vitro*, qui pourraient stimuler la prolifération des cellules des muscles lisses des voies respiratoires. Le LTC_4, un important médiateur des réactions allergiques des voies respiratoires, peut stimuler la libération par les macrophages du facteur de croissance dérivé des plaquettes (PDGF), qui est un puissant stimulateur de la prolifération. La présente étude visait à examiner le rôle des macrophages dans la réaction de prolifération des cellules des muscles lisses des voies respiratoires; cette prolifération entraîne le remodelage des voies respiratoires. Des macrophages ont été recueillis sur des rats bruns de Norvège de sept à neuf semaines, laissés à adhérer à des plats de culture de plastique de 25 cm^2 pendant 25 minutes, rincés au PBS stérile à 37°C afin de lessiver les cellules autres que les macrophages et maintenus en culture à court terme dans un milieu exempt de sérum. Des cellules de muscles lisses des voies respiratoires ont également été recueillies et mises en culture. Le milieu de macrophage a été recueilli après 12 heures et congelé. Les cellules de muscles lisses ont été cultivées jusqu'à l'état de confluence, sous-cultivées à une densité de 10^4 cellules par puits, laissées à croître pendant 72 heures dans un milieu additionné de FBS à 10%, après quoi la croissance a été stoppée pendant 72 heures dans un milieu additionné de FBS à 1%. Pendant les six heures suivant l'arrêt de la croissance, les cellules de muscles lisses ont été traitées uniquement au milieu de macrophages recueilli, après quoi de la [^{3}H]-thymidine a été ajoutée afin qu'elle s'intègre aux cellules et permette

de mesurer la stimulation de la prolifération. Le produit surnageant des macrophages a stimulé de façon significative la prolifération des cellules des muscles lisses des voies respiratoires, qui se sont multipliées par cinq, par comparaison au groupe témoin ($p<0.05$). Cette stimulation n'a été affectée ni par l'ajout de l'inhibiteur de cyclo-oxygénase ASA, ni par l'ajout de l'inhibiteur de LTD_4 MK-571. Toutefois, la stimulation a été réduite par l'ajout de suramine, un inhibiteur de PDGF, ainsi que par des anticorps polyclonaux anti-PDGF. L'inhibition maximale observée aux anticorps a été de 34% ($p<0.01$). Nous en concluons que les macrophages ont la capacité de stimuler la prolifération des muscles lisses des voies respiratoires en libérant des facteurs de croissance, que l'un de ces facteurs de croissance est la PDGF, et que la PDGF compte pour 34% de la prolifération totale des cellules des muscles lisses des voies respiratoires. Il est donc vraisemblable que les macrophages jouent un rôle important dans le remodelage des voies respiratoires et que ce remodelage puisse constituer un élément important de la pathologie de l'asthme.

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Abbreviations

ASA	Acetyl salicylic acid
BAL	Bronchoalveolar lavage
bFGF	Basic fibroblast growth factor
ConA	Concanavalin A
DAG	Diacyl glycerol
ECF-A	Eosinophil chemotactic factor of anaphylaxis
EGF	Epidermal growth factor
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
FBS	Fetal bovine serum
GM-CSF	Granulocyte/macrophage colony-stimulating factor
HBSS	Hank's balanced salt solution
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
IP_3	Phosphatidylinositol 3,4,5-triphosphate
IPF	Idiopathic pulmonary fibrosis
LCF	Lymphocyte chemoattractant factor
LPS	Lipopolysaccharide
LT	Leukotriene
MBP	Major basic protein
NCF	Neutrophil-chemotactic factor
PAF	Platelet activating factor
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PG	Prostaglandin
PKC	Protein kinase C
PLC	Phospholipase C
SF-DMEM	Serum-free Dulbecco's modified Eagle's medium
TNF-α	Tumour necrosis factor-α
TX	Thromboxane

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Asthma

Asthma is a chronic disease of the airways characterized by increased bronchoconstrictive responses to various stimuli which induce paroxysmal airflow limitation, marked dyspnea, and wheezing (Robbins and Kumar, 1987). It is defined as a disease of increased airway hyperresponsiveness to a variety of stimuli, with variable airway obstruction and is usually accompanied by histological evidence of inflammatory changes of the airway wall (American Thoracic Society, 1987). Airway obstruction is reversible at least in part with the symptoms resolving either spontaneously or with treatment, although in some cases the attack fails to remit (status asthmaticus) and may prove to be fatal. The airway narrowing is due to the constriction of airway smooth muscle as well as due to accumulation of shed epithelial cells, leukocytes, secretions, and proteinaceous exudate in the airways.

An integral part of asthma is the airway inflammation in which there is a characteristic eosinophil infiltrate. This infiltrate serves to distinguish asthma from other airway inflammatory diseases (Kirby *et al.*, 1987). A feature of untreated asthma is inflammation of the airways which contributes directly and indirectly to the obstructive process (Olivieri & Foresi, 1992). Another feature of asthma is the increased numbers and activation of mast cells which contribute to the allergic response. The pathological changes that have been described in the lung and result in significant remodelling of the airway wall include hypertrophy of airway smooth muscle cells, thickening of the subepithelial membrane, disruption of the airway epithelium, prominence of the mucous glands and goblet cells, an inflammatory infiltrate, partial occlusion of the lumen with

cellular debris and mucus, and increased airway wall area (James *et al.*, 1989; Laitinen & Laitinen, 1991; Katz & Beer, 1993).

1.1.1 The Epidemiology of Asthma

1.1.1.1 The Factors Affecting the Prevalence of Asthma

The factors affecting the prevalence of asthma include age, sex, race, heredity, occupational environment, air pollution, respiratory infections, smoking, and breast feeding (Bonner, 1984; Office of Health Economics, 1990).

Asthma is most prevalent among children and older adults. On average, approximately 10% of children have asthma (Gergen *et al.*, 1988). Childhood asthma tends to develop in the first few years of life with 33% of asthmatic children having an onset before two years of age and 80% having an onset before five years of age. Most children cease having exacerbations by age 12 and the number of adolescents having asthma drops to 6%. Prevalence of asthma in older adults peaks in the population aged 70 or more with 6.5% of individuals exhibiting symptoms. Although some individuals have asthma throughout their entire lives, the onset and remission of asthma can occur at any age.

The second factor affecting the prevalence of asthma is gender. In children with asthma, boys outnumber girls by a ratio of 3:2 in the 6 to 11 year old group and by a ratio of 8:5 in the 11 to 17 year old group. This ratio is lost in early adulthood as asthma becomes as common among women as men, and the ratio changes to 5:2 in older adults as asthma again becomes more prevalent among men (Burr *et al.*, 1979).

Race has an influence on the prevalence of asthma. For all ages in the U.S., asthma is less prevalent among whites than among other races. This difference may be partly due to environmental factors as the prevalence is low in non-white developing countries (Cookson & Makoni, 1980).

Heredity appears to play a role in the development of asthma. Twin studies show that the predisposition to asthma is heritable although concordance is incomplete. This incompleteness may be due to environmental or other factors which influence the development of the disease (Siegel & Rachelefsky, 1985).

The occupational environment influences the development of asthma. One of six asthmatic adults attributes their illness to exposure to factors at their place of work. In addition to this, the risk of occupational asthma is highest among industrial and agricultural workers (Chan-Yeung *et al.*, 1985). Occupational exposures are recognized as being more and more important to the prevalence of asthma. The pathogenic mechanism may be toxic, pharmacologic, or immunologic (Davies *et al.*, 1983). Substances causing occupational asthma include metal salts, wood dust, vegetable dusts, industrial chemicals, pharmaceutical agents, and biologic enzymes. The underlying mechanism involves the development of immunologic sensitivity or nonspecific triggering of bronchospasm. Pre-existing atopy increases the probability of sensitization to certain substances such as proteolytic enzymes and flour. Usually, improvement is noted when subjects change jobs, however it may require months (Chan-Yeung *et al.*, 1982).

Air pollutants such as sulfur dioxide and ozone are present in high enough concentrations in the air to cause increased airway resistance. If the airways of an

asthmatic are hyperreactive or hypersensitive, then it is likely that pollution may contribute to the severity or frequency of attacks. The incidence of asthma has increased in relation to increases in air pollution in cities such as London, Tokyo, and Los Angeles (Bonner, 1984).

Respiratory infections, especially viral infections, may initiate an asthmatic attack and are more likely to do so in children than in adults (Hudgel *et al.*, 1979). Viruses are believed to cause attacks by several mechanisms including damaging the respiratory epithelium, changing the function of β-receptors, stimulating the synthesis of antiviral immunoglobulin E (IgE), or enhancing the release of histamine from mast cells (Frick, 1983). Viruses may also contribute to allergic sensitization since acute viral infection often coincides with the onset of allergic sensitization (Frick *et al.*, 1979).

Smoking may affect the prevalence of asthma as parental smoking correlates with wheeziness in children (Gortmaker *et al.*, 1982). Cigarette smoke is a bronchial irritant and may promote allergic sensitization by leading to increased levels of IgE (Gerrard *et al.*, 1980).

Feeding breast milk instead of cow's milk reduces the incidence of atopic disease and infections in infants (Chandra, 1979). Breast-feeding appears to be protective against the development of allergies in susceptible children especially when the infant's diet is restricted (Schatz *et al.*, 1983). Breast-feeding directly affects allergic sensitization through avoidance of foreign antigens found in cow's milk; and indirectly by supplying high levels of secretory IgA which not only bind antigens in the gut and lumen, but also absorb into the circulation where they bind antigens (Bonner, 1984).

1.1.1.2 The Morbidity and Mortality of Asthma

In Canada, deaths from asthma as a percentage of total deaths increased by 50% from 1974 (0.194) to 1984 (0.292). This increase was most noteworthy in the 25 to 34 year age group (Bates & Baker-Anderson, 1987). Asthma patients comprise a considerable part of a general practitioner's patients. In the U.S., nearly 33% of all patients with asthma are confined to bed for some period and 33% frequently or always exhibit symptoms. Of asthmatic children approximately 50% miss 6 or more days of school per year and approximately 10% are admitted to hospital for up to 10 days per year (Wilder, 1973).

More than 33% of asthmatic patients have 2 or more severe asthma attacks in a 6-month period. The morbidity of asthma is augmented by higher than normal levels of anxiety and restrictions on lifestyle (White *et al.*, 1989). The quality of life is affected by factors such as 1) disturbed sleep which results not only in increased tiredness, but may also disrupt normal development due to disturbance of the normal biological rhythms, and 2) restricted physical and sporting activities.

1.1.1.3 Asthma Therapies

Asthma therapies fall into two categories: those that prevent or reverse airway inflammation and hyperresponsiveness, and those that relieve the airway smooth muscle contraction (Cockcroft, 1990). Environmental control is perhaps the most obvious route of therapy for extrinsic asthma, although it may be difficult to achieve (Platts-Mills *et al.*, 1982). Removal of the causes of airway inflammation is critical to the management of disease. Once the causes are removed, improvement of symptoms may occur immediately

or may require weeks or months, reflecting the slow improvement of inflammation.

Inhaled cromolyn is a drug used to treat asthma and it acts prophylactically by preventing allergens and sensitizing agents from producing airway inflammation (Cockcroft and Murdock, 1987). If given before allergen exposure, cromolyn prevents the early asthmatic response, the late asthmatic response and airway hyperresponsiveness. If given following allergen exposure, it does not have this effect. Cromolyn appears to act by aborting airway inflammation and airway hyperresponsiveness and induces spontaneous resolution of the latter. This, along with cromolyn's ability to prevent the early response and the late response, suggests that this drug inhibits mediator release from mast cells, basophils, and eosinophils. The way in which it inhibits bronchospasm is not known, but is probably by indirect means through inhibition of the release and/or synthesis of bronchoactive mediators.

Corticosteroids do not inhibit the early response, therefore they do not prevent acute bronchoconstriction. They do, however, have anti-inflammatory effects on the late response and reduce airway hyperresponsiveness (Cockcroft and Murdock, 1987).

Bronchodilators serve to relieve bronchoconstriction but do not influence long-term airway hyperresponsiveness (Cockcroft *et al.*, 1989). Although they alleviate airway smooth muscle contraction, they do not affect the late response or the allergen-induced increase in nonallergic airway hyperresponsiveness. β_2-agonists, for example, affect airway smooth muscle directly and lead to bronchodilation, inhibition of bronchospasm, or both. Anticholinergic drugs such as ipratropium and atropine moderately inhibit most bronchoconstrictive triggers. They are relatively ineffective in inhibiting the allergen-

induced asthmatic response. Theophylline is an effective bronchodilator, however it does not inhibit most bronchospastic triggers.

1.1.2 Intrinsic and Extrinsic Asthma

Asthma can be divided into two distinct types based on the underlying cause of symptoms. Intrinsic asthma, also known as nonallergic, nonatopic or postinfectious asthma, is the form of asthma that is not obviously the outcome of external allergen exposure (Rackermann, 1947). Although the etiology of intrinsic asthma remains somewhat unknown, several possible underlying causes have been postulated: bacterial or viral infections, pollution, parasympathetic nervous system abnormalities, hidden allergens or auto-immune phenomena (Walker, 1993).

Intrinsic and extrinsic asthma have distinct clinical and laboratory characteristics. Intrinsic asthma usually starts in adulthood, is perennial, is more severe, is exacerbated by viral respiratory infections and is often associated with chronic rhino-sinusitis and nasal polyposis (Walker, 1993). Intrinsic asthmatics, by definition, have negative skin prick test responses to common aeroallergens and normal levels of total and specific serum IgE. In contrast, extrinsic asthma usually begins during childhood, is a disease of Type I hypersensitivity and is mediated by IgE and allergy. It is characterized by allergen-dependent, seasonal symptoms with positive skin tests, elevated total and allergen-specific serum IgE, and a positive family history. One feature shared by both types of asthma is eosinophilia in the blood, BAL fluid and sputum.

Extrinsic asthmatics show a pattern of acute T-helper cell activation in

bronchoalveolar lavage (BAL) fluid and peripheral blood. As this T-cell activation correlates with numbers of low affinity IgE-bearing B-cells, extrinsic asthma is an IgE-mediated disease (Walker, 1993). Intrinsic asthmatics, however, show activation of both CD4+ and CD8+ T-cells in blood and BAL fluid (Walker *et al.*, 1991).

Both extrinsic and intrinsic asthmatics have increased amounts of interleukin-3 (IL-3), IL-5, and granulocyte/macrophage colony-stimulating factor (GM-CSF) in their sera with the concentrations being sufficiently high to prolong eosinophil survival (Walker, 1993). Extrinsic asthmatics, however, show increased levels of IL-4 and IL-5 in BAL fluid and this elevated IL-4 presumably contributes to the elevated IgE levels found in the allergic subjects (Walker *et al.*, 1991). Intrinsic asthmatics, however, show elevated levels of IL-2, interferon-γ (IFN-γ), and IL-5. In both types of asthma, there is a close correlation between levels of IL-5 and eosinophilia, suggesting that IL-5 is the main cytokine responsible for the eosinophil infiltration in asthmatic airways. Although there are more similarities than differences between extrinsic and intrinsic asthma, the differences suggest a basic immunological difference between the two.

1.1.3 The Early and the Late Response

Allergic factors are frequently found in association with asthma. Responses to allergen exposure result in three characteristic events, the early asthmatic response, the late asthmatic response, and bronchial hyperreactivity (Johnston & Holgate, 1991). Allergen challenge in atopic subjects elicits an early response which occurs maximally within 10 to 15 minutes following introduction of specific allergen into the skin or

airways and resolves spontaneously within one to three hours (Busse *et al.*, 1993). In the skin, there is an immediate reaction consisting of pruritis, erythema and edema, accompanied histologically by mast cell degranulation and increased microvascular permeability (Ting *et al.*, 1980). In the upper and lower airways, there is an immediate allergic reaction producing symptoms of rhinoconjunctivitis, and, in a subset of atopic subjects, asthma involving smooth muscle contraction, plasma exudation and mucus production. Bronchoconstriction is induced by spasmogens such as histamine, eicosanoids, and platelet activating factor (PAF) which are released from IgE-receptor-bearing cells such as mast cells, macrophages, platelets and eosinophils. Various resident cells, including mast cells, macrophages and airway epithelial cells secrete potent inflammatory cell chemotactic factors such as LTB_4, hydroxy-eicosatetraenoic acid, eosinophil chemotactic factor of anaphylaxis (ECF-A), and PAF. The secretion of spasmogens and chemotaxins may be stimulated in an IgE-dependent (immunologic) manner or IgE-independent (nonimmunologic) manner. The simultaneous release of spasmogens and chemotaxins induces airway inflammation and may be the link between the early response and late response (Katz & Beer, 1993).

Following the early response, there is a recovery period of one to two hours which is often followed by a secondary bronchoconstriction termed the late response. The late response appears from four to 12 hours following allergen challenge and may last for more than 24 hours. Most allergic asthmatics who develop an early response also develop a late response, although occasionally only an early response or only a late response is experienced (Busse *et al.*, 1993). Subjects who exhibit only the early response show lower

levels of specific IgE antibodies than subjects experiencing both the early response and late response (Crimi *et al.*, 1992). Therefore, IgE appears to be at least partly associated with the late response. Microscopically, there is edema and a mixed inflammatory cell infiltrate consisting of polymorphonuclear and mononuclear leukocytes (Charlesworth *et al.*, 1989). The late response is frequently seen in a number of animal models and in the Brown Norway rat. The response is attributable to airway smooth muscle constriction in the large airways in the Brown Norway rat (Du *et al.*, 1991), although microvascular leak may also contribute in part in other species (Erjefalt *et al.*, 1993). This IgE-dependent, allergic, late-phase response manifests clinical signs in the skin as erythema and edema, in the nose as congestion and pruritis, in the conjunctiva as edema and pruritis, and in the lungs as airway obstruction.

Bronchial hyperresponsiveness begins within three hours of allergen challenge and may last for several weeks (Johnston & Holgate, 1991). Tissue events involving airway inflammation may occur before the late response is apparent and continue for some time thereafter. Bronchial hyperresponsiveness, which does not correlate well with the severity of disease, does show a general relationship with several indices of disease severity (Cockcroft *et al.*, 1977).

1.2 The Pathology of Allergic Asthma

1.2.1 Airway Inflammation

Evidence for inflammation as a cause of airway obstruction comes from the association of asthma with atopy, the presence of eosinophils in sputum and blood, and

the beneficial effect of steroids in the treatment of asthma (Vrugt & Aalbers, 1993). The diathesis of asthma features airway inflammation and airway hyperresponsiveness to exogenous and endogenous stimuli. Pathological changes in the lung include smooth muscle hypertrophy/hyperplasia, subepithelial membrane thickening, disruption of airway epithelium, prominence of mucous glands and goblet cells, airway inflammation associated with infiltration of neutrophils, eosinophils, mononuclear phagocytes and lymphocytes, partial occlusion of the lumen with cellular debris and mucus, and an overall increase in airway wall area demonstrable by morphometry (Dunnill *et al.*, 1969; Heard & Hossain, 1972; James *et al.*, 1989; Laitinen & Laitinen, 1991; Saetta *et al.*, 1992; Katz & Beer, 1993).

The airway tissue comprises the epithelial lining, secretory glands, cartilage, smooth muscle, bronchial vessels, and connective tissue. These tissues are arranged such that airway lumen narrowing results if there is smooth muscle spasm, submucosal connective tissue swelling, or exudation onto the airway lumen surface.

Asthma is a chronic inflammatory disease which contributes directly and indirectly to the obstructive process. A constant feature of untreated asthma is inflammation of the airways, regardless of the clinical state of the disease (Olivieri & Foresi, 1992). Even patients with extremely mild asthma show an abnormal mucosa, mast cell degranulation, widespread eosinophil infiltration and increases in mucosal neutrophils and mononuclear cells (Beasley *et al.*, 1989). Mast cells and basophils may not always be visible once they degranulate (Rankin, 1989). BAL fluid is characterized by elevated numbers of eosinophils and mast cells during mild to moderate episodes of disease (Wardlaw *et al.*,

1988; Olivieri & Foresi, 1992). Sputum features similar increases in eosinophils and mast cells during mild exacerbations (Gibson *et al.*, 1989). Morphologically, neutrophils, mononuclear cells and especially eosinophils are associated with mucosal edema in the airways in active asthma as well as during remission (Djukanovic *et al.*, 1990). Inflammatory cells and mediators in the airways determine epithelial shedding, eosinophil infiltrate in the submucosa, edema, airway smooth muscle hypertrophy, and thickening of the basement membrane. Subjects having a thickened basement membrane have significantly more inflammatory cells and eosinophils in their airways than subjects having a normal basement membrane, thus indicating that the degree of basement membrane thickening is related to mucosal inflammation (Olivieri & Foresi, 1992).

The inflammatory response commences with an imbalance between plasma exudation and lymphatic drainage which determines the severity of edema. Following exudation of fluid, platelets, polymorphonuclear leukocytes, monocytes, and lymphocytes appear at the site of inflammation (Laitinen & Laitinen, 1991). This cellular infiltrate is responsible for the structural changes, the first of which is epithelial shedding. The shedding may occur in clumps called Creola bodies. This is followed by mucus hypersecretion from epithelial cells and submucosal glands. Mucous plugs form when fluid exudate, cells from the microvasculature, cells shed from the epithelium, and mucus fill the airways. During repair, epithelial cells divide to cover the denuded surface which may be responsible for another feature of asthma, the thickened basement membrane. In addition to this, proliferation of microvessels, connective tissue and muscle causes a thickening of the submucosa (James *et al.*, 1989).

1.2.1.1 Inflammatory Mediators

The mediators of the inflammatory response fall into three categories: tissue-derived mediators, plasma-derived mediators, and migrating cell-derived mediators. Tissue-derived mediators are derived from the metabolism of arachidonic acid via the cyclooxygenase or 5-lipoxygenase pathway. Thromboxane A_2 (TXA_2) is one of the mediators derived from the cyclooxygenase pathway and is a platelet activator, whereas LTC_4 is one derived from the 5-lipoxygenase pathway and is able to activate macrophages. Histamine, which is important in airway narrowing, is synthesized in the granules of mast cells (reviewed by Hogg, 1990).

Plasma-derived mediators such as the Hageman factor are activated when plasma exudate comes into contact with a foreign surface. The Hageman factor converts plasma proenzymes to their active form which generates inflammatory mediators via the activation of the complement pathway, coagulation, kinin cascade, and fibrinolytic processes (Colman, 1993).

Mediators derived from migrating cells contribute a variety of inflammatory mediators. Neutrophils, for example, release cationic proteins, acid and neutral proteases from granules, and various toxic oxygen metabolites including the superoxide anion, the hydroxyl radical, and hydrogen peroxide. Monocytes migrate into areas of inflammation and transform into macrophages which process antigen and generate cytokines that stimulate T-cell and B-cell proliferation. Monokines and lymphokines affect the function of neutrophils, eosinophils, monocytes, and lymphocytes in inflammation (reviewed by Hogg, 1990).

1.2.2 Airway Hyperresponsiveness

Airway hyperresponsiveness is associated with airway inflammation which is also generally believed to be responsible for the late response (Cockcroft, 1990). Hyperresponsiveness begins within three hours of allergen challenge and may last for several weeks. Tissue events involving airway inflammation may occur before the late response is apparent and continue for some time thereafter (Johnston & Holgate, 1991). Although asthma symptoms and airway hyperresponsiveness vary in the atopic subject, the variation correlates with exposure to different triggering factors. These factors are classified into two groups: the inflammatory triggers and the bronchospastic triggers (Cockcroft, 1990).

1.2.2.1 Inflammatory Triggers

Inflammatory triggers, such as allergen, increase airway hyperresponsiveness by evoking an inflammatory response in the airways and airway hyperresponsiveness correlates with the prevalence of atopy. Bronchospastic triggers, such as dust and cold air, cause bronchospasm alone and are regarded as producing symptoms and not asthma itself. These triggers do not cause airway inflammation and the degree to which they induce airway smooth muscle contraction depends on the existing level of airway hyperresponsiveness (Cockcroft, 1990).

Other inflammatory triggers include occupational sensitizing chemicals, viruses, and irritant gases (Cockcroft, 1990; Busse *et al.*, 1993). Allergens are responsible for transient, acute increases in airway hyperresponsiveness and repeated allergen exposure

may lead to permanent hyperresponsiveness that characterizes the perennial allergic asthmatic.

Low molecular weight chemicals such as toluene diisocyanate and plicatic acid may produce asthma in otherwise nonatopic individuals (Lam *et al.*, 1979). Like allergens, low molecular weight chemicals produce large and long-lasting changes in airway hyperresponsiveness, and prolonged exposure to these triggers may lead to permanent airway hyperresponsiveness. Whether the early response, late response, or airway hyperresponsiveness observed in these patients is IgE-mediated is not clear, however, 90% of sensitive subjects exhibit a late response compared to 50% of allergen-sensitive subjects.

Viral respiratory infections often exacerbate asthma and may elicit an inflammatory response in the airways which may induce airway hyperresponsiveness (Cockcroft, 1990). Symptoms may last for weeks in normal, nonatopic individuals, however, the increased airway hyperresponsiveness is less than that observed in asthmatics. In children, viral infections are often precipitants of asthma.

High concentrations of irritant gases such as sulfur dioxide and nitrogen dioxide induce inflammatory exacerbations caused by the infiltration of neutrophils (Lam *et al.*, 1979). Exposure to low concentrations of these noxious gases, however, usually induces bronchospastic and not inflammatory changes. Irritant gas-induced changes are small, short-lived and quickly lead to tolerance as compared to allergen-induced changes.

1.2.2.2 Bronchospastic Triggers

Bronchospastic triggers such as histamine, methacholine, cholinergic agonists, cholinesterase inhibitors and β-adrenergic antagonists cause bronchospasm without leading to airway inflammation. Other bronchospastic triggers include cold air, emotional stress, inhaled particles, exercise, low concentrations of irritant gas, hyperventilation, cigarette smoke, and the isolated early response (Cockcroft, 1990; Busse *et al.*, 1993). These triggers cause airway smooth muscle contraction in various ways, including neural mechanisms involving tachykinin release and cholinergic reflexes.

1.2.2.3 Airway Hyperresponsiveness

A characteristic feature of asthma is increased bronchial hyperresponsiveness to non-specific stimuli which is used as a diagnostic criterion for asthma. Although histamine and cholinergic drugs are the most commonly used stimuli for the quantitation of airway hyperresponsiveness, others include slow-reacting substance of anaphylaxis, $PGF_{2\alpha}$, β-adrenergic blocking drugs, cold air, sulfur dioxide, dust, and exercise (Benson, 1975). Usually, subjects showing hyperresponsiveness for one stimulus also show hyperreponsiveness to other stimuli, although concordance is not the rule among different stimuli. Airway hyperresponsiveness varies diurnally within subjects and peaks at night. Hyperresponsiveness is also increased during or immediately following an asthma attack and decreases with clinical improvement.

Although hyperresponsiveness is the underlying cause for asthmatic symptoms including nocturnal symptoms, early morning symptoms, and wheeziness due to exercise,

cold air, fumes, smoke, and aerosol sprays, it is also experienced under other conditions. For example, transient hyperresponsiveness is experienced by normal subjects following upper respiratory tract infections and subjects suffering allergic rhinitis during the pollen season, and longstanding hyperresponsiveness is found among subjects with chronic obstructive pulmonary disease and cystic fibrosis (Kay *et al.*, 1989).

1.2.2.4 Inflammation and Airway Hyperresponsiveness

There is strong evidence that airway inflammation is directly related to the development and maintenance of airway hyperresponsiveness. Airway inflammation is linked to hyperresponsiveness through bronchial edema, mucosal permeability, sensory nerve exposure, and proinflammatory mediator release (Chung, 1986). In asthmatics, mast cells, eosinophils, and total cell number in BAL fluid is significantly correlated with measurements of airway hyperresponsiveness (Kirby *et al.*, 1987). Stimuli such as allergen challenge are associated with the induction of hyperresponsiveness and cause airway inflammation.

Following the allergic response in the airways, asthmatic subjects frequently experience an increase in airway responsiveness to methacholine, histamine, or exercise (Mussaffi *et al.*, 1986). In addition to this, allergen-induced late responses are often associated with increased airway responsiveness (Cartier *et al.*, 1982), and non-allergic bronchial reactivity is worsened by prior exposure to allergen (Cockcroft *et al.*, 1977). This suggests that inflammatory responses play a role in airway hyperresponsiveness.

Airway inflammation is the mechanism underlying the development of

hyperresponsiveness as mediator release from inflammatory cells, infiltration of peripheral blood leukocytes, and eosinophilia in the blood and airways are observed during the late response (Durham *et al.*, 1988). The mechanism of hyperresponsiveness may be linked to inflammation in the airways since the development of airway hyperresponsiveness is closely related to the presence of a late response and the late response is closely related to the appearance of inflammatory cells and inflammatory responses in the airways of Brown Norway rats (Sapienza *et al.*, 1991).

1.2.3 Increased Airway Smooth Muscle Content

Studies using point counting methods show that there is increased airway smooth muscle content in the airways of patients dying of status asthmaticus as compared to patients dying of other causes (Dunnill *et al.*, 1969). Other studies using the point counting method have found similar increases and it appears that increased airway smooth muscle is a typical feature of asthma (Takizawa & Thurlbeck, 1971). Airways of asthmatic subjects show an increase in airway wall area due to an increase in the area of epithelium, the area of airway smooth muscle, and the area of submucosa (James *et al.*, 1989). There is increased airway smooth muscle content in the large and small airways except for membranous airways of internal perimeter less than 2 mm.

Airway smooth muscle content can be measured using point counting and this technique shows the airway smooth muscle content to be tripled in asthmatic subjects as compared to normal subjects (Heard & Hossain, 1972). Counting the individual muscle cells reveals an increase in cell number. Therefore, it is likely that the increased airway

smooth muscle content is due to hyperplasia of muscle cells in both the proximal and distal bronchi.

The airways of asthmatic human subjects show increased airway smooth muscle content which is a result of hyperplasia or hypertrophy of the smooth muscle cells (Hossain, 1973; Ebina *et al.*, 1993). Using a new technique of three-dimensional morphometry, some asthmatics are shown to have a thickened smooth muscle layer in only the central bronchi and due only to hyperplasia of the airway smooth muscle cells, whereas others have a thickened smooth muscle layer in the bronchi (due to hyperplasia) and to a lesser extent in the bronchioles (due to hypertrophy) (Ebina *et al.*, 1993). This increased airway smooth muscle content is a potential cause of the increased responsiveness in asthmatic airways and may account for excessive airway narrowing when a bronchoconstrictive stimulus is presented. Brown Norway rats sensitized to allergen show a similar increase in airway smooth muscle content in the airways following repeated allergen challenges (Sapienza *et al.*, 1991). In addition to increased airway smooth muscle mass, Brown Norway rats exhibit significantly increased responsiveness to aerosolized methacholine.

1.2.4 Altered Airway Mechanics

Asthmatic subjects display excessive airway narrowing to both specific and non-specific stimuli. The airway tissues which comprise the epithelium, the secretory glands, the cartilage, the smooth muscle, the bronchial vessels, and the supporting connective tissue function together such that airway narrowing may result from bronchial smooth

muscle spasm, submucosal connective tissue swelling, or exudation onto the airway lumen surface (Moreno *et al.*, 1986). Quantitating airway obstruction is done by measuring airway or pulmonary resistance or by measuring change in maximal expiratory flow. Maximal expiratory flow is related to the mechanical properties of the airways at the sites of flow limitation, the calibre of airways upstream from the points where airway collapse occurs, and the elastic recoil of the lung (Paré *et al.*, 1991). In asthma, lung elastic recoil and airway closure are believed to be less important than airway narrowing. Pulmonary and airway resistance, which are measured during tidal breathing, quantitate airway narrowing more specifically than maximal expiratory flow, which is measured during maximal forced expiration. During tidal breathing, there is less influence of lung recoil and no influence of airway collapse as compared to during forced expiration.

Airway narrowing is believed to be caused by the increased airway smooth muscle content which contributes to airway wall thickening. If the airway wall is thicker, less airway smooth muscle shortening is required for a given degree of airway narrowing. This is influenced by the proportion of the airway circumference that is occupied by airway smooth muscle, the airway wall thickness, the presence of exudate in the airways, and the mechanical advantage of the airway smooth muscle (Moreno *et al.*, 1986).

Models of the tracheobronchial tree permit the simulation of the mechanisms underlying airway narrowing, and allow the examination of the interaction between airway wall thickening and airway smooth muscle shortening on airway resistance (Wiggs *et al.*, 1990). When morphometric methods are used to generate values for airway wall thickening and these values are used with the models, the effect of wall thickening on

airway narrowing can be calculated (Wiggs *et al.*, 1992). In peripheral airways showing small increases in airway wall thickness which do not affect baseline resistance, there can be a marked increase in airway resistance due to airway smooth muscle shortening. It is believed that airway wall thickening is partly responsible for the airway hyperresponsiveness observed in asthmatic subjects.

A more recent computational model investigates the effect of increased airway smooth muscle mass, increased adventitial mass, and increased submucosal mass on the increased airway resistance to bronchoconstrictors. Using morphometry to obtain values for these three compartments of the airway, the model shows that increased adventitial mass may increase bronchoconstriction by decreasing parenchymal interdependence, that increased submucosal mass leads to increased luminal occlusion for all degrees of airway smooth muscle shortening, and that increased airway smooth muscle mass results in increased smooth muscle shortening (Lambert *et al.*, 1993). These results suggest that the increased airway smooth muscle content is the most important abnormality in the airways of asthmatic subjects, and is responsible for the increased bronchoconstrictor response as increased airway smooth muscle thickness permits increased muscle tension and hence permits increased airway constriction.

1.2.5 Animal Models of Allergic Asthma

Asthma is a disease of human airways, and as such, does not occur naturally in animals as far as we know. Therefore, inducing a syndrome in animals which resembles the human airway disease is sufficient to establish an animal model of the disease. While

animal models fail to reproduce the spontaneous, sustained airflow obstruction, bronchospasm can be induced. In addition to this, the three most important features of airway disease are exhibited by the animal model. These include airway smooth muscle contraction, epithelial hypersecretion, and microvascular leakage (Wanner, 1990).

In general, animal models have both strengths and weaknesses. Their strengths include the feasibility of invasive methods, the availability of tissue for *in vitro* studies, and the ability to control and manipulate genetic and environmental factors. Their weaknesses include the rarity of natural hyperresponsiveness in animals, the variability between species in their lung anatomy, and the differences between species in the pathogenesis of hyperresponsiveness (Wanner, 1990).

1.2.5.1 The Brown Norway Rat

Brown Norway rats which are actively sensitized to allergen develop allergic bronchoconstriction. The changes characteristic of this animal model of allergic bronchoconstriction are similar to the changes observed in the human with allergic asthma (Tarayre *et al.*, 1992). As in asthmatic humans, actively sensitized Brown Norway rats exhibit early and late airway responses to inhaled allergen (Eidelman *et al.*, 1988). Following the early response, the BAL fluid shows increased numbers of polymorphonuclear leukocytes (Blythe *et al.*, 1986). During the late response, there is a mononuclear as well as a polymorphonuclear cell infiltrate. Histologically, there is an inflammatory reaction which closely parallels that seen in humans (Blythe *et al.*, 1986). When Brown Norway rats are actively sensitized to allergen and repeatedly challenged

with allergen, airway hyperresponsiveness develops (Bellofiore & Martin, 1988) which is a phenomenon observed in human asthmatics. Two-dimensional morphometrical analysis of the airways shows that allergen challenged rats have twice as much airway smooth muscle as control rats (Sapienza *et al.*, 1991) although the overall area of the airway wall does not change as it does in humans. More recent studies have shown that the airway smooth muscle content in the large airways increases by more than 50% following allergen challenge whereas the increase in the small airways is less (Wang *et al.*, 1993). Immunocytochemical techniques show that there is an increase in the number of airway smooth muscle cells in Brown Norway rats that develop increased airway smooth muscle content. As in humans, hyperplasia appears to be the underlying mechanism for the increased airway smooth muscle content observed in the Brown Norway rat (Panettieri *et al.*, 1994). Therefore, active sensitization and exposure to allergen induces airway hyperresponsiveness as well as increased airway smooth muscle content, suggesting that the two phenomena may be causally related.

1.2.5.2 Other Animal Models

Other animal models of airway hyperresponsiveness include the guinea pig, the rabbit, the dog, and the primate. The guinea pig model of airway hyperresponsiveness shares features with human asthmatics. For example, guinea pigs which have been sensitized to ovalbumin develop bronchial hyperreactivity as well as eosinophilia and neutrophilia in the airways following aerosolized challenge (Watson *et al.*, 1993; Van Oosterhout *et al.*, 1993), and eosinophil activation rather than accumulation is believed

to be essential for the development of airway hyperresponsiveness (Pretolani *et al.*, 1994). In addition to these characteristics, guinea pigs display bronchoconstrictive responses to adenosine (Thorne & Broadley, 1994), and elevated levels of major basic protein (MBP) in BAL fluid as asthmatics do (Pretolani *et al.*, 1994). In the rabbit model, the induction of sterile sinusitis results in the development of airway hyperresponsiveness in the lower airways (Brugman *et al.*, 1993). The association between asthma and sinusitis is clinically recognized although its exact nature is controversial. The Basenji-greyhound is an animal model of nonspecific airway hyperresponsiveness which bears similarities to that observed in humans. Studies in humans suggest a defect in the β-adrenergic pathway underlying asthma and the Basenji-greyhound shows an altered response to β-adrenergic agonists (Emala *et al.*, 1993). Mongrel dogs sensitized and exposed repeatedly to ragweed pollen develop allergic airway hyperresponsiveness (Jiang *et al.*, 1992). Studies in primates show that following allergen sensitization and challenge, there is eosinophil activation and influx into the airways, neutrophil influx into the airways, and an allergen-induced late response. These changes are characteristic of the events occurring in the asthmatic airways (Gundel *et al.*, 1992). Therefore, syndromes can be induced in animals which resemble the allergic asthmatic condition in humans.

1.3 Inflammatory Cells in Allergic Asthma

When an allergen first arrives at the mucosal surface or lymph node, it is taken up by an antigen-presenting cell which presents the allergen to a B-cell. The B-cell becomes activated and produces IgE antibodies against the allergen. IgE acts locally first

and sensitizes local mast cells by binding to high affinity receptors on their surface. Excess IgE enters the circulation and binds to its receptors on tissue-fixed mast cells and circulating basophils. During allergen challenge, the allergen binds to IgE molecules on the surface of mast cells and crosslinks IgE molecules. Ultimately, mast cells degranulate and release preformed and newly synthesized mediators which are chemotactic (Roitt *et al.*, 1989).

IgE molecules are induced by allergens and bind specifically to high affinity receptors on the surface of tissue mast cells and circulating basophils, and low affinity receptors on the surface of B-cells, T-cells, eosinophils, platelets and macrophages. The challenge with allergen crosslinks the IgE molecules and causes mediator release which plays a role in asthma.

Since repeated allergen challenges in the Brown Norway rat model of allergic asthma result in increased airway smooth muscle mass, it follows, therefore, that cells of the immune system could also be responsible for causing the changes in airway smooth muscle in human asthmatic subjects.

1.3.1 Lymphocytes

The cellular infiltrate which is a hallmark of asthma consists of neutrophils, eosinophils, mononuclear phagocytes and lymphocytes. The presence of activated T-cells in the blood, bronchial biopsies and BAL from asthmatics shows that T-cells play a central role in the pathogenesis of asthma and in propagating the chronic inflammation seen in asthma. Evidence for this is the presence of activated peripheral blood T-cells

expressing increased MHC class II antigens and IL-2 receptors in the airways during acute exacerbations (Brown *et al.*, 1991). Airway allergen studies in asthmatics show that CD4+ T-cells are found in BAL fluid 24 hours following the challenge, thereby associating T-cells and the late phase response (Robinson *et al.*, 1991).

1.3.1.1 Th_1 and Th_2 Cells

The type of immune response elicited by a foreign antigen depends on the cytokine profile of the T-helper cell. T-helper cells can be divided into two groups based on their cytokine profile: the Th_1 group which produces predominantly IL-2, IFN-γ, and lymphotoxin; and the Th_2 group which produces predominantly IL-4, IL-5, IL-6, and GM-CSF. Th_1 cells are involved in cellular responses whereas Th_2 cells are involved in humoral responses. Following allergen challenge, T-cells in nonatopic subjects produce a Th_1-type of cytokine pattern whereas T-cells in atopic subjects produce a Th_2-type of cytokine pattern (Parronchi *et al.*, 1991; Robinson *et al.*, 1993). Within the atopic subject, however, allergen-specific T-cell clones produce a Th_2-type of cytokine pattern whereas other antigen-specific T-cell clones in the same subject produce a Th_1-type of cytokine pattern (Wieranga *et al.*, 1990). Comparing the distinct responses between atopic and nonatopic individuals, it has been shown that clones specific for dust mite allergen produce IL-4 in allergic asthmatics and produce IFN-γ in nonatopic, nonasthmatic individuals. This difference is important as IL-4 is critical for the synthesis of IgE whereas IFN-γ inhibits the synthesis of IgE (Romagnani, 1990; Robinson *et al.*, 1993). IL-4 leads to isotype switching in B-cells so that B-cells switch from expressing one type

of immunoglobulin to expressing IgE. This usually requires other B-cell activation signals such as LPS stimulation in murine B-cells and contact with Th-cells in humans (Zubler *et al.*, 1993).

Finally, *in situ* hybridization studies in cutaneous tissue illustrate the association between atopy and Th_2 cells. In allergen-induced late phase airway reactions, infiltrating cells express mRNA for the Th_2-type cytokines IL-3, IL-4, IL-5, and GM-CSF, but not for the Th_1-type cytokines IL-2 and IFN-γ (Kay *et al.*, 1991).

1.3.1.2 The Role of T-lymphocytes in the Asthmatic Process

The T-cell participates in three major processes contributing to asthma: 1) the regulation of IgE synthesis (Saxon *et al.*, 1980; Saryan *et al.*, 1983), 2) the regulation of eosinophil production and function (Metcalf *et al.*, 1986; Lopez *et al.*, 1987; Lopez *et al.*, 1988), and 3) the generation of the late response (Frew & Kay, 1990; Frew *et al.*, 1990).

IgE production is regulated by two distinct T-cell populations, the T-helper cells and the T-suppressor cells. Abrogating the T-helper cell population results in decreased IgE levels and abrogating the T-suppressor cell population results in increased IgE levels (Taniguchi & Tada, 1971). Eosinophil production and function are also regulated by T-cells. There are several T-cell-derived cytokines, including GM-CSF, IL-3 and IL-5 which modulate eosinophilopoiesis. GM-CSF and IL-3 are multilineage hematopoietic regulators that stimulate eosinophil growth as well as promote differentiation of neutrophils and macrophages. IL-5 selectively stimulates eosinophil proliferation, differentiation, survival and function. It induces shape changes, membrane ruffling and granule polarization and

these morphologic changes are accompanied by augmented cellular cytotoxicity, phagocytosis and superoxide generation (Katz & Beer, 1993).

Finally, the T-cell is suspected to be involved in the generation of the late response. Adoptive transfer of antigen-primed T-lymphocytes increases airway hyperresponsiveness in unsensitized Brown Norway rats challenged with allergen, whereas adoptive transfer of unsensitized T-lymphocytes does not (Watanabe *et al.*, 1994). The cell differentials of the BAL fluid from the rats receiving sensitized T-lymphocytes does not vary from those of the rats receiving unsensitized T-lymphocytes. Furthermore, T-lymphocytes can be induced to produce lymphocyte chemoattractant factor (LCF) by histamine or specific antigen. LCF binds to CD4 molecules on the surface of eosinophils and enhances chemokinetic and chemotactic migration of eosinophils but does not stimulate degranulation, superoxide generation, LTC_4 production or cell surface receptor expression (Rand *et al.*, 1991).

Th_2 cells also produce IL-3 and IL-5 which play an important part in allergic asthma. IL-3 stimulates proliferation and growth of mucosal mast cells, and IL-5 stimulates production, activation, and recruitment of eosinophils (Sperber, 1993).

1.3.2 Mast Cells

Another feature of asthma is the increased numbers and activation of mast cells (Gibson *et al.*, 1993). Mast cells are located in the lumen of the airways, in the bronchial epithelium, in the submucosa, and in the lung parenchyma (reviewed by Kay *et al.*, 1989). The mast cell is predominantly responsible for the early response and plays a pivotal role

in airway inflammation as it degranulates and releases mediators. Immediately following allergen challenge, there is an increase in histamine and tryptase in BAL fluid which is proportional to the amount of allergen administered (Busse *et al.*, 1993). Forty-eight hours later, BAL fluid does not contain tryptase thus mast cells are no longer secreting mediators. Histamine, however, remains slightly elevated. One possible explanation for this is that basophils, which contain histamine but not tryptase and which increase in number during the late response, participate in the late response (Rankin, 1989).

1.3.2.1 Mucosal Mast Cells and Connective Tissue Mast Cells

There exist two major types of mast cells: the mucosal mast cell and the connective tissue mast cell (Sperber, 1993). Both are found in the intestinal submucosa, the intestinal mucosa, the bronchi, the bronchioles, the nasal mucosa, and the conjunctiva but only the mucosal mast cell is found in the alveolar wall and only the connective tissue mast cell is found in the skin. Other differences include: enzyme content with mucosal mast cells having tryptase and connective tissue mast cells having tryptase as well as chymase; T-cell dependency, with mucosal mast cells being T-cell dependent and connective tissue mast cells being T-cell independent; granule morphology with mucosal mast cell granules having a lattice structure and grading crystal formation and connective tissue mast cells having a scroll-like appearance; histamine content with mucosal mast cells having less histamine than connective tissue mast cells and the latter releasing histamine more readily in response to IgE and other stimuli; and LTC_4 synthetic capacity with mucosal mast cells having a greater capacity to synthesize LTC_4 than connective

tissue mast cells (Sperber, 1993).

1.3.2.2 Mast Cell Activation and Mediator Release

Mast cell activation occurs following antigen recognition by IgE on high affinity IgE receptors on the surface of pulmonary mast cells and crosslinking of IgE antibodies by allergen molecules. These cells become activated and release newly synthesized lipid mediators (e.g. prostaglandin D_2 (PGD_2), leukotriene C_4 (LTC_4), and PAF) and preformed mediators (e.g. histamine) (Page, 1993).

5-Lipoxygenase products are important as they can induce airway smooth muscle contraction, airway hyperresponsiveness, mucus release, microvascular leakage, edema, eosinophil and neutrophil chemotaxis and activation. The leukotrienes LTC_4, LTD_4 and LTE_4 are the main leukotrienes associated with asthma and not only are they 1000 times more potent than histamine in causing bronchoconstriction, but they also potentiate the effect of histamine (Sperber, 1993). LTC_4 has particular importance in the pathogenesis of asthma and particular relevance to the current studies. Once LTC_4 has been released, it binds to its receptor on macrophages and specifically activates them to synthesize platelet-derived growth factor (PDGF) (Phan *et al.*, 1987), and PDGF stimulates proliferation of various cells including airway smooth muscle cells (Noveral & Grunstein, 1992).

Activated mast cells also release a variety of mediators including platelet-activating factor (PAF) which is a potent bronchoconstrictor and can cause microvascular leakage, eosinophil and neutrophil chemotaxis, mucous secretions and edema which contribute to

airway obstruction (Cuss *et al.*, 1986). Intragranular mediators are released, for example histamine which causes vasodilation, vasopermeability, pruritis, mucus secretion, nerve stimulation and bronchoconstriction; proteases which degrade the basement membrane of blood vessels; heparin which forms a complex with proteases; eosinophil-chemotactic factor-A (ECF-A) which causes eosinophil chemotaxis; and neutrophil-chemotactic factor (NCF) which causes neutrophil chemotaxis (Serafin & Austen, 1987). Mast cells also release PGD_2, LTB_4, LTC_4, and chymase which cause vasodilation, edema, eosinophil and neutrophil chemotaxis and activation, mucus secretion, nerve stimulation, and bronchoconstriction (Sperber, 1993).

Mast cells synthesize and release many mediators which play a role in inflammation and are partly responsible for the pathological changes seen in asthma. During the early response, IgE binds to receptors on the mast cell surface which causes mast cell degranulation and activation. This results in the release of the preformed and newly-synthesized mediators discussed above which causes the generation of proinflammatory mediators and the recruitment of eosinophils and neutrophils into the airways. The late phase plays a greater role in the propagation of airway inflammation as there is movement of inflammatory cells to the site of inflammation, release of mediators from these cells, and recruitment of more inflammatory cells (Sperber, 1993).

1.3.3 Eosinophils

The eosinophil is often considered the effector cell in asthma and probably has the most important role in developing airway inflammation. This is supported by both

epidemiological as well as biochemical data (Busse *et al*, 1993). Eosinophilia in the blood and airways is frequently but not always associated with asthma. However, when there is infiltration, the increase in eosinophils is in proportion to the intensity of airway obstruction (Bousquet *et al.*, 1990). Immediately following allergen challenge, there is no increase in the numbers of eosinophils in the airways. Forty-eight hours later, there is leukocyte influx predominantly made up of eosinophils. Whereas eosinophils increase to 80% of the total leukocyte count in the airways, circulating eosinophils increase by a factor of less than two (Busse *et al.*, 1993).

1.3.3.1 Eosinophil Maturation and Activation

Eosinophil maturation is under the control of IL-3, IL-4, IL-5, and GM-CSF which regulate eosinophil function and phenotypic features (Robinson *et al.*, 1993a). These cytokines are released by various immune cells including activated Th_2 cells and mast cells (Johnston & Holgate, 1991). IL-5 has the greatest specificity for eosinophils, therefore an increase in IL-5 may account for changes in eosinophil function and density. IL-5 is not found in the BAL fluid collected during the early response, but is found in BAL fluid collected during the late response. There is a strong correlation between airway eosinophilia and IL-5 concentration following allergen challenge in human subjects (Lopez *et al.*, 1988).

Following eosinophil recruitment, two events are required to generate airway inflammation: eosinophil migration to the lung and eosinophil activation. Eosinophil adhesion to endothelium and airway epithelium are involved in eosinophil activation.

Blocking adhesion to the endothelium or epithelium is believed to significantly reduce the allergic reaction in the primate model of airway hyperresponsiveness (Wegner *et al.*, 1990).

1.3.3.2 Eosinophil Mediators

During the early response, there is no accumulation of granule-associated eosinophil products including MBP, eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), and eosinophil peroxidase. During the late response, there is a marked increase in all four with MBP increasing in relation to the amount of allergen given (Busse *et al.*, 1993).

Eosinophil-derived mediators have the potential to exert deleterious effects on airway and cell function (Weller, 1991). MBP injures the airway epithelium directly, promotes bronchial responsiveness, causes airway smooth muscle contraction, and activates mast cell and basophil mediator release. There is a significant correlation between the level of MBP and the percentage of eosinophils in BAL fluid (Kay *et al.*, 1989). ECP is also cytotoxic to the respiratory epithelium, and may contribute to the airway hyperresponsiveness observed in asthma (Gleich *et al.*, 1979). There is a correlation between the percentage of eosinophils and the degree of airway hyperresponsiveness, suggesting that hyperresponsiveness may be secondary to epithelial damage (Wardlaw *et al.*, 1988).

Eosinophil activation results in the release of LTC_4 which causes airway smooth muscle contraction, and PAF which may contribute to the asthmatic symptoms by causing

increased vascular permeability, eosinophil chemotaxis, eosinophil adherence to endothelial cells, increased mucus secretion, and increased airway hyperresponsiveness (Cuss *et al.*, 1986) although the direct proof of many of these processes is lacking.

In addition to MBP, ECP, EDN, PAF, and LTC_4, eosinophils release LTB_4, PGE_2, oxygen radicals and eosinophil peroxidase which cause bronchoconstriction, epithelial damage, and recruitment and priming of other inflammatory cells (Johnston & Holgate, 1991). Thus, eosinophils can directly as well as indirectly cause airway obstruction and bronchial hyperresponsiveness. Additionally, the eosinophil's ability to generate chemoattractants and IL-5 could result in further eosinophil migration and a self-perpetuating process (Busse *et al.*, 1993).

1.3.4 Neutrophils

Neutrophils potentially play an important role in allergic asthma as neutrophil influx into the airways is believed to be associated with the occurrence of the late response in the primate model of airway hyperresponsiveness (Gundel *et al.*, 1992). Neutrophils are also found in BAL fluid and airway biopsies of asthmatic subjects (White & Kaliner, 1987). The neutrophil has a great ability to cause inflammation by releasing lysosomal enzymes, oxygen metabolites, and LTB_4; and by generating histamine-releasing factor. Histamine-releasing factor has been postulated to amplify the allergic reaction by initiating mediator release from basophils which appear during the late response (White & Kaliner, 1987).

Neutrophils are a normal resident of larger airways in both asthmatics and

nonasthmatics. They become activated following allergen-induced early and late responses, suggesting that they are exposed to an activating stimulus (Moqbel *et al.*, 1986). In association with the late response, the number of neutrophils in the airways increases significantly (Diaz *et al.*, 1989). The accumulation of neutrophils in the airways results in tissue damage due to the release of oxygen metabolites, proteases, and cationic materials. Neutrophils also release various immune mediators such as prostaglandins, thromboxanes, LTB_4, and PAF which may contribute to airway responses and exacerbate inflammation.

PAF is released from neutrophils within a few minutes following activation. PAF is also released from platelets and activated eosinophils, although in lesser amounts than from neutrophils. Activated macrophages are capable of releasing PAF, and alveolar macrophages from asthmatic subjects spontaneously release PAF, unlike those from normal subjects (Barnes, 1991). Mucosal mast cells may synthesize and release PAF, but the greatest source of PAF remains the neutrophil. PAF may be important in asthma as it can induce clinical symptoms and pathological changes observed in asthmatic airways such as edema, eosinophilia, and airway hyperresponsiveness (Chung & Barnes, 1991). However, PAF antagonists do not appear to be effective in the treatment of asthma (Freitag *et al.*, 1993). PAF is highly chemotactic and stimulatory for eosinophils, and this interaction may be essential in the pathogenesis of airway hyperresponsiveness in asthma.

1.3.5 Platelets

Although it is not always possible to identify platelets clearly by morphological

methods, they are found in BAL fluid following allergen challenge (Metzger *et al.*, 1985). Following activation, platelets synthesize thromboxane and could participate in transcellular metabolism of LTA_4 to LTC_4 which may activate macrophages. Human platelets release factors from the granules that are chemotactic for eosinophils (Burgers *et al.*, 1993). The migration of platelets into the airways is not understood and may reflect loss of vessel wall integrity. Platelets possess IgE receptors on their surface, and might participate intravascularly in the allergic response. However, there is little evidence to date that platelets represent an important source of inflammatory mediators in the airways or that they play an essential role in asthma or airway hyperresponsiveness.

1.3.6 Mononuclear Cells

Mononuclear cells are the most common cells recovered from BAL, are integral to the inflammatory process, and comprise macrophages, monocytes, and dendritic cells.

1.3.6.1 Alveolar Macrophages, Interstitial Macrophages, and Dendritic Cells

Macrophages are found virtually in all organs, and, in the lung, are divided into two groups based on their distribution: alveolar macrophages and interstitial macrophages. Alveolar macrophages reside in the alveoli on top of the epithelial cells. Due to this strategic position, alveolar macrophages are one of the first phagocytic cells encountered by an inhaled particle and thus comprise an important part of the first line of defense of the body (Fels & Cohn, 1986).

Dendritic cells are tissue-fixed macrophages which are able to process inhaled

antigen and present it to T-cells to effect an allergic tissue response (Johnston & Holgate, 1991). They function as highly specialized accessory cells in the induction of immunity and tolerance. They are the most potent inducers of IL-2 production in T-cells, and also induce the production of IL-3, IL-4, and IFN-γ (Ellis *et al.*, 1993). Dendritic cells can be found in three different compartments of the body. Interstitial dendritic cells of the lung and heart and the Langerhans cells of the skin are found in non-lymphoid tissue, the veiled cells of the afferent lymph are found in the circulation, and the interdigitating cells of the T-cell areas and thymic medulla are found in the lymph organs (Steinman *et al*, 1993). In the lung, dendritic cells are dense in the subepithelial region of the airways and their processes pass between the epithelial cells into the lumen (Holt, 1993). They are ideally located for the uptake, processing, and presentation of foreign antigen.

1.3.6.2 Mononuclear Cell Activation

Activation is achieved in two different but equally important manners. Firstly, macrophages may become activated following IgE binding to low-affinity IgE receptors on their surfaces when IgE is released during the allergic response (Thorel *et al.*, 1988). Secondly, antigen recognition by high affinity IgE receptors on the surface of pulmonary mast cells activates mast cells to release LTC_4 and LTD_4. Once LTC_4 has been released, it may bind to its receptor on macrophages and activate them (Gibson *et al.*, 1993).

1.3.6.3 Alveolar Macrophage-Derived Mediators

Alveolar macrophages release a variety of mediators, of which only a few play a

potential role in airway inflammation in asthma. Being the first phagocytic cell to encounter allergen in the airways, the macrophage participates in airway inflammation by presenting allergen to lymphocytes and releasing monokines such as IL-1 that stimulate lymphocytes for diverse effector functions (Rankin, 1989). Furthermore, activated macrophages release several neutrophil chemotaxins including LTB_4, PAF, tumour necrosis factor-α (TNF-α), C5a, and PDGF which initiate the neutrophil infiltration observed in asthma. (Rankin, 1989). In addition to releasing neutrophil chemotaxins, macrophages release LTB_4 and PAF which are chemotaxins for eosinophils. Macrophages are also capable of modifying eosinophil participation in the inflammatory response by releasing monokines which enhance the synthesis of 5-lipoxygenase products, regulate the metabolism of endogenous arachidonic acid, and enhance IgG-dependent LTC_4 release (Rankin, 1989). Therefore, there is an important relationship between macrophages and eosinophils with macrophages influencing the participation of eosinophils in the airway inflammatory response. Finally, macrophages spontaneously synthesize and release a product which may cause mast cells and basophils to release histamine which contributes to the inflammatory response (Rankin, 1989).

Activated macrophages are capable of releasing a variety of cytokines and growth factors such as IL-1, PDGF, macrophage colony stimulating factor, granulocyte colony stimulating factor, granulocyte-monocyte colony stimulating factor, bombesin, transforming growth factor-α, transforming growth factor-β, TNF-α, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and acidic fibroblast growth factor (Nathan, 1987; Rappolee *et al.*, 1988; Higashiyama *et al.*, 1991). The macrophage-derived

factors which are capable of stimulating the proliferation of airway smooth muscle cells are PDGF, bFGF, LTD_4, TXA_2, and somatomedins (Yang, 1990; Noveral & Grunstein, 1992; De *et al.*, 1993).

1.3.6.4 Alveolar Macrophages as a Source of PDGF

It is postulated that LTC_4 specifically activates macrophages to release macrophage-derived growth factor (Phan *et al.*, 1987) which comprises at least two forms of PDGF. Proteins of two sizes are examined: those in the 37-39 kilodalton range and those in the 12-17 kilodalton range. Mitogenically active proteins which are PDGF-like are detected using antigenic similarity with PDGF and ability to compete with ^{125}I-PDGF for high-affinity PDGF receptor sites. Furthermore, PDGF-B mRNA can be detected in the macrophage using Northern analysis, and *de novo* synthesis of PDGF-B proteins by activated macrophages can be detected using immunoprecipitation of ^{35}S-labelled proteins with anti-PDGF IgG. Therefore, macrophages are able to synthesize PDGF and upregulate *de novo* synthesis of PDGF-B following activation by adherence (Shimokado *et al.*, 1985).

Alveolar macrophages from normal individuals spontaneously release small amounts of PDGF. Resting monocytes constitutively transcribe PDGF-A and PDGF-B, and constitutively express PDGF-A and PDGF-B mRNAs. When monocytes mature into macrophages, the transcription of both genes increases, however, only the expression of PDGF-B mRNAs increases (Nagaoka *et al.*, 1992). Alveolar macrophages also constitutively transcribe PDGF-A and PDGF-B genes at almost the same rate. However, the levels of PDGF-B mRNAs in these cells are approximately five times greater than

PDGF-A mRNAs. Furthermore, PDGF-BB homodimeric proteins are more abundant than PDGF-AA homodimers or PDGF-AB heterodimers. Therefore, alveolar macrophages preferentially express PDGF-B mRNAs and PDGF-BB proteins despite the equal transcription rates of the PDGF-A and PDGF-B genes.

The enhanced release of PDGF may lead to disorders such as idiopathic pulmonary fibrosis (IPF). IPF is a chronic disorder of the lung characterized by inflammation in the interstitial and intra-alveolar spaces, and fibrotic thickening of the alveolar wall. Interstitial macrophages from patients with idiopathic pulmonary fibrosis spontaneously release increased amounts of biologically active PDGF which is believed to stimulate the growth of fibroblasts and is believed to be responsible for the fibrotic thickening (Nagaoka *et al.*, 1990). The increased release of PDGF is believed to be due to increased transcription of the PDGF-A and PDGF-B genes with PDGF-B maintaining a ten-fold dominance over PDGF-A. Hence, it is speculated that most of the PDGF released from alveolar macrophages is of the PDGF-BB isotype which is the most potent growth stimulator of the three isotypes. PDGF appears to be specifically associated with macrophages and PDGF accumulation appears to precede the fibrotic process (Vignaud *et al.*, 1991). Therefore, if alveolar macrophages can be stimulated by LTC_4 to release increased amounts of PDGF, and if the PDGF is localized so that it can act on airway smooth muscle cells, then PDGF may be able to stimulate the proliferation of airway smooth muscle cells and cause the increased airway smooth muscle content observed in asthma.

1.4 Platelet-Derived Growth Factor

PDGF is a cationic glycoprotein with a molecular weight of approximately 30 kilodaltons (Ross *et al.*, 1986). PDGF is a potent mitogen for smooth muscle cells and fibroblasts and is believed to play a role in tissue repair, development and cell growth regulation (Bonner *et al.*, 1991).

1.4.1 The Role of PDGF in the Pathogenesis of Asthma

Various pathologic processes including neoplasia, atherosclerosis and pulmonary fibrosis are possibly due to abnormal expression and secretion of PDGF and PDGF-like molecules (Bonner *et al.*, 1991).

Some recent evidence reports the proliferative effect of IL-1β on guinea pig tracheal smooth muscle proliferation to be mediated through PDGF (De *et al.*, 1993). Airway smooth muscle cells isolated from guinea pig trachea and exposed to recombinant IL-1β show significant increases in the number of cells as compared to controls. In fact, the increase in cell number is dose-dependent. When cells are treated with polyclonal antibodies to PDGF-BB, the IL-1β-induced proliferation is attenuated. When cells are treated with PDGF-BB, the proliferation is potentiated in a dose-dependent manner. The importance of this is that an inflammatory cytokine such as IL-1β is produced in asthma and may provide a link between the inflammatory process and the stimulation of airway smooth muscle cell proliferation through a PDGF-dependent mechanism. The problem with these experiments, however, is that the IL-1β-dependent cell proliferation is only observed in the presence of indomethacin which inhibits cyclooxygenase from

synthesizing PGE_2 which is a potent inhibitory regulator of airway smooth muscle proliferation. As indomethacin is not naturally found in the human body, this study does not emulate well what may be occurring in the asthmatic airway and the results may not be relevant to what is observed *in vivo*.

1.4.2 Isoforms of PDGF and its Receptor

1.4.2.1 The Two PDGF Genes

There are two genes encoding human PDGF, denoted A and B. The two genes are arranged in the same pattern of exons with most of the differences in amino acid sequence occurring in exons III and VI. Both genes produce transcripts with long 5' and 3' untranslated regions (Fabisiak and Kelley, 1993). PDGF-A is located on chromosome 7 and occupies a 24kb region at p21-p22, whereas PDGF-B is located on chromosome 22 and occupies a 24kb region at q12.3-q13.1 (Nagaoka *et al.*, 1990). PDGF-A is a seven exon gene and alternative splicing of exon VI yields two polypeptides differing by 15 amino acids, PDGF-SA and PDGF-LA. The most common form of PDGF-A mRNA is the PDGF-SA transcript which lacks exon VI. The PDGF-SA homodimer is predominantly secreted from the cells. The PDGF-LA mRNA includes exon VI and its homodimer is secreted much less efficiently (Raines & Ross, 1992; Kelley *et al.*, 1993).

PDGF-B is also a seven exon gene but codes for a single 27-kilodalton precursor protein. This latter gene is also known as the *c-sis* proto-oncogene which is a normal cellular gene of close homology to the *v-sis* transforming gene of the simian sarcoma virus (Nagaoka *et al.*, 1990). PDGF-BB is found localized in the Golgi, lysosomes and

endoplasmic reticulum of Chinese hamster ovary cells (Thyberg *et al.*, 1990). PDGF-SA, which is present in lower levels, is packaged in locations consistent with protein secretion.

1.4.2.2 PDGF-AA, -AB, and -BB

PDGF is stable to agents such as acetic acid and boiling but loses its biological activity in the presence of reducing agents which destroy its disulfide bonds. Reduction results in the liberation of 14 and 17 kilodalton protein species (Ross *et al.*, 1986). PDGF is a dimeric protein made up of two subunits (A and B) which give rise to homodimers PDGF-AA and PDGF-BB as well as the heterodimer PDGF-AB. Both the PDGF-A chain and the PDGF-B chain are synthesized as high molecular weight precursors which are processed extensively before secretion. Processing includes post-translational glycosylation and proteolytic cleavage, both of which contribute to PDGF peptides of varying weights (Fabisiak and Kelley, 1993). PDGF-BB and PDGF-AB are consistently mitogenic whereas the mitogenicity of PDGF-AA varies between species (Buch *et al.*, 1991). In most non-human mammals including the rat, PDGF-BB is the predominant isoform found in the serum, whereas human serum is made up of mostly PDGF-AB peptides. Human serum also has relatively more PDGF than the serum of other mammals (Fabisiak and Kelley, 1993).

1.4.2.3 The PDGF Receptor

Each PDGF isoform binds to a different dimeric cell-surface receptor. The combination of the two subunits α and β determines the receptor type ($\alpha\alpha$, $\alpha\beta$, and $\beta\beta$)

and receptor type determines the specificity of the receptor. The α subunit can recognize both PDGF-A and PDGF-B, whereas the β subunit can recognize PDGF-B only. Therefore, the ββ-type receptor recognizes PDGF-BB only, the αβ-type receptor recognizes PDGF-BB as well as PDGF-AB, and the αα-type receptor recognizes PDGF-BB, PDGF-AB and PDGF-AA. The response of the target cell to PDGF may be further modulated by complex formation with α_2-macroglobulin, an endoprotease inhibitor. If PDGF is bound to the receptor-recognized form of α_2-macroglobulin, proliferation is enhanced, whereas if PDGF is bound to the non-receptor form of α_2-macroglobulin, proliferation is inhibited. The total number of receptors and the relative ratio of receptor types differ greatly from cell type to cell type and may account for the heterogeneous responses of cells to the three isoforms. The mitogenic response of the target cells to the three isoforms depends on expression of the appropriate PDGF receptor subtypes. Response patterns can be autocrine (the same cell secretes and responds to PDGF), paracrine (PDGF-secreting cells are near or adjacent to the target cells) or intracrine (PDGF-secreting cells have intracellular receptors which bind PDGF before it is secreted) (Bonner *et al.*, 1991; Fabisiak and Kelley, 1993).

1.4.2.4 The PDGF-Related Microenvironment

Normal levels of PDGF in human plasma are undetectable and PDGF injected intravenously into normal baboons is rapidly cleared ($t_{1/2}$= 2 minutes). Therefore, it is the local synthesis and secretion of PDGF and not the circulating levels which are responsible for cell proliferation (Ross *et al.*, 1986). In addition to this, it is possible that the local

concentrations and ratios of PDGF isoforms may differ greatly from microenvironment to microenvironment as effector cells may differ (by secreting different ratios of PDGF isoforms) and responder cells may differ (by possessing different ratios of PDGF receptors). The result is that the microenvironment may be very unique (Bonner *et al.*, 1991).

1.4.3 Cellular Sources of PDGF

Cells which are sources of PDGF are endothelial cells which express PDGF-A and PDGF-B mRNA and which release biologically active PDGF-like proteins, fibroblasts which release more PDGF when they come from newborn subjects as opposed to adult subjects, smooth muscle cells which express little or no PDGF-A or PDGF-B mRNA in the intact blood vessel wall but which express PDGF-A when they are cultured, epithelial cells which express PDGF-B mRNA in the lung and which express PDGF-A and PDGF-B mRNA in malignant tumours, and macrophages which can secrete or store PDGF and which can secrete PDGF in response to various stimuli (Fabisiak and Kelley, 1993) including activation by LTC_4 released from degranulating mast cells (Phan *et al.*, 1987).

1.4.4 PDGF Signalling Pathways

Cells which possess PDGF receptors include fibroblasts, vascular smooth muscle cells and related mesenchymal cells, epithelial cells and endothelial cells (Fabisiak and Kelley, 1993). Mitogens for airway smooth muscle cells are divided into two general categories based on the way in which tyrosine kinase is activated. There are mitogens that

activate receptors with intrinsic tyrosine kinase activity, and there are mitogens that activate receptors coupled to G-proteins which activate cytoplasmic, non-receptor linked tyrosine kinases. PDGF, along with EGFand bFGF, elicit their responses through the receptor-linked tyrosine kinase which generates phosphatidylinositol 3,4,5-triphosphate (IP_3) and diacyl glycerol (DAG) from phosphatidylinositol 3,4-bisphosphate. These are among the most potent airway smooth muscle mitogens (Banskota *et al.*, 1989). It is not known exactly how these growth factors activate the receptor tyrosine kinase, but it is postulated that the growth factor binds to the receptor and induces the oligomerization of receptor monomers, or forms a receptor-growth factor complex which is then internalized (Cadena & Gill, 1992).

The step following activation is the autophosphorylation of the receptor which removes inhibitory substrates and reveals high affinity sites containing phosphotyrosine residues. Substrates having particular binding sites named SH2 domains bind to these autophosphorylated tyrosine residues, and couple the activated receptor to the intracellular signalling pathways involved in various cell functions including cell proliferation (Carpenter, 1992).

Phospholipase-γ (PLC-γ) is the next enzyme to become activated in this signalling pathway as compared to PLC-β1 which becomes activated by non-receptor linked tyrosine kinase. PLC-γ hydrolyzes phosphoinositides to generate IP_3 and DAG. DAG activates protein kinase C (PKC) which, in conjunction with cytosolic calcium, stimulates Na+/H+ exchange and the phosphorylation of specific substrates associated with cell proliferation (Bobik *et al.*, 1990). In addition to this, the expression of *c-fos* and *c-myc* is stimulated

and these proto-oncogenes regulate many cellular functions including gene transcription and cell proliferation (Komuro *et al.*, 1988).

Either direct or indirect activation of PKC is an important step in the signalling pathway leading to cell proliferation. PKC activated by calcium and DAG induces the phosphorylation of threonine and serine residues of various proteins which leads to the transcription of the proto-oncogenes *c-fos*, *c-myc*, and *c-jun*. This is followed by translation of the newly synthesized mRNA and the resulting protein products move to the cell nucleus where they bind to the DNA and activate genes which are responsible for cell proliferation (Magnaldo *et al.*, 1986).

The receptor-dependent tyrosine kinase signalling pathway and the G-protein dependent pathway both lead to PLC activation which generates IP_3 and DAG and ultimately results in gene transcription. However, these two pathways are different enough to explain how concomitant stimulation of both pathways may show an additive effect. In asthma, for example, the synergy between growth factors may be a result of this (Paris & Pouysségur, 1993).

1.5 Objectives of This Study

The purpose of the experiments described in this thesis was to investigate the role of the macrophage in airway smooth muscle cell proliferation, and to determine whether PDGF, bFGF, cyclooxygenase products and leukotrienes are released from macrophages and promote airway smooth muscle cell proliferation.

CHAPTER 2

PROLIFERATIVE RESPONSE OF AIRWAY SMOOTH MUSCLE CELLS TO SUBSTANCES RELEASED BY ACTIVATED MACROPHAGES

2.1 Introduction

Asthmatic subjects exhibit increased airway smooth muscle content (Hossain, 1973; Ebina *et al.*, 1993) which is a potential cause of the hyperresponsiveness of asthmatic airways and may account for excessive airway narrowing when a bronchoconstrictive stimulus is presented. Along with the increased airway smooth muscle, there is an inflammatory infiltrate comprised of neutrophils, eosinophils, mononuclear phagocytes, mast cells and lymphocytes (Cockcroft, 1990). Brown Norway rats sensitized to allergen show a similar increase in airway smooth muscle content and a similar inflammatory response in the airways following repeated allergen challenges (Sapienza *et al.*, 1991).

It follows, therefore, that inflammatory cells could be responsible for the changes in airway smooth muscle. The macrophage is the most common cell recovered from BAL and it possesses low-affinity IgE receptors on its surface. Therefore, it may become activated during the allergic response which induces the release of IgE (Thorel *et al.*, 1988). Furthermore, there is antigen recognition by high affinity IgE receptors on the surface of pulmonary mast cells following allergen challenge in sensitized rats. The mast cells become activated and release LTC_4 which binds to its receptor on the macrophage surface and activates them (Gibson *et al.*, 1993). LTC_4 has been shown to activate macrophages to specifically release PDGF (Phan *et al.*, 1987) which is a potent mitogen for airway smooth muscle cells. Activated macrophages are able to produce a variety of growth factors including bFGF, thromboxane A_2, LTD_4, IL-1β, and somatomedins which potentially promote the proliferation of airway smooth muscle cells (Yang, 1990; Noveral

& Grunstein, 1992; De *et al.*, 1993). Macrophage activation *in vitro* may be achieved by treatment with LPS (Tobias & Ulevitch, 1993), exposure to particulates which are engulfed (Burns & Zarkower, 1983), or adherence to a surface (Shaw *et al.*, 1990).

To examine the proliferative response of airway smooth muscle cells to some of these macrophage-derived growth factors, various antagonists and inhibitors were used. To determine the contribution of LTD_4, MK-571 was used. It is an antagonist for the LTD_4 receptor and binds irreversibly to prevent LTD_4 binding (Jones *et al.*, 1988; Cirino *et al.*, 1992). Acetyl salicylic acid (ASA), which irreversibly acetylates cyclooxygenase (Hirsch *et al.*, 1992), was given to inhibit the synthesis of thromboxane A_2. To examine the contribution of macrophage-derived PDGF on the proliferation of airway smooth muscle cells, the nonspecific PDGF inhibitor suramin was given to the macrophage cultures. Suramin acts by binding several PDGF molecules together and prevents them from binding to their receptors on the surface of airway smooth muscle cells either by sterically occluding the receptor binding region or by inducing a conformational change in the growth factor (Westphai *et al.*, 1991; Middaugh *et al.*, 1992). Neutralizing antibodies to PDGF and bFGF were used as specific inhibitors of these growth factors to assess their participation in the proliferative response of airway smooth muscle cells.

2.2 Materials and Methods

2.2.1 Animals

In all experiments, 7- to 9-week old male Brown Norway rats were used (Harlan Sprague Dawley, Charles River). Animals were housed in a conventional animal facility

at McGill University and allowed to feed and drink *ad libidum*.

2.2.2 Surgery and Harvest of Required Tissue

Sodium pentobarbital overdose (1 ml of 65 mg/ml per rat) was used to euthanize the rat and its body was wetted with 70% ethanol from the chin to the lower abdomen. This latter step served to sterilize the area of surgery as well as to mat down the fur which tends to contaminate the required tissues. The rat was then placed in a sterile laminar flow hood. Using surgical instruments which were soaked in 70% ethanol for 20 minutes to sterilize them, an incision was made in the skin above the lower abdomen extending to the chin and the skin was pulled away from the midline to remove the fur from the immediate area of surgery. An incision was made in the musculature of the abdomen at the midline and extended to the tip of the sternum. At this point, the rib cage was cut along the sternum and the ribs removed to expose the lungs. Using fine autoclaved sterile instruments, the trachea was located and cut at the cricoid cartilage, and the trachea and lungs were removed.

2.2.3 Macrophage Culture

The lungs were aseptically excised and all excess tissue was removed. The lungs were placed in 60x15mm sterile Petri dishes (Fisher Scientific, Montreal, PQ) containing 7 mls of 0.1% collagenase IV in serum-free Dulbecco's modified Eagle's medium (SF-DMEM) supplemented with penicillin (10 000 U/ml) and streptomycin (10 000 U/ml) (all supplied by Gibco, Burlington, ON). Using sterile forceps to grasp the bronchi, the lung

tissue was scraped away from the bronchi using a sterile scalpel blade. The bronchi were discarded and the lung tissue was minced. The homogenate was transferred into a sterile 15 ml conical tube (Sarstedt, St. Laurent, PQ) and incubated in a horizontal shaking water bath at 37°C for 30 minutes. Following the incubation, the homogenate was pipetted up and down a sterile 10 ml pipette (Costar, Montreal, PQ) approximately 50 times to mince any remaining tissue pieces. The homogenate was incubated for another 30 minutes at 37°C in a shaking water bath. Following this incubation, the homogenate was allowed to rest for several minutes to permit the pieces of tissue to settle on the bottom of the tube. Without disturbing the settled pieces, as much homogenate as possible was collected into a sterile 15 ml conical tube. Seven mls of SF-DMEM were added to the first tube to rinse the leftover pieces of tissue and enough SF-DMEM was collected into the second tube to fill it. The collected homogenate was centrifuged at 1300 rpm at 20°C for 10 minutes. The supernatant was discarded and the pellet was resuspended in 12 mls SF-DMEM at 37°C containing 0.2% trypsin. Cells were divided among 25 cm^2 culture flasks (Corning, Kirkland, PQ) and incubated at 37°C for 25 minutes. Flasks were rinsed with sterile phosphate-buffered saline (PBS) at 37°C to remove all cells except adherent macrophages. SF-DMEM with or without various substances or inhibitors was given to the macrophage cultures, collected after 8 or 12 hours and frozen at -80°C until needed.

2.2.4 Activation of the Macrophages

Shimokado and coworkers (1985) showed that human alveolar macrophages which were allowed to adhere to plastic tissue culture plates and maintained in serum-free

medium with or without concanavalin A (Con A) were able to release mitogenic activity. Although the mitogenic activity varied from being lesser than that of Con A, to being equal to that of Con A, to being greater than that of Con A, it was determined that the mere adherence of the macrophage to the plastic surface of the culture dish is sufficient to activate the macrophage to release mitogenic substances.

2.2.5 Characterization of the Macrophages

To assess whether the cells isolated from the lungs were macrophages, a non-specific esterase stain was used (Sigma Diagnostics, Mississauga, ON). The cells stained positively for the presence of esterase granules, confirming that the cells were macrophages.

2.2.6 Treatment of Macrophage Cultures

Macrophage cultures were either untreated or treated with 10^{-6}M MK-571 (a gift from Merck-Frosst, Montreal, PQ), $5x10^{-4}$M ASA (Merck-Frosst, Montreal, PQ), or 10^{-4}M suramin (a gift from the Centres for Disease Control, Atlanta, GA).

2.2.7 Airway Smooth Muscle Culture

The trachea was cut longitudinally on the anterior side to allow full exposure of the posteriorly-located smooth muscle cells to the digesting enzymes (Devore-Carter *et al.*, 1988). The trachea was incubated in 1.5 mls Hank's balanced salt solution (HBSS) at 37°C containing 0.2% collagenase IV and 0.05% elastase IV (both supplied by Sigma

Biochemicals, St. Louis, MO) for 30 minutes in a shaking water bath. Following the incubation, the supernatant was collected into a sterile 15 ml conical tube. The trachea was rinsed with 1.5 mls sterile HBSS and this was added to the previously collected supernatant. The supernatant was centrifuged at 1300 rpm (Model # Centra-8R, International Equipment Co., MA) at 20°C for 10 minutes. The supernatant was discarded and the pellet was resuspended in 6 mls of Dulbecco's modified Eagle's medium-Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), penicillin (10 000 U/ml) and streptomycin (10 000 U/ml) at 37°C. Cells were seeded in 2 wells of a 6-well culture plate (Corning, Kirkland, PQ) and allowed to grow to confluence. Medium was changed every 2 to 3 days. Cells grew to confluence in 2 to 3 weeks. Confluent cells were removed from the wells using HBSS containing 0.25% trypsin (Worthington, Freehold, NJ) and 0.02% EDTA (Sigma Biochemicals, St. Louis, MO) and suspended in medium supplemented with 10% FBS. Cells were seeded onto 24-well plates at a density of 10^4 cells/ml and at a volume of 1 ml/well.

2.2.8 Characterization of Airway Smooth Muscle Cells

To assess whether the cells isolated from the trachea were smooth muscle cells, morphologic and immunologic techniques were used. Phase-contrast microscopy determined that confluent cells were spindle-shaped and had a typical "hill-and-valley" appearance which is characteristic of cultured smooth muscle cells (Chamley-Campbell *et al.*, 1979; Ross and Vogel, 1978). Immunofluorescence staining for smooth muscle specific anti-α-actin monoclonal antibodies was performed to confirm that isolated cells

were indeed airway smooth muscle cells. Harvested cells were allowed to reach confluence and were then subcultured and grown to 70% confluence on glass coverslips in 6-well culture plates (Corning, Kirkland, PQ). Cells were rinsed with PBS at room temperature, cooled and dried at -20°C for 10 minutes, fixed in 100% acetone at -20°C for 10 minutes, rinsed in 70% ethanol at room temperature for 5 minutes and rinsed three times with PBS at room temperature. Cells were then incubated with a mouse anti-α-actin antibody (1:30 dilution in PBS) at room temperature for 60 minutes, rinsed three times with PBS at room temperature, incubated with an FITC-conjugated secondary goat anti-mouse IgG antibody (1:40 dilution with PBS) at room temperature for 60 minutes in the dark and rinsed with PBS. Cover slips were fixed with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and sealed before observing immunofluorescence with an inverted microscope equipped for epifluorescence (Nikon, Montreal, PQ).

2.2.9 [^{3}H]-Thymidine Incorporation

[^{3}H]-Thymidine incorporation was used to evaluate the proliferative response of airway smooth muscle cells. Cells were incubated for 72 hours in medium supplemented with 10% FBS and then for 72 hours in medium supplemented with 1% FBS to growth arrest cells in the S phase of the cell cycle. Macrophage supernatant was applied to the cells six hours before adding 1uCi/ml [^{3}H]-thymidine and incubating an additional 18 hours. Anti-PDGF or anti-bFGF antibody was added to some of the cells receiving macrophage supernatant (Fisher Scientific, Montreal, PQ). Following the last incubation, wells were rinsed four times with 1 ml ice-cold PBS, and 0.5 ml 0.1 N NaOH with 1%

SDS (Sigma Biochemicals, St. Louis, MO) was added to each well at room temperature for 20 minutes. The liquid from each well was transferred into a 20 ml scintillation vial (Sarstedt, St. Laurent, PQ) and neutralized with 100 ul of 1.0N HCl before adding 15 mls of scintillation fluid (SX12-4, Fisher Scientific, Montreal, PQ). Radioactivity was measured in a liquid scintillation counter (Model #LS6000SC, Beckman, Mississauga, ON). Samples were done in triplicate.

2.2.10 Statistical Analysis

Data are expressed as the mean ± SEM. Comparisons between means were analyzed using the paired Student's t test and the Dunnett's t test as appropriate. Results were considered significantly different when $p<0.05$.

2.3 Results

2.3.1 Time Course of Macrophage Activation

To assess at what time point the macrophage supernatant should be collected and what amount of supernatant should be given to the airway smooth muscle cells, we established certain criteria to aid us in selecting these values. We wished the time point of macrophage supernatant collection to be late enough to allow PDGF or other factors to be synthesized *de novo* and released but not too late to allow PDGF degradation or metabolism of other mitogenic substances. The amount of supernatant given should stimulate airway smooth muscle cells neither minimally nor maximally thus allowing increases as well as potential decreases in stimulation to be detected. Macrophage

supernatant was collected following 1-, 2-, 4-, 8-, 16- and 24-hour incubations of macrophages and given in amounts of 100ul, 300ul, 500ul and 700ul per 1ml to the airway smooth muscle cells. Figure 1 shows the stimulation for proliferation of airway smooth muscle cells for varying amounts of macrophage supernatant collected at various times during 24 hours. There is a direct relationship between the amount of macrophage supernatant given and airway smooth muscle cell stimulation for proliferation. A dose-dependent increase is seen in Figure 1 as well as in Figure 2, therefore the macrophages are releasing growth stimulatory mediators. We chose to use 500ul macrophage supernatant per ml as the results for this quantity fit the criteria established earlier. The incubation time we chose was 8 hours as a peak in stimulation occurred at this time for 3 out of 4 curves. This choice also fulfilled the criteria established earlier.

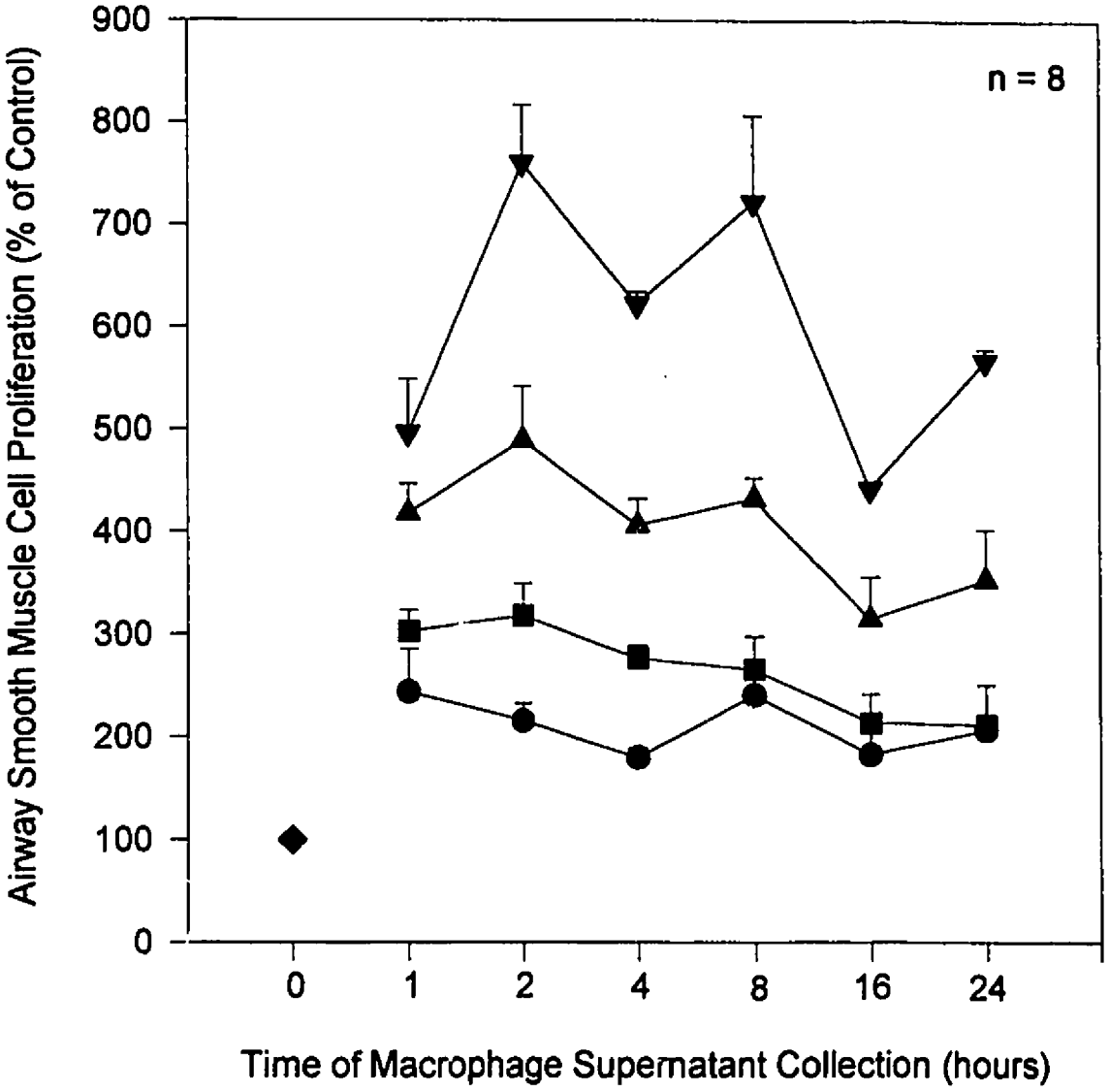

Fig. 1. The time course of macrophage supernatant collection and proliferative effect of increasing volumes of macrophage supernatant are shown. Supernatant was collected at 1, 2, 4, 8, 16 and 24 hours and added to airway smooth muscle cells in volumes of 100ul (circles), 300ul (squares), 500ul (upward triangles) and 700ul (downward triangles). Airway smooth muscle cell proliferation was increased above controls (1%FBS) (diamond) at all times studied and there was a dose-dependent increase in proliferation.

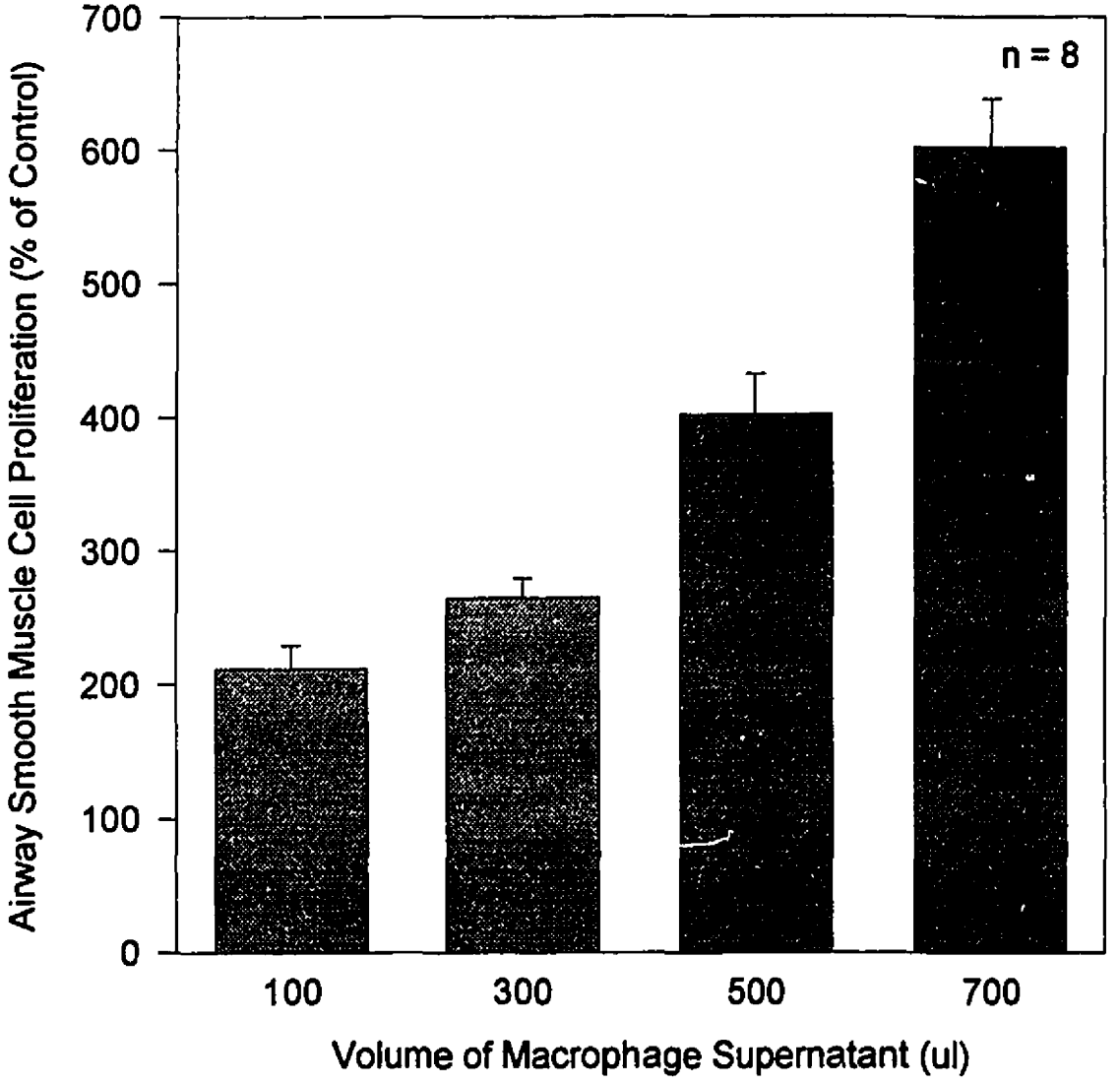

Fig. 2. The dose-response curve for macrophage supernatant averaged for all incubation times. Airway smooth muscle cells were treated with 100ul, 300ul, 500ul or 700ul of supernatant. There is a direct relationship between volume of supernatant received by airway smooth muscle cells and airway smooth muscle cell proliferation.

2.3.2 Macrophage Activation

To determine whether macrophage adherence to the plastic surface of the tissue culture flask was sufficient to activate the macrophages to release growth factors, macrophage supernatant was collected following a 12-hour incubation and given to airway smooth muscle cells. Media supplemented with either 1% or 10% FBS were used as 1% FBS is the minimal requirement for survival, and 10% is the supplement for the medium used to grow the cells. Figure 3 compares the stimulation of proliferation by 1% FBS, 10% FBS and macrophage supernatant. Treatment of airway smooth muscle cells with macrophage supernatant results in stimulation of airway smooth muscle cells significantly greater than 1% FBS ($p<0.05$) and almost as much as 10% FBS.

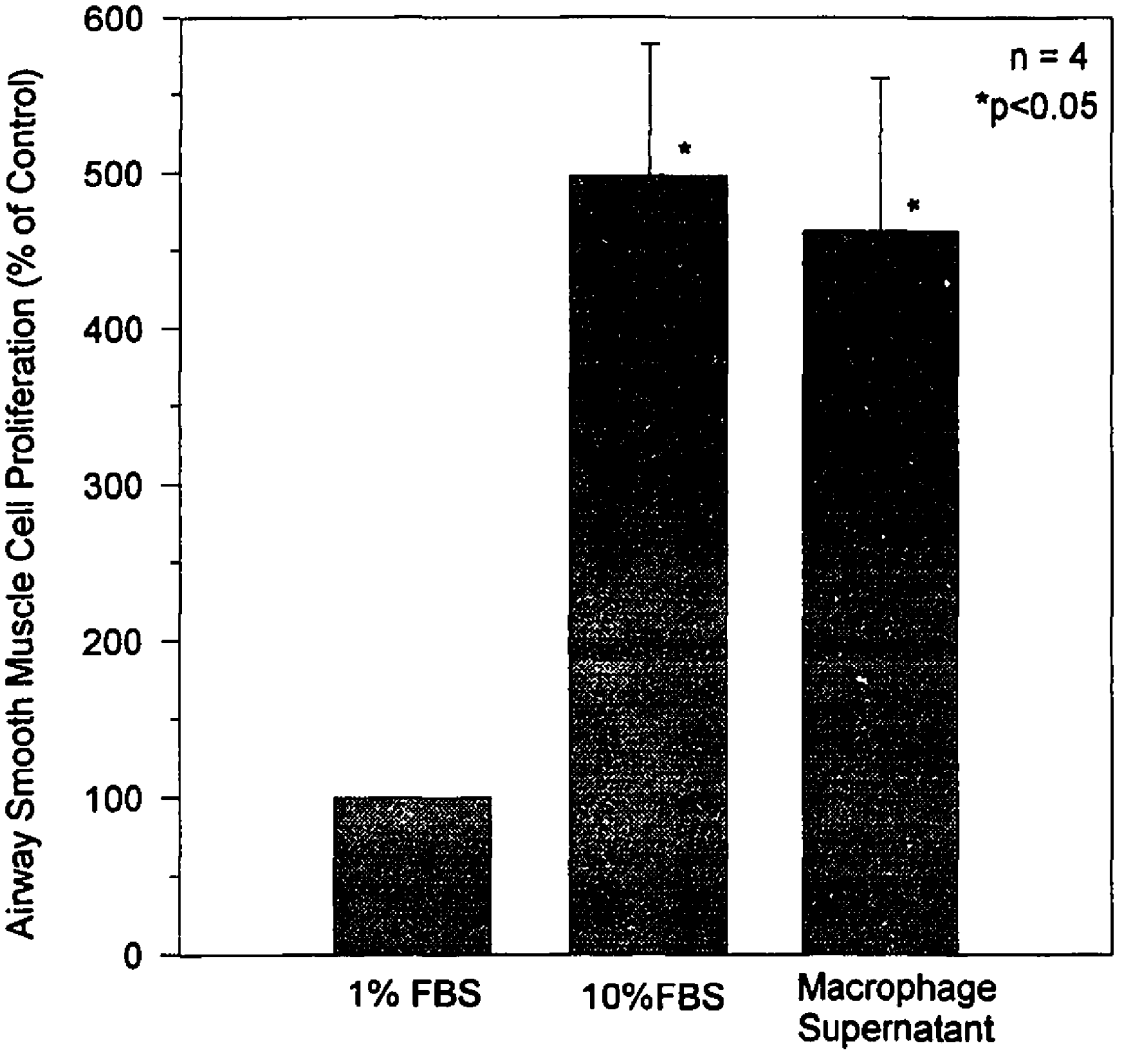

Fig. 3. The effect of 10% FBS and 500ul of macrophage supernatant collected at 12 hours on airway smooth muscle cell proliferation as compared to 1% FBS. Macrophage supernatant significantly promoted airway smooth muscle cell proliferation as did 10% FBS, and macrophage supernatant promoted airway smooth muscle cell proliferation just as much as 10% FBS.

2.3.3 The Effects of ASA, MK-571, and Suramin

The cyclooxygenase inhibitor ASA, the LTD_4 inhibitor MK-571, and the PDGF inhibitor suramin were used to assess the role of cyclooxygenase products (thromboxanes and prostaglandins), the role of LTD_4, and the role of PDGF in the macrophage-mediated stimulation for proliferation of airway smooth muscle cells. Neither ASA nor MK-571 significantly altered airway smooth muscle cell stimulation, whereas suramin significantly inhibited airway smooth muscle cell stimulation ($p<0.05$).

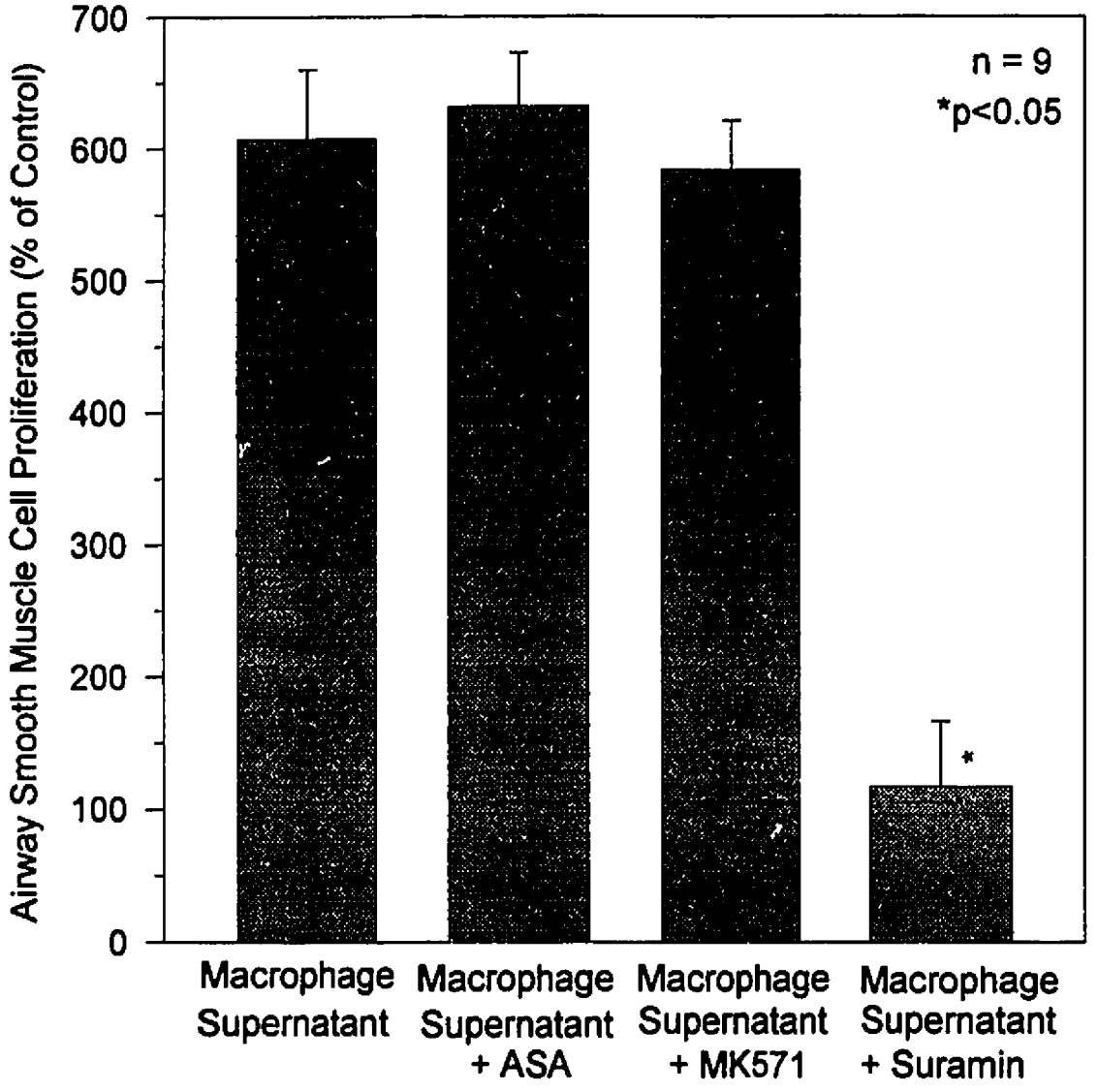

Fig. 4. Comparison of airway smooth muscle cell proliferation when macrophage cultures were treated with ASA, MK-571 or suramin. ASA and MK-571 had no effect on airway smooth muscle cell proliferation whereas suramin significantly inhibited airway smooth muscle cell proliferation.

2.3.4 The Effect of a Neutralizing Antibody to PDGF

A neutralizing antibody to PDGF was used in an attempt to evaluate the contribution of PDGF to growth stimulation. Macrophage supernatant was treated with three doses of anti-PDGF antibody (2.5, 5.0 and 10.0 ug/ml) before being added to airway smooth muscle cells. Figure 5 shows the inhibitory effect of the neutralizing antibody on the stimulation of airway smooth muscle cells. Significant decreases were observed with 2.5 and 5.0 ug/ml (19.4%, $p<0.05$ and 21.7%, $p<0.01$), although an even greater decrease was observed with 10.0 ug/ml (34%, $p<0.01$).

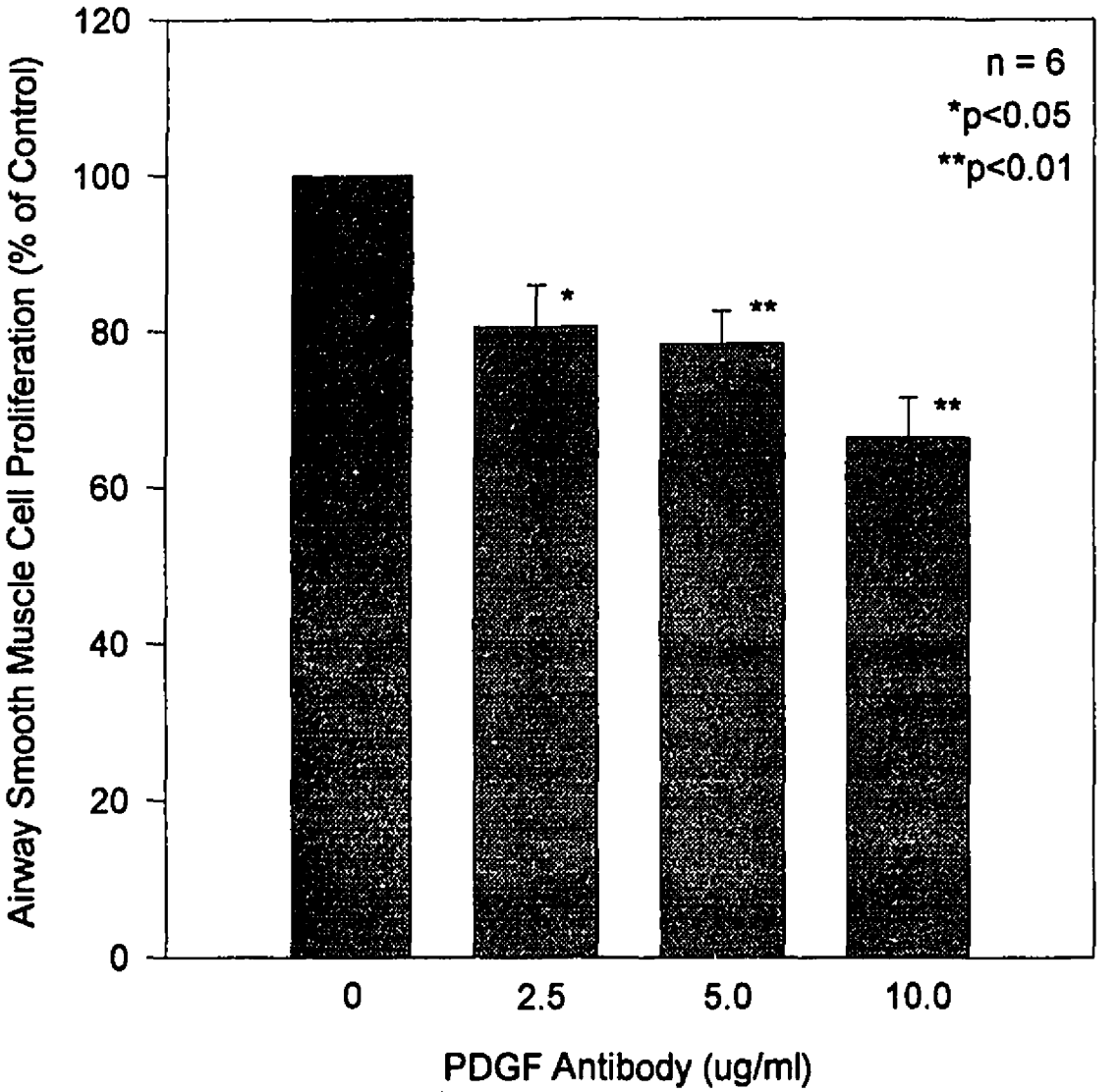

Fig. 5. The effect of increasing concentrations of anti-PDGF antibody on the ability of macrophage supernatant to promote airway smooth muscle cell proliferation. There is a 34% decrease in airway smooth muscle cell proliferation when macrophage supernatant is treated with 10.0ug/ml anti-PDGF antibody.

2.3.5 The Effect of a Neutralizing Antibody to bFGF

A neutralizing antibody to bFGF was used to examine whether bFGF (which is also secreted by macrophages) was being secreted along with PDGF and causing part of the remaining 66% stimulation of airway smooth muscle cells. In addition to this, we wanted to exclude the possibility that the neutralizing antibodies were non-specifically inhibiting the proliferation of the airway smooth muscle cells by acting on the cells directly. Macrophage supernatant was treated with various doses of anti-bFGF antibody: 0.1, 1.0, 10.0, 30.0 and 50.0 ug/ml. Figure 6 shows that the neutralizing antibody to bFGF does not inhibit the proliferation of airway smooth muscle cells.

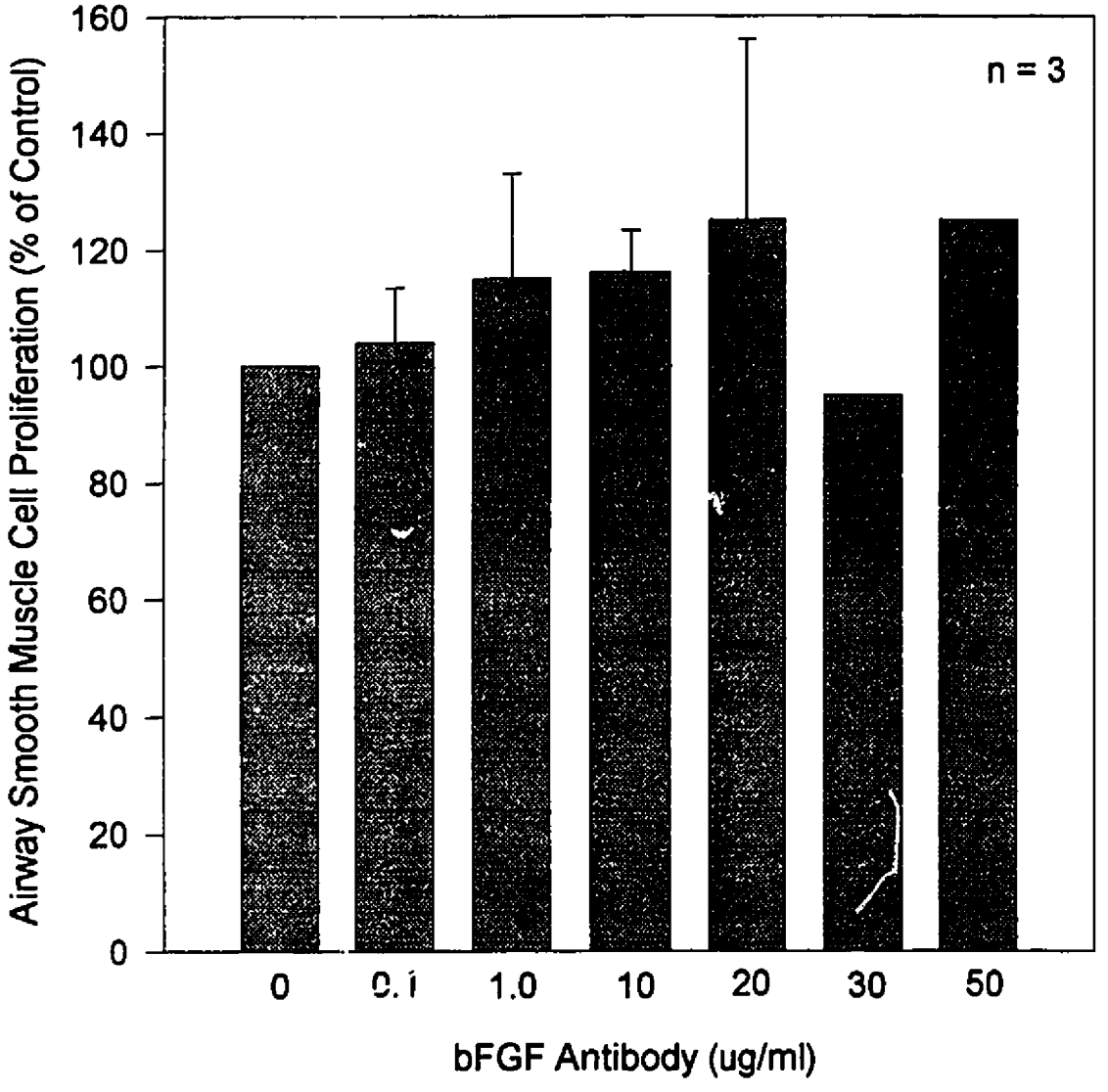

Fig. 6. The effect of increasing concentrations of anti-bFGF antibody on the ability of macrophage supernatant to promote airway smooth muscle cell proliferation. There was no significant change in airway smooth muscle cell proliferation for any of the antibody concentrations studied.

CHAPTER 3

GENERAL DISCUSSION

Discussion

Asthmatic subjects exhibit several characteristic features in the airways including airway inflammation, airway hyperresponsiveness, and increased airway smooth muscle content (James *et al.*, 1989; Laitinen & Laitinen, 1991; Katz & Beer, 1993). These changes can be evoked in the airways of guinea pigs (Watson *et al.*, 1993), rabbits (Brugman *et al.*, 1993), dogs (Jiang *et al.*, 1992), primates (Gundel *et al.*, 1992) and Brown Norway rats (Sapienza *et al.*, 1991) by sensitization and subsequent challenge with foreign antigen. The airway inflammation comprises both a mononuclear and a polymorphonuclear infiltrate (Laitinen & Laitinen, 1991). This association between airway inflammation and increased airway smooth muscle content led us to postulate that mediators released during the inflammatory response could participate in the proliferation of airway smooth muscle cells. The cell we chose to investigate was the macrophage as it is the most common cell found in BAL fluid, and it can become activated during the allergic response. Activation can occur either when IgE binds to low-affinity receptors on the macrophage (Thorel *et al.*, 1988), or when LTC_4 is released from degranulating mast cells and binds to LTC_4 receptors on the macrophage (Gibson *et al.*, 1993). This latter activation is postulated to result specifically in the release of PDGF (Phan *et al.*, 1987) which is a potent growth factor for airway smooth muscle cells.

Macrophage cultures and airway smooth muscle cell cultures were established from Brown Norway rat lung and tracheal tissue in order to determine whether macrophage-derived mediators could promote the proliferation of airway smooth muscle cells, and to partly characterize the macrophage-derived mediators. There are advantages

to using cultures of airway smooth muscle cells over whole organ or tissue strip studies. First, it is possible to grow large populations of airway smooth muscle cells which are free of other cell types, thereby allowing the dissection of mechanisms in the absence of the influences of other cell types. Second, cell cultures make it possible to growth arrest all the cells in a culture to synchronize them. Third, cell cultures grow in monolayers therefore all the cells have equal access and exposure to any substance added to the medium.

The disadvantages of using cell cultures are that data must be extrapolated from the *in vitro* situation to *in vivo* conditions when the comparability between the two is unknown, the setup is artificial as it eliminates all other cell types which interact with one another in the whole organism, and the phenotype of the airway smooth muscle cells is different *in vitro* as compared to *in vivo*. *In vitro*, cells are actively synthesizing proteins and dividing to establish a monolayer on the surface of the culture dish whereas *in vivo*, cells are quiescent but actively contracting. If the airway smooth muscle cell phenotype can vary from being contractile to synthetic, then it is possible that there are other important differences between airway smooth muscle cells *in vivo* as compared to *in vitro*.

The supernatant collected from macrophage cultures at various times was able to promote the proliferation of airway smooth muscle cells. This stimulation peaked at eight hours' incubation time which was in concordance with the criteria we established beforehand. At this time, *de novo* synthesis of PDGF and other growth factors probably had occurred but the degradation of PDGF had not, nor had the metabolism of other mitogenic substances. The ideal amount of macrophage supernatant to be added to airway

smooth muscle cultures was determined to be 500ul per well of 1ml to allow the proper monitoring of increases and decreases in promotion of airway smooth muscle cell proliferation. There was a direct relationship between the amount of macrophage supernatant added to airway smooth muscle cultures and amount of airway smooth muscle proliferation.

Figure 1 shows that macrophage supernatant is able to promote airway smooth muscle proliferation at all time points of supernatant collection. These experiments focussed on partially characterizing the macrophage supernatant collected following an 8-hour incubation. Therefore, the macrophage supernatant collected at other times should be characterized to determine how PDGF influences airway smooth muscle proliferation at the other time points. To examine the time course of the synthesis and secretion of PDGF, further experiments are required. *In situ* hybridization techniques could be used to trace the PDGF-A and PDGF-B mRNAs in the macrophage cultures to determine whether there is upregulation of mRNA synthesis at any point during the 24-hour time course. Additionally, immunological techniques could be used to locate the PDGF proteins within the macrophages and to follow the secretion of the PDGF proteins. Tracing the synthesis and secretion of PDGF allows us to establish to what extent it contributes to the total proliferation of airway smooth muscle cells at each time point.

To evaluate the extent of airway smooth muscle proliferation induced by macrophage supernatant, 500ul of macrophage supernatant was added to airway smooth muscle cells and proliferation was compared to proliferation induced by medium supplemented with 1% FBS or 10% FBS. Macrophage supernatant as well as 10% FBS

significantly promoted the proliferation of airway smooth muscle cells as compared to 1% FBS, and macrophage supernatant and 10% FBS stimulated airway smooth muscle cells equally well. We concluded that adherence to the plastic of the cell culture dish was sufficient to activate macrophages to release growth-promoting substances. This was not surprising since adherence is an important first step in the transition of a circulating monocyte to a tissue macrophage (Shaw *et al.*, 1990). Additionally, it has been previously shown that alveolar macrophages which are allowed to adhere to plastic tissue culture plates are able to release mitogenic activity. Furthermore, activation by adherence is believed to be sufficient to upregulate *de novo* synthesis of PDGF-B proteins in macrophages (Shimokado *et al.*, 1985).

Stimulation was not affected by the addition of the cyclooxygenase inhibitor ASA, therefore, it was not likely that the cyclooxygenase product thromboxane A_2 contributed to airway smooth muscle proliferation in this study. ASA also inhibited the synthesis of PGE_2 which has been shown to inhibit the growth of guinea pig airway smooth muscle cells in culture (Florio *et al.*, 1994). Since the synthesis of TXA_2 and PGE_2 was inhibited simultaneously, we can not exclude the possibility that both may have been present but their effects offset, one by the other. By inhibiting the synthesis of thromboxane A_2 only, the contribution of PGE_2 could be determined, and by inhibiting the synthesis of PGE_2 only, the contribution of TXA_2 could be determined. Analyzing these two cyclooxygenase products separately would allow for a better understanding of the complex interaction involving the macrophage-derived mediators.

The response of airway smooth muscle cells to macrophage supernatant was not

affected by the addition of MK-571, therefore LTD_4 did not contribute to the total proliferation of airway smooth muscle cells. We do not know whether adherence to the plastic tissue plates was insufficient to activate macrophages to release LTD_4, or whether LTD_4 was not stimulatory for Brown Norway airway smooth muscle cells.

The PDGF inhibitor suramin significantly decreased the proliferative response of airway smooth muscle cells to macrophage supernatant. However, we could not conclude from this that PDGF was released from macrophages and promoted airway smooth muscle cell proliferation because suramin is a non-specific inhibitor of various peptide growth factors including PDGF (Middaugh *et al.*, 1992). Therefore, a neutralizing antibody to PDGF was used as a specific inhibitor of PDGF. The anti-PDGF antibody significantly inhibited the proliferative response of the airway smooth muscle cells to the macrophage supernatant. It is likely that PDGF released from macrophages accounts for at least a third of airway smooth muscle stimulation.

The anti-bFGF antibody did not affect the proliferative response of airway smooth muscle cells to macrophage supernatant. It is possible that either 1) bFGF is not released by the activated macrophages or that 2) bFGF is released but the airway smooth muscle cells do not respond.

Since the antibody to bFGF had no effect on the proliferation of the airway smooth muscle cells, we concluded that the anti-PDGF antibody did not non-specifically act on the airway smooth muscle cells themselves, and did indeed inhibit the proliferation of the airway smooth muscle cells by acting on PDGF molecules.

Macrophage cultures were stained with a non-specific esterase stain and the cells

stained as macrophages should. In addition to this, the cells had the morphological appearance of macrophages, and cells failed to divide or survive in culture for more than four days. However, performing a more specific stain such as an immunocytochemical stain, for example, would give greater confidence as to the identity of the cells. The airway smooth muscle cell cultures were characterized using immunofluorescence staining for smooth muscle α-actin. The cultures, however, were not counterstained to determine what percentage of the cells were airway smooth muscle cells. We estimate the percentage to be high as the cells of the airway smooth muscle cell cultures were morphologically similar.

Unfortunately, we never assessed how much PDGF activity was coming from the airway smooth muscle cells themselves. Airway smooth muscle cells have the ability to synthesize and release their own PDGF (Rossi *et al.*, 1992), therefore it is possible that the proliferative effect of macrophage supernatant is partly due to the autocrine action of PDGF.

A disadvantage of this study is that the airway smooth muscle cell cultures were obtained from the trachea of the Brown Norway rats. It is not known how closely these smooth muscle cells resemble the smooth muscle cells located more distally in the airways. The most obvious difference in smooth muscle in these two areas is their morphological appearance. The smooth muscle exists in a strip located posteriorly on the trachea whereas the smooth muscle cells from the airways is more diffuse and exists in smaller bundles. In human airways, the musculature of the first and second order bronchi resembles that of the trachea while the fourth to seventh order airways show different size

of muscular bundles, number of gap junctions, size of gap junctions, arrangement of muscle bundles, and appearance of contractile filaments (Daniel *et al.*, 1986). Therefore, it is possible that the airway smooth muscle cells of the trachea in the Brown Norway may rat respond differently to macrophage-derived mediators than airway smooth muscle cells from smaller airways.

Furthermore, only two cell types were investigated in this study. In the intact organism, there is a complex interaction between the inflammatory cells and the various cell types found in the airways. Although macrophages were studied, the experimental setup did not take into account the contribution of lymphocytes, neutrophils, eosinophils, mast cells, and platelets to the mediators released in the proximity of the airway smooth muscle cells. Perhaps the most important of all the inflammatory mediators is PGE_2 which has been shown to inhibit the proliferation of airway smooth muscle cells in culture. Perhaps PGE_2 modulates the response of the airway smooth muscle cells *in vivo* such that the airway smooth muscle cells do not proliferate as much as they do *in vitro*.

Additionally, only airway smooth muscle cells were studied in these experiments. Airways in human asthmatics and in animal models do not exhibit only increased airway smooth muscle content. There is also increased adventitial mass, increased submucosal mass, and secretory gland hypertrophy. Although it is postulated that the increase in airway smooth muscle mass may be the most important change in the airways, we can not ignore the other changes which contribute to increased airway hyperresponsiveness as the organism functions as a whole.

Airway smooth muscle cells are acted upon by the products of various cells other

than macrophages *in vivo*. In the guinea pig model of airway hyperresponsiveness, there is evidence suggesting that airway epithelial cells are an important source of PGE_2 which exerts an antiproliferative effect on the airway smooth muscle cells (Florio *et al.*, 1994). A disadvantage of these experiments is that they do not take into account the effects of cells other than macrophages, and do not emulate well what occurs *in vivo*.

We conclude that macrophages display potent growth promoting activity for airway smooth muscle cells in culture, that one of the growth factors released by activated macrophages is PDGF which contributes to the total airway smooth muscle cell proliferation, and that macrophages may play an important role in allergen induced airway remodelling.

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