

**EOSINOPHILS AND BONE MARROW PROGENITORS IN ALLERGEN-INDUCED
AIRWAY HYPERRESPONSIVENESS IN DOGS**

By

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ABSTRACT

The pathogenesis of airway hyperresponsiveness, a characteristic feature of asthma, is uncertain. Current evidence suggests a pathogenic role for immature (progenitors) and mature inflammatory cells, particularly eosinophils. The aim of this thesis was to examine the role of eosinophils and bone marrow progenitors in the development of allergen-induced airway hyperresponsiveness in dogs.

Random source mongrel dogs demonstrating skin test reactivity to *Ascaris suum* allergen were studied. Acetylcholine airway responsiveness was measured before and 24 hours after inhalation of *Ascaris* allergen. For the second and third studies, dogs were pre-treated with inhaled budesonide twice daily for one week prior to allergen challenge. Airway eosinophils were enumerated from bronchoalveolar lavage samples and eosinophil activation was assessed by measurement of eosinophil peroxidase levels in bronchoalveolar lavage fluid. Bone marrow was obtained 24 hours after allergen inhalation with progenitors counted after 8 days in culture.

In the first study, dogs that developed airway hyperresponsiveness were found to have a greater number of and more activated airway eosinophils before allergen inhalation than dogs that did not develop airway hyperresponsiveness.

In the second study, pre-treatment with inhaled budesonide reduced the number of eosinophils present in the airways before allergen inhalation. This reduction was associated with an attenuation of allergen-induced airway hyperresponsiveness.

In the third study, allergen inhalation increased bone marrow progenitor production in dogs that developed allergen-induced airway hyperresponsiveness. Furthermore, the increases in progenitors and airway hyperresponsiveness were reduced by pre-treatment with inhaled budesonide.

These results suggest that pre-existing airway eosinophilia influences the development of airway hyperresponsiveness after allergen inhalation. In addition, the results from this thesis provide the first direct evidence that allergen inhalation can increase bone marrow progenitor production and suggest that such increases may contribute to the development of airway hyperresponsiveness in asthma.

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PREFACE

Three papers, prepared for publication, form the basis of Chapters 2, 3 and 4 in this thesis. These papers have been submitted as:

Role of airway eosinophils in the development of allergen-induced airway hyperresponsiveness in dogs.

MJ Woolley, CG Lane, R Ellis, WH Stevens, KL Woolley and PM O'Byrne.

Submitted to: American Journal of Physiology: Lung Cellular and Molecular Physiology.

Effect of inhaled corticosteroid on airway eosinophils and allergen-induced airway hyperresponsiveness in dogs.

MJ Woolley, J Wattie, R Ellis, CG Lane, WH Stevens, KL Woolley, M Dahlback and PM O'Byrne.

Submitted to: Journal of Applied Physiology

Allergen-induced changes in bone marrow progenitors and airway hyperresponsiveness in dogs: Effects of inhaled budesonide.

MJ Woolley, JA Denburg, R Ellis, M Dahlback and PM O'Byrne.

Submitted to: Journal of Clinical Investigation

These studies could not have been completed without the technical help of CG Lane, J Wattie, R Ellis, WH Stevens, KL Woolley and M Dahlback. However, each of these papers represents my work. Under the guidance of my supervisor and supervisory committee, I was responsible for reviewing the literature, formulating the research questions and designing the studies. I was involved in the day to day collection of data during each study. I performed the analysis of data and was responsible for writing the papers and this thesis.

In addition, the studies involving the use of inhaled budesonide necessitated the development of a system which could deliver inhaled budesonide to the dogs while they were conscious and spontaneously breathing. This system involved the development of individually fitted plastic muzzle masks and the use of a Wright Dust Feeder, both of which were supplied by Astra Draco AB. Several pilot studies were then conducted by myself to establish the correct dose for the dogs based on budesonide levels in the blood and on expiratory filters.

In addition to the methods utilized in this thesis, I also explored several additional techniques in order to further examine the role of the eosinophil in the dog model. Although unsuccessful, these techniques included attempts to: isolate canine circulating and BAL eosinophils using either density gradient separation, magnetic activated cell sorting or fluorescent activated cell sorting; develop a canine eosinophil survival assay; develop a clonal growth assay for the growth of canine eosinophil/basophil progenitors.

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LIST OF ABBREVIATIONS

BAL:	Bronchoalveolar lavage
BALF:	Bronchoalveolar lavage fluid
CFU:	Colony-forming unit
CSF:	Colony-stimulating factor
ECP:	Eosinophil cationic protein
EDN:	Eosinophil-derived neurotoxin
DS:	Dog serum
FCS:	Fetal calf serum
Eo\B-CFU:	Eosinophil\basophil colony-forming unit
EPO:	Eosinophil peroxidase
G-CSF:	Granulocyte colony-stimulating factor
GM-CFU:	Granulocyte-macrophage colony-forming unit
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
ID:	Internal diameter
IL:	Interleukin
MBP:	Major basic protein
MMAD:	Mass median aerodynamic diameter
OD:	Optical density

LIST OF ABBREVIATIONS (Continued)

PBS:	Phosphate buffered saline
SCF:	Stem cell factor
SD:	Standard deviation
SEM:	Standard error to the mean
WDF:	Wright dust feeder
w/v:	Weight per volume

CHAPTER 1: INTRODUCTION

This thesis comprises five chapters. This chapter provides a review of topics that are addressed in the experimental studies conducted for the thesis. Specifically, an overview of asthma and airway hyperresponsiveness is followed by a discussion of the *Ascaris* dog model of airway hyperresponsiveness. A summary of the role of airway inflammation in the pathogenesis of airway hyperresponsiveness then leads to a review of hemopoiesis and the characteristics of the eosinophil. Each of the next three chapters presents an experimental study prepared for publication. The final chapter, Chapter 5, presents a summary of findings and a general discussion.

Asthma

Definition

Asthma has been defined in terms of characteristic symptoms (Godfrey, 1985), abnormalities in airway physiology (American Thoracic Society, 1962) or histology (Reed, 1986) and on the basis of the suspected etiology (Pepys, 1967). Although no definition has been universally accepted, an international expert panel has recently recommended (Expert Panel, 1991) that asthma be defined as a lung disease with the following characteristics:

1. Airway obstruction that is often reversible either spontaneously or with treatment,
2. Airway inflammation
3. Airway hyperresponsiveness to a variety of stimuli.

Diagnosis

A diagnosis of asthma has traditionally been based upon objective measurements of the variability of airway obstruction together with a clinical history of episodic symptoms. Symptoms typically associated with asthma include wheezing, breathlessness, chest tightness and cough (McFadden et al., 1992). Airway obstruction has usually been evaluated by tests of forced expiration or measurements of airway resistance (Pride, 1979; Dubois et al., 1956). The reversibility of the airway narrowing has typically been determined from lung

function tests before and after inhalation of a beta-adrenergic agonist. The inflammatory component of asthma is harder to evaluate and has often been inferred from the therapeutic response of the patient to anti-inflammatory treatments (Busse et al., 1993). Evidence of airway inflammation may also be obtained through bronchoalveolar lavage, bronchial biopsy or sputum sampling, although, these techniques are used primarily for research purposes. While measurement of airway responsiveness was also initially restricted to research studies, it is being increasingly used as an aid in the diagnosis of asthma.

Airway Hyperresponsiveness

Definition and measurement

Airway responsiveness refers to the ability of the airways to constrict to non-sensitizing physical (e.g. exercise, hyperventilation) or chemical (e.g. histamine, cholinergic agonists) stimuli (Cockcroft et al., 1991). Airway hyperresponsiveness, therefore, is an increase above the normal responsiveness of the airways to these stimuli (Cockcroft et al., 1991). Airway hyperresponsiveness is characterized by an increase in the sensitivity of the airways to constrictor stimuli, as indicated by a left shift of the dose-response curve, and a greater maximal bronchoconstrictor response (Sterk et al., 1985; Woolcock et al., 1984).

Airway responsiveness is commonly measured by inhalation tests with histamine or a cholinergic agonist such as methacholine. Typically, increasing concentrations of the agonist are inhaled until a predetermined degree of airway constriction, as indicated by lung function tests, is attained or the concentration is high enough to conclude that responsiveness is not increased above normal levels (Dolovich et al., 1992). The level of responsiveness is then expressed as the concentration of agonist sufficient to cause the preset level of airway constriction. The lower the concentration of agonist required, the greater the responsiveness. There is, however, no clear division between normal and increased responsiveness since airway responsiveness is distributed continuously in a log normal manner in humans (Cockcroft et al., 1983) and animals (Snapper et al., 1978). Evaluation of the maximal bronchoconstrictor response is often not undertaken due to the potential hazards associated with its measurement (Sterk et al., 1991).

Numerous studies have demonstrated that airway responsiveness in asthmatic subjects may increase transiently following inhalation of air pollutants (Orehek et al., 1976), sensitizing occupational chemicals (Lam et al., 1979) and allergens (Cockcroft et al., 1977; Cartier et al., 1982). Inhalation of these agents, therefore, provides a means of investigating the pathogenesis of airway hyperresponsiveness and asthma. Studies in asthmatic subjects, however, are often limited due to practical and safety reasons. Thus, animal models of airway hyperresponsiveness have been developed. In dogs, inhalation of ragweed

(Chung et al., 1985) or *Ascaris suum* (Krell et al., 1976) allergens have been used to induce airway hyperresponsiveness. In this thesis, the *Ascaris* allergen model was used to examine the mechanism of airway hyperresponsiveness.

Ascaris allergen model

Studies in the early 1970's were the first to demonstrate that inhalation of an extract of the pig nematode, *Ascaris suum*, by dogs with skin reactivity to the antigen, caused airway obstruction which was maximal 3 to 10 minutes after challenge and spontaneously resolved over the next hour (Booth et al., 1970; Gold et al., 1972; Patterson et al., 1974). The change in lung function was believed to be a result of an antigen-IgE mediated release of bronchoconstricting mediators from mast cells (Patterson et al., 1974). *Ascaris suum* was thought to trigger mediator release due to cross reactivity of the *Ascaris* antigen with those of *Toxocara canis*, a parasitic nematode commonly found in dogs (Patterson et al., 1974). Subsequent studies have implicated several mediators, including histamine (Chiesa et al., 1975; Gold et al., 1977), prostaglandins (Kleeberger et al., 1986) and thromboxane (Kleeberger et al., 1987), as the cause of the immediate bronchoconstriction to *Ascaris*. The changes in lung function in the dogs and the putative mediators responsible for the bronchoconstriction are similar to findings in asthmatic subjects during the allergen-induced early asthmatic response.

The occurrence of a late bronchoconstrictor response in dogs following

allergen inhalation, as observed in asthmatic subjects, has been more difficult to demonstrate. In approximately half of the asthmatic subjects with an early asthmatic response, bronchoconstriction may recur 3 to 4 hours after allergen challenge (Herxheimer, 1952; Booij-Noord et al., 1972). This late asthmatic response may persist for over 24 hours (O'Byrne et al., 1987). An *Ascaris*-induced late response has been observed in dogs following pretreatment with metyrapone, a cortisol synthesis inhibitor (Sasaki et al., 1987), or after local *Ascaris* instillation in a peripheral airway (Turner et al., 1990). In contrast, no late response has been reported in untreated dogs after whole lung *Ascaris* inhalation (Stevens et al., 1989).

Initial studies examining the effect of *Ascaris* inhalation on the development of airway hyperresponsiveness in dogs were conducted by Krell and associates (1976). They found no significant increases in the airway responsiveness of skin test positive dogs either 60 minutes or one week after allergen inhalation when compared to the baseline responsiveness of a group of control dogs. Similarly, Sasaki et al. (1987) found no significant increases in the airway responsiveness of 10 skin test positive dogs 6 hours after *Ascaris* inhalation. In contrast, several studies have reported increases in airway responsiveness of skin test positive dogs after *Ascaris* inhalation (Kannan et al., 1986; Dollery et al., 1987; Stevens et al., 1989). Airway hyperresponsiveness was found to develop as early as one hour after allergen inhalation (Kannan et al., 1986) and persisted for at least one

week (Stevens et al., 1989). However, the increase in airway responsiveness after *Ascaris* inhalation did not occur in all dogs. Stevens and colleagues (1989) reported that only 4 of their 9 skin test positive dogs developed airway hyperresponsiveness after *Ascaris* inhalation. An examination of the data of Sasaki et al. (1987) reveals that 6 of the 10 skin test positive dogs they studied had greater than a twofold increase in airway responsiveness after allergen inhalation. This variability between dogs may explain the discrepancy between studies which found no significant changes in airway responsiveness after *Ascaris* inhalation (Krell et al., 1976; Sasaki et al., 1987) and those that did (Kannan et al., 1986; Dollery et al., 1987; Stevens et al., 1989).

Although these studies indicate that allergen inhalation can transiently increase airway responsiveness in dogs, the pathogenesis of this hyperresponsiveness remains to be clarified. However, findings from several studies suggest that airway inflammation may play a central role.

Airway inflammation in the pathogenesis of airway hyperresponsiveness

Indirect evidence that airway inflammation is involved in the pathogenesis of airway hyperresponsiveness has been obtained from bronchial biopsy (Azzawi et al., 1990; Bousquet et al., 1990; Laitinen et al., 1991; Jeffery et al., 1989) and bronchoalveolar lavage (Wardlaw et al., 1988; Kirby et al., 1987; Adelroth et al., 1990; Bousquet et al., 1990) studies of asthmatic subjects with persistent airway

hyperresponsiveness. Airway inflammation in these patients was characterized by the presence of epithelial shedding, edema, subepithelial fibrosis, hyperplasia of mucous glands and increased numbers of airway inflammatory cells including eosinophils, mast cells and lymphocytes. Several studies have also demonstrated significant correlations between airway responsiveness in asthmatic subjects and the number or state of activation of eosinophils (Wardlaw et al., 1988; Kirby et al., 1987), mast cells (Wardlaw et al., 1988; Kirby et al., 1987) and lymphocytes (Azzawi et al., 1990).

Further evidence of a link between airway inflammation and airway hyperresponsiveness has come from allergen inhalation studies in animals. These studies have shown a temporal relationship between inflammatory events in the airways and the development of allergen-induced airway hyperresponsiveness. In animals, increases in eosinophils (Dollery et al., 1987; Gundel et al., 1989; Elwood et al., 1992), neutrophils (Chung et al., 1985; Elwood et al., 1992; Murphy et al., 1986) and lymphocytes (Elwood et al., 1992) after a single allergen inhalation have been associated with the development of allergen-induced airway hyperresponsiveness. Longer term animal studies using repeated allergen inhalation have also shown an association between the development of chronic airway hyperresponsiveness and a sustained increase in the number of eosinophils (Gundel et al., 1990; Becker et al., 1989) and mast cells (Becker et al., 1989). More direct evidence of a link between airway inflammation and airway

hyperresponsiveness comes from a study where inhibition of cell infiltration into the airways was associated with a significant reduction of allergen-induced airway hyperresponsiveness (Wegner et al., 1990).

Few studies, examining both airway inflammation and airway hyperresponsiveness after allergen inhalation, have been conducted in asthmatic subjects. Increases in airway responsiveness after allergen inhalation have been associated with the influx of several inflammatory cell types into the airways including eosinophils (Brusasco et al., 1990; Rossi et al., 1991; Smith et al., 1992), lymphocytes (Brusasco et al., 1990), metachromatic cells (mast cells/basophils) (Pin et al., 1992) and neutrophils (Smith et al., 1992). Allergen inhalation has also been associated with cell activation and the release of preformed and newly generated mediators (Sedgwick et al., 1991; Diaz et al., 1989). Inhalation of several of these mediators, such as leukotriene E₄ (Arm et al., 1988), prostaglandin D₂ (Fuller et al., 1986) and platelet activating factor (Cuss et al., 1986), has been shown to cause transient increases in airway responsiveness in asthmatic and normal subjects.

Although the evidence is not causal, results from the above studies suggest that airway inflammation may contribute to the pathogenesis of airway hyperresponsiveness. Since a consistent finding of these studies has been an increase in the number and activation of airway eosinophils, particular attention has been directed toward the role of eosinophils in the mechanism of airway

hyperresponsiveness. Before describing the characteristics of the eosinophil, a general review of the bone marrow production of inflammatory cells will be undertaken.

Inflammatory Cell Hemopoiesis

Stem cells and progenitors

Inflammatory cells are thought to develop from a very small number of pluripotent stem cells located primarily in the bone marrow (Quesenberry et al., 1979). Pluripotent stem cells have the ability to reproduce (self-renewal) and to differentiate along either lymphoid or myeloid lineages (Abramson et al., 1977; Fowler et al., 1967). As the stem cell differentiates, there is a progressive lineage restriction, as evidenced by the expression of specific cell surface receptors (Goldschneider et al., 1980; Lopez et al., 1988) and a decreased capacity for self-renewal (Suda et al., 1983). Commitment of stem cells to specific lineages may proceed through numerous differentiation stages and is believed to occur in a stochastic (random) manner (Suda et al., 1984; Leary et al., 1984). Eventually, committed stem cells or progenitors become restricted to a single lineage with limited capacity for self-renewal (Quesenberry et al., 1979). The cells then undergo morphological changes developing into mature effector cells which are then

released into the circulation (Cannistra et al., 1988).

In vitro clonal growth assays have been used to identify inflammatory cell progenitors (Bradley et al., 1966). In these assays, putative progenitors are placed in a semisolid matrix of agar or methylcellulose and allowed to differentiate and grow into colonies of hemopoietic cells, termed colony-forming units (CFU). Colonies can then be removed from the matrix and the cells identified by morphological and biochemical analysis. Enumeration of colonies also provides an estimate of progenitor cell frequency (Bainton, 1988). Along the myeloid lineage, eosinophils and basophils are believed to be derived from eosinophil-basophil progenitors (Eo/B-CFU) (Denburg et al., 1985) while neutrophils and macrophages are thought to arise from a common bipotential progenitor cell termed the granulocyte-macrophage colony-forming unit (GM-CFU) (Moore et al., 1971; Metcalf, 1970). Along the lymphoid lineage, T and B lymphocytes are thought to develop from a common lymphoid progenitor (Abramson et al., 1977)

Hemopoietic cytokines

Cytokines are a diverse group of signaling proteins produced by a wide variety of cells. Cytokines serve an important role in cell communication and regulation through their ability to bind to specific high affinity receptors expressed on the surface of target cells (Pugh-Humphreys et al., 1991). Cytokines are thought to play a critical role in many normal and pathological processes, including

the generation of immune responses, tissue repair and inflammation (Kelley, 1990). A role for cytokines in promoting airway inflammation in asthma has been proposed based on studies reporting the presence of several cytokines, including granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-3 (IL-3) and interleukin-5 (IL-5), in the airways of asthmatic subjects (Broide et al., 1991; Robinson et al., 1992). These cytokines are of interest due to their ability to enhance the recruitment, survival and function of eosinophils *in vitro* (Lopez et al., 1986; Warringa et al., 1991; Begley et al., 1986).

Cytokines may also be involved in regulating the growth and differentiation of inflammatory cells. Cytokines which regulate progenitor colony growth and differentiation have been identified through the use of the clonal growth assays. Colony formation has been found to be critically dependent on the presence of hemopoietic cytokines termed colony-stimulating factors (CSF) (Metcalf, 1986). These factors are produced by a variety of cells, including stromal and inflammatory cells (Sieff, 1987) and may exert effects on hemopoietic cells at various stages of differentiation (Ogawa, 1993). Although, CSFs exhibit some target cell selectivity, there is overlap in the actions of the different factors (Kelso et al., 1990). For example, both of the CSFs, granulocyte-colony stimulating factor (G-CSF) and GM-CSF can stimulate granulocyte-macrophage colony formation (Sieff et al., 1985; Eliason, 1986). In addition, hemopoietic growth factors may have synergistic effects in stimulating colony formation. For example, GM-CSF or

IL-3 can act synergistically with stem cell factor (SCF), a recently described hemopoietic cytokine (Matsui et al., 1990), in stimulating GM-CFU colony formation (Brandt et al., 1992; McNiece et al., 1991).

Eosinophils

Life cycle

After differentiation in the bone marrow, eosinophils are released into the blood where they circulate for approximately 12 hours (Parwaresch et al., 1976). They then migrate into tissues, preferentially residing in the skin, lung and gastrointestinal tract (Beeson et al., 1977). Eosinophils are predominantly a tissue cell with circulating eosinophils representing only one percent of the total number of mature eosinophils in the body (Rytomaa, 1960). Once in the tissues, the lifespan of the eosinophil is uncertain but has been reported to be 2 to 4 days (Cohen et al., 1967). In vitro studies have recently suggested that cytokines, such as GM-CSF, IL-3 and IL-5, can prolong the survival of eosinophils in culture, possibly by inhibiting apoptosis (Yamaguchi et al., 1991; Stern et al., 1992). Whether cytokines enhance eosinophil tissue survival in vivo remains to be determined. At the end of their lifecycle, eosinophils are thought to undergo either cytolytic degranulation or apoptosis followed by phagocytosis by macrophages

and, perhaps, other phagocytic cells (Ross et al., 1966; Speirs et al., 1962).

Morphology and mediators

Mature eosinophils are approximately 10 to 15 μm in diameter and typically possess a bilobed nucleus (Miller et al., 1966). Eosinophils are characterized by the presence of cytoplasmic granules which stain a distinctive orange-red colour with acid dyes such as eosin (Weller, 1991). Several types of eosinophil granules have been described. The specific or secondary granules contain an electron dense, crystalloid core surrounded by a less dense matrix (Hardin et al., 1970). The granule core is composed of an alkaline protein termed major basic protein (MBP) while the matrix contains the proteins eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) (Peters et al., 1986). The alkalinity of these proteins accounts for the avidity of the granules for the acidic dye, eosin (Gleich et al., 1986). Additional cytoplasmic granules identified in eosinophils include (a) primary granules which are found in immature eosinophils and may be the precursor of the specific granules (Hardin et al., 1970), (b) small granules which are present in tissue eosinophils only and contain the enzymes arylsulfatase and acid phosphatase (Parmley et al., 1974) and (c) specific microgranules whose function and biochemical contents remain to be determined (Zucker-Franklin, 1980). Eosinophils also contain a plasma membrane-bound enzyme, lysophospholipase, which crystallizes to form the Charcot-Leyden

crystals characteristic of tissue eosinophilia (Weller et al., 1980).

Eosinophils possess a variety of cell membrane receptors including immunoglobulin (Capron et al., 1981), complement (Anwar et al., 1977), cytokine (Lopez et al., 1991) and adhesion molecule receptors (Hartnell et al., 1990). The cell membrane also serves as a source of lipids for de novo synthesis of potent inflammatory mediators such as leukotrienes C₄ and D₄, platelet activating factor, prostaglandin D₂ and thromboxane B₂ (Weller et al., 1983; Lee et al., 1984; Parsons et al., 1988). Following cell activation, these mediators, along with oxygen radicals (Kanofsky et al., 1988) and the preformed granule proteins, are released extracellularly (Weller, 1991). In addition, recent studies have demonstrated that eosinophils may produce a variety of cytokines including GM-CSF (Ohno et al., 1991), IL-3 (Kita et al., 1991) and IL-5 (Desreumaux et al., 1992).

Function

Eosinophils are an important component of the body's innate immune system. Traditionally, the eosinophil has been considered to be important in the defense against parasitic helminth infections (Butterworth, 1984). Following their migration to areas of infestation, eosinophils attach to the parasite and degranulate (Kephart et al., 1984). The release of their granule proteins may kill the parasite through a direct cytotoxic effect (Butterworth et al., 1979). In vitro studies have also suggested that eosinophils are phagocytic and microbicidal, although the

importance of these functions in vivo is uncertain (Zucker-Franklin, 1990). Based on findings that eosinophils can be induced to synthesize and express Class II proteins of the major histocompatibility complex (Lucey et al., 1989), eosinophils may also function as antigen presenting cells (Weller, 1991). Thus, eosinophils may be able to initiate antigen-specific lymphocyte responses at mucosal surfaces (Weller, 1991).

The common association between eosinophils and allergic disease suggests that eosinophils may also play a functional role in allergic asthma. The precise role of the eosinophil in asthma, however, has yet to be established. Eosinophils have been reported to be involved in the down regulation of the inflammatory response following immediate hypersensitivity reactions (Goetzi et al., 1975). An anti-inflammatory role for the eosinophil is supported by findings that eosinophil enzymes inactivate several mast cell-derived inflammatory mediators including histamine (Zieger et al., 1976) and the leukotrienes (Henderson et al., 1982). Moreover, prostaglandins released by eosinophils have been reported to inhibit mast cell histamine release (Hubscher, 1975). As discussed previously, however, eosinophils also have the capacity to release potent pro-inflammatory cytokines and preformed and newly generated mediators. The ability of the cytokines to increase inflammatory cell chemotaxis (Warringa et al., 1991), mediator release (Tai et al., 1990) and adhesion molecule expression (Lopez et al., 1986) and the capacity of the mediators to damage pulmonary epithelium (Frigas et al., 1980),

increase mucus production (Marom et al., 1982) and cause edema (Dahlen et al., 1981) has implicated the eosinophil as a pro-inflammatory cell in asthma.

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Overview of Study for Chapter 2

The studies reviewed in Chapter 1 implicate inflammatory cells, particularly eosinophils, in the pathogenesis of the airway hyperresponsiveness associated with asthma. The role of inflammatory cells in the development of *Ascaris*-induced airway hyperresponsiveness in dogs is not clear. The first study in this thesis, therefore, examined whether the development of allergen-induced airway hyperresponsiveness in dogs was associated with increases in the number and level of activation of airway eosinophils before and after allergen inhalation. We were particularly interested in examining the role of the eosinophil before allergen inhalation since several studies had suggested that the cellular composition of the airways before allergen inhalation may influence the development of post-allergen airway responses.

CHAPTER 2: ROLE OF AIRWAY EOSINOPHILS IN THE DEVELOPMENT OF ALLERGEN-INDUCED AIRWAY HYPERRESPONSIVENESS IN DOGS

Abstract

The role of the eosinophil in the development of allergen-induced airway hyperresponsiveness is uncertain. We examined whether the development of airway hyperresponsiveness in 17 dogs after inhalation of *Ascaris suum* allergen (10^{-6} - 10^{-2} w/v) was associated with increases in the number and level of activation of eosinophils before and after allergen inhalation. Airway responsiveness to inhaled acetylcholine was measured before and 24 hours after *Ascaris* inhalation. Eosinophil number and levels of eosinophil peroxidase (EPO), used as a marker of eosinophil activity, were assessed by bronchoalveolar lavage (BAL) performed one week before allergen inhalation and 15 minutes after the 24 hour acetylcholine challenge. Dogs which developed allergen-induced airway hyperresponsiveness (n=8) had a greater number of BAL eosinophils before allergen inhalation (mean (standard error of the mean, SEM): $4.6 (1.94) \times 10^4$ cells/ml) than dogs that did not become hyperresponsive (n=9) ($1.2 (0.81) \times 10^4$ cells/ml) ($p = 0.034$). Eosinophil peroxidase levels were also significantly higher before allergen inhalation in dogs

that developed allergen-induced airway hyperresponsiveness (0.044 (0.013) optical density/100 μ l BAL fluid) than in dogs that did not become hyperresponsive (0.020 (0.014) optical density/100 μ l BAL fluid) ($p = 0.034$). Allergen-induced airway hyperresponsiveness was not associated with increases in eosinophil number or BAL EPO levels 24 hours after allergen inhalation. These results suggest that the presence of airway eosinophils before allergen inhalation is necessary for the development of allergen-induced airway hyperresponsiveness.

Introduction

Airway hyperresponsiveness is a characteristic feature of patients with asthma (Hargreave et al., 1981). Airway hyperresponsiveness may develop in allergic asthmatics following inhalation of specific allergen (Cockcroft et al., 1977). The mechanism(s) underlying allergen-induced airway hyperresponsiveness has been the focus of numerous studies. Several studies have reported that the recruitment to and activation of inflammatory cells in the airways after allergen inhalation is associated with the development of airway hyperresponsiveness (Brusasco et al., 1990; Smith et al., 1992; Pin et al., 1992). While a variety of inflammatory cells have been implicated in the pathogenesis of airway hyperresponsiveness, current evidence suggests that eosinophils play a key role.

This is because eosinophils are found in increased numbers in the airways of asthmatic subjects after allergen challenge (De Monchy et al., 1985; Aalbers et al., 1993; Brusasco et al., 1990) and are capable of producing mediators which can induce airway hyperresponsiveness (Gundel et al., 1991). The precise role of the eosinophil and other inflammatory cells in the mechanism of allergen-induced airway hyperresponsiveness, however, has yet to be established.

In dogs, naturally sensitized to the roundworm *Ascaris suum*, inhalation of an extract of this allergen results in an increase in pulmonary resistance which reaches a maximum 3 to 10 minutes post-allergen and usually resolves within one hour (Gold et al., 1972). Approximately 50% of sensitized dogs will also develop airway hyperresponsiveness after inhalation of *Ascaris suum* (Stevens et al., 1989; Sasaki et al., 1987). The role of inflammatory cells in the development of *Ascaris*-induced airway hyperresponsiveness in dogs is not clear. Although increases in airway eosinophils (Dollery et al., 1987; Johnson et al., 1992) or eosinophils and neutrophils (Oostveen et al., 1986) have been found after *Ascaris suum* challenge in dogs, only one study (Dollery et al., 1987) has measured both airway cellular changes and airway responsiveness. This study reported that lung instillation of *Ascaris suum* caused an increase in airway hyperresponsiveness at 2 hours and an increase in BAL eosinophils at 24 hours after allergen.

The above studies have focussed on cellular events occurring in the airways after allergen challenge. However, the cellular composition and level of activation

of inflammatory cells in the blood or airways *before* allergen challenge may also influence post-allergen airway responses. This hypothesis is supported by results from animal (Gundel et al., 1992) and human (Venge et al., 1988; Aalbers et al., 1993) studies examining the late asthmatic response, an event associated with the development of airway hyperresponsiveness (Cockcroft et al., 1977). Gundel et al. (1992) reported that monkeys who develop a late-phase response after allergen inhalation had a significantly greater number of eosinophils in the airways prior to allergen challenge compared to monkeys with an isolated early response. Similarly, in asthmatic patients, high blood (Venge et al., 1988) or airway (Aalbers et al., 1993) eosinophil numbers and eosinophil-derived protein levels before allergen inhalation were associated with the development of an allergen-induced late asthmatic response. While these findings raise the possibility that increased numbers and/or activation of eosinophils in the airways or blood prior to allergen challenge may contribute to the development of a late response, the effect on airway hyperresponsiveness has not been examined.

The purpose of this study was to test the hypothesis that the presence of eosinophils are required in the airways prior to allergen inhalation in order to develop allergen-induced airway hyperresponsiveness. Therefore, we investigated whether differences in the number and level of activation of eosinophils *before* allergen inhalation existed between dogs that developed airway hyperresponsiveness and those that did not. We also sought to determine

whether the development of *Ascaris*-induced airway hyperresponsiveness in dogs was associated with post-allergen increases in the number and state of activation of eosinophils in the airways. Measurement of the eosinophil-derived granule protein, eosinophil peroxidase (EPO), in BAL fluid was used as a marker of eosinophil activation.

Methods

Study design

Seventeen random-source mongrel dogs (17 to 34 kg), demonstrating a positive skin test reaction to *Ascaris suum* extract, were studied on two randomly assigned occasions separated by six weeks. On each occasion, measurements of baseline pulmonary resistance and acetylcholine airway responsiveness were made 45 minutes before and 24 hours after dogs inhaled either *Ascaris suum* antigen or diluent. Pulmonary resistance was also measured immediately before and for 30 minutes after allergen or diluent challenge. A BAL was performed one week prior to each occasion and 15 minutes after the 24 hour acetylcholine challenge. The first lavage was performed one week before allergen or diluent challenge to avoid a marked lung neutrophilia which is caused by the BAL procedure.

Procedures

Dogs were anesthetized with intravenous pentobarbital sodium (30 mg/kg; Somnotol, MTC Pharmaceuticals, Mississauga, Canada). Additional anesthetic was administered as required during the experiment. An endotracheal tube (10 mm ID) was inserted and connected to a constant volume ventilator (Model 551, Harvard Apparatus, South Natick, MA) set at a tidal volume of 10 ml/kg and at a rate of 30 breaths/min. An esophageal balloon catheter was inflated as previously described (Lemen et al., 1974) and was placed in the esophagus at the point of most negative end expiratory pressure. The esophageal catheter and a port at the proximal end of the endotracheal tube were connected to a differential pressure transducer (Hewlett Packard 267, Palo Alto, CA).

Measurement of total pulmonary resistance

Transpulmonary pressure was measured as the differential pressure between the mouth and esophageal pressure. Flow was measured by use of a pneumotach (Fleisch no. 1, Instrumentation Associates, New York, NY), a differential pressure transducer (Hewlett Packard 270) and a pressure amplifier (Hewlett Packard 8805C). A continuous measurement of total pulmonary resistance was computed from the flow and transpulmonary pressure using a Respiratory Analyzer (Hewlett Packard 8816A), which utilizes the method described by Mead and Whittenberger (1953). The pulmonary resistance was corrected for

the tare resistance of the system. Pulmonary resistance, transpulmonary pressure and flow were recorded on a paper chart recorder (Hewlett Packard 7758A).

Measurement of airway responsiveness

Airway responsiveness was determined by obtaining a dose-response curve of pulmonary resistance plotted against doubling concentrations of acetylcholine (0.7 to 80.0 mg/ml; Sigma Chemicals, St. Louis, MO). After baseline pulmonary resistance was measured, the dogs inhaled normal saline, followed by the increasing concentrations of acetylcholine. The acetylcholine aerosol was generated from a Bennett Twinjet nebulizer (Bennett Respiration Products, Los Angeles, CA) and delivered via the endotracheal tube. The aerosol was administered as five inhalations, each of 3 seconds duration. The nebulized output was 0.196 ml/min with droplets of a mass median aerodynamic diameter (MMAD) of 2.5 μm (geometric standard deviation (SD), 2.3). Increasing concentrations of acetylcholine were administered at 5 minute intervals until an increase in pulmonary resistance of at least 5 cm $\text{H}_2\text{O}/\text{l}/\text{s}$ above the baseline value was obtained. The response was expressed as the concentration of acetylcholine causing an increase in pulmonary resistance of 5 cm $\text{H}_2\text{O}/\text{l}/\text{s}$ above the baseline measurement and was termed the acetylcholine provocative concentration. A decrease in this value represents an increase in airway responsiveness. Dogs which became hyperresponsive to acetylcholine after allergen inhalation (ie greater than a twofold

decrease in the acetylcholine provocative concentration from pre-allergen values and less than a twofold decrease after allergen diluent challenge) were termed responders. Dogs whose airway responsiveness changed by less than twofold after allergen and diluent challenges were termed non-responders.

Ascaris suum challenge

Ascaris suum challenge was performed according to a method previously described (Sasaki et al., 1987) with some modifications. Increasing concentrations of Ascaris suum (10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} , 10^{-2} w/v) were prepared by serial dilution, with saline, of a stock solution of Ascaris suum extract (10^{-1} w/v; Greer Laboratories, Lenoir, NC). After baseline pulmonary resistance was measured, the dogs inhaled the increasing concentrations of Ascaris suum until an increase in pulmonary resistance of at least 10 cm H₂O/l/s above baseline resistance was obtained or the highest concentration administered. Ascaris suum aerosol was generated by the same type of nebulizer used for the acetylcholine challenge and was delivered into the lungs via the endotracheal tube. Each concentration of Ascaris suum was administered for 50 inhalations, each inhalation of 3 seconds duration. A 10 minute interval separated the inhalation of each concentration. The diluent of the Ascaris suum extract, 0.4% phenol, was prepared and inhaled in the same manner as the allergen. Each dog inhaled the same concentrations of diluent and Ascaris suum.

Skin testing

Skin testing of dogs was performed on the day of the initial BAL. Thirty minutes after the lavage was completed, 0.1 ml of 0.9% saline and *Ascaris suum* extract (10^{-2} w/v) were injected intracutaneously at shaved, separate (4 cm apart) sites adjacent to the spine. Twenty minutes later, the area of the wheal was determined by planimetry. The skin test reaction was scored positive when the area of the wheal induced by *Ascaris suum* was greater than twice the area of the wheal induced by saline.

Bronchoalveolar lavage

A flexible fiberoptic bronchoscope (Model BF-B2, Olympus, Tokyo, Japan) with an outer diameter of 6 mm was positioned at the level of the third airway generation in the middle lobe of the right lung. Five 20 ml aliquots of phosphate buffered saline (PBS), warmed to 37°C, were injected into the lung via the bronchoscope. Immediately after each aliquot, the PBS was aspirated into a sterile trap using a suction pressure of 70-90 mm Hg. The fluid was then centrifuged at 200 g for 10 minutes at 4°C. The supernatant was decanted and stored at -70°C for measurement of EPO levels. The cell pellet was washed in PBS and a total cell count performed using a hemocytometer. The cells were then diluted with PBS to a concentration of 1×10^6 cell/ml. Cytocentrifuge slides were prepared and stained with either a modified Wright-Giemsa (Diff-Quik, Baxter, McGraw Park, IL)

for a differential cell count (400 cells counted) or toluidine blue (pH = 0.5) for a metachromatic cell count (10,000 cells counted). Cell counts were performed by one investigator blinded to the experimental day. Cells were classified as macrophages, neutrophils, eosinophils, lymphocytes and metachromatic cells using standard morphological criteria (Rebar et al., 1980). Total and differential cell counts were expressed as the number of cells per milliliter of fluid recovered.

Eosinophil peroxidase assay

Eosinophil peroxidase levels in BAL fluid were measured using a colorimetric assay as described by Strath et al. (1985). Briefly, 100 μ l of a substrate solution consisting of 0.1 mM o-phenylenediamine dihydrochloride (OPD, Sigma) in Tris buffer pH 8.0 (0.05 M Tris-HCl and 0.05 M Tris base) containing 0.1% Triton X-100 (Sigma) and 1 mM hydrogen peroxide (Fischer Scientific, Fair Lawn, NJ) was added to 100 μ l of unconcentrated BAL fluid in a microplate well and left at room temperature for 30 minutes. The reaction was stopped by the addition of 50 μ l of 4 M sulphuric acid. Absorbance at 492 nm was measured using a microplate reader (MR 600, Dynatech Instruments, Torrance, CA). All lavage samples were assayed in duplicate on one day with optical density readings corrected for a blank (PBS and substrate) sample. The intra-assay coefficient of variation was 10.0%. Results were expressed as the optical density per 100 μ l of BAL fluid (OD/100 μ l BALF).

Analysis

Analysis of acetylcholine provocative concentrations were performed using \log_{10} transformed data. These results were summarized as geometric means with percent SEM in parentheses. All other values were reported as arithmetic means with SEM in parentheses. The magnitude of the effect of *Ascaris suum* inhalation on acetylcholine airway responsiveness was determined as the pre-allergen - post-allergen differences in the log-transformed acetylcholine provocative concentrations. Effects of allergen and diluent challenge on acetylcholine provocative concentration and pulmonary resistance were analyzed using t tests for paired observations. Differences in pulmonary resistance between responders and non-responders were compared by t tests for unpaired observations. Baseline acetylcholine provocative concentrations and pulmonary resistance were compared between challenges and between responders and non-responders using two-way analysis of variance. Nonparametric tests were used to analyze the BAL cell counts and EPO levels since the data were not normally distributed. The effects of *Ascaris suum* and diluent challenges on BAL measures were evaluated by Wilcoxon matched-pairs signed-rank test and Kruskal-Wallis analysis of variance. Mann-Whitney U tests were used to compare BAL cell counts and EPO levels between responders and non-responders. The relationship between BAL cell counts or EPO levels and airway responsiveness was examined using Spearman's rank correlation. Differences were considered statistically significant

when $p < 0.05$ (two-tailed).

Results

Airway hyperresponsiveness developed in eight dogs (responders) 24 hours after *Ascaris suum* inhalation. Nine dogs did not develop airway hyperresponsive (non-responders). In the responders, the acetylcholine provocative concentration decreased from 7.47 (1.51) mg/ml before allergen to 2.09 (1.42) mg/ml after allergen ($p < 0.001$). In these dogs, diluent inhalation did not significantly change airway responsiveness. The acetylcholine provocative concentration was 5.54 (1.44) mg/ml before diluent inhalation and 6.20 (1.50) mg/ml after diluent ($p = 0.440$). The mean log difference in the acetylcholine provocative concentration pre-allergen - post-allergen was 0.55 (0.06) and pre-diluent - post-diluent was -0.05 (0.06) ($p < 0.001$) (Figure 2.1). In the non-responder dogs, neither allergen nor diluent inhalation had a significant effect on airway responsiveness. The acetylcholine provocative concentration was 3.65 (1.67) mg/ml before allergen and 3.27 (1.63) mg/ml after allergen ($p = 0.659$). The acetylcholine provocative concentration was 5.62 (1.65) mg/ml before diluent and 4.22 (1.70) mg/ml after diluent ($p = 0.175$). The mean log difference in the acetylcholine provocative concentration pre-allergen - post-allergen was 0.05 (0.11) and pre-diluent - post-

diluent was 0.12 (0.08) ($p = 0.503$) (Figure 2.1). There were no significant differences in baseline acetylcholine provocative concentrations between allergen and diluent challenges or between responders and non-responders.

All dogs had a positive skin test response to *Ascaris suum* injection indicating sensitivity to *Ascaris*. There was no significant difference in mean skin wheal area following allergen injection between responders and non-responders.

Ascaris suum inhalation significantly increased pulmonary resistance in both responders ($p = 0.001$) and non-responders ($p = 0.004$) compared to diluent inhalation (Figure 2.2). However, there was no significant difference in the magnitude of the allergen-induced increase in pulmonary resistance between responders and non-responders ($p = 0.826$). Baseline pulmonary resistance before acetylcholine inhalation and before diluent and allergen challenges was also not statistically different between responders and non-responders. In addition, there was no difference in the mean dose of inhaled *Ascaris suum* between responders and non-responders.

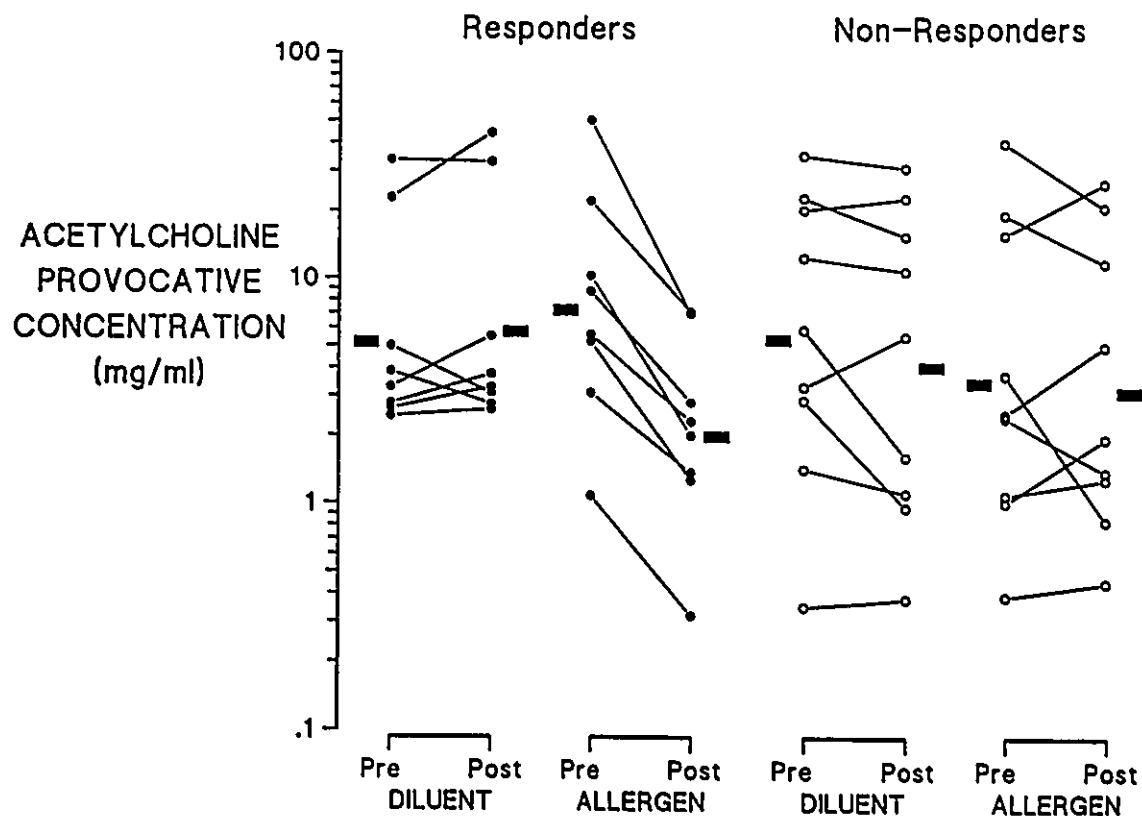


Figure 2.1. Acetylcholine airway responsiveness before and after diluent and allergen inhalation in dogs that developed airway hyperresponsiveness (responders) and those that did not (non-responders). In the responder dogs (closed circles), there was a significant reduction in acetylcholine provocative concentration after allergen inhalation ($p < 0.001$) but not after diluent challenge. In the non-responder dogs (open circles), no significant changes in acetylcholine provocative concentration occurred after allergen or diluent inhalation. Solid bars represent geometric means.

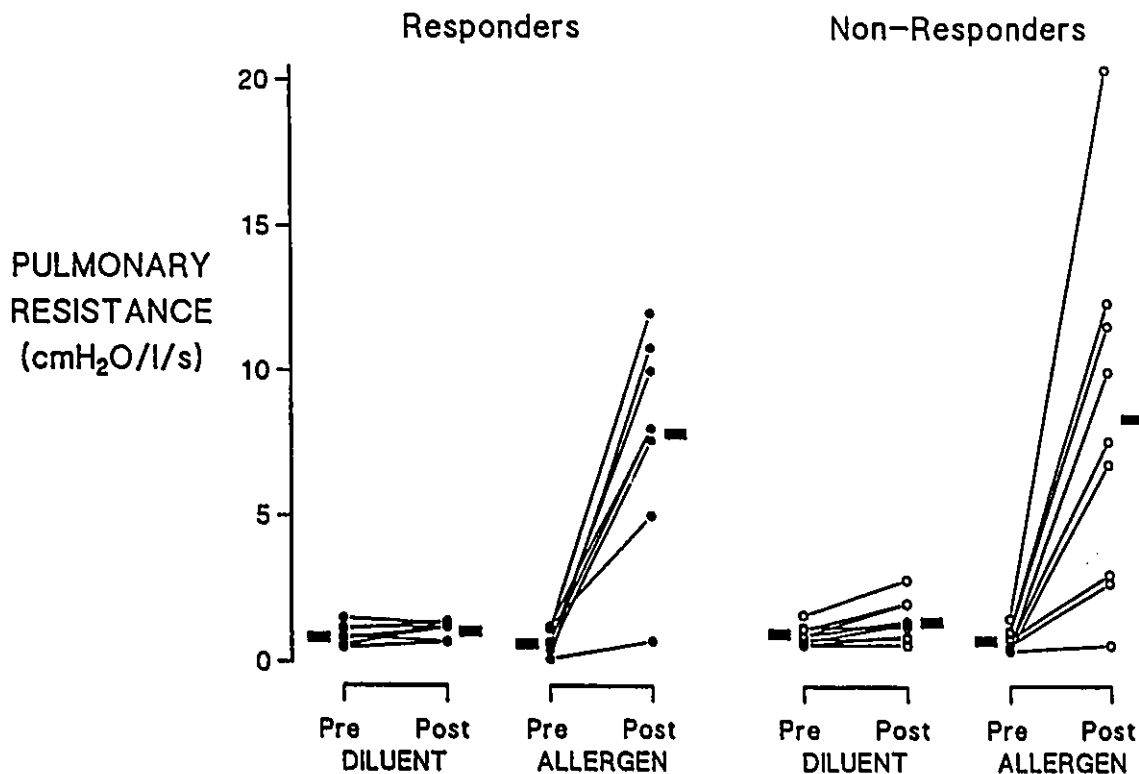


Figure 2.2. Pulmonary resistance before and after diluent and allergen inhalation in dogs that developed airway hyperresponsiveness (responders) and those that did not (non-responders). Allergen inhalation significantly increased pulmonary resistance in responders (closed circles) ($p = 0.001$) and non-responders (open circles) ($p = 0.004$) compared to diluent inhalation. There was no significant difference in the magnitude of the allergen-induced increase in pulmonary resistance between responders and non-responders. Solid bars represent mean values.

The number of eosinophils in BAL fluid obtained one week prior to allergen challenge was significantly higher in the responders, with a mean value of $4.6 (1.94) \times 10^4$ cells/ml, than in the non-responders, with a mean value of $1.2 (0.81) \times 10^4$ cells/ml ($p = 0.034$). No significant differences were found in the volume of BAL fluid recovered, total cell counts or the number of macrophages, neutrophils, lymphocytes and metachromatic cells between responders and non-responders one week prior to allergen inhalation (Table 2.1). Eosinophil peroxidase levels in the BAL were also significantly higher in responders before allergen inhalation, with a mean value of $0.044 (0.013)$ OD/100 μ l BALF, than in non-responders, with a mean value of $0.020 (0.014)$ OD/100 μ l BALF ($p = 0.034$). No significant correlations were observed between the pre- to post-allergen change in airway responsiveness and any pre-allergen cell counts or BAL EPO levels in the responder dogs.

In the responder dogs, allergen inhalation did not cause any significant change in BAL cell numbers or EPO levels compared to the diluent inhalation (Table 2.2). By contrast, in the non-responder dogs, allergen inhalation induced a significant increase in the number of BAL eosinophils. The mean change in eosinophil number from before allergen to after allergen was $5.7 (3.10) \times 10^4$ cells/ml, while the before to after diluent change in eosinophil number was $-2.1 (3.49) \times 10^4$ cells/ml ($p = 0.038$). There were no significant differences in the pre- to post-challenge number of neutrophils, lymphocytes and metachromatic cells

between allergen and diluent inhalations in the non-responder dogs (Table 2.2). Levels of BAL EPO were also significantly increased in non-responders dogs after allergen inhalation. The mean change in EPO levels from before allergen to after allergen was 0.029 (0.009) OD/100 μ l BALF while the mean change in EPO levels after diluent inhalation was 0.002 (0.006) OD/100 μ l BALF ($p = 0.008$) (Table 2.2). In both responders and non-responders, there were no significant differences between allergen and diluent lavages in the volume of BAL fluid recovered or total cell counts.

Table 2.1. Cell counts in BAL fluid of responder and non-responder dogs before allergen inhalation

Cell Type	Responder	Non-Responder
Macrophages	18.4 (4.50)	23.9 (3.81)
Neutrophils	1.9 (0.78)	0.8 (0.15)
Lymphocytes	1.3 (0.43)	0.9 (0.30)
Eosinophils	4.6 (1.94)*	1.2 (0.81)
Metachromatic cells	0.2 (0.05)	0.1 (0.03)
Total	26.4 (7.32)	26.9 (7.83)

Values are mean (SEM) number of cells x 10⁴ per ml of recovered bronchoalveolar lavage fluid. * significantly different from non-responders (p = 0.034)

Table 2.2. Changes in BAL cell counts and EPO levels in responder and non-responder dogs after diluent and allergen inhalation

Cell Type	Responder		Non-Responder	
	Diluent	Allergen	Diluent	Allergen
Macrophages	10.3 (4.5)	5.1 (5.40)	3.0 (6.26)	7.1 (3.87)
Neutrophils	8.3 (3.86)	3.1 (1.94)	4.8 (3.07)	8.6 (4.14)
Lymphocytes	1.2 (0.69)	0.4 (0.72)	1.1 (0.72)	1.2 (0.63)
Eosinophils	3.3 (2.25)	2.1 (1.28)	-2.1 (3.49)	5.7 (3.10)*
Metachromatic cells	0.1 (0.07)	0.0 (0.06)	0.0 (0.02)	0.0 (0.01)
Total	23.2 (10.56)	10.7 (7.47)	6.8 (16.65)	22.6 (10.61)
EPO (OD/100 μ l BALF)	0.008 (0.009)	-0.003 (0.011)	0.002 (0.006)	0.029 (0.009)*

Values are before to after challenge changes expressed as means with SEM in parentheses. Cell counts are number of cells x 10⁴ per ml of recovered bronchoalveolar lavage fluid. * significantly different from non-responder diluent change (p < 0.05).

Discussion

This study demonstrated that dogs which develop airway hyperresponsiveness after *Ascaris* inhalation have a greater number of and more activated airway eosinophils, as indicated by significantly increased BAL EPO levels, before allergen inhalation than dogs that do not develop airway hyperresponsiveness. However, *Ascaris*-induced airway hyperresponsiveness in dogs was not associated with an increase in the number of airway eosinophils nor increased eosinophil activation 24 hours after allergen inhalation. In contrast, increases in the number and activity of airway eosinophils after allergen inhalation were found in dogs which did not develop airway hyperresponsiveness. These findings suggest that the prior presence of airway eosinophils may be an important determinant of whether dogs develop allergen-induced airway hyperresponsiveness, but eosinophil influx and activation in the airways is not sufficient to cause allergen-induced airway hyperresponsiveness.

The criteria used to define airway hyperresponsiveness in this study was based on the less than twofold variability in acetylcholine responsiveness measures observed in our and other (Holtzman et al., 1983) laboratories when two measures are performed 24 hours apart. Greater than a twofold decrease in acetylcholine provocative concentration after allergen and less than a twofold decrease after diluent is outside the variability of the measurement and, therefore,

was thought to represent a true increase in airway responsiveness. Only one dog had more than a twofold decrease in acetylcholine provocative concentration after allergen and after diluent. This dog was classified as a non-responder.

In this study, measurement of EPO was used as an indicator of eosinophil activation. Selection of EPO was based on several factors (i) release of EPO is indicative of eosinophil degranulation (ii) technical expertise and equipment was available for the measurement of this mediator and (iii) although a variety of highly sensitive immunological assays are currently available for measuring other mediators specifically derived from human eosinophils, these cannot be used with dog eosinophils due to limited cross-reactivity of the antibodies to canine antigens.

Increased pre-allergen eosinophil numbers have been previously associated with the development of allergen-induced late asthmatic responses (Gundel et al., 1992; Venge et al., 1988; Aalbers et al., 1993). In addition, in asthmatic subjects, baseline airway eosinophil numbers and mediator levels have been reported to be positively correlated to the degree of airway responsiveness (Wardlaw et al., 1988) and the severity of asthma (Bousquet et al., 1990). Recent studies have also shown that chronic airway eosinophilia resulting from repeated allergen exposure in primates (Gundel et al., 1990) or neonatally sensitized dogs (Becker et al., 1989) leads to the development of airway hyperresponsiveness. These observations, taken together with our current findings, support the hypothesis that the presence of airway eosinophils, while not important for the initiation of acute

bronchoconstrictor responses to inhaled allergen, may be necessary for the development of more delayed allergen-induced responses, such as late responses or airway hyperresponsiveness. Whether these responses are mediated by the eosinophil alone or require the collaboration of other cells and/or cytokines or mediators remains to be determined.

The difference in the development of allergen-induced airway hyperresponsiveness between the groups was not due to differences in the level of sensitization of the dogs to *Ascaris suum*, as indicated by skin test responses, the dose of inhaled *Ascaris*, the magnitude of airway narrowing caused by *Ascaris* inhalation, pre-allergen acetylcholine airway responsiveness or baseline airway resistance before acetylcholine or allergen challenges.

An increase in airway eosinophils after *Ascaris suum* challenge in dogs has been reported previously (Johnson et al., 1992; Dollery et al., 1987), and this has been associated in one study with an increase in airway responsiveness (Dollery et al., 1987). Discrepancies between our findings and these investigators may relate to differences in the methods used to measure airway hyperresponsiveness or administer the allergen. Dollery et al (1987) measured airway responsiveness to intravenous histamine 2 hours after local lung instillation of *Ascaris suum*, and quantified airway eosinophils at 24 hours. In the present study, measurements of airway responsiveness to inhaled acetylcholine were made 24 hours after inhaled *Ascaris suum*, immediately before the BAL. We did demonstrate a significant

increase in BAL eosinophils at this time point, but only in the dogs who did not develop allergen-induced airway hyperresponsiveness at 24 hours.

Although our findings suggest that post-allergen increases in eosinophil number or levels of EPO may not be involved in *Ascaris*-induced airway hyperresponsiveness 24 hours after *Ascaris* inhalation in dogs, a role for the eosinophil cannot be entirely discounted for several reasons. First, we performed the BAL 24 hours after allergen inhalation. This time point was selected as earlier studies had reported increases in eosinophils 20 to 24 hours after allergen challenge in dogs (Oostveen et al., 1986; Dollery et al., 1987). Changes in eosinophil numbers and levels of EPO may have occurred earlier in the responder dogs and, therefore, not have been detected at 24 hours. Evidence for this is suggested from human studies where increases in BAL eosinophils (Rossi et al., 1991) and serum EPO (Durham et al., 1989) have been found 4-12 hours after allergen inhalation but not at 24 hours. Second, increases in eosinophils may have occurred in the tissues rather than the airway lumen of the responder dogs and, therefore, were not detected with bronchoalveolar lavage. Previous studies have documented the insensitivity of BAL to detect tissue cellular changes (Frew et al., 1990; Iijima et al., 1987). Third, eosinophil-derived mediators other than EPO may be responsible for the increase in airway hyperresponsiveness in responder dogs. For example, the eosinophil mediator, major basic protein, can cause airway hyperresponsiveness following its inhalation in vivo (Gundel et al., 1991) and has

been found in increased levels after allergen challenge in allergic rhinitic subjects (Sedgwick et al., 1991).

In conclusion, we have shown that the development of airway hyperresponsiveness is associated with a greater number of airway eosinophils and higher levels of BAL EPO. These findings suggest that the presence of airway eosinophils before allergen inhalation is necessary for the development of allergen-induced airway hyperresponsiveness. However, airway hyperresponsiveness measured 24 hours after *Ascaris suum* inhalation is not associated with post-allergen increases in eosinophil number or level of activation.

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Overview of Study for Chapter 3

The previous study indicated that the development of allergen-induced airway hyperresponsiveness in dogs was associated with increases in the number and level of activation of airway eosinophils before, but not after, allergen inhalation. In the second study, the role of pre-allergen airway eosinophils in the pathogenesis of allergen-induced airway hyperresponsiveness was further evaluated. This study examined whether reductions in airway eosinophil number and/or level of activation before allergen inhalation, as a result of budesonide treatment, would prevent allergen-induced airway hyperresponsiveness.

CHAPTER 3: EFFECT OF AN INHALED CORTICOSTEROID ON AIRWAY EOSINOPHILS AND ALLERGEN-INDUCED AIRWAY HYPERRESPONSIVENESS IN DOGS

Abstract

The presence of airway eosinophils before allergen inhalation may contribute to the development of allergen-induced airway responses. We examined whether a reduction in airway eosinophil numbers and/or level of activation before allergen inhalation as a result of inhalation of the corticosteroid, budesonide, would prevent allergen-induced airway hyperresponsiveness in 7 dogs. Acetylcholine airway responsiveness was measured before and 24 hours after inhalation of either *Ascaris suum* allergen (10^{-6} - 10^{-2} w/v) or its diluent on 4 test days separated by at least 4 weeks. Dogs were pretreated for 7 days before and on the morning of each test day with inhaled budesonide (mean daily dose 2.69 mg) or a placebo (lactose). Airway eosinophil number and levels of EPO, used as marker of eosinophil activation, were assessed by BAL. Inhaled budesonide significantly reduced the number of airway eosinophils before allergen inhalation (mean (SEM): $0.3 (0.21) \times 10^4$ cells/ml) compared to inhaled lactose (3.6

(2.38×10^4 cells/ml) ($p = 0.028$) but had no effect on pre-allergen BAL EPO levels ($p = 0.554$). The decrease in eosinophil number was associated with a significant reduction in allergen-induced airway hyperresponsiveness ($p = 0.005$). These findings suggest that the number of eosinophils present in the airways before allergen inhalation is an important determinant in the development of allergen-induced airway hyperresponsiveness.

Introduction

Eosinophils are implicated in the pathogenesis of the airway hyperresponsiveness associated with asthma (Brusasco et al., 1990). We have found that allergen-induced airway hyperresponsiveness in dogs is associated with increases in the number and activity of airway eosinophils before allergen inhalation (Woolley et al., submitted). The development of an allergen-induced late asthmatic response has also been associated with increased airway eosinophil numbers before allergen inhalation (Gundel et al., 1992; Venge et al., 1988; Aalbers et al., 1993). These findings suggest that the presence of airway eosinophils before allergen inhalation may contribute to the development of allergen-induced airway responses. On this basis, we speculated that a reduction in airway eosinophil number or activation before allergen inhalation may decrease

allergen-induced airway hyperresponsiveness.

One method of decreasing eosinophil number and activity in the airways is through corticosteroid inhalation. Decreases in BAL (Duddridge et al., 1993) and bronchial tissue (Jeffery et al., 1992; Djukanovic et al., 1992) eosinophil numbers have been demonstrated in asthmatic subjects following treatment with inhaled corticosteroids. Corticosteroid inhalation by asthmatic subjects has also been reported to reduce BAL levels of the eosinophil-derived protein, eosinophil cationic protein (Adelroth et al., 1990), and the frequency of free eosinophil granules in the bronchial mucosa (Jeffery et al., 1992) suggesting an inhibitory effect on eosinophil activity.

Few studies have examined the effect of a corticosteroid-induced decrease in eosinophil number and activity prior to allergen challenge on airway responses after allergen inhalation. Corticosteroid treatment has been associated with decreases in pre-allergen eosinophil numbers (Gundel et al., 1992) and eosinophil-derived mediators (Bisgaard et al., 1990; Gronborg et al., 1993) and inhibition of an allergen-induced late-phase response. The contribution of increased eosinophil numbers and mediators before allergen inhalation to the development of late responses in these studies is uncertain, however, since steroid treatment also prevented post-allergen increases in inflammatory cell numbers and mediator levels. To our knowledge, no studies have directly investigated the effects of decreasing airway eosinophil numbers and level of activation prior to allergen

inhalation on allergen-induced airway hyperresponsiveness. Our canine model enables the effect of decreasing pre-allergen eosinophils on allergen-induced airway hyperresponsiveness to be examined since post-allergen changes in airway inflammatory cell numbers have not been observed.

The purpose of this study, therefore, was to examine whether a reduction in airway eosinophil numbers and/or level of activation before allergen inhalation as a result of inhalation of a potent and topically active corticosteroid, budesonide, would prevent the development of allergen-induced airway hyperresponsiveness in dogs. Measurement of the eosinophil-derived granule protein, EPO, in BAL fluid was used as a marker of eosinophil activation.

Methods

Study design

Seven random-source mongrel dogs (21 to 36 kg), demonstrating a positive skin test reaction to *Ascaris suum* extract and a history of airway hyperresponsiveness after *Ascaris suum* inhalation, were studied under four conditions. For the first condition, dogs inhaled lactose followed by *Ascaris suum* diluent. For the second condition, dogs inhaled lactose followed by *Ascaris suum* allergen. For the third condition, dogs inhaled budesonide followed by *Ascaris*

suum diluent. For the fourth condition, dogs inhaled budesonide followed by *Ascaris suum* allergen. Initially, the first and second conditions, were completed in random order. Subsequently, the third and fourth conditions were completed in random order. Each condition was separated by at least a four week interval which served as a drug washout period and allowed the dogs to recover from the allergen challenge.

For each condition, dogs inhaled budesonide or lactose twice a day (morning and afternoon) for seven days. On the morning of the eighth day, dogs received a final budesonide or lactose inhalation and a venous blood sample was obtained to allow measurement of budesonide plasma levels. Dogs were then anesthetized and measurements of baseline pulmonary resistance and acetylcholine airway responsiveness were made. After a 45 minute wait to allow pulmonary resistance to return to baseline levels, dogs inhaled either *Ascaris suum* or diluent. Pulmonary resistance was then recorded for 30 minutes. Twenty four hours after allergen or diluent challenge, acetylcholine airway responsiveness was again measured and, 15 minutes later, a BAL was performed.

Airway eosinophil number could not be assessed by BAL immediately before allergen and allergen diluent inhalations due to a confounding lavage-induced neutrophilia which can persist for up to 2 days after BAL (Damiano et al., 1980). Therefore, the number of eosinophils observed in the BAL for the first condition was assumed to represent the baseline number of eosinophils in the

airways before allergen inhalation after lactose treatment. Similarly, eosinophil number in the BAL for the third condition was taken as a baseline measure of the number of eosinophils in BAL before allergen inhalation after budesonide treatment.

Procedures

Dogs were anesthetized with intravenous pentobarbital sodium (30 mg/kg; Somnotol, MTC Pharmaceuticals, Mississauga, Canada). Additional anesthetic was administered as required during the experiment. An endotracheal tube (10 mm ID) was inserted and connected to a constant volume ventilator (Model 551, Harvard Apparatus, South Natick, MA) set at a tidal volume of 10 ml/kg and at a rate of 30 breaths/min. An esophageal balloon catheter was inflated as previously described (Lemen et al., 1974) and was placed in the esophagus at the point of most negative end expiratory pressure. The esophageal catheter and a port at the proximal end of the endotracheal tube were connected to a differential pressure transducer (Hewlett Packard 267, Palo Alto, CA).

Measurement of total pulmonary resistance

Transpulmonary pressure was measured as the differential pressure between the mouth and esophageal pressure. Flow was measured by use of a pneumotach (Fleisch no. 1, Instrumentation Associates, New York, NY), a

differential pressure transducer (Hewlett Packard 270) and a pressure amplifier (Hewlett Packard 8805C). A continuous measurement of total pulmonary resistance was computed from the flow and transpulmonary pressure using a Respiratory Analyzer (Hewlett Packard 8816A), which utilizes the method described by Mead and Whittenberger (1953). The pulmonary resistance was corrected for the tare resistance of the system. Pulmonary resistance, transpulmonary pressure and flow were recorded on a paper chart recorder (Hewlett Packard 7758A).

Measurement of airway responsiveness

Airway responsiveness was determined by obtaining a dose-response curve of pulmonary resistance plotted against doubling concentrations of acetylcholine (0.7 to 80.0 mg/ml; Sigma Chemicals, St. Louis, MO). After baseline pulmonary resistance was measured, the dogs inhaled normal saline, followed by the increasing concentrations of acetylcholine. The acetylcholine aerosol was generated from a Bennett Twinjet nebulizer (Bennett Respiration Products, Los Angeles, CA) and delivered via the endotracheal tube. The aerosol was administered as five inhalations, each of 3 seconds duration. The nebulized output was 0.196 ml/min with droplets of a mass median aerodynamic diameter (MMAD) of 2.5 μm (geometric standard deviation (SD), 2.3). Increasing concentrations of acetylcholine were administered at 5 minute intervals until an increase in pulmonary resistance of at least 5 cm $\text{H}_2\text{O}/\text{l/s}$ above the baseline value was obtained. The

response was expressed as the concentration of acetylcholine causing an increase in pulmonary resistance of 5 cm H₂O/l/s above the baseline measurement and was termed the acetylcholine provocative concentration. A decrease in this value represents an increase in airway responsiveness.

Budesonide inhalation

Micronized dry powder of either budesonide or lactose was compressed to a tablet in brass cups supplied by Astra Draco AB (Lund, Sweden). The cups were connected to a modified Wright Dust Feeder (WDF, Adams Ltd., London, England) fitted with a speed control motor (Motomatic II, Electro-Craft, South Eden Prairie, MN). Powder was scraped off the tablets by the WDF and aerosolized in a current of air (airflow 10 liters/min). The aerosol was directed to a plastic nose mask fitted tightly on spontaneously breathing, conscious dogs. The nose mask permitted nose or mouth breathing. The concentration of particles generated by the WDF was monitored by a light scattering instrument (AMS 950, Casella, Bedford, England) and the speed of the WDF adjusted if necessary to maintain a constant particle flow predetermined to deliver the dose over a five minute inhalation. Budesonide aerosol of a MMAD of 1.19 μm (geometric SD 2.6) and lactose aerosol of a MMAD of 2.3 μm (geometric SD 1.8) were generated. Particle size was measured with an aerodynamic particle sizer (APS 33B, TSI, St. Pauls, MN) in a vertical wind tunnel. The dose inhaled for each exposure was estimated

from the concentration of budesonide measured on a expiration filter. The amount of budesonide on the filter was analyzed by reversed phase high performance liquid chromatography at Astra Draco AB, Sweden.

A separate study was performed before the start of the main study to investigate the amount of budesonide deposited on inspiration and expiration filters during continuous exposure. We found that of the total dose administered to dogs with a body weight between 20 kg and 30 kg, approximately 46% was deposited on the inspiration filter and 54% was deposited on the expiration filter. To calculate the inhaled dose, the amount of budesonide found on the expiration filter was multiplied by a factor of 0.85 ($46/54 = 0.85$). The mean daily (two inhalations) amount of budesonide on the expiration filter during the study was 3.16 (standard error of the mean, SEM: 0.10) mg. Thus, the dogs inhaled a mean dose of $3.16 \times 0.85 = 2.69$ mg of budesonide per day except on the eighth day when half this dose was inhaled. This dose is equivalent to $100.0 \mu\text{g}/\text{kg}$ for the dog's average bodyweight of 26.9 kg. These values are an approximate of the inhaled dose since breathing patterns may vary between dogs.

Budesonide plasma level measurement

Twenty mls of heparinized blood was centrifuged at 750 g for 20 minutes at 4°C. The plasma was withdrawn and separated into aliquots of 3.5 mls which were stored at -70°C. Coded plasma samples were then shipped on dry ice to

Astra Draco AB, Sweden for measurement of budesonide levels. Budesonide levels in plasma were measured by mass spectrometry and results were expressed in nmol/l of blood (Lindberg et al., 1992).

Ascaris suum challenge

Ascaris suum challenge was performed according to a method previously described (Sasaki et al., 1987) with some modifications. Increasing concentrations of Ascaris suum (10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} , 10^{-2} w/v) were prepared by serial dilution, with saline, of a stock solution of Ascaris suum extract (10^{-1} w/v; Greer Laboratories, Lenoir, NC). After baseline pulmonary resistance was measured, each dog inhaled pre-determined concentrations of Ascaris suum which had been shown during a earlier study to cause airway hyperresponsiveness. Ascaris suum was generated by the same type of nebulizer used for the acetylcholine challenge and was delivered into the lungs via the endotracheal tube. Each concentration of Ascaris suum was administered for 50 inhalations, each inhalation of 3 seconds duration. A 10 minute interval separated the inhalation of each concentration. The diluent of the Ascaris suum extract, 0.4% phenol, was prepared and inhaled in the same manner as the allergen. For each dog, the same concentrations of Ascaris suum and diluent were inhaled for all conditions.

Skin testing

Skin testing of dogs was performed at least four weeks before the study. After dogs were anaesthetized, 0.1 ml of 0.9% saline and *Ascaris suum* extract (10² w/v) were injected intracutaneously at shaved, separate (4 cm apart) sites adjacent to the spine. Twenty minutes later, the area of the wheal was determined by planimetry. The skin test reaction was scored positive when the area of the wheal induced by *Ascaris suum* was greater than twice the area of the wheal induced by saline.

Bronchoalveolar lavage

A flexible fiberoptic bronchoscope (Model BF-B2, Olympus, Tokyo, Japan) with an outer diameter of 6 mm was positioned at the level of the third airway generation in the middle lobe of the right lung. Five 20 ml aliquots of phosphate buffered saline (PBS), warmed to 37°C, were injected into the lung via the bronchoscope. Immediately after each aliquot, the PBS was aspirated into a sterile trap using a suction pressure of 70-90 mm Hg. The fluid was then centrifuged at 200 g for 10 minutes at 4°C. The supernatant was decanted and stored at -70°C for measurement of EPO levels. The cell pellet was washed in PBS and a total cell count performed using a hemocytometer. The cells were then diluted with PBS to a concentration of 1 x 10⁶ cell/ml. Cytocentrifuge slides were prepared and stained with either a modified Wright-Giemsa (Diff-Quik, Baxter, McGraw Park, IL)

for a differential cell count (400 cells counted) or toluidine blue (pH = 0.5) for a metachromatic cell count (10,000 cells counted). Cell counts were performed by one investigator blinded to the experimental day. Cells were classified as macrophages, neutrophils, eosinophils, lymphocytes and metachromatic cells using standard morphological criteria (Rebar et al., 1980). Total and differential cell counts were expressed as the number of cells per milliliter of fluid recovered.

Eosinophil peroxidase assay

Eosinophil peroxidase levels in BAL fluid were measured using a colorimetric assay as described by Strath et al. (1985). Briefly, 100 μ l of a substrate solution consisting of 0.1 mM o-phenylenediamine dihydrochloride (OPD, Sigma) in Tris buffer pH 8.0 (0.05 M Tris-HCl and 0.05 M Tris base) containing 0.1% Triton X-100 (Sigma) and 1 mM hydrogen peroxide (Fischer Scientific, Fair Lawn, NJ) was added to 100 μ l of unconcentrated BAL fluid in a microplate well and left at room temperature for 30 minutes. The reaction was stopped by the addition of 50 μ l of 4 M sulphuric acid. Absorbance at 492 nm was measured using a microplate reader (MR 600, Dynatech Instruments, Torrance, CA). All lavage samples were assayed in triplicate on one day with optical density readings corrected for a blank (PBS and substrate) sample. The intra-assay coefficient of variation was 10.0%. Results were expressed as the optical density per 100 μ l of BAL fluid (OD/100 μ l BALF).

Analysis

Analysis of acetylcholine provocative concentrations were performed using \log_{10} transformed data. These results were summarized as geometric means with percent SEM in parentheses. All other values were reported as arithmetic means with SEM in parentheses. The magnitude of the effect of *Ascaris suum* inhalation on acetylcholine airway responsiveness was determined as the pre-allergen - post-allergen differences in the log-transformed acetylcholine provocative concentrations. Effects of treatment conditions on acetylcholine provocative concentration, baseline pulmonary resistance and the change in pulmonary resistance after allergen were analyzed using analysis of variance for repeated measures. Post hoc comparisons were performed when indicated using the Newman-Keuls test. Effects of treatments on BAL cell counts and EPO levels were evaluated by Friedman analysis of variance for repeated measures and Wilcoxon matched-pairs signed-rank test. Differences were considered statistically significant when $p < 0.05$ (two-tailed).

Results

Inhaled budesonide significantly reduced the number of pre-allergen eosinophils in BAL fluid compared to inhaled lactose ($p = 0.028$). After budesonide, the mean number of eosinophils before allergen diluent inhalation was $0.3 (0.21) \times 10^4$ cells/ml. After lactose, the mean number of eosinophils before allergen diluent inhalation was $3.6 (2.38) \times 10^4$ cells/ml. Inhaled budesonide did not significantly decrease the numbers of other cell types in the BAL before allergen inhalation, including metachromatic cells (Table 3.1). Also, budesonide did not cause any significant change in pre-allergen BAL EPO levels compared to lactose ($p = 0.554$) (Table 3.1).

Allergen inhalation caused a significant increase in the number of neutrophils in the BAL compared to allergen diluent inhalation after treatment with lactose ($p = 0.018$) or budesonide ($p = 0.043$) (Table 3.1). However, the magnitude of the allergen-induced BAL neutrophilia did not differ significantly between lactose and budesonide treatments ($p = 0.612$). There were no significant differences in the volume of BAL fluid recovered, total cell counts or the number of macrophages, lymphocytes and metachromatic cells between conditions (Table 3.1). Also, there was no significant difference in BAL EPO levels between allergen and allergen diluent inhalations after treatment with lactose ($p = 0.311$) (Table 3.1).

Table 3.1. Cell counts and EPO levels in BAL fluid obtained after allergen diluent and allergen inhalation following treatment with lactose or budesonide

Cell Type	Lactose + Diluent	Lactose + Allergen	Budesonide + Diluent	Budesonide + Allergen
Macrophages	24.3 (4.19)	24.6 (6.42)	29.0 (4.26)	23.7 (3.47)
Neutrophils	1.8 (0.69)	12.7 (4.59)*	0.5 (0.15)	16.3 (11.78) [†]
Lymphocytes	1.8 (0.67)	1.5 (0.26)	1.1 (0.32)	0.9 (0.26)
Eosinophils	3.6 (2.38)	5.8 (3.88)	0.3 (0.21) [‡]	0.7 (0.54)
Metachromatic cells	0.2 (0.03)	0.2 (0.06)	0.2 (0.03)	0.1 (0.02)
Total	31.7 (7.85)	44.8 (14.75)	31.1 (5.57)	41.7 (12.66)
Fluid returned (mls)	74.6 (1.48)	70.4 (3.81)	75.4 (1.97)	71.1 (3.63)
EPO (OD/100 μ l BALF)	0.065 (0.024)	0.097 (0.037)	0.043 (0.025)	0.036 (0.021)

Values are means with SEM in parentheses. Cell counts are number of cells $\times 10^4$ per ml of recovered bronchoalveolar lavage fluid. * significantly greater than lactose and allergen diluent neutrophil number ($p = 0.018$). [†] significantly greater than budesonide and allergen diluent neutrophil number ($p = 0.043$). [‡] significantly less than lactose and allergen diluent eosinophil number ($p = 0.028$).

Inhaled budesonide did not alter the baseline acetylcholine airway responsiveness; however, budesonide significantly, but not completely, attenuated the allergen-induced increase in airway responsiveness. After lactose, the mean acetylcholine provocative concentration fell from 5.06 (1.54) mg/ml before allergen to 0.86 (1.60) mg/ml after allergen ($p < 0.001$) (Figure 3.1). After budesonide, the mean acetylcholine provocative concentration was 3.61 (1.29) mg/ml before allergen and 1.55 (1.55) mg/ml after allergen ($p = 0.048$) (Figure 3.1). The mean log difference in the acetylcholine provocative concentration pre- to post-allergen was 0.77 (0.11) after lactose, which was significantly greater than the pre- to post-allergen value of 0.37 (0.15) after budesonide ($p = 0.005$). There was no significant difference in the baseline acetylcholine provocative concentrations between the four conditions.

Allergen inhalation significantly increased pulmonary resistance after treatment with either lactose ($p = 0.001$) or budesonide ($p = 0.002$) (Figure 3.2), but there was no difference in the magnitude of the increase between the treatments ($p = 0.297$). The baseline pulmonary resistance before acetylcholine inhalations and before diluent and allergen challenges was also not statistically different between conditions.

Mean budesonide plasma levels were 16.6 (1.91) nmol/l before allergen inhalation and 13.3 (1.44) nmol/l before diluent inhalation.

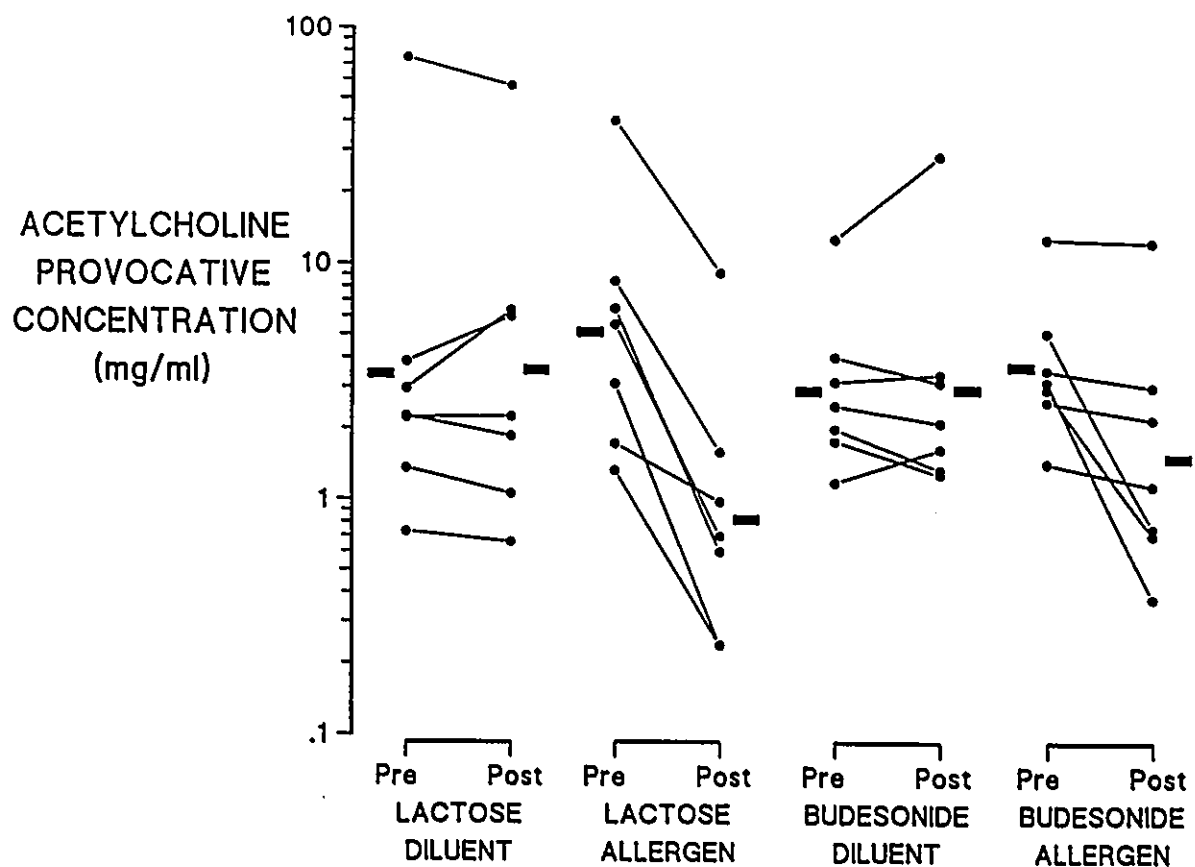


Figure 3.1. Acetylcholine airway responsiveness before and after allergen diluent and allergen inhalation following treatment with lactose or budesonide. After lactose, there was a significant reduction in acetylcholine provocative concentration after allergen inhalation ($p < 0.001$) but not after allergen diluent challenge. After budesonide, the allergen-induced decrease in acetylcholine provocative concentration was significantly, but not completely, attenuated ($p = 0.005$). Solid bars represent geometric means.

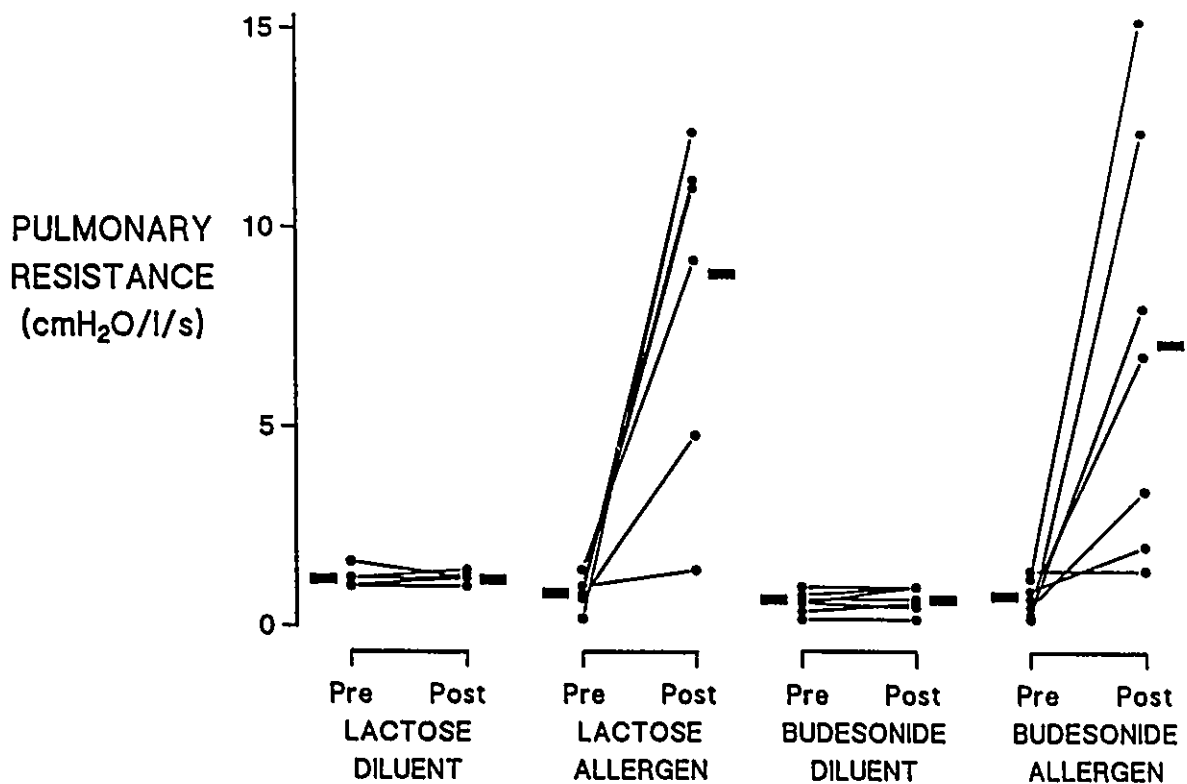


Figure 3.2. Pulmonary resistance before and after allergen diluent and allergen inhalation after treatment with lactose or budesonide. Allergen inhalation significantly increased pulmonary resistance after treatment with either lactose ($p = 0.001$) or budesonide ($p = 0.002$), but there was no difference in the magnitude of the increase between treatments ($p = 0.297$). Solid bars represent mean values

Discussion

This study has demonstrated that inhaled budesonide decreased the number of airway eosinophils, but no other cells, before allergen inhalation, and that this was associated with a significant reduction in allergen-induced airway hyperresponsiveness. This finding supports our earlier study (Woolley et al., submitted) that the presence of airway eosinophils before allergen inhalation contributes to the development of allergen-induced airway hyperresponsiveness in dogs.

Decreases in pre-allergen eosinophil numbers and eosinophil-derived mediators following corticosteroid treatment have previously been associated with the inhibition of an allergen-induced late-phase response (Gundel et al., 1992; Bisgaard et al., 1990; Gronborg et al., 1993), an event implicated in the development of airway hyperresponsiveness (Cockcroft et al., 1977). However, in these studies, steroid treatment also inhibited post-allergen increases in inflammatory cell numbers and mediator levels. Thus, the importance of pre-allergen eosinophils in the development of allergen-induced late responses in these studies is uncertain.

In our study, the decrease in airway hyperresponsiveness after budesonide treatment was not due to a reduction in the magnitude of the immediate bronchoconstrictor response to allergen, changes in baseline airway

responsiveness or baseline airway resistance or to a reduction of post-allergen increases in BAL cell numbers or EPO levels. Post-allergen increases in neutrophil number, however, were found after treatment with either lactose or budesonide. Increases in neutrophils after allergen inhalation have not been observed previously in our canine model, suggesting that the neutrophilia was due to factors other than allergen inhalation. Since the only method difference between this study and our previous study was powder inhalation, the neutrophilia may be a consequence of an irritant effect of inhaled powder on airway inflammatory or structural cells. Once irritated, these cells may prime neutrophils for an enhanced chemotactic response to allergen inhalation.

Although budesonide treatment decreased airway hyperresponsiveness and the number of eosinophils in the BAL before allergen inhalation, no change in eosinophil activation, as indicated by BAL EPO levels, was found. This suggests that the number of eosinophils present in the airways before allergen inhalation rather than their level of activation may be the primary determinant of whether dogs develop allergen-induced airway hyperresponsiveness. Assessment of eosinophil activation in our study, however, was based solely on measurements of EPO in BAL. Decreases in other markers of eosinophil activation following steroid treatment have been reported (Evans et al., 1993; Cox et al., 1991; Altman et al., 1981). The effect of budesonide treatment on pre-allergen eosinophil activation based on markers of cell function other than EPO remains to be

determined.

Studies in asthmatic subjects (Cockcroft et al., 1987; De Marzo et al., 1988) and animals (Abraham et al., 1986) have reported that corticosteroid inhalation reduces allergen-induced airway hyperresponsiveness. The beneficial effect of inhaled corticosteroids on airway hyperresponsiveness has been attributed to their anti-inflammatory effects in the airways (Cockcroft et al., 1987). Corticosteroids have been shown to prevent post-allergen increases in airway inflammatory cell numbers (Gundel et al., 1992; Abraham et al., 1988; Elwood et al., 1992) and the release of mediators (Bisgaard et al., 1990; Wong et al., 1992), events implicated in the pathogenesis of allergen-induced airway hyperresponsiveness (Brusasco et al., 1990). However, to our knowledge, our study is the first to examine the effects of prolonged corticosteroid inhalation on allergen-induced changes in both airway inflammation and airway hyperresponsiveness. The results of our study suggest that an additional mechanism by which inhaled corticosteroids may reduce allergen-induced airway hyperresponsiveness is by decreasing the number of eosinophils in the airways before allergen inhalation.

In our study, budesonide treatment significantly reduced, but did not completely prevent, the increase in airway responsiveness after allergen inhalation. The persistence of allergen-induced airway hyperresponsiveness after budesonide treatment may be due to eosinophils remaining in the airways after steroid treatment, albeit in greatly reduced numbers. Alternatively, we cannot exclude the

possibility that post-allergen changes in BAL cell numbers may have occurred earlier than 24 hours after allergen inhalation or that cell changes may have occurred in the tissues rather than the lumen. In addition, mediators other than EPO may have contributed to the development of airway hyperresponsiveness. The inability of budesonide to completely prevent airway hyperresponsiveness is unlikely to reflect an inadequate dosage since plasma levels of budesonide in our study were higher than levels reported in humans (Thorsson et al., 1993) following inhalation of doses known to inhibit allergen-induced airway hyperresponsiveness (De Baets et al., 1990; Abraham et al., 1986).

The corticosteroid treatment used in this study had no effect on the numbers of BAL metachromatic cells or the allergen-induced increase in pulmonary resistance. This was unexpected, since corticosteroids can significantly reduce the immediate bronchoconstrictor response after allergen inhalation in asthmatics (Burge et al., 1982; Dahl et al., 1982) and sensitized animals (Gundel et al., 1992). The failure of budesonide to affect the bronchoconstrictor response while reducing airway hyperresponsiveness suggests that the mechanism underlying the increase in pulmonary resistance after allergen differs from that involved in the development of airway hyperresponsiveness in dogs. In support of this, we have shown that dogs which do not develop allergen-induced airway hyperresponsiveness experience an increase in pulmonary resistance after allergen inhalation to the same magnitude as dogs that become hyperresponsive (Woolley

et al., submitted).

In conclusion, our findings demonstrate that budesonide treatment significantly reduced the number of eosinophils in BAL fluid before allergen inhalation but had no effect on pre-allergen BAL levels of EPO. The decrease in eosinophil number was associated with a significant, but not complete, reduction in allergen-induced airway hyperresponsiveness. These findings suggest that the number of eosinophils present in the airways before allergen inhalation is an important determinant in the development of allergen-induced airway hyperresponsiveness in dogs.

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Overview of Study of Chapter 4

In the previous studies, mature inflammatory cells were implicated in the development of allergen-induced airway hyperresponsiveness. The third study of this thesis examined whether allergen inhalation and the development of airway hyperresponsiveness was associated with changes in immature (progenitor) inflammatory cells. As technical difficulties precluded the examination of eosinophil progenitors, granulocyte-macrophage inflammatory cell progenitors were investigated. In addition, the effect of budesonide treatment on allergen-induced changes in progenitors was examined.

CHAPTER 4: ALLERGEN-INDUCED CHANGES IN BONE MARROW PROGENITORS AND AIRWAY RESPONSIVENESS IN DOGS AND THE EFFECTS OF INHALED BUDESONIDE ON THESE PARAMETERS

Abstract

Although the number of circulating inflammatory cell progenitors in asthmatic subjects is increased after allergen inhalation, no direct evidence exists for increased bone marrow progenitor production. This study examined the effect of allergen inhalation on bone marrow progenitor production in 7 dogs that develop allergen-induced airway hyperresponsiveness. The effect of inhaled budesonide on allergen-induced bone marrow progenitor production and airway responses was also investigated. Acetylcholine airway responsiveness was measured before and 24 hours after inhalation of either *Ascaris suum* allergen (10^{-6} - 10^{-2} w/v) or its diluent on 4 test days separated by at least 4 weeks. Dogs were pretreated for 7 days before and on the morning of each test day with inhaled budesonide (mean daily dose 2.69 mg) or a placebo (lactose). Bone marrow aspirates were obtained 24 hours after allergen or diluent challenge. Granulocyte-macrophage colony-forming units (GM-CFU), cultured in methylcellulose, were

counted on day 8. Allergen inhalation significantly increased airway responsiveness ($p < 0.001$) and the number of GM-CFU when cultured with dog serum and either SCF ($p < 0.001$) or G-CSF ($p = 0.035$). Budesonide treatment significantly reduced the allergen-induced increases in airway responsiveness ($p = 0.005$) and abolished the allergen-induced increases in the numbers of GM-CFU cultured with dog serum and either SCF ($p < 0.001$) or G-CSF ($p = 0.009$). These findings provide direct evidence that allergen inhalation increases bone marrow progenitor production in association with the development of airway hyperresponsiveness. Furthermore, inhaled corticosteroids can attenuate allergen-induced bone marrow progenitor production and airway hyperresponsiveness.

Introduction

Airway inflammation is an important feature of asthma (Dunnill, 1960; Dunnill et al., 1969) and is thought to contribute to the development of allergen-induced airway hyperresponsiveness (Brusasco et al., 1990; Smith et al., 1992), as well as persistent asthma (Bousquet et al., 1990; Wardlaw et al., 1988). Airway inflammation in asthma is characterized by the presence of several cell types including mast cells, lymphocytes and, in particular, eosinophils (Azzawi et al.,

1990; Kirby et al., 1987; Wardlaw et al., 1988).

Recently, increased interest has been directed towards the role that inflammatory cell progenitors may have in contributing to the airway inflammation present in asthma (Denburg et al., 1989). This interest has been stimulated by findings that circulating progenitors for eosinophils-basophils are present in increased numbers in allergic individuals (Gibson et al., 1991; Denburg et al., 1985a), fluctuate in relation to exacerbation and resolution of clinical asthma (Gibson et al., 1990) and are increased after allergen inhalation in association with the development of airway hyperresponsiveness (Gibson et al., 1991). The increased production of inflammatory cell progenitors could lead to the enhanced accumulation of inflammatory cells in the airways of asthmatic subjects (Gibson et al., 1991).

Based on changes in circulating progenitors, the above studies suggest that bone marrow progenitor production is increased in clinical asthma or after allergen inhalation. However, no direct evidence exists for increased bone marrow progenitor production. The purpose of this study, therefore, was to examine the effect of allergen inhalation on bone marrow progenitor production in dogs that develop allergen-induced airway hyperresponsiveness. We hypothesized that progenitor production would be increased after allergen inhalation. To further examine allergen-induced bone marrow progenitor production and to explore the mechanism of action of a drug known to be effective in the treatment of asthma,

we also investigated the effect of inhalation of a potent and topically active corticosteroid, budesonide, on allergen-induced bone marrow progenitor production and airway responses.

Methods

Study design

Seven random-source mongrel dogs (21 to 36 kg), demonstrating a positive skin test reaction to *Ascaris suum* extract and with previously demonstrated airway hyperresponsiveness after *Ascaris suum* inhalation, were studied under four conditions. For the first condition, dogs inhaled lactose followed by *Ascaris suum* diluent. For the second condition, dogs inhaled lactose followed by *Ascaris suum* allergen. For the third condition, dogs inhaled budesonide followed by *Ascaris suum* diluent. For the fourth condition, dogs inhaled budesonide followed by *Ascaris suum* allergen. Initially, the first and second conditions, were completed in random order. Subsequently, the third and fourth conditions were completed in random order. Each condition was separated by at least a four week interval which served as a drug washout period and allowed the dogs to recover from the allergen challenge.

For each condition, dogs inhaled budesonide or lactose twice a day (morning and afternoon) for seven days. On the morning of the eighth day, dogs received a final budesonide or lactose inhalation and a venous blood sample was obtained. Dogs were then anesthetized and measurements of baseline pulmonary resistance and acetylcholine airway responsiveness were made. After a 45 minute wait to allow pulmonary resistance to return to baseline levels, dogs inhaled either *Ascaris suum* or diluent. Pulmonary resistance was then recorded for 30 minutes. Twenty four hours after allergen or diluent challenge, a second venous blood sample and a bone marrow aspirate were obtained and acetylcholine airway responsiveness measured.

Procedures

Dogs were anesthetized with intravenous pentobarbital sodium (30 mg/kg; Somnotol, MTC Pharmaceuticals, Mississauga, Canada). Additional anesthetic was administered as required during the experiment. An endotracheal tube (10 mm ID) was inserted and connected to a constant volume ventilator (Model 551, Harvard Apparatus, South Natick, MA) set at a tidal volume of 10 ml/kg and at a rate of 30 breaths/min. An esophageal balloon catheter was inflated as previously described (Lemen et al., 1974) and was placed in the esophagus at the point of most negative end expiratory pressure. The esophageal catheter and a port at the proximal end of the endotracheal tube were connected to a differential pressure

transducer (Hewlett Packard 267, Palo Alto, CA).

Measurement of total pulmonary resistance

Transpulmonary pressure was measured as the differential pressure between the mouth and esophageal pressure. Flow was measured by use of a pneumotach (Fleisch no. 1, Instrumentation Associates, New York, NY), a differential pressure transducer (Hewlett Packard 270) and a pressure amplifier (Hewlett Packard 8805C). A continuous measurement of total pulmonary resistance was computed from the flow and transpulmonary pressure using a Respiratory Analyzer (Hewlett Packard 8816A), which utilizes the method described by Mead and Whittenberger (1953). The pulmonary resistance was corrected for the tare resistance of the system. Pulmonary resistance, transpulmonary pressure and flow were recorded on a paper chart recorder (Hewlett Packard 7758A).

Measurement of airway responsiveness

Airway responsiveness was determined by obtaining a dose-response curve of pulmonary resistance plotted against doubling concentrations of acetylcholine (0.7 to 80.0 mg/ml; Sigma Chemicals, St. Louis, MO). After baseline pulmonary resistance was measured, the dogs inhaled normal saline, followed by the increasing concentrations of acetylcholine. The acetylcholine aerosol was generated from a Bennett Twinjet nebulizer (Bennett Respiration Products, Los

Angeles, CA) and delivered via the endotracheal tube. The aerosol was administered as five inhalations, each of 3 seconds duration. The nebulized output was 0.196 ml/min with droplets of a mass median aerodynamic diameter (MMAD) of 2.5 μm (geometric standard deviation (SD), 2.3). Increasing concentrations of acetylcholine were administered at 5 minute intervals until an increase in pulmonary resistance of at least 5 cm $\text{H}_2\text{O}/\text{l/s}$ above the baseline value was obtained. The response was expressed as the concentration of acetylcholine causing an increase in pulmonary resistance of 5 cm $\text{H}_2\text{O}/\text{l/s}$ above the baseline measurement and was termed the acetylcholine provocative concentration. A decrease in this value represents an increase in airway responsiveness.

Budesonide inhalation

Micronized dry powder of either budesonide or lactose was compressed to a tablet in brass cups supplied by Astra Draco AB (Lund, Sweden). The cups were connected to a modified Wright Dust Feeder (WDF, Adams Ltd., London, England) fitted with a speed control motor (Motomatic II, Electro-Craft, South Eden Prairie, MN). Powder was scraped off the tablets by the WDF and aerosolized in a current of air (airflow 10 litres/min). The aerosol was directed to a plastic nose mask fitted tightly on spontaneously breathing, conscious dogs. The nose mask permitted nose or mouth breathing. The concentration of particles generated by the WDF was monitored by a light scattering instrument (AMS 950, Casella,

Bedford, England) and the speed of the WDF adjusted if necessary to maintain a constant particle flow predetermined to deliver the dose over a five minute inhalation. Budesonide aerosol of a MMAD of 1.19 μm (geometric SD 2.6) and lactose aerosol of a MMAD of 2.3 μm (geometric SD 1.8) were generated. Particle size was measured with an aerodynamic particle sizer (APS 33B, TSI, St. Pauls, MN) in a vertical wind tunnel. The dose inhaled for each exposure was estimated from the concentration of budesonide measured on a expiration filter. The amount of budesonide on the filter was analyzed by reversed phase high performance liquid chromatography at Astra Draco AB, Sweden.

A separate study was performed prior to starting the main study to investigate the amount of budesonide deposited on inspiration and expiration filters during continuous exposure. We found that of the total dose administered to dogs with a body weight between 20 kg and 30 kg, approximately 46% was deposited on the inspiration filter and 54% was deposited on the expiration filter. To calculate the inhaled dose, the amount of budesonide found on the expiration filter was multiplied by a factor of 0.85 ($46/54 = 0.85$). The mean daily (two inhalations) amount of budesonide on the expiration filter during the study was 3.16 (standard error of the mean (SEM): 0.10) mg. Thus, the dogs inhaled $3.16 \times 0.85 = 2.69$ mg of budesonide per day except on the eighth day when half this dose was inhaled. This dose is equivalent to 100.0 $\mu\text{g}/\text{kg}$ for the dog's average bodyweight of 26.9 kg. These values are an approximate of the inhaled dose

since breathing patterns may vary between dogs.

Budesonide plasma level measurement

Twenty mls of heparinized blood was centrifuged at 750 g for 20 minutes at 4°C. The plasma was withdrawn and separated into aliquots of 3.5 mls which were stored at -70°C. Coded plasma samples were then shipped on dry ice to Astra Draco AB, Sweden for measurement of budesonide levels. Budesonide levels in plasma were measured by mass spectrometry and results were expressed in nmol/l of blood (Lindberg et al., 1992).

Ascaris suum challenge

Ascaris suum challenge was performed according to a method previously described (Sasaki et al., 1987) with some modifications. Increasing concentrations of Ascaris suum (10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} , 10^{-2} w/v) were prepared by serial dilution, with saline, of a stock solution of Ascaris suum extract (10^{-1} w/v; Greer Laboratories, Lenoir, NC). After baseline pulmonary resistance was measured, each dog inhaled pre-determined concentrations of Ascaris suum which had been shown during a earlier study to cause airway hyperresponsiveness. Ascaris suum was generated by the same type of nebulizer used for the acetylcholine challenge and was delivered into the lungs via the endotracheal tube. Each concentration of Ascaris suum was administered for 50 inhalations, each inhalation of 3 seconds duration.

A 10 minute interval separated the inhalation of each concentration. The diluent of the *Ascaris suum* extract, 0.4% phenol, was prepared and inhaled in the same manner as the allergen. For each dog, the same concentrations of *Ascaris suum* and diluent were inhaled for all conditions.

Skin testing

Skin testing of dogs was performed at least four weeks before the study. After dogs were anaesthetized, 0.1 ml of 0.9% saline and *Ascaris suum* extract (10² w/v) were injected intracutaneously at shaved, separate (4 cm apart) sites adjacent to the spine. Twenty minutes later, the area of the wheal was determined by planimetry. The skin test reaction was scored positive when the area of the wheal induced by *Ascaris suum* was greater than twice the area of the wheal induced by saline.

Bone marrow aspiration

Dogs were sedated with intramuscular meperidine hydrochloride (3 mg/kg; Demerol, Abbott Laboratories, Toronto, Canada) and acepromazine maleate (0.05 mg/kg; Atravet, Ayerst Laboratories, Montreal, Canada). Twenty minutes later, a venous blood sample was obtained for blood cell counts and to provide serum for progenitor cultures. Dogs were then anesthetized with intravenous thiopental sodium (11 mg/kg; Pentothal, Abbott Laboratories, Montreal, Canada). Bone

marrow (3-4 ml) was then aspirated aseptically from the iliac crest using a 16 gauge Rosenthal needle and a 10 ml syringe containing 1 ml of heparin (1000 units/ml).

Cell counts and progenitor cultures

Total white blood cell counts were obtained using a Neubauer hemocytometer. Differential counts (400 cells counted) were obtained from two blood smears stained with Diff Quik (Baxter, McGraw Park, IL). Blood cell counts were expressed as the number of cells per liter of blood. Methylcellulose cultures of low-density nonadherent bone marrow cells were performed as previously described (Denburg et al., 1983; Gibson et al., 1990) with some modifications. Briefly, heparinized bone marrow was diluted to 50 mls with McCoy's 5A medium and separated by density gradient centrifugation over 65% Percoll (Pharmacia, Uppsala, Sweden). Cells at the interface were washed in McCoy's 5A medium and then incubated in McCoy's 5A medium supplemented with 15% fetal calf serum (FCS), 1% penicillin-streptomycin (Gibco, Grand Island, NY) and 5×10^{-5} M 2-mercaptoethanol (2-ME) (final concentration) for 2 hours in plastic flasks at 37°C and 5% CO₂. Non-adherent cells were then cultured (1×10^5 per 35 x 10 mm tissue culture dish, Falcon Plastics, Oxnard, CA), in duplicate, in supplemented Iscove's modified Dulbecco's medium (with 1% penicillin-streptomycin and 2-ME), 0.9% methylcellulose and either 20% FCS alone or in combination with one of the

following growth factors: 10% of the dog's own serum (DS); DS and recombinant canine stem cell factor (rcSCF, 5 ng/ml; AMGEN, Thousand Oaks, CA); DS and rc granulocyte-macrophage colony-stimulating factor (rcGM-CSF, 1 μ g/ml; AMGEN) or DS and rc granulocyte colony-stimulating factor (rcG-CSF, 1 μ g/ml; AMGEN). Colonies of \geq 40 cells were counted after 8 days incubation at 37°C and 5% CO₂ and identified by their morphology under inverted microscopy as granulocyte-macrophage (GM) type colonies (Denburg et al., 1985a; Denburg et al., 1985b). The identity of the colonies was corroborated by Diff Quik staining of 5 to 10 colonies picked at random from each culture dish. Evidence as to the identity of the colonies was also obtained from measurements of histamine content in 5 to 10 randomly picked colonies using a radioenzymatic, single isotope assay sensitive to 0.2 ng/ml. Granulocyte-macrophage colonies, in contrast to eosinophil-basophil type colonies, rarely contain histamine (Denburg et al., 1983; Bousquet et al., 1990). All colonies tested were histamine negative suggesting that the colonies were of the GM type. Colony counts were expressed as GM-colony forming units (CFU) per 10⁵ non-adherent mononuclear cells (NAMC). All counts were performed by one investigator blinded to the experimental day.

Analysis

Analysis of marrow colony counts and acetylcholine provocative concentrations were performed using \log_{10} transformed data. These results were summarized as geometric means with percent SEM in parentheses. All other values were reported as arithmetic means with SEM in parentheses. The magnitude of the effect of *Ascaris suum* inhalation on acetylcholine airway responsiveness was determined as the pre-allergen - post-allergen differences in the log-transformed acetylcholine provocative concentrations. Effects of treatment conditions on marrow colony counts, acetylcholine provocative concentration, baseline pulmonary resistance and the change in pulmonary resistance after allergen were analyzed using analysis of variance for repeated measures. Post hoc comparisons were performed when indicated using the Newman-Keuls test. Given the a priori hypothesis that progenitors would be increased after allergen inhalation compared to diluent, a one tailed analysis was used for colony counts. Effects of treatments on blood cell counts were evaluated by Friedman analysis of variance for repeated measures and Wilcoxon matched-pairs signed-rank test. Differences were considered statistically significant when $p < 0.05$.

Results

Allergen inhalation significantly increased bone marrow progenitor production. After lactose treatment, the mean number of bone marrow GM-CFU stimulated by DS and rcSCF was 14.1 (1.33) after allergen inhalation compared to 3.3 (1.51) after diluent inhalation ($p < 0.001$) (Figure 4.1). The number of marrow GM-CFU stimulated by DS and rcG-CSF was also significantly higher after allergen inhalation, being 27.8 (1.26), compared to 12.5 (1.2) after diluent inhalation ($p = 0.035$) (Figure 4.1). There were no significant differences in the number of GM-CFU stimulated by the other growth conditions between allergen and diluent (Figure 4.1).

Inhaled budesonide prevented the allergen-induced increase in bone marrow GM-CFU. After budesonide, the post-allergen number of GM-CFU stimulated by DS and rcSCF was 3.2 (1.49), which was significantly less than the mean post-allergen counts after lactose ($p < 0.001$). Similarly, the post-allergen number of GM-CFU stimulated by DS and rcG-CSF, with a mean value of 10.4 (1.52) was significantly less than the mean post-allergen counts after lactose ($p = 0.009$) (Figure 4.1). Moreover, the number of GM-CFU stimulated by the two growth conditions after budesonide and allergen inhalation were not significantly different from the baseline colony counts after budesonide and allergen diluent inhalation (Figure 4.1). Mean budesonide plasma levels were 16.6 (1.91) nmol/l

before allergen inhalation and 13.3 (1.44) nmol/l before diluent inhalation.

The increases in bone marrow GM-CFU after lactose and allergen inhalation were accompanied by the development of allergen-induced airway hyperresponsiveness (Figure 4.2). After lactose, the mean acetylcholine provocative concentration fell from 5.06 (1.54) mg/ml before allergen to 0.86 (1.60) mg/ml after allergen ($p < 0.001$). Allergen diluent inhalation, however, did not significantly change airway responsiveness. The acetylcholine provocative concentration was 3.30 (1.75) mg/ml before diluent inhalation and 3.48 (1.75) mg/ml after diluent ($p = 0.73$). The mean log difference in the acetylcholine provocative concentration pre- to post-allergen after lactose was 0.77 (0.11) which was significantly greater than the pre- to post-allergen diluent value of -0.02 (0.06) ($p < 0.001$).

Inhaled budesonide did not alter the baseline acetylcholine airway responsiveness; however, budesonide significantly, but not completely, attenuated the allergen-induced increase in airway responsiveness (Figure 4.2). The mean log difference in the acetylcholine provocative concentration pre- to post-allergen was 0.37 (0.15) after budesonide which was significantly less than the pre- to post-allergen value of 0.77 (0.11) after lactose ($p = 0.005$). There were no significant differences in baseline acetylcholine provocative concentrations between the four conditions.

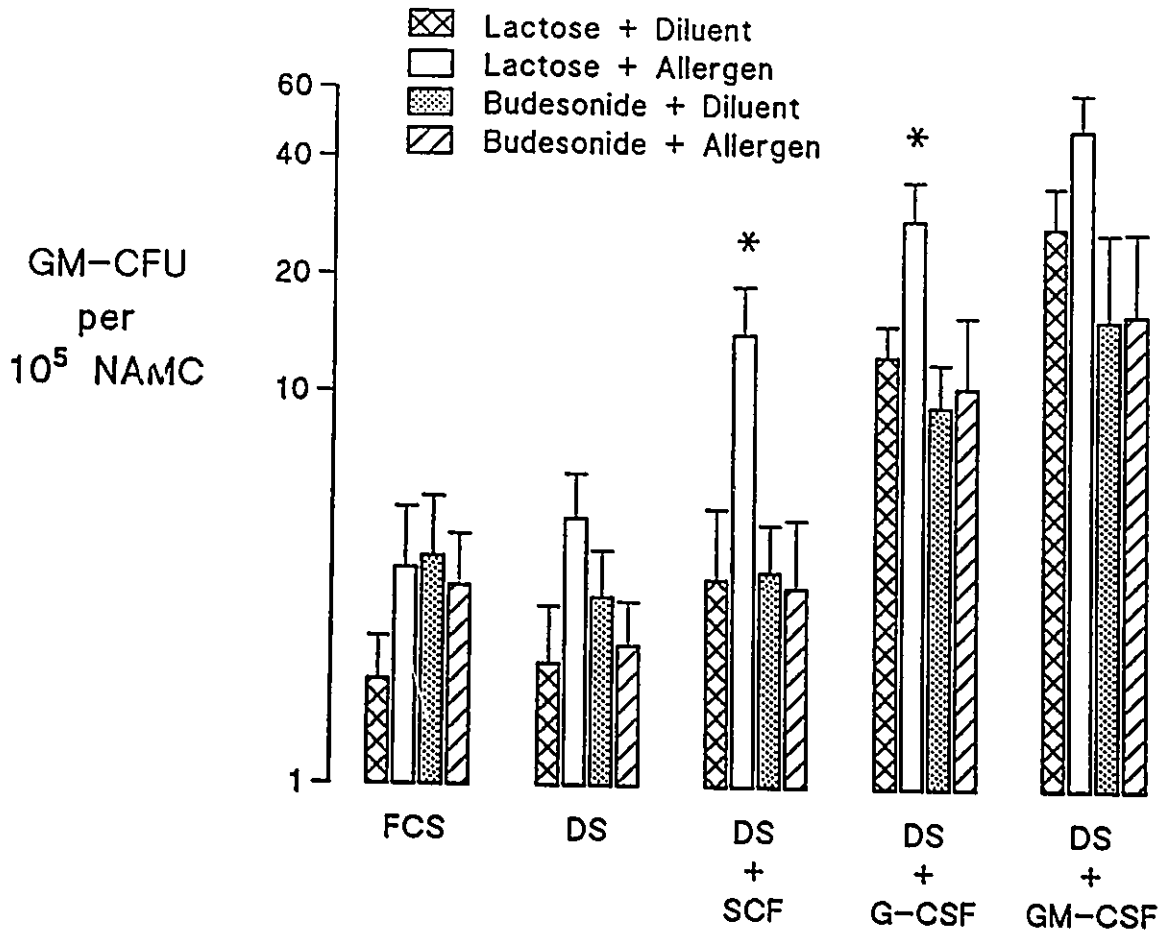


Figure 4.1. Bone marrow GM-CFU per 10^5 NAMC after allergen diluent and allergen inhalation following treatment with lactose or budesonide. Colonies were grown in the presence of fetal calf serum (FCS) alone or in combination with one of the following growth factors: 10% of the dog's own serum (DS); DS and recombinant canine stem cell factor (rcSCF); DS and rc granulocyte colony-stimulating factor (rcG-CSF) or DS and rc granulocyte-macrophage colony-stimulating factor (rcGM-CSF). After lactose, allergen inhalation significantly increased GM-CFU stimulated by DS and rcSCF ($p < 0.001$) or rcG-CSF ($p = 0.035$). Inhaled budesonide prevented the allergen-induced increase in GM-CFU stimulated by each growth condition.

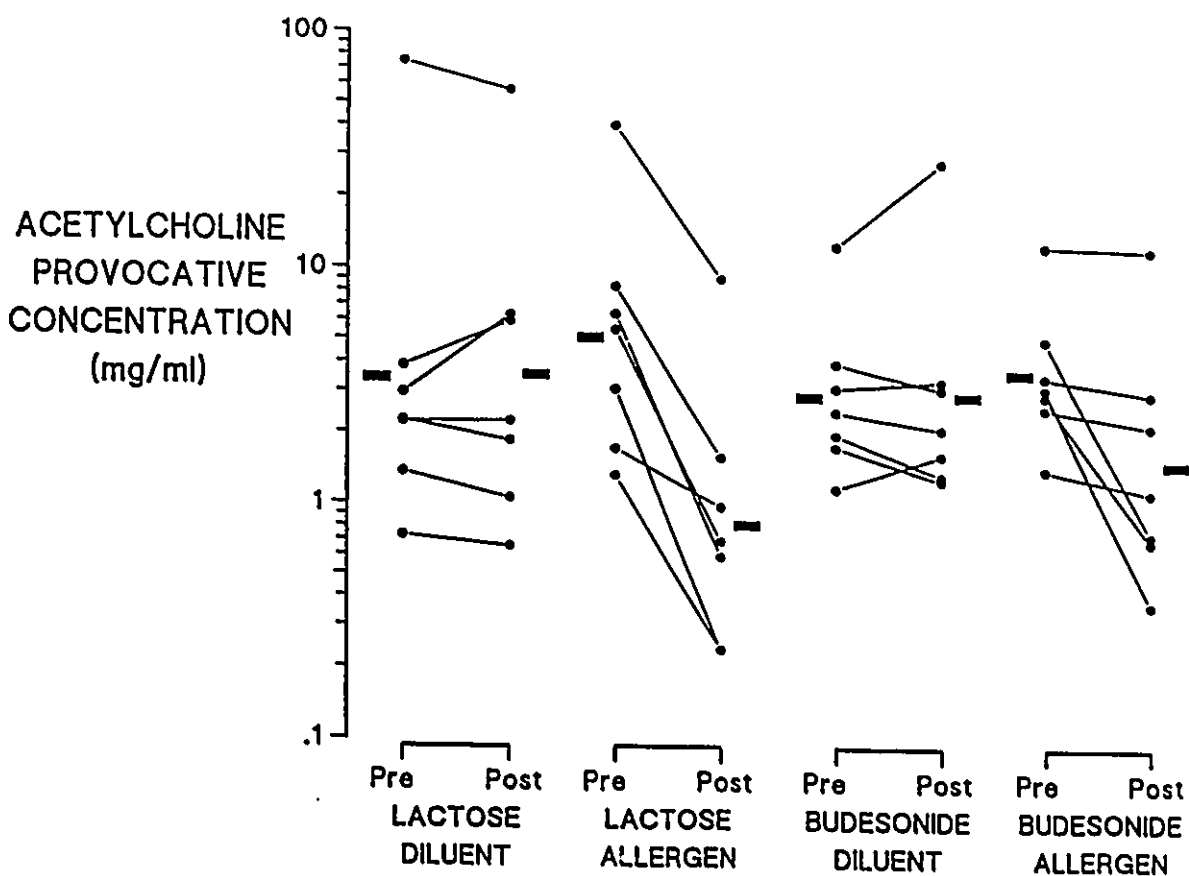


Figure 4.2. Acetylcholine airway responsiveness before and after allergen diluent and allergen inhalation following treatment with lactose or budesonide. After lactose, there was a significant reduction in acetylcholine provocative concentration after allergen inhalation ($p < 0.001$) but not after allergen diluent challenge. After budesonide, the allergen-induced decrease in acetylcholine provocative concentration was significantly, but not completely, attenuated ($p = 0.005$). Solid bars represent geometric means.

There were no significant differences in circulating cell counts between allergen and allergen diluent challenges either immediately after lactose treatment (Table 4.1) or 24 hours after challenge (Table 4.2). Inhaled budesonide significantly reduced the number of circulating eosinophils ($p = 0.018$) and tended to reduce the number of circulating lymphocytes ($p = 0.063$) compared to lactose inhalation (Table 4.1). There were no significant differences in total blood cell counts or the number of circulating neutrophils and monocytes between lactose and budesonide treatment (Table 4.1). In addition, there were no significant differences in blood cell counts between lactose and budesonide treatments 24 hours after allergen inhalation (Table 4.2).

Allergen inhalation significantly increased pulmonary resistance after treatment with either lactose ($p = 0.001$) or budesonide ($p = 0.002$) (Figure 4.3), but there was no difference in the magnitude of the increase in pulmonary resistance between treatments ($p = 0.297$). The baseline pulmonary resistance before acetylcholine inhalations and before diluent and allergen challenges was also not statistically different between conditions.

Table 4.1. Circulating cell counts obtained immediately after the final lactose or budesonide treatments

Cell Type	Lactose + Diluent	Lactose + Allergen	Budesonide + Diluent	Budesonide + Allergen
Monocytes	0.3 (0.14)	0.2 (0.05)	0.3 (0.05)	0.2 (0.04)
Neutrophils	8.3 (1.95)	6.9 (0.85)	10.3 (1.18)	6.9 (0.76)
Lymphocytes	2.7 (0.45)	2.7 (0.49)	2.8 (0.40)	1.8 (0.17) [†]
Eosinophils	1.8 (1.04)	0.9 (0.32)	0.6 (0.17)	0.1 (0.05) [*]
Total	13.1 (3.42)	10.7 (1.51)	14.0 (1.07)	9.1 (0.82)

Values are means with SEM in parentheses. Cell counts are number of cells x 10⁹ per liter of blood.

† tended to be less than lactose and allergen lymphocyte number ($p = 0.063$).

* significantly less than lactose and allergen eosinophil number ($p = 0.018$).

Table 4.2. Circulating cell counts obtained 24 hours after allergen diluent and allergen inhalation following treatment with inhaled lactose or budesonide

Cell Type	Lactose + Diluent	Lactose + Allergen	Budesonide + Diluent	Budesonide + Allergen
Monocytes	0.2 (0.07)	0.2 (0.5)	0.2 (0.06)	0.2 (0.04)
Neutrophils	5.9 (1.36)	7.4 (0.42)	7.0 (1.47)	8.2 (1.02)
Lymphocytes	3.2 (0.42)	3.3 (0.52)	2.6 (0.35)	2.8 (0.62)
Eosinophils	0.6 (0.14)	0.8 (0.16)	0.3 (0.13)	0.5 (0.18)
Total	9.9 (1.55)	11.7 (0.85)	10.1 (1.59)	11.7 (1.56)

Values are means with SEM in parentheses. Cell counts are number of cells x 10⁹ per liter of blood.

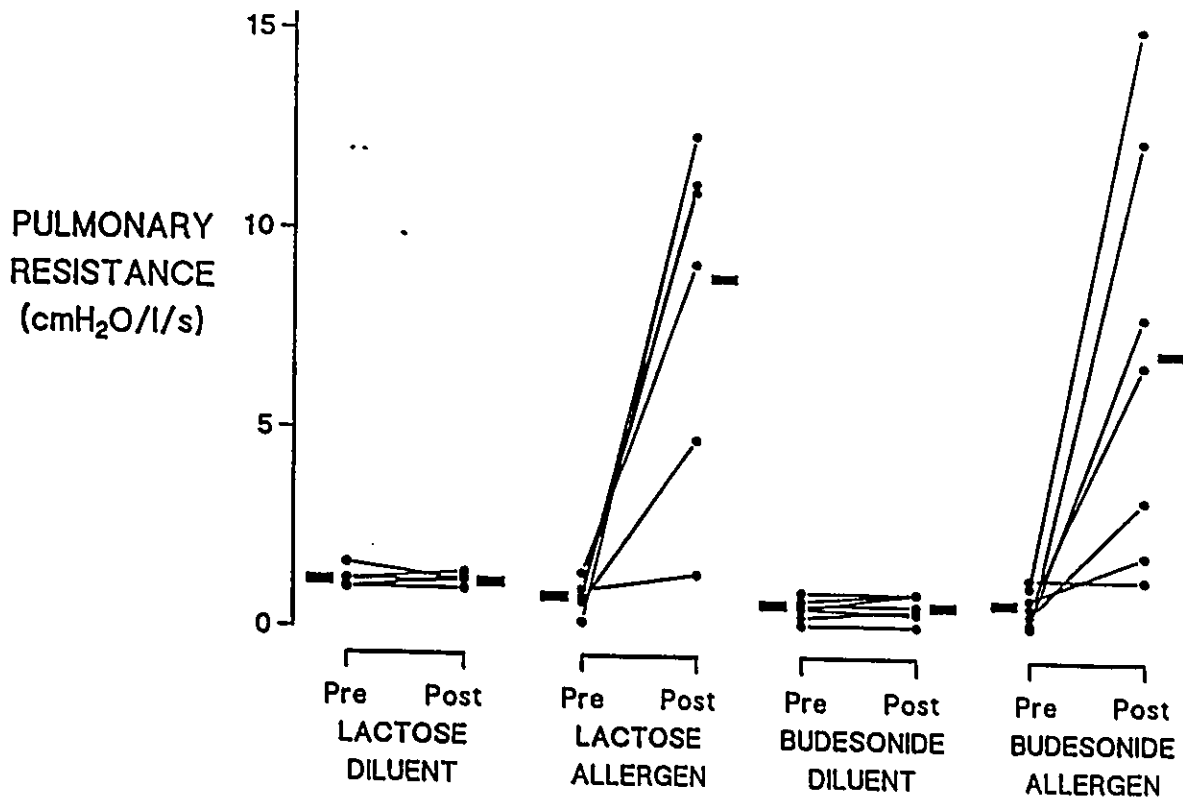


Figure 4.3. Pulmonary resistance before and after allergen diluent and allergen inhalation after treatment with lactose or budesonide. Allergen inhalation significantly increased pulmonary resistance after treatment with either lactose ($p = 0.001$) or budesonide ($p = 0.002$), but there was no difference in the magnitude of the increase between treatments ($p = 0.297$). Solid bars represent mean values.

Discussion

This study demonstrated that allergen inhalation significantly increased bone marrow granulocyte-macrophage progenitor production in dogs that developed allergen-induced airway hyperresponsiveness. Furthermore, the allergen-induced increases in progenitor production and airway hyperresponsiveness were significantly reduced by inhaled budesonide. These findings provide the first direct evidence that allergen inhalation can increase bone marrow progenitor production and suggest that such increases may contribute to the development of airway hyperresponsiveness in asthma.

Previous studies examining the role of inflammatory cell progenitors in asthmatic subjects have reported increases in the number of circulating progenitors after allergen inhalation (Gibson et al., 1991) and during asthma exacerbations (Gibson et al., 1990). These studies have focused on changes in the circulation, rather than the bone marrow, because of the ease of sampling and greater variability of bone marrow colony counts when sample volumes differ (Gordon et al., 1977). Our canine model, however, enabled us to directly examine bone marrow changes after allergen inhalation while potential problems due to differences in sample volumes were avoided since samples of 3 to 4 mls were consistently obtained.

The magnitude of the allergen-induced increase in progenitors in our study (approximately threefold) is similar to the increase in circulating progenitors observed in asthmatic subjects after allergen inhalation (Gibson et al., 1991). The increase in progenitors after allergen inhalation that we observed was unlikely to have occurred as a result of an allergen-induced decrease in the number of circulating cells as no changes in blood cell numbers were found 24 hours after allergen challenge. Given that peripheral blood neutropenia may stimulate bone marrow progenitor proliferation (Rickard et al., 1971), further study is needed to determine whether hemopoiesis was stimulated by an allergen-induced decrease in circulating cell numbers at some time before 24 hours. The effects of airway constriction per se was also unlikely to account for the increase in progenitors since acetylcholine inhalation induced bronchoconstriction on the allergen diluent day without a concomitant increase in progenitors.

Although several growth conditions were used in our study, only stimulation with DS and rcSCF or rcG-CSF significantly increased colony formation after allergen inhalation. Stem cell factor (also termed c-kit ligand, mast cell growth factor and steel factor) is a recently identified hemopoietic growth factor thought to be produced by a wide variety of tissues, including bone marrow stromal cells (Matsui et al., 1990). Studies using the Sl/Sl^d and W/W^v mast cell-deficient mouse models have shown that SCF plays a crucial role in mast cell growth and differentiation (Zsebo et al., 1990; Huang et al., 1990). In addition, SCF supports

the growth and differentiation of granulocyte-macrophage progenitors. By itself, SCF has little (Broxmeyer et al., 1991) or no (McNiece et al., 1991; Shull et al., 1992) stimulatory effect on GM-CFU formation in vitro, a finding supported by our results. However, when used in combination with other cytokines, such as IL-3 or GM-CSF, rcSCF has a potent synergistic effect in stimulating formation of GM-CFU in humans (Broxmeyer et al., 1991; Brandt et al., 1992) and dogs (Shull et al., 1992). Based on these findings, we postulate that the allergen-induced increase in colony formation stimulated by rcSCF in our study may result from a synergistic effect of SCF with hemopoietic cytokines released after allergen inhalation. Likely candidates for the released cytokines are GM-CSF and IL-3, hemopoietic growth factors which stimulate production of granulocyte-macrophage colonies (Burgess et al., 1977) and which are found in increased levels in the airways (GM-CSF) or nose (IL-3) of allergic patients after allergen inhalation (Broide et al., 1991; Durham et al., 1992). A similar explanation may explain our findings with rcG-CSF since a combination of G-CSF and GM-CSF or IL-3 also has a synergistic effect on bone marrow colony formation (McNiece et al., 1988).

Inhaled budesonide significantly reduced the allergen-induced increase in bone marrow progenitor production and airway hyperresponsiveness. This novel finding, together with previous reports that inhaled corticosteroids can significantly decrease circulating inflammatory cell progenitors in association with resolution of asthma exacerbations (Gibson et al., 1990), suggests that the effectiveness of

inhaled steroids in asthma may result, at least in part, from their ability to suppress the bone marrow production of cells which contribute to airway inflammation.

The mechanism of action of corticosteroids in preventing the allergen-induced increase in bone marrow colony formation remains uncertain. The mechanism may involve a direct systemic effect of the budesonide on bone marrow colony formation. Alternatively, Gibson and coworkers (1990) have hypothesised that the decreases in circulating progenitors in asthmatic subjects after steroid inhalation in their study resulted from a local effect of inhaled steroids in reducing airway-derived hemopoietic cytokines. Further study is needed to determine whether these hypotheses account for the inhibitory effect of budesonide on colony formation after allergen inhalation.

An unexpected finding in this study was that one week of corticosteroid inhalation had no effect on the allergen-induced increase in pulmonary resistance. This contrasts with earlier studies in humans (Burge et al., 1982; Dahl et al., 1982) which found that one week of corticosteroid inhalation, using doses similar to that employed in our study, significantly decreased the immediate bronchoconstrictor response after allergen inhalation. Since budesonide significantly reduced bone marrow colony formation after allergen inhalation, our findings suggest that the mechanism underlying the increase in pulmonary resistance after allergen differs from that involved in the allergen-induced increase in colony formation.

In summary, this study has demonstrated that allergen inhalation significantly increased the number of bone marrow granulocyte-macrophage progenitors in association with the development of airway hyperresponsiveness. Following inhalation of budesonide, the allergen-induced increase in progenitors and airway hyperresponsiveness were significantly attenuated. These findings suggest that increased bone marrow production of inflammatory cell progenitors after allergen inhalation may be important in the pathogenesis of airway hyperresponsiveness in asthma.

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CHAPTER 5: SUMMARY AND GENERAL DISCUSSION

Summary of Findings

In the following section, the major findings from each of the studies in the thesis, are summarized.

The first study examined whether the development of allergen-induced airway hyperresponsiveness in skin test positive dogs was associated with increases in the number and level of activation of eosinophils before and after allergen inhalation. The results demonstrated that dogs which develop allergen-induced airway hyperresponsiveness have a greater number of and more activated airway eosinophils before allergen inhalation than dogs that do not develop airway hyperresponsiveness. However, allergen-induced airway hyperresponsiveness was not associated with an increases in the number and activity of airway eosinophils after allergen inhalation.

In the second study, the importance of increased numbers and activity of airway eosinophils before allergen inhalation in the pathogenesis of allergen-induced airway hyperresponsiveness in dogs was further evaluated. We examined whether reductions in airway eosinophil number and/or levels of BAL EPO, a

putative indicator of activation, before allergen inhalation, as a result of inhalation of budesonide, would prevent allergen-induced airway hyperresponsiveness. Indeed, inhaled budesonide, in association with a significant attenuation of airway hyperresponsiveness, significantly reduced the number of airway eosinophils, but not the level of EPO in the BAL. Taken together, the findings of the first two studies suggest that eosinophils present in the airways before allergen inhalation contribute directly or indirectly to the development of allergen-induced airway hyperresponsiveness in dogs.

In the third study, the effect of allergen inhalation on bone marrow progenitor production was examined in dogs that developed allergen-induced airway hyperresponsiveness. The study demonstrated that allergen inhalation increased bone marrow progenitor production and that the increase was inhibited by budesonide inhalation. These findings suggest that allergen-induced bone marrow production of inflammatory cell progenitors may be an important mechanism contributing to the development of the airway inflammation and airway hyperresponsiveness associated with asthma.

In summary, the findings in this thesis suggest a potential role for airway eosinophils in the development of allergen-induced airway hyperresponsiveness in dogs. In addition, the ability of inhaled allergen to increase bone marrow progenitors highlights a potential mechanism by which inflammatory cells may accumulate in the airways of asthmatics. Furthermore, the ability of inhaled

budesonide to reduce pre-allergen eosinophils and bone marrow progenitors provides insight into potential mechanisms of action of corticosteroids.

General Discussion

Increasing evidence suggests that airway inflammation contributes to the airway hyperresponsiveness associated with asthma. The precise pathogenic role of airway inflammation, however, has yet to be established. The aim of this thesis was to examine the role of airway eosinophils, a cell implicated in the pathogenesis of asthma, in the development of allergen-induced airway hyperresponsiveness in dogs. In addition, this thesis examined whether inflammatory cell progenitors may contribute to the airway inflammation associated with asthma by investigating the effect of allergen inhalation on bone marrow progenitor production in dogs. In this thesis, the use of inhaled budesonide provided a means by which eosinophils and bone marrow progenitors in the dog allergen model could be further investigated.

The results of this thesis suggest that pre-existing airway eosinophilia influences the development of airway hyperresponsiveness after allergen inhalation. This finding supports previous studies which showed that increased baseline airway inflammation was associated with increased severity of allergen-induced late asthmatic responses (Rossi et al., 1991; Gundel et al., 1992; Venge et al., 1988).

Although the mechanism(s) through which eosinophils may cause allergen-induced airway hyperresponsiveness have not been determined, eosinophil-derived mediators have been implicated (Diaz et al., 1989; Gundel et al., 1991). The release of inflammatory mediators from eosinophils may occur directly by allergen binding to the low affinity IgE receptors on the surface of eosinophils (Capron et al., 1984). Alternatively, mediators may be released from eosinophils secondary to the release of secretagogues from degranulating mast cells (Pincus et al., 1982). Once released, eosinophil-derived mediators may exert a direct effect on airway smooth muscle, enhancing its contractile response (Hallahan et al., 1990). The ability of eosinophil-derived mediators to cause epithelial damage (Frigas et al., 1980) may also result in airway hyperresponsiveness. Increased responsiveness after epithelial injury may potentially occur through the loss of epithelial products which can directly relax airway smooth muscle (Flavahan et al., 1988) or because of an increase in permeability of the mucosal surface to inhaled agonists (Dolovich et al., 1992). In this thesis, BAL levels of EPO did not significantly increase after allergen inhalation in dogs which developed airway hyperresponsiveness, suggesting that EPO does not play a major, direct role in the increase in airway responsiveness. Other eosinophil granule proteins or mediators may, however, have pathogenic effects; these remain to be studied.

Eosinophil-derived cytokines may also contribute to development of airway hyperresponsiveness. For example, the release of GM-CSF from eosinophils may facilitate the proliferation and maturation of bone marrow eosinophil progenitors (Sieff et al., 1985) and, also, their recruitment and activation within the airways (Warringa et al., 1991; Lopez et al., 1986). Eosinophil-derived cytokines may also enhance the survival and activation of airway eosinophils in an autocrine manner, thereby, contributing to the persistent airway eosinophilia and airway hyperresponsiveness associated with asthma (Moqbel et al., 1991).

Studies in this thesis support a role for the bone marrow in the accumulation of inflammatory cells in the airways of atopic asthmatics. Conceivably, asthmatics continually exposed to allergen may have ongoing increased bone marrow production of inflammatory cells. The accumulation of these cells in the airways may predispose asthmatic subjects to develop airway hyperresponsiveness. The possibility also exists that with chronic allergen exposure and the release of hemopoietic cytokines, the airway microenvironment of the allergic asthmatic may become upregulated compared to the airways of normals, leading to a sustained, high level of progenitor production (Denburg et al., 1990). This proposal is supported by findings of increased numbers of circulating progenitors in asthmatics and atopic subjects compared to normals (Denburg et al., 1985).

With the recognition that asthma is an inflammatory disease of the airways, anti-inflammatory drugs such as inhaled steroids are increasingly used in the treatment of asthma. However, the mechanism by which corticosteroids decrease airway hyperresponsiveness is not known. Results from this thesis suggest that the corticosteroid, budesonide, attenuates allergen-induced airway hyperresponsiveness by decreasing the number of pre-allergen eosinophils. As previously mentioned, eosinophils have the capacity to release a variety of mediators and cytokines which may be associated with the development of airway hyperresponsiveness. Conceivably, a decrease in eosinophil mediators and/or cytokines, secondary to a steroid-induced reduction of eosinophil numbers before allergen inhalation, could attenuate allergen-induced airway hyperresponsiveness.

The precise mechanism through which steroids decrease airway eosinophil numbers can not be determined from the present studies. However, in vitro findings that corticosteroids can inhibit the release of eosinophil chemotactic factors (Flower, 1988), prevent eosinophil chemotaxis (Altman et al., 1981) and reduce cytokine enhanced eosinophil survival (Cox et al., 1991) may account for the decreased number of airway eosinophils. Several studies have also reported that corticosteroids block cytokine production by a variety of cells found in the airways (Vecchiarelli et al., 1992; Corrigan et al., 1991). Thus, local inhibition of airway cell hemopoietic cytokine release after steroid inhalation could result in decreased bone marrow production of eosinophil progenitors and, eventually,

reduced accumulation of eosinophils in the airways (Gibson et al., 1990). Alternatively, the reduction in airway eosinophil numbers in our study may have resulted from a direct inhibitory effect of systemic budesonide on bone marrow eosinophil production (Butterfield et al., 1986; Bjornson et al., 1985).

Directions for Future Research

Although the findings in this thesis suggest a role for pre-allergen airway eosinophils in the development of allergen-induced airway hyperresponsiveness, further research is required.

In this thesis, measurement of EPO was used as a marker of eosinophil activation as this was the only index of canine eosinophil activity available to us. Development of assays to assess eosinophil activity using other markers (eg: ECP, GM-CSF) may provide additional information on the state of activation of eosinophils before and after allergen inhalation in dogs. There is also a need to identify if the levels of other eosinophil-derived mediators or cytokines are increased before or after allergen challenge. If increased, studies employing specific synthesis blockers, receptor antagonists or cytokine antagonists are required to determine whether these mediators or cytokines have a role in allergen-induced airway hyperresponsiveness.

As further independent evidence that eosinophils play a crucial role in allergen-induced airway hyperresponsiveness, studies inducing airway eosinophilia in dogs that do not develop airway hyperresponsiveness could be conducted.

Additional research into the effects of allergen inhalation on bone marrow progenitor production is also required. In this thesis, granulocyte-macrophage bone marrow colonies were grown and counted as a measure of progenitor production. At present, technical difficulties preclude the growth of pure and distinct eosinophil colonies in the dog. As circulating eosinophil/basophil progenitors have been implicated in the pathogenesis of asthma (Gibson et al., 1990; Gibson et al., 1991), the development of techniques to grow eosinophil colonies in dogs would be useful.

The finding that allergen inhalation increases bone marrow inflammatory cell progenitor production also provides a model for studying progenitor and mature inflammatory cell trafficking. For example, labelling of inflammatory cell progenitors may enable the monitoring of the kinetics and migration of cells from the bone marrow to the airways. Information gained from such studies may provide further insight into the regulation of inflammatory process occurring in asthma.

In addition, further research is required to identify the specific hemopoietic cytokine(s) responsible for the increase in bone marrow progenitor production and their cellular source(s). Once identified, these cytokines may provide potential targets for therapeutic intervention. For example, the effects of a specific cytokine

could be neutralized with the use of either monoclonal antibodies or soluble receptors for the target cytokine. To minimize in vivo side effects, these agents could be delivered locally to the airways.

The novel demonstration in this thesis of a direct effect of allergen inhalation on bone marrow progenitor production highlights the potential importance of hemopoietic mechanisms in asthma. Although limited by potential species differences, the results of this thesis provide a theoretical basis to investigate allergen-induced changes in bone marrow progenitors in human asthmatics. The use of an animal model also limits our finding that airway eosinophils before allergen inhalation may contribute to the development of allergen-induced airway hyperresponsiveness. Verification of this finding in asthmatic subjects may provide further understanding of the pathogenesis of airway hyperresponsiveness. Furthermore, establishing a role for pre-allergen eosinophils and bone marrow progenitors in human asthma may provide new therapeutic targets for the treatment of this disease.

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