MECHANISMS OF AIRWAY NARROWING DURING ALLERGIC RESPONSES IN THE RAT

by

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To my beloved wife, parents and parents-in-law for their patience, understanding and unreserved support.

To my beloved uncle, C.F. Hsu who gave me all the support needed to complete this thesis.

ABSTRACT

The mechanisms of allergen induced airway narrowing, during allergen induced early (ER) and late (LR) responses, and airway hyperresponsiveness were investigated in vivo using the ovalbumin (OA) sensitized Brown Norway (BN) rat. ER and LR were induced by aerosolized OA. The lungs were fixed using a quick-freezing technique to maintain the pathological alterations as close as possible to their in vivo conditions. The results from both morphologic and morphometric studies showed that during the ER and LR, airway narrowing was mainly caused by airway smooth muscle contraction, but not by airway wall thickening or mucus. These findings were supported by intravenous administration of Evans blue which was used as a measure of vascular leakage. The degree of airway narrowing during the early response was greater in the large intrapulmonary airways than in the small intrapulmonary airways. Airway narrowing during the late response was limited to the large intrapulmonary airways. These findings were closely related to the mast cell distribution which progressively increased from small to large intrapulmonary airways in normal BN rats. These findings suggests that mast cells may play an important role in both early or late responses.

In order to study structural mechanisms of airway hyperresponsiveness, increased airway responses to inhaled methacholine were induced by repeated inhalational challenge of

sensitized BN rats with OA . Quantitation of airway smooth muscle, airway wall area and epithelial area demonstrated that increased airway hyperresponsiveness was associated with the increased amount of airway smooth muscle but not with airway wall thickening. There was a correlation between the quantity of airway smooth muscle in large airways and the change in airway hyperresponsiveness. Hence, it is possible that an altered quantity of airway smooth muscle is an important mechanical contributor to airway narrowing in human allergic asthma.

RESUME

Les mécanismes de rétrécissement de voies aeriennes provoque par l'inhalation d'allergènes ainsi que l'hypersensibilité à la méthacholine causée par ces allergènes ont éte etudiés in vivo tant au niveau de la réponse immédiate (I) que de la reponse semi-retardée (SR) chez des rats Brown Norway (BN) présensibilisés à ovalbumine (OA). Les poumons ont ete fixes par une technique de congélation rapide afin de maintenir les alterations pathologiques aussi semblables que possible aux conditions in vivo. Les résultats morphologiques et morphometriques indiquent que le rétrécissement des voies aériennes observé lors des reponses I et SR est principalement causé par la contraction des muscles lisses, et non par l'épaississement de la paroi des voies aériennes ni par l'apparition de mucus. Cette conclusion est supportée par des résultats démontrant l'étanchéite vasculaire etudiee à l'aide d'injections intraveineuses de teinture Evans blue. Le degré de rétrécissement lors de la réponse I est plus elevé au niveau des voies aériennes intrapulmonaires larges qu'au niveau des étroites. Le rétrécissement associé à la réponse SR est observe seulement au niveau des voies aériennes intrapulmonaires larges. Ces derniers résultats sont en accords avec la distribution du nombre de mastocytes progressivement accru au niveau des voies aeriennes intrapulmonaires larges, chez le rat BN. Ces résultats suggerent

que les mastocytes peuvent jouer un rôle important lors des réponses I et SR. Afin d'étudier les mécanismes structuraux de l'hyperréactivité des voies aeriennes, des rats (BN) préalablement sensibilisés à l'OA ont éte exposés de façon répétitive à ce dernier allergene pour ensuite mesurer les changements au niveau de leur sensibilité à la methacholine. La quantification des surfaces représentant les muscles lisses, la paroi et la région épithéliale des voies aériennes démontre qu'une hyperréactivité élevée serait associée à une augmentation de la quantité des muscles lisses, et non à celle d'un épaississement de la paroi des voies aériennes. Une corrélation est établie entre la quantité de muscles lisses au niveau des voies aériennes larges et un changement d'hyperréactivité. Il est donc possible qu'un changement de la quantité de muscles lisses soit un facteur mécanique important pouvant contribuer au rétrécissement des voies aériennes associé à l'asthme allergique chez l'homme.

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ABBREVIATIONS

ER	Early response
LR	Late response
OA	Ovalbumin
MCh	Methacholine
ASM	Airway smooth muscle
$\dot{V}$	Airflow
Ptp	Transpulmonary pressure
R_L	Pulmonary resistance
BM	Basement membrane of epithelium
LuB	Internal airway lumen defined by BM
Ae	External area of airway defined by outer boundary of airway wall
AW	Airway wall
EB	Evans blue dye
IL	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous
LuE	Airway lumen defined by luminal surface of epithelium
Ae	Area of airway defined by outer border of airway wall
AW	Area of airway wall
Ep	Area of airway epithelium
LT	Leukotriene
PAF	Platelet-activating factor
PG	Prostaglandins
TX	Thromboxane
MBP	Major basic protein derived from eosinophils
BAL	Bronchcalveolar lavage
HE	Hematoxylin & eosin
HPS	Hematoxylin-phloxine-saffron (HPS)

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PROLOGUE

The thesis has been divided into six chapters. The first chapter provides background information and a review of the literature related to this thesis. The experiments in Chapter II have already been published in the American Review of Respiratory Diseases, 1991, 143: 132-137. Chapter V, dealing with structural changes in the rats with airway hyperresponsiveness induced by repeated allergen inhalation, has been submitted to the same journal, reviewed and have been accepted for publication pending approval of the reviewers with revisions which have been incorporated into this thesis. Chapter III, dealing with morphometric changes during the allergen induced early response, and Chapter IV, dealing with microvascular leakage during the allergen induced early and late responses have been submitted to the same journal. Chapter VI contains the general conclusions and the claims of originality.

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CHAPTER I

GENERAL INTRODUCTION

Asthma is a common disease; it affects between 3 to 10 percent people in the United States, depending on the definition (Evans et al. 1987). Millions suffer from this disease which may lead to absence from school and work. The characteristic feature of asthma is reversible airways obstruction. The pathogenesis of asthma is complex and can be contributed to by many factors. It is likely that it is not a single entity but may consist of several diseases which have a common clinical expression.

1.1. ASTHMA

1.1.1. Definition and Classification of Asthma

An often-quoted conceptual definition was proposed by the American Thoracic Society in 1962 (Committee on Diagnostic Standards for Nontuberculosis Respiratory Disease, American Thoracic Society): " Asthma is a disease characterized by an increased responsiveness of the trachea and bronchi to a variety of stimuli and manifested by widespread narrowing of the airways that changes in severity either spontaneously or as a result of therapy".

The classification of asthma is still controversial. One acceptable classification was described by Robbins et al. (1984). Two major categories are extrinsic and intrinsic asthma.

Extrinsic asthma can be subcategorized into atopic extrinsic and nonatopic extrinsic groups. Atopic extrinsic asthma is caused by inhalation of allergens from the environment. The host responds

to the allergen with synthesis of specific IgE. Since atopic extrinsic asthma is induced by allergen, clinically, it is also often called " allergic asthma". This type of asthma is the most common (Robbins et al. 1984). So-called nonatopic extrinsic asthma is also induced by environmental allergens. The mechanism of nonatopic extrinsic asthma is not thought to be mediated by IgE, but by other types of antibody (Scadding 1985).

Intrinsic asthma appears to be caused by factors other than inhaled allergen. This type of patient shows a negative skin test to allergens (Dunnill 1987).

A mixed pattern of asthma has been postulated by Robbins et al. (1984). Both extrinsic and intrinsic factors are involved in the mechanism of this type of asthma.

Several chemicals can cause sensitization with IgE or other immunoglobulin responses. Asthma resulting from such sensitization is called occupational asthma. If asthma is triggered by exercise, it is often referred to as exercise induced asthma (Scadding 1985).

The classification, referring to allergic (extrinsic) and nonallergic (intrinsic) forms of asthma, has been popular for many years. However, a recent population study showed that the prevalence of asthma was closely related to the serum IgE level standardized for age and sex (Burrows et al. 1989). The authors concluded that asthma is almost always associated with IgE-mediated reactions, challenging the concept that there are basic differences between " extrinsic" and " intrinsic" forms of asthma.

1.1.2. Epidemiology of Asthma

Based on the morbidity, mortality, and economic impact of asthma, it is a serious and common disease at the present time. It is prevalent in many industrialized as well as nonindustrialized countries (Gregg 1977; Turner 1987). In the United States, approximately 9 million Americans suffer from and thousands of people die from this clinical disorder (Iafrate et al. 1986). The national cost for the treatment of asthma in the United States is extremely high and has reached 2.6 billion dollars in 1985 (National Health, Lung, and Blood Institute. 1988).

In the United States, both the prevalence of asthma and death rates from asthma increased between 1965 to 1984 (Evans et al. 1987). The prevalence of asthma is greater in males, blacks and people with lower socioeconomic background; it is slightly higher in the southern and western region of the United States (Weiss and Speizer 1985; Evans et al. 1987). The morbidity of asthma varies with age. It is higher in childhood, declining in adolescence and young adulthood and then increasing during middle age and late years (Weiss and Speizer 1985). One third to one fourth of American asthmatic patients are children (Evans et al. 1987). It has been reported that the prevalence of asthma in children was significantly increased between 1971 to 1980 and it was significantly higher in black compared to white children, in boys compared to girls, and in urban compared to rural areas; asthma in children is usually of the allergic type (Gergen et al. 1988).

The prevalence of asthma in New Zealand, Australia and England has also increased during the past twenty years. The prevalence and morbidity rates of asthma in these countries are even greater than in the United States (Woolcock 1988). The prevalence of asthma has also increased in some developing countries during recent years. (Turner 1987).

Several useful drugs, such as theophylline, $beta_2$-agonists, and corticosteroids have been applied to the treatment of asthma. Although much effort has been devoted to the treatment of this disease, the death rate from asthma has still increased during the past ten years (Sly 1988; Barbee 1987).

Several endogenous and environmental factors may influence the incidence of asthma. One of the most important endogenous factor is atopy, including a family and personal background of allergic disease. The prevalence of asthma in the parents of asthmatics is higher than in others (Charpin and Charpin 1987) and two thirds of asthmatics have a personal history of allergic disorder (Hendrick et al. 1975). Both phenomena are more obviously present in allergic asthma (Sibbald and Turner-Warwick 1979; Hendrick et al. 1975). Positive skin tests to common allergens (Hagy and Settipane 1976) and higher level of serum IgE (Charpin and Charpin 1987; Pollart et al. 1989; Burrows et al. 1989) may also relate to the prevalence of allergic asthma.

Several environmental factors may be important for the development of asthma, such as air pollution, allergen inhalation and socioeconomic status (Charpin and Charpin 1987; Pollart et al.

1989).

In summary, asthma is an important disease for adults and children. The prevalence of asthma has progressively increased in many countries during recent years . As a subtype, allergic asthma has also increased and may be related to the morbidity and mortality of asthma. In order to treat this disease effectively, an understanding of the pathogenesis of allergic asthma will have important implication for this major public health problem.

1.1.3. Pathology of Asthma

Pathological findings in different types of asthma are said to be similar (Hogg 1985). The recognized pathological alterations in asthma are mainly dependent upon observations on postmortem lungs. Since these patients are often at the end stage of the disease, the morphological changes may be more pronounced and may not be representative of patients with mild or moderate asthma. In postmortem specimens, the lungs are normally immersed in or infiltrated with fixatives. On these tissues, several morphological changes can be observed, including hypertrophy and hyperplasia of airway smooth muscle, airway mucous plugs, enlarged mucous glands and airway wall edema associated with inflammatory cell infiltration. Other pathological changes, such as thickening of the basement membrane of the epithelium, lung hyperinflation, epithelial damage as well as increased goblet cell numbers in the epithelium are also often detected (Hogg 1985; Dunnill 1987).

The reversibility of asthmatic attacks suggests that excessive

airway smooth muscle contraction is an important pathological feature of asthma. In patients who died from asthma, both hypertrophy and hyperplasia of airway smooth muscle have been reported (Heard and Hossain 1973; Kleinerman and Adelson 1987). Increased airway muscle is present in the entire airway tract (Hogg 1985). This abnormal airway smooth muscle could contribute to the severity of asthma. However, muscle contraction is unlikely to be maintained in lungs fixed in inflation. Therefore its contribution to airway narrowing in vivo is unknown.

Mucous plugs appear in small and large bronchi of the lungs and can be observed even with the naked eye. However, they are not easily seen following inflation with fixative through the tracheobronchial tree (Dunnill 1987). Mucous plugging of airways is due to mucous hypersecretion. The mucous is secreted from both mucous glands and goblet cells. The excess of mucus in the airway may decrease the air flow through the airways (Hogg 1985). It has also been suggested that extravasation of protein rich fluid into the airway lumen may also contribute to these" mucous" plugs (Persson 1988).

Airway edema and inflammatory cell infiltration are caused by vasodilation, vascular leakage, increased microvascular permeability and emigration of inflammatory cells from the blood circulation (Hogg 1985). The eosinophil is a predominant infiltrating cell in the airway wall (Dunnill 1960). Increased eosinophils in allergic asthmatic patients have been further confirmed by BAL during the late asthmatic responses (De Monchy

1985, 1986). Degranulation of mast cells can be detected on biopsy tissue from asthmatic patients. However, it is difficult to observe mast cells in postmortem tissue from asthmatics. These findings suggest that the degranulation of mast cells is contributing to the pathogenesis of asthma (Dunnill 1987). Inflammatory cells can produce and release many potent mediators such as leukotrienes, prostaglandins, platelet-activating factor and histamine. In asthmatic patients, these mediators may cause several important pathological changes, including muscle contraction, edema, mucous hypersecretion and epithelial damage. The mediators will be discussed in a subsequent section of this thesis. By causing airway wall thickening, it has been suggested that increase in airway wall thickness from edema could contribute to the airway narrowing in asthmatic patients (James et al. 1989).

1.2. MECHANISM OF ALLERGIC ASTHMA

Allergic asthma is a classic example of a type I IgE- mediated hypersensitivity reaction. Exposure to an allergen can stimulate the production of specific IgE by B lymphocytes. The mast cells bind to the IgE molecule by high affinity IgE receptors. When IgE-coated mast cells are re-exposed to the same antigen, these mast cells are stimulated to release chemical mediators such as leukotrienes, platelet-activating factor, prostaglandins, histamine and chemotactic factors (Roitt et al. 1989a; Reviewed in 1.3 and 1.4). In allergic asthma, most if not all of the pathological changes can be attributed to either the mast cell derived mediators

or to the inflammatory cells attracted by mast cell-derived chemotactic factors.

Allergic asthma is characterized by reversible airflow obstruction which is due to airway narrowing. Four major features of allergic asthma are the early airway response, late airway response, airway inflammation and airway hyperreactivity (Kay 1986). Several cell types are involved in airway inflammation and each of them can release potentially important mediators. The cells and mediators involved in the pathogenesis of allergic asthma will be particularly discussed in the sections 1.3 and 1.4. In this section, I will focus on the IgE and IgE receptors, early and late airway responses, and airway hyperreactivity.

1.2.1. IgE and IgE Receptors

The central role of IgE antibody in allergic asthma is no longer in dispute. Increased IgE levels in asthmatic patients has been demonstrated by many investigators (Criqui et al. 1990; Pollart et al. 1988 and 1989). Indeed at any age the absence of IgE virtually precludes the presence of asthma (Burrows et al. 1989).

IgE was discovered in 1966 (Ishizaka et al. 1966). It is produced by plasma cells and present in very low concentration in normal individuals (180-350 ng/ml) (Johansson et al. 1972). IgE consists of two heavy and two light chains covalently linked by disulfide bonds (Bennich and Bahr-Lindstrom 1974). IgE is able to bind with high affinity receptors on the mast cells and

basophils.

There are large numbers of IgE receptors (FcR1) on the surface of mast cells and basophils. It has been reported that there are 300,000 high affinity receptors per rat mast cell (Conrad et al. 1975) and 40,000 to 100,000 receptors per human basophil (Ishizaka et al. 1973). The cross-linking of IgE FcR1 receptors triggers the cells to initiate the allergic reaction by synthesizing and releasing their mediators (Kulczycki 1981).

It has been shown that there is another type of IgE receptor, a low affinity IgE receptor (FcR2) which is present on the surface of macrophages (Anderson and Spiegelberg 1981), eosinophils (Capron et al. 1981), platelets (Capron et al. 1983) T- (Yodoi and Ishizaka 1979) and B-lymphocytes (Gonzalez-Molina and Spiegelberg 1977). However, neutrophils do not have IgE receptors (Capron et al. 1984). Antigenically, the IgE FcR2 is different from the IgE receptor (IgE FcR1) on mast cell and basophil; goat anti-FcR1 antibody does not block the binding of IgE to IgE FcR2 (Spiegelberg 1984). The stimulation of FcR2 receptors may also contribute to the allergic reaction. Further details will be reviewed in other parts of my thesis.

1.2.2. Early and Late Airway Responses

Two phases of airway airflow obstruction have been recognized in sensitized human subjects after challenge with inhaled allergen, namely early and late responses. Early responses (ER) appear within 10-15 minutes after the allergen exposure and resolve

spontaneously within 1 to 3 hours (O'Byrne et al. 1987). Late responses (LR) may develop from 4 to 12 hours after allergen inhalation and can last for more than 24 hours (Hargreave et al. 1974; Cartier et al. 1982; Booij-Noord et al. 1972; Pelikan and Pelikan-Filipek 1986). Approximately 50% of adults and 80% of children manifest both early and late responses (O'Byrne et al. 1987). Other asthmatic subjects show either isolated early or isolated late responses (Rocklin and Findlay 1985). In allergic asthmatics, the evidence in the literature suggests that both early and late airway responses are IgE-mediated and related to the mast cell degranulation (O'Byrne et al. 1987).

The early airway response is mainly due to airway smooth muscle spasm which is caused by mast cell derived mediators (Kay 1986). Traditionally, histamine is described as a potent airway smooth muscle constrictor during the early response (Kay 1986). The contribution of leukotrienes (LTs) to early responses has been documented in allergic sheep (LTD_4/LTE_4; Abraham et al. 1986) and Brown Norway rat model (LTD_4; Sapienza et al. 1990). Both LTD_4 and LTE_4 can also induce airway smooth muscle contraction (Cross 1986). Besides the airway bronchospasm, in allergic asthmatic patients, airway hyperemia and edema have also been detected by bronchoscopy after local allergen challenge (Metzger et al. 1987). Direct histological evidence has been obtained in allergic rabbits by Behrens et al. (1987). Thirty minutes after inhalational challenge of allergen, edema and vascular dilation can be observed in the large airways of rabbits. This evidence suggests

that both airway smooth muscle contraction and edema may be involved in the pathogenesis of the early airway response and contribute to airway narrowing.

Late responses are often associated with airway hyperreactivity and may cause more severe clinical symptoms of asthma (Cartier et al. 1982). Recently, the late response has become the subject of more intensive investigation. The early response to antigen can be inhibited by $beta_2$-agonists and disodium cromoglycate (Booij-Noord et al. 1971, 1972), whereas the late response is not well antagonized by $beta_2$-agonists but is reduced by corticosteroids (Booij-Noord et al. 1971). Therefore, it has been argued that the late airway response in humans is not only caused by airway smooth muscle constriction, but also by airway inflammation, edema and mucous hypersecretion (O'Byrne et al. 1987).

Mast cells may play an important role in both early and late airway responses by release of their mediators. It has been reported that nonimmunologic stimulation with compound 48/80 may cause mast cell degranulation and produce both early and late responses in allergic sheep (Russi et al. 1984). The occurrence of mast cell degranulation during the early and late responses has been observed after local challenge of asthmatic patients with allergen (Metzger et al. 1987). The activation of mast cells during the early response and re-activation of mast cells during the late response has been suggested by Durham et al. (1984), although there is evidence to the contrary. However, mast cell

activation during the ER is certainly present (Keyzer et al. 1984). The production of leukotrienes during the early response contributing to subsequent late responses has been reported in sheep (Abraham 1988). Therefore, mast cell activation likely contributes indirectly if not directly to the development of late responses.

Other inflammatory cells potentially also contribute to the late responses. Increased inflammatory cells following allergen challenge have been demonstrated by bronchoalveolar lavage (BAL). Elevated eosinophil levels appear at 6 to 7 hours (De Monchy et al. 1985) and neutrophils, lymphocytes and eosinophils are increased at 48 hours after allergen challenge (Metzger et al. 1987). The mechanism by which these cells are attracted into the airways has not been completely elucidated but the mast cell synthesizes chemotactic factors and cytokines which could account for all of the observed inflammation. The mediators derived from these inflammatory cells cause the pathological alterations involved in the late responses.

Although the early and late responses have been studied for many years, from the standpoint of the immunologic and biochemical reactions involved, two basic questions still remain unanswered. First, what is the predominant site of airway narrowing (small or large airways) during the early and late airway responses. Second, among airway smooth muscle contraction, mucus hypersecretion, airway wall edema and inflammation, which is the most important contributor to airway narrowing during the early and late

responses.

1.2.3 Airway Hyperresponsiveness

Airway hyperresponsiveness to various physical, chemical and pharmacologic stimuli is one of the major characteristics of asthma. In asthmatics, the degree of airway narrowing in response to the stimulant agents is greater than that in normal subjects (Nadel and Sheppard 1985; Woolcock et al. 1984). The mechanism of airway hyperresponsiveness has been explored by inducing changes in a variety of animals by exposing them to ozone and allergen. Changes on exposure to allergen have been produced in several allergic animal models, such as sheep, dog, monkey, rabbit, guinea pig and rat (Reviewed in section 1.2.4.).

Airway hyperresponsiveness is determined by constructing dose-response curves to a stimulus (Cockroft et al. 1977a). The usual chemicals to test nonspecific airway hyperreactivity are histamine (Woolcock et al. 1984)and methacholine (Sterk et al. 1985). Other stimuli such as cold air have also been used but show less sensitivity and are cumbersome.

The precise mechanism of airway hyperreactivity is unknown. The reversibility of airway narrowing to the constrictive stimuli suggests that airway smooth muscle plays the most important role in the mechanism of airway hyperreactivity (Macklem 1988). Whether smooth muscle is abnormal or is responding excessively because of other factors, such as decreased loads on airway smooth muscle, abnormalities of autonomic regulation, airway wall edema and

inflammation is uncertain.

In postmortem asthmatic airways, both hypertrophy and hyperplasia of airway smooth muscle have been described (Heard and Hossain 1973; Kleinerman and Adelson 1987). An increased amount of airway smooth muscle is present throughout the entire airway tree (Hogg 1985). As suggested by Moreno et al. (1986), increased airway smooth muscle strength and an increase in airway smooth muscle proportion in the airway circumference are potential mechanisms of airway hyperresponsiveness.

Decreased load on airway smooth muscle may theoretically permit an increased degree of airway narrowing. This load is generated by the airway structure, the surrounding parenchyma and the transmural pressure of the airways. The load on airway smooth muscle in cartilaginous airways is provided mainly by airway cartilage, whereas the major loads in membranous airways are provided by surrounding parenchyma and the transmural pressure (Moreno et al. 1986). In rabbits, the softening of cartilage by intravenously administrated papain increases the airway resistance and the degree of airway narrowing (McCormack et al. 1986; Moreno et al. 1988). However, whether this mechanical change occurs in humans is still not clear.

Post mortem asthmatic airway walls usually demonstrate thickening caused by edema and inflammatory cell infiltration (Hogg 1985). The observation that airway hyperresponsiveness to methacholine more often follows the appearance of a late rather than an early asthmatic response (Cockcroft et al. 1977b; Machado

1985), also suggests that airway inflammation may play an important role in airway hyperreactivity. Increased airway wall thickness could contribute substantially to exaggerated airway narrowing (James et al. 1989). Therefore, airway edema and airway inflammation may be involved in the mechanism of airway hyperresponsiveness (Moreno et al. 1986).

The contribution of inflammatory cells to airway hyperreactivity is not due only to the passive effects of infiltration, but also to their mediators. Leukotrienes and PAF are potent mediators of vascular leakage which may thicken airway wall (Reviewed in 1.4.1. & 1.4.2.). Recently, it has been reported that inhaled LTD_4 and PAF induced airway hyperresponsiveness to methacholine in six of eight normal human subjects (Kaye and Smith 1990). This suggests that both LTD_4 and PAF may contribute to airway hyperreactivity.

Hypersecretion of mucus in the airway lumen could also cause airway narrowing which may be a further mechanical factor in airway hyperresponsiveness (Moreno et al. 1986).

Autonomic nervous regulation may play some role in the mechanism of airway hyperreactivity . The autonomic innervation of the airways includes the sympathetic, parasympathetic and nonadrenergic inhibitory divisions (Nadel and Sheppard 1985). The sympathetic system consists of sympathetic nerves and adrenal medulla. Post ganglionic nerves release norepinephrine close to the target tissue and the medullary cells secrete catecholamines into the circulation. Two types of adrenergic receptors, alpha and beta,

are present in the airway. Stimulation of alpha receptors causes the contraction of airway smooth muscle, whereas beta receptors mediate airway smooth muscle relaxation (Nadel et al. 1985). The parasympathetic innervation can cause airway smooth muscle contraction by the release of acetylcholine (Nadel and Sheppard 1985). There is another nervous system that regulates airway smooth muscle activation, the so called nonadrenergic inhibitory nervous system. According to current knowledge, vasoactive intestinal peptide (VIP) may be the transmitter for this system (Barnes 1986). Possibly, airway narrowing can be caused by an increase in alpha-adrenergic or parasympathetic nervous activity, and airway dilatation can be caused by an increase in beta-adrenergic or nonadrenergic inhibitory nervous activity (Nadel and Sheppard 1985). However, the reports from different laboratories or on different species are quite controversial (Barnes 1986; Leff 1988; Nadel and Sheppard 1985). The contribution of autonomic innervation to airway hyperreactivity is not clearly understood.

1.2.4 Animal Models for the Study of Allergic Asthma

Obviously, it is difficult to study the pathogenesis of asthma in allergic asthmatics. Postmortem studies of allergic asthma focus mainly on the end stage of the disease (James et al. 1989; Hogg 1985). The development of the disease and factors contributing to the disease are not easily studied in human subjects. In order to investigate the mechanism of this disease, several animal models have been developed during the past ten years, such as rabbit,

guinea pig, rat, sheep, monkey and dog. Animals models offer great advantages in the study of the pathophysiology of allergic asthma.

In sensitized rabbits, following allergen inhalation, early and late airway responses and increased airway hyperresponsiveness have been demonstrated (Behrens et al. 1987; Richerson et al. 1986; Shampain et al. 1982).

Guinea pigs have been utilized by several groups for the study of allergic asthma. In the ovalbumin sensitized guinea pig, the timing and the pattern of airway responses is slightly different from other animals and human allergic asthmatics. Three types of airway responses occur. After allergen inhalation, the early airway response appears at 2 hours and two late phase responses occur at 17 and then at 72 hours (Hutson et al. 1988, 1990). In sensitized guinea pigs, airway hyperresponsiveness can be demonstrated to methacholine challenge, but not to histamine challenge (Andersson and Bergstrand 1981).

Monkey, sheep and dog are commonly used large animal models. After sensitization, an early airway response can be induced in Macaca arctoides monkey and rhesus monkeys (Patterson et al. 1983; Richards et al. 1983). The former also shows airway hyperresponsiveness to histamine challenge (Richards et al. 1983). Recently, it has been reported that allergen inhalation can induce both early and late airway responses in sensitized squirrel monkeys (Hamel et al. 1986).

The allergic sheep model has been intensively studied. Inhalational challenge with allergen can induce early and late

airway responses in actively sensitized sheep (Abraham and Perruchoud 1986, 1989), whereas airway hyperresponsiveness can be observed at 24 hours after allergen challenge (Abraham et al. 1988a).

Following allergen inhalation, the sensitized dog also showed some aspects in common with human allergic asthma, such as airway hyperresponsiveness and early airway response. However, the late airway responses to allergen challenge can not be consistently produced in dogs (Becker et al. 1989; Mapp et al. 1985; Peters et al. 1982a). However, when endogenous corticosteroid production is blocked by metyrapone, late responses are noted (Sasaki et al. 1987).

The animal model that I have used for my research is the Brown Norway rat (BN). The life span of this highly inbred rat is 29 months for males and 31 months for females (Festing 1979). In this animal, sensitization induces IgE production and the level of IgE in the serum peaks around 14 days after sensitization (Murphey et al. 1974; Pauwels et al. 1979; Smith and Petillo 1976). The report of this model for the study of allergic asthma was initially published in 1988 by Eidelman et al. Active sensitization of the eight to ten weeks old rats was carried out by a single subcutaneous injection of a suspension of 1 mg ovalbumin and 200 mg of aluminium hydroxide in 1 ml of 0.9% saline. At the same time, 1 ml of Bordetella pertussis vaccine containing 6 x 10^9 heat-killed bacilli was given intraperitoneally as an adjuvant. Two weeks after sensitization, inhalational challenge with aerosolized ovalbumin

induced both early and late responses in 7 out of 15 rats. Other animals showed either isolated early or isolated late responses (Eidelman et al. 1988). In this animal model, airway hyperresponsiveness to methacholine challenge can be demonstrated following repeated inhalation of ovalbumin aerosol (Bellofiore and Martin 1988).

The above reports indicate that sensitized BN rats show similar pathophysiological phenomena present in allergic asthmatic patients. In order to study the pathogenesis of allergic asthma using morphometric techniques, this animal model has been used in my studies. From the histological point of view, a major advantage of this model is that is easier to examine entire lungs and all of the airways in the small sized lungs.

1.3. CELLULAR POPULATIONS INVOLVED IN THE PATHOGENESIS OF ALLERGIC ASTHMA

Several cell types may be contributing to the pathogenesis of allergic asthma. However, based on their location, IgE receptors and ability to release mediators, mast cells must be considered one of the most important cells in initiating allergic asthma. Following mast cell activation, many inflammatory cells infiltrate the respiratory tree. Currently, the presence of eosinophils, macrophages and lymphocytes have been clearly demonstrated in the airways of allergic asthma . In this section of the thesis, the possible contribution of each cellular component will be discussed.

1.3.1. Mast Cells

The mast cell is a pivotal cell in the pathogenesis of allergic asthma. The mast cell was discovered in the 19th century and named by Ehrlich (Janes and McDonald 1948). These cells are present in greatest number in the respiratory system, the gastrointestinal tract and the skin (Wasserman 1984). In the monkey, the total amount of mast cells is between 10^7 and 10^8 cells per lung and the percentage of the cells in the airways and lung parenchyma is approximately 83% and 17%, respectively (Guerzon et al. 1979). This indicates that most mast cells of the respiratory system are located in the airways.

In rodents, two distinct mast cell types have been recognized. They have been termed mucosal and connective tissue mast cells. The difference between these two types of mast cells is mainly dependent on their morphological characteristics after histological preparation. Connective tissue mast cells can be observed after formalin fixation. In contrast, mucosal mast cells can not be seen in formalin fixed tissue because of the dissolution of the granule content by the formalin. By using fixatives such as Carnoy's, Mota's and basic lead acetate, stained mucosal mast cells are visualized (Friedman and Kaliner 1987). The mucosal mast cells are prominent in the mucosal surface and for their proliferation, they are dependent on T-lymphocyte derived lymphokines (IL-3 and IL-4). Connective tissue mast cells are more often located in the connective tissue and they are T-lymphocyte independent for their proliferation (Roitt et al 1989b).

In addition to the differences in the staining characteristics, location and sensitivity to lymphokines, these two types of mast cells also demonstrate differences in their ultrastructure, chemical components, response to mast cell triggering agents and the effect of antiallergic compounds. These differences include: 1) the major granule proteoglycans of mucosal mast cells differ from connective tissue mast cell in their degree of sulfation and in their solubility; 2) heparin is a major component in secretory granules of connective tissue mast cells, but not of mucosal mast cells; 3) compound 48/80 is a potent stimulus for connective tissue mast cell degranulation, but not for mucosal mast cells; 4) IgE antibody can bind to both the membrane surface and cytoplasm of mucosal mast cells, but only binds to the membrane surface of connective tissue mast cells (Friedman and Kaliner 1987).

In the normal Lewis rat, comparison of formalin and Mota's solution fixed tissues shows that most mast cells of the respiratory system are of the connective tissue type. Mucosal mast cells reportedly were only identified in the trachea, especially in the mucosa of the cartilaginous portion (Goto et al 1984).

The heterogeneity of mast cells in human lung is related to their size (9.9-18.4 microns in diameter), histamine content and the ability to release mediators (Schulman et al. 1983). However, studies of human lung and intestine demonstrate similarity of many of the mast cell characteristics (Fox et al. 1985). So it is still unclear if there are two distinct subtypes

of human mast cells (Bienenstock et al. 1989).

The relationship between asthma and mast cell activation has been demonstrated in the results of bronchoalveolar lavage (BAL) and autopsy studies (Connell 1971; Tomioka et al. 1984). The number of mast cells in BAL fluid is greater in asthmatic patients than that in normal subjects (0.25% vs 0.08%). However, the histamine content of mast cells from asthmatic patients is lower than that from controls (8.2±6.0 pg/cell vs 15.3±10.8 pg/cell; Tomioka et al. 1984). The number of mast cells in bronchial walls of patients who died from asthma is lower than that of patients who died from other causes (Connell 1971). These observations suggest that in asthmatic patients, the mast cells are activated to degranulate and release their chemical mediators.

Mast cells have Fc epsilon receptors (FcR1) on their membrane surface and these receptors have a high affinity for IgE molecules. In atopic humans, after exposure to an allergen, the plasma cells produce a specific IgE which binds to FcR1 on the mast cell surface. Re-exposure to the same allergen can stimulate the mast cell through cross-linking of two adjacent IgE molecules on the mast cell surface by the allergen. Activation of mast cells leads to cell degranulation and the release of chemical mediators. (Roitt et al 1989a).

The mast cell may have a role in inducing bronchoconstriction through mechanisms other than allergen. Physical stimuli and mediators can also stimulate the mast cell and its circulating precursor, the basophil. Major basic protein, derived from

eosinophils, can activate rat mast cell and human basophils to release histamine (O'Donnell et al. 1983). Peptide triggers such as C3a (Glovsky et al. 1979) and C5a (Grant et al. 1975) and neurotransmitters, including gastrin (Tharp et al. 1984) and substance P (Mazurek et al. 1981) can also stimulate mast cell degranulation. Interestingly, lipid products derived from arachidonic acid, including 5-hydroperoxyeicosatetraenoic acid, 15-hydroxyeicosatetraenoic acid, prostaglandin D_2 and leukotriene B_4 are also able to stimulate mast cells and basophils (Peters et al. 1982b; 1982c and 1984).

Activated mast cells generate and release several important mediators such as histamine, adenosine, prostaglandins, leukotrienes, platelet-activating factor (PAF), chemotactic factors and various enzymes (Wasserman 1987). These mediators can cause many pathological changes, including airway smooth muscle contraction, vascular leakage and inflammatory cell accumulation in the lungs. Such pathological alterations may directly or indirectly contribute to nonspecific airway hyperresponsiveness and both the early and late allergic airway responses (Wasserman 1987; Kaliner 1989).

In conclusion, there seems to be little doubt that the mast cells play an important role in the pathogenesis of allergic asthma, because of their high affinity IgE receptors, strategic anatomical location in the tracheobronchial tree and content of bronchoactive and proinflammatory mediators.

1.3.2. Eosinophils

During the past ten years, the role of the eosinophil in the pathogenesis of asthma has been intensively studied. Eosinophils have low affinity IgE receptors (FcR2) on their surface (Capron et al. 1981). It has been reported that stimulated eosinophils can generate many chemical substances, such as major basic protein (MBP; Gleich et al. 1973), eosinophil cationic protein (Olsson and Venge 1974), eosinophil peroxidase (Takenaka et al. 1977), leukotriene C4 (Jorg et al. 1982 and Weller et al. 1983) and PAF (Lee et al. 1984). As will be discussed below, these mediators could contribute to the pathology of asthma.

The molecular weight of MBP is 9300 in humans. The eosinophil granule contains a high concentration of major basic protein, which can damage the respiratory epithelium (Frigas and Gleich 1986) and induce the mast cell and basophil to release chemical mediators (O'Donnell et al. 1983). During an acute attack of asthma, the concentration of MBP in the sputum is significantly increased and after treatment, the sputum concentration of MBP is significantly decreased (Dor and Frigas 1983; Frigas et al 1981). In patients who died from asthma, eosinophil disruption and extracellular MBP has been demonstrated in the lung tissue by an immunofluorescence technique (Filley et al. 1982).

Apart from MBP, two other eosinophil derived substances, eosinophil peroxidase (EPO) and eosinophil cationic protein (ECP) may also play a role in the pathogenesis of allergic asthma. EPO is present in the eosinophil granule matrix (Enomoto and Kitani

1966). The presence of eosinophil peroxidase together with the eosinophil's capacity to produce H_2O_2 and to utilize physiological concentrations of bromide can lead to the generation of large amounts of tissue-damaging brominated products (Weiss et al. 1986). Furthermore, under certain conditions, EPO can also induce mast cell degranulation (Henderson 1980). The intracellular location of ECP in eosinophil is not clear. However, when released ECP can cause epithelial damage (Olsson and Venge 1974). There is evidence that serum ECP is increased in asthmatic patients (Dahl et al. 1978) and treatment with beta-agonists and theophylline may reduce the level of ECP in the serum (Dahl and Venge 1978).

Eosinophils may also contribute to the pathophysiology of allergic asthma by their ability to synthesize leukotrienes (Jorg et al. 1982 and Weller et al. 1983) and PAF (Lee et al. 1984). Leukotrienes are potent bronchoconstrictors and PAF can induce bronchial hyperresponsiveness (Chung et al. 1986). Recently, an in vitro study showed that the supernatant from activated eosinophils contains leukotrienes which cause hyperresponsiveness of guinea pig airway smooth muscle (Aizawa et al 1990).

In human subjects, after allergen challenge, an increased number of eosinophils has been recovered from the airways by BAL during the late response (De Monchy et al. 1985, 1986). It has been suggested that human bronchoalveolar eosinophilia is closely associated with late but not early asthmatic reactions. A close relationship between the late phase response and eosinophilia has been found also in allergic sheep. In this animal model of allergic

asthma, after allergen inhalation, the percentage of eosinophils in BAL fluid is elevated in animals with dual responses, but is not increased in the animals with isolated early responses. When the late responsewas blocked by methylprednisolone succinate, the eosinophilia was also lowered to a similar level as that in animals with an early response (Abraham et al. 1988b). A similar result has been observed in allergen sensitized guinea pigs. In this animal, an elevated eosinophil amount in BAL fluid is associated with the appearance of the late airway response after allergen challenge (Hutson et al. 1988).

1.3.3. Mononuclear Cells

Mononuclear cells in respiratory tract tissue include macrophages, monocytes and dendritic cells. Macrophages and their monocyte precursors are considered to be important cells for airway inflammation. The lung macrophages have been traditionally classified into two categories based on their anatomical distribution: interstitial macrophages and alveolar macrophages. Interstitial macrophages are present in the interstitium. Alveolar macrophages are located predominantly in the alveolar air space and airway lumen (Reynolds 1987; Eschenbacher and Gravelyn 1987). In the normal or asthmatic subjects, the fluid from both bronchoalveolar lavage and airway lavage reveals that macrophages are the predominant cellular population of both the alveolar air space and the airways (Eschenbacher and Gravelyn 1987). The cells in the latter location are potentially important in allergic

asthma.

In both humans and rats, it has been demonstrated that the macrophages and their monocyte precursors have low affinity IgE receptors (FcR2) on their membrane surface (Findbloom and Metzger 1981; Joseph et al. 1983; Melewicz et al. 1982). FcR2 positive monocytes in the circulation are increased in allergic disorders, including allergic asthma (Melewicz et al. 1981). The results of bronchoalveolar lavage have shown that macrophages are increased after allergen challenge (Metzger et al. 1987), suggesting the emigration of monocytes from peripheral blood into the lung tissue after allergen inhalation.

The macrophage can release its chemical mediators by cross-bridging its surface IgE FcR2 (Joseph et al. 1980). The macrophage is stimulated when the antigen is presented as antigen-IgE-complex. However, the concentration of allergen required to stimulate th macrophage is necessarily higher than that required to stimulate mast cells or basophils. (Rouzer et al. 1982a).

The stimulated macrophage is able to produce and release several important mediators, such as leukotrienes, prostaglandins, PAF, TxB_2 and lysosomal enzymes (Godard et al. 1987). Other immunological functions of the macrophage, including antigen presentation (Rosenstreich and Mizel 1978) and lymphocyte activation (Thiele and Lipski 1982), may be also involved in allergic asthma.

Since macrophages have low affinity IgE receptors, they could

be activated following the inhalation of allergen by sensitized subjects. It is possible, given the very large number of these cells in the alveoli and airways, that the macrophage may be an important cell in the pathogenesis of allergic asthma.

Another interesting cell type is the dendritic cell. In the rat, two types of dendritic cell have been identified in the respiratory tract tissue using an immunohistochemical technique. They are located in the airway epithelium and alveolar septal walls respectively (Holt and Schon-Hegrad 1987). It has been shown that dendritic cells can transport antigen from the gut wall and subsequently present it to T-lymphocytes in the draining lymph nodes (Mayrhofer et al. 1986). In the lung tissue and upper respiratory tract, it has been reported that dendritic cells are the major antigen-presenting-cells (APCs; Sertl et al. 1986; Holt et al. 1985). It is still unknown how the dendritic cell contributes to the inflammatory response in the airway wall following the inhalation of antigen.

1.3.4. Lymphocytes, Neutrophils and Platelets

It has been well recognized that B lymphocytes contribute to allergic asthma through the production of IgE antibody. In general, when an allergen is inhaled by a sensitized subject, the allergen binds to antigen-presenting cells (APCs) which might be either dendritic cells or macrophages. APCs then present the processed allergen to lymphocytes. With T cell regulation, B lymphocytes are stimulated to synthesize and secrete IgE (Roitt et

al. 1989a). Currently, the role of T lymphocytes in the pathogenesis of allergic asthma is receiving considerable attention. The process of IgE production is enhanced by T-helper cells and controlled by T-suppressor cells (Ricci et al. 1987).

T lymphocytes are not only involved in the mechanism of allergic asthma by their regulation of IgE production, but potentially also by their production of lymphokines. Interleukin-3 (IL-3), IL-4 and IL-5 from T lymphocytes act as growth factors for mast cells and basophils (Ihle et al 1983; Hamaguchi et al. 1987). IL-3 and IL-5 can initiate and support eosinophil differentiation (Campbell et al. 1987; Sanderson et al. 1985; Vadas et al. 1983).

It has been reported that after exposure to an allergen, T lymphocytes from the blood of an asthmatic patient can produce a large amount of IL-2 which is a potent T-lymphocyte growth factor (Hsieh 1985). The effects of IL-2 have been studied in rats. In both Lewis and Brown Norway rats, injections of IL-2 significantly increase the percentage of lymphocytes in the BAL fluid. In Lewis rats, IL-2 treatment significantly enhances airway responsiveness to inhaled aerosolized methacholine (Renzi et al. 1989). Interestingly, after allergen inhalation, the magnitudes of both early and late airway responses in allergic Brown Norway rats are significantly higher in IL-2 treated rats compared with controls (Renzi et al. 1990). These results indicate that T-lymphocytes have the potential to play an important role in the pathogenesis of allergic asthma.

It has been documented that stimulated neutrophils can produce many important mediators such as prostaglandins (Goldstein et al. 1987), leukotrienes (Lee et al. 1983) and PAF (Lotner et al. 1980). However, neutrophils do not have IgE receptors on their membrane surface (Capron et al. 1984). It is unlikely that allergen can directly activate neutrophils to release such mediators. In asthmatic patients, after allergen challenge, late airway responses (LR) are accompanied by elevated neutrophils and eosinophil levels in BAL fluid. Neutrophil infiltration into the lung appears much earlier than eosinophils (Metzger et al. 1986). Neutrophilic infiltration into the lungs following allergen challenge has been also demonstrated in the guinea pig (Hutson et al. 1988) and rabbit models (Marsh et al. 1985). However, depletion of neutrophils can only prevent LR in the rabbit (Murphy et al. 1986), but not in the guinea pig (Hutson et al. 1990). Whether the neutrophil is contributing to human allergic asthma is still unclear.

Platelets can produce and release platelet-activating factor (PAF; Chignard et al. 1979; Benveniste et al. 1982), However the possible role of platelets in the pathogenesis of allergic asthma is also uncertain.

1.4. MEDIATORS INVOLVED IN THE PATHOGENESIS OF ALLERGIC ASTHMA

As I discussed in the previous section, diverse cellular populations appear to be involved in allergic asthma. The contribution of the cells to allergic asthma is mainly due to their

production of chemical mediators. The potential importance of several major mediators such as leukotrienes, platelet-activating-factor, histamine, prostaglandins and thromboxanes has been well documented in the literature. The components uniquely produced by different cells, such as eosinophil derived major basic protein, have already been mentioned in relation to their cells in origin (Reviewed in 1.3.2.). I shall focus on the major biological effects of the most important mediators which may be involved in the pathological changes of allergic asthma, including leukotrienes, platelet-activating-factor, histamine, prostaglandins and thromboxanes. Other mediators such as serotonin (Popovich et al. 1988), or minor mediator metabolite subgroups, such as 9a-11b-PGF_2 derived from PGD_2 (Beasley 1987) and 15-HETE, 12-HETE, 5-HETE derived from the lipoxygenase pathway (Yanni et al. 1989), may also play some role in the pathogenesis of allergic asthma. Since the biological activities of these compounds are less well documented, they will not be considered in this section.

1.4.1. Leukotrienes

Leukotrienes (LTs) were named by Samuelsson and his co-workers in the early of 1980s (Samuelsson 1983). These substances are biologically active fatty acids derived from arachidonic acid. Arachidonic acid is derived from cell membrane phospholipids. Two enzymes, phospholipase A_2 and phospholipase C, are involved in the production of arachidonic acid. Once released from membrane phospholipids, arachidonic acid (AA) may undergo oxidative

metabolism by cyclooxygenase to form prostaglandins and thromboxanes or by a series of lipoxygenase enzymes to form leukotrienes (Samuelsson 1983).

Leukotrienes are generated and released by mast cells (Peters et al. 1984; Mencia-Huerta et al. 1983), eosinophils (Weller et al. 1983), macrophages (Fels et al. 1982; Wasserman 1986) and neutrophils (Lee et al. 1983). They can be operationally divided into two groups: LTB_4 and its isomers and metabolites; and the sulfidopeptide leukotrienes, including LTC_4, LTD_4 and LTE_4. The sulfidopeptide leukotrienes were previously known as "slow reacting substance of anaphylaxis (SRS-A)".

Currently, it has been documented that LTs play an important role in the pathogenesis of allergic asthma and other allergic diseases. LTB_4 is one of the most potent chemoattractant substances for human eosinophils (Nagy et al. 1982), polymorphonuclear leukocytes (Ford-Hutchinson et al. 1980) and monocytes (Palmer et al. 1980). Sulfidopeptide leukotrienes may cause airway smooth muscle contraction, increased mucus secretion, increased microvascular permeability and retarded mucociliary clearance (Cross 1986).

In allergic asthmatics, the concentrations of sulfidopeptide leukotrienes and leukotriene B_4 in the bronchoalveolar lavage fluid are significantly higher than in normal subjects (Pliss et al. 1989; Wardlaw et al. 1989; Taylor et al. 1989). Urinary LTE_4 is increased in allergic asthmatics after allergen challenge (Taylor et al. 1989).

Pharmacologic experiments suggest that peptide leukotrienes mediate allergic bronchoconstriction in the sheep and the rat. An orally administrated LTD_4/LTE_4 antagonist, LY171883 can significantly reduce the early response and block the late response (Abraham et al. 1986). In sensitized Brown Norway rats, pretreatment with LTD_4 antagonists reduces allergen-induced early response and completely inhibits late responses (Sapienza et al. 1990).

In the allergic sheep model, inhaled LTD_4 can induce both early and late responses (Abraham et al. 1985). LTD_4 not only contributes to allergen-caused dual responses, but also contributes to airway hyperreactivity. Recently, it has been reported by Kaye and Smith that inhaled LTD_4 induced airway hyperresponsiveness to methacholine in six of eight normal human subjects (Kaye and Smith 1990).

Based on the above reports, it is likely that LTs are important mediators of allergic asthma.

1.4.2. Platelet-Activating Factor

Platelet-activating factor (PAF or PAF-acether) is a unique phospholipid substance. It was initially isolated from rabbit basophils and it is capable of aggregating rabbit platelets (Benveniste et al. 1972; Cazenave et al. 1979).

PAF is derived from membrane phospholipids. The biosynthetic pathway for PAF has been established. Several enzymes may be involved in the synthesis of PAF. Two principal enzymes are

phospholipase A_2 and acetyltransferase. Activated phospholipase A_2 acts on the phospholipids to produce lyso PAF-acether. As an intermediate precursor, lyso PAF-acether is then acetylated to form PAF acether (Arnoux et al. 1987; Braquet et al. 1987). PAF can be produced and released by several cell types, such as mast cells (Camussi et al. 1977), eosinophils (Lee et al. 1984), macrophages (Arnoux et al. 1980), neutrophils (Lotner et al. 1980) and platelets (Chignard et al. 1979).

In sheep and normal human subjects, PAF can induce bronchoconstriction (Soler et al. 1990; Abraham et al. 1989; Adamus et al. 1990). In sheep (Soler et al. 1990) and guinea pigs (Robertson et al. 1988), PAF may also cause airway hyperreactivity. The results from different laboratories are somewhat contradictory concerning the induction of hyperresponsiveness in human subjects. Kaye and Smith (1990) reported that inhaled PAF could induce airway hyperreactivity in normal human subjects. However, evidence from other investigators does not support this conclusion (Hopp et al. 1989; Lai et al. 1990).

In allergen induced bronchoconstriction, PAF may be more closely associated with late but not early airway responses. In sheep, a PAF antagonist only blocks the allergen induced late response but does not block the early response (Tomioka et al. 1989).

It is still not clear if PAF has a direct effect on airway smooth muscle. In vitro PAF and platelets can cause canine

tracheal muscle contraction, but PAF alone does not have this action. The PAF- and platelet-induced muscle contraction can be reversed by a serotonin antagonist (Popovich et al. 1988). It has been reported that PAF can induce the release of LTB4 from polymorphonuclear leukocytes in human and rabbit (Chilton et al. 1982; Lin et al. 1982). In allergic sheep, PAF aerosol induced bronchoconstriction can be blocked by a leukotriene antagonist (Abraham et al. 1989), suggesting that PAF causes bronchoconstriction indirectly through the action of leukotrienes from different cellular populations.

It has been reported previously that PAF could cause the release of prostaglandins from alveolar macrophages in mice (Poitevin et al. 1984). However, at least in sheep (Abraham et al. 1989) and human subjects (Smith et al. 1988), PAF induced bronchoconstriction is not inhibited by a cyclooxygenase blocker (indomethacin), suggesting that products from the cyclooxygenase pathway are not involved in PAF induced bronchoconstriction.

It has been also demonstrated that subcutaneously injected PAF is able to cause plasma exudation in the human, rat and guinea pig (Pirotzky et al. 1984; Humphrey et al. 1982). In guinea pigs, both inhaled and intraperitoneally injected PAF can induce pulmonary eosinophilia (Coyle et al. 1988; Sanjar et al. 1989). An in vitro study showed that in guinea pig, PAF-activated eosinophils, but not PAF alone may lead to airway epithelial disruption (Yukawa et al. 1990). These results support the idea that PAF may be related to other pathological alterations in

allergic asthma, such as edema, epithelial damage and inflammatory cell infiltration.

PAF is a potent mediator of mucus secretion from porcine trachea (Steiger et al. 1987). The induction of mucus secretion is a further reason to believe that PAF may be contributing to the pathogenesis of allergic asthma.

Finally other PAF-induced effects that may also contribute to the pathogenesis of allergic asthma, include neutrophil aggregation (Camussi et al. 1981), chemotactic activation (Czarnetzki and Benveniste 1981), lysosomal enzyme and oxygen radical release (O'Flaherty et al. 1981; Poitevin et al. 1984).

1.4.3. Histamine, Prostaglandins and Thromboxanes

Histamine is one of the important substances associated with mast cells and basophils (Bhat et al. 1976; Ishizaka et al. 1972). Histamine is produced by the action of histidine decarboxylase (Campbell and Smith 1988). This mediator is intracellularly bound to proteoglycan, heparin in mast cells and chondroitin sulfate in basophils. The concentration of histamine is approximately 2-10 pg per mast cell and 1pg per basophil, respectively (Metcalf et al. 1979; Schulman et al. 1983; Wasserman 1983). The interaction between antigen and specific IgE FcR1 can trigger mast cells to release their mediators, including histamine (Ishizaka 1981).

The effect of histamine is through its action on specific receptors. Three different types of histamine receptors are

present on different tissues, H_1, H_2 and H_3 receptors (Black et al. 1977; Ishikawa and Sperelakis 1987). In the human respiratory system, one of the effects of histamine is to induce airway smooth muscle contraction and inhaled histamine can induce bronchospasm (Curry 1946; Cockcroft 1985a; Nelson 1983). Histamine causes airway smooth muscle contraction through the interaction with the H_1 receptor (Rosa et al. 1951; Douglas 1985). On the other hand, histamine induced airway smooth muscle contraction may also result from its ability to promote prostaglandin release (Platshon and Kaliner 1978).

A second major effect of histamine is to induce microvascular leakage through the H_1 receptor. Studies on both human nasal mucosa and dog lung suggest that histamine may increase microvascular permeability (Propst et al. 1978; Raphael et al. 1986).

Histamine can also contribute to mucous hypersecretion in the airways. In vitro studies have shown that histamine is able to induce mucous glycoprotein release from cultured human bronchi. This effect can be inhibited by cimetidine, a H_2 antagonist (Shelhamer et al. 1980). It suggests that histamine stimulates mucus secretion through its H_2 receptor.

It is still not clear if the interaction between histamine and its H_3 receptor is involved in the mechanism of human allergic asthma. As reported recently by Ichinose et al. (1990), H_3-agonists can inhibit neurogenic microvascular leakage in guinea pigs.

Both prostaglandins (PGs) and thromboxanes (Txs) are

derived from arachidonic acid through the cyclooxygenase pathway. The initial product in prostaglandin metabolism from arachidonic acid is PGG_2, which is converted to PGH_2 by hydroperoxidase. Through different enzymatic or nonenzymatic procedures, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin) and TXA_2 are formed from PGH_2. PGD_2 and $PGF_{2\alpha}$ are bronchoconstrictors. PGE_2 and PGI_2 are bronchodilators (Henderson 1987).

Prostaglandins and thromboxanes can be produced by the mast cell (Lewis et al. 1982; Schulman et al. 1981), macrophage (Rouzer et al 1982b), neutrophil (Goldstein et al. 1987), eosinophil (Horn et al. 1975) and platelets (Hamberg and Samuelsson 1974).

PGD_2 is of particular interest because it is a mast cell product. In humans, acute antigen challenge causes the release of PGD_2 into the airways (Murray et al. 1986). Furthermore, inhaled PGD_2 may induce bronchoconstriction and airway hyperreactivity (Fuller et al. 1984; Hardy et al 1984). PGD_2 is able also to stimulate bovine tracheal glands to secrete mucus (Sommerhoff et al. 1987).

It has been reported that thromboxane A_2 has been implicated in the late cutaneous allergic reaction (Dorsch et al. 1983) and a metabolite of TxA_2, 12-(S)-hydroxy-5,8,10- heptadecatrienoic acid (HHT) is a weak chemoattractant for leukocytes (Hamberg and Samuelsson 1974). However, whether Txs are involved in the pathogenesis of allergic asthma is unknown.

Currently, the effects of cycloxygenase products in the

pathogenesis of allergic asthma has been largely discounted. In asthmatic patients, orally administered indomethacin, an antagonist of cycloxygenase inhibits airway hyperresponsiveness, but it does not block both early and late responses after allergen inhalation (Kirby et al. 1989).

1.5. DIAGNOSIS OF ALLERGIC ASTHMA

The diagnosis of allergic asthma is based mainly on the demonstration of reversible airway obstruction. The patient's history is very important. The appearance of symptoms is often seasonal or related to exposure to certain materials, such as chemicals, pets and foods. A family background of asthma or allergic diseases also suggests the diagnosis (Wahn 1987; Williams and Shim 1985).

The symptoms of allergic and nonallergic asthma do not differ. Cough and wheeze are the most common symptoms, followed by dyspnea and chest tightness (Daniele 1982; Fry and Sandler 1988; Williams and Shim 1985).

Bronchial provocation tests are very often used to confirm the diagnosis of allergic asthma. Currently, most allergen extracts are commercially available, such as pollens, dusts, molds and animal danders. If the disease is caused by a common allergen, inhalational challenge with aerosolized allergen can be used to evoke airway responses. Allergen challenge by the inhalational route is usually reserved for cases of occupational asthma where identification of the causitive allergen requires a change of work.

The avoidance of specific allergen exposure benefits such patients. Since allergic asthmatics usually have an increased airway responsiveness to many nonspecific stimuli, nonspecific airway hyperreactivity is tested by inhalational challenge with several chemicals. Histamine and methacholine provocation challenges are most often used. The dose response curve of the chemicals reflects the degree of airway hyperreactivity in each patient (Spector 1985 and 1987).

Since IgE plays an important role in allergic asthma, serum IgE level is generally increased in allergic asthmatic patients (Witting et al. 1978).

1.6. OBJECTIVES OF THESE STUDIES

Allergic asthma, a common disease, has been studied for many years, However, a clear picture of the pathogenesis of allergic asthma has never been drawn. The basic mechanical abnormality of allergic asthma is an increased airway resistance which is due to airway narrowing (Macklem 1988). Theoretically, airway narrowing may be attributed to the airway smooth muscle contraction, airway wall thickening caused by edema and inflammation, and mucus (Moreno et al. 1986). One of important features of allergic asthma is allergen induced early and late responses. It is still unknown which is the most important factor and where is the predominant site for the airway narrowing during the allergen induced early and late airway responses. The understanding of mechanical contributors and the location of airway narrowing in the airway tree during the

early and late response may benefit the prevention and treatment of this disease.

On the other hand, the biological functions of mast cells have caused them to be considered as an primary trigger cells for the pathogenesis of allergen induced airway narrowing. The degranulation of mast cells during the early response and late responses have been reported (Holgate 1988; Metzger et al. 1987; Keyer et al. 1984). In allergic sheep, stimulation of mast cell degranulation by inhaled compound 48/80 produces both early and late responses (Russi et al. 1984). However, there are no studies which address the relationship between the distribution of mast cell in the airway tree and the location of airway narrowing during the early and late airway responses.

Another important feature of allergic asthma is airway hyperesponsivness to different stimuli. The mechanism of airway hyperreactivity is unknown. The reversibility of airway narrowing to the constrictive stimuli as well as increased amount of airway smooth muscle suggest the contribution of airway smooth muscle in the mechanism of airway hyperreactivity (Macklem 1988; Heard and Hossain 1973; Kleinerman and Adelson 1987). However, there is no animal model to study this particular feature of allergic asthma and the relationship between the increased amount of airway smooth muscle following allergen challenge and airway hyperreactivity has not yet been reported.

The aims of the experiments of my thesis are to study the mechanisms of allergen induced airway narrowing and airway

hyperresponsiveness using the Brown Norway rat model. The studies were undertaken to investigate the following questions:

(1) What are the morphometric changes during the allergen induced early and late responses in sensitized Brown Norway rats?

(2) What is the relationship between the site of airway narrowing and mast cell distribution in the airway tree during the allergen induce early and late responses in sensitized Brown Norway rats?

(3) Does microvascular permeability increase during the allergen induced early and late responses in sensitized Brown Norway rats?

(4) What is the relationship between airway structural changes and airway hyperresponsiveness in sensitized Brown Norway rats following repeated allergen challenges?

The experimental results in the thesis are presented in Chapters II to V. Each chapter contains abstract, introduction, materials and methods, results, discussion as well as quoted references.

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CHAPTER II

MORPHOMETRY OF THE AIRWAYS DURING LATE RESPONSES TO ALLERGEN CHALLENGE IN THE RAT

2.1. ABSTRACT

To quantitate the structural changes in the airways that contribute to the late bronchial response (LR) to allergen challenge we killed six Brown-Norway rats, sensitized to ovalbumin (OA) and challenged by aerosol, during the LR and compared the dimensions of the intraparenchymal airways to those of six control animals. Lungs were rapidly frozen with liquid nitrogen and fixed in Carnoy's solution. Paraffin sections were stained with hematoxylin-phloxine-saffron. At the time of the LR (382 $\pm$ 39 mins after OA challenge), pulmonary resistance (R_L) increased from the baseline value (0.067 $\pm$ 0.034 $cmH_2O.ml^{-1}.s$) by 0.107 $\pm$ 0.03 $cmH_2O.ml^{-1}.s$ ($p<0.05$). R_L did not change significantly in the control rats. The lumen size and the wall area of all membranous airways were measured and were corrected for airway size by dividing by the basement membrane length squared (BM^2). There was no increase in airway wall area in OA challenged animals. However, the lumen of large airways (BM: 2.0 - 2.99 mm) was significantly less for the OA-challenged animals (0.039$\pm$0.0055mm^2) than for the control animals (0.058$\pm$0.0063 mm^2 $p<0.05$). In six additional rats, the distribution of mast cells (MC) in the bronchial tree was determined. Tissues were fixed with Carnoy's solution and stained with a modified May-Grunwald Giemsa stain. There were significantly more MC in the large airways than in medial or small airways. We conclude that smooth muscle constriction of large airways and not airway wall edema accounts for the LR in the rat. The distribution of the mast cells corresponds closely to the site

of bronchoconstriction.

2.2. INTRODUCTION

Two distinct responses of the airways of sensitized subjects to inhaled allergen have been recognized, namely, early and late responses. Early airway responses (ERs) appear within 10-15 mins after the allergen exposure and resolve spontaneously within 1 to 3 hrs (O'Byrne et al. 1987). Late airway responses (LRs) may develop from 4 to 12 hrs after allergen inhalation and last for more than 24 hrs (Hargreave et al. 1974; Cartier et al. 1982; Booij-Noord et al. 1972; Pelikan et al. 1986). LRs are found not only in humans but in other animals. To date such responses have been demonstrated in sensitized rabbits (Shampain et al. 1982), sheep (Abraham et al. 1983), guinea pigs (Brattsand et al. 1985; Iijima et al. 1987; Hutson et al. 1988a), squirrel monkeys (Hamel et al. 1986), and rats (Eidelman et al. 1988) undergoing allergen provocation. Because the LR frequently is associated with an increase in airway responsiveness (Altounyan 1970; Cockcroft 1977), it is of particular importance to understand its pathogenesis.

The early response to allergen, which is caused by IgE mediated triggering of mast cells, is easily inhibited by β-agonists and disodium cromoglycate (Booij-Noord et al. 1972; Pepys and Hutchcroft 1975; Booij-Noord et al. 1971). The LR is not well antagonized by β-agonists but is reduced by corticosteroids (Booij-Noord et al. 1971). In contrast to the ER, the

participation of mast cells in the LR is not clearly defined. The refractoriness of the LR to bronchodilators has led to the suggestion that the LR is caused by airway inflammation with edema, rather than by bronchospasm (O'Byrne et al. 1987). There are several potentially important pathological alterations that could cause airway obstruction following airway challenge with allergen. Mucous secretions, airway smooth muscle spasm, and increase in the thickness of the airway wall as a result of inflammatory cell accumulation and edema could singly or in combination account for all of the physiologic changes.

The potential importance of structural changes of the airway wall to airway resistance has been pointed out by Moreno et al. (Moreno et al. 1986). Although not necessarily related to allergy, airway wall thickening has been described in asthmatic subjects (James et al. 1989) and could contribute importantly to airway narrowing by amplifying the changes in resistance for any given degree of airway smooth muscle contraction (James et al. 1989). The primary objective of our study was to identify and quantitate the structural changes in the airways that might contribute to the LR. Experiments were performed on actively sensitized Brown Norway rats, which develop early and late airway responses in high prevalence following allergen challenge (Eidelman et al. 1988). Experimental rats were killed during late airway responses and the lungs were rapidly frozen using liquid nitrogen in an attempt to retain the structural and functional state of the airways as close as possible to that in vivo. Morphometric measurements were

performed on lungs from test animals and also on those from control animals that were killed at identical time points. A secondary objective was to relate the morphometric changes associated with the late response to the distribution of airway mast cells. To do this we quantitated mast cells as a function of airway size.

2.3. MATERIALS AND METHODS

2.3.1. Animals and sensitization:

Sixteen Brown Norway rats 6 to 7 weeks of age and weighing from 130 to 180 g, (Charles River, St. Constant, Quebec, Canada) were housed in a conventional rat colony in the McGill University Animal Centre. Animals undergoing allergen challenge were actively sensitized with a single subcutaneous injection of 1 ml of a sterile suspension of 1 mg ovalbumin (OA) and 200 mg of aluminium hydroxide in 0.9% saline. At the same time, 1 ml of Bordetella pertussis vaccine containing 6 x 10^9 heat-killed bacilli was given intraperitoneally as an adjuvant.

2.3.2. Measurement of airway responses:

Fourteen to eighteen days after sensitization, rats were anesthetized with urethane (1.1 g. kg^{-1} intraperitoneally). Blind orotracheal intubation was performed using a 6-cm-long polyethylene catheter (PE 240). Animals were placed on a heating blanket and rectal temperature was monitored with an electronic thermometer throughout the experiment. The lateral decubitus posture was alternated every hour. Airway responses were determined from

measurements of pulmonary resistance (R_L), using methods that we have previously described in detail (Eidelman et al. 1988). Briefly, the end of the endotracheal tube was inserted into a small Plexiglas® box (volume: 265ml) for the delivery of aerosols and for the measurement of airflow. A Fleisch no. 0 pneumotachograph coupled to a differential pressure transducer (MP 45±2cm H_2O; Validyne Corp., Northridge, CA) was attached to another end of the box. Changes in esophageal pressure were measured using a saline-filled catheter and differential pressure transducer (Sanborn 267 BC; Hewlett-Packard, Waltham, MA). Another port of the transducer was connected to the box. The esophageal catheter consisted of polyethylene tube (PE 240) 20 cm long attached to a shorter length tubing (6 cm; PE-100) that was advanced into the esophagus of the rats until a clear cardiac artifact was discernible. Transpulmonary pressure (Ptp) was computed as the difference between esophageal and box pressure. Pulmonary resistance was calculated using multiple linear regression by obtaining the best fit for the equation: $P_u=\dot{V}R_L+VE_L+K$. where P_u is tracheal pressure, $\dot{V}$ is flow, V is volume, R_L is pulmonary resistance, E_L is elastance and K is a constant (Bates et al. 1989). The resistance of the endotracheal tube was measured as 0.11 cm H_2O/ml/s. R_L is reported after subtraction of tube resistance.

Aerosol was generated using a Hudson nebulizer with an output of 0.18 ml/min connected to one side port of the box. During the aerosolization, the pneumotachograph was occluded and airflow was

diverted through a second side port. The box was flushed with a stream of fresh air between each measurement in order to prevent the accumulation of CO_2.

We defined an early airway response (ER) as an increase in R_L to at least 150% of the post-saline value within 30 min of challenge. A late airway response (LR) was considered as an increase in R_L to at least 150% of the post-saline value for a minimum period of 45 min between 3 and 8 hrs after OA challenge. Animals were studied in pairs, one receiving a challenge with OA (test animal) and the other receiving a saline aerosol (control). When a LR was identified in the test animal, both the test animal and the corresponding control were killed.

2.3.3. Tissue Preparation:

Anesthetized animals were killed by opening the abdominal cavity and by cutting the aorta, the inferior vena cava and the portal vein. The trachea was clamped at end-expiration and the thorax was opened through the diaphragm. The lungs were removed intact, immediately immersed in liquid nitrogen for 30 minutes and subsequently fixed in Carnoy's solution at 4°C. After two hours of fixation, the lungs were transferred to cold absolute alcohol for 12-16 hr. Paraffin blocks were made from mid-sagittal slices of the lungs. Sections of 5 microns in thickness were cut and stained with hematoxylin-phloxine-saffron (HPS).

2.3.4. Morphologic and Morphometric Studies:

HPS-stained sections from both the left and right lungs were examined by light microscopy. Measurements were made by either projecting photographic slides of small airways or projecting large airways directly from the glass slide using a microprojector (Zeiss, W. Germany) onto a computer-controlled digitizing board (Jandel Scientific, Corte Madera, CA), respectively. A digitizing program (Sigma Scan, Jandel Scientific, Corte Madera, CA) was used for all measurements. Because measurements were made on mid-sagittal sections, relatively few cartilaginous airways were observed, and we therefore confined the analysis to membranous airways. Extrapulmonary airways were not studied because of potential artifactual distortion of these airways caused by handling of the tissues. Airways whose ratio of maximal to minimal internal diameter was equal to or larger than 2 were considered to be cut obliquely and were not measured. The remaining airways were evaluated for the following: 1) the airway lumen both defined as the area circumscribed by the luminal surface of the airway epithelium (LuE), and the area circumscribed by the basement membrane of epithelium (LuB), 2) the length of the airway basement membrane (BM), and 3) the area defined by the outer border of the airway wall (Ae). The area of the airway wall (AW) and the area of airway epithelium (Ep) were calculated as the difference between Ae and LuB and LuB and LuE, respectively.

Light microscopy revealed areas of atelectasis in both control and test animals. The possible association between atelectasis and airway obstruction caused by the LR prompted us to quantitate the

degree of atelectasis. This was done by determining the percentages of the sections occupied by lung tissue and air, respectively, using a computer image analysis program developed by Dr. Harold Riml of the Medical Physics Unit, McGill University. Briefly, the microscopic images were captured by a video camera (Newvicon, Panasonic PV 1450), digitized by a real time frame grabber (Coreco Oculus 200) and stored in a microcomputer (IBM PC AT). Lung parenchyma and airspace could be identified by characteristic grey levels. The proportion of the image occupied by lung parenchyma or airspace was quantitated by counting the number of picture elements with the corresponding grey level. At least 44 areas from each section of lung representing between 82 mm^2 and 150 mm^2 were analyzed.

In order to examine the relationship between mucus within the airways and the LR, we counted the number of airways that were positive for mucus and expressed the result as a function of the total number of airways.

2.3.5. Quantitation of mast cells:

Six 8-10 week old Brown Norway rats were used to study the distribution of mast cells in the bronchial tree. Animals were anesthetized, the lungs were removed, and fixed with Carnoy's solution under 25 cmH_2O pressure and processed as above. Paraffin embedded 5 micron sections were stained with a modified May-Grunwald Giemsa stain. We examined a midsagittal and a parahilar section of lung from each animal. The numbers of mast

cells within the airway walls were counted for all intraparenchymal airways using oil immersion microscopy and the length of the basement membrane was measured by digitization.

2.3.6. Drugs and chemicals:

Ovalbumin and urethane were purchased from Sigma Chemicals (St. Louis, MO), Bordetella pertussis vaccine from Connaught Laboratories (Willowdale, Ontario), aluminum hydroxide from Anachemia Chemical Ltd. (Montreal, Quebec), Jenner and Giemsa stains from BDH Chemicals (Montreal, Quebec).

2.3.7. Data Analysis:

All morphometric measurements were made by a single observer (T.D.) blinded to the group status. Airways were divided into three groups based on the length of the basement membrane: small (BM: 0-0.99 mm), medium (BM: 1.0-1.99 mm) and large (BM: 2.0-2.99 mm). Since the largest membranous airways (BM > 3.0 mm) were not always present in all of the lung sections, the occasional airway that had a BM in excess of 3 mm was excluded from analysis. We analyzed an average of 26 airways/animal (range, 17 to 33). LuB, Ep and AW from each airway were normalized to the BM^2 to adjust for differences in airway size. The mast cells counts in the intraparenchymal airways were expressed as a function of BM length to correct for differences related to airway size. Comparisons of means between experimental and control groups were made by t-tests for unpaired data. Linear regression analysis was

performed by the method of least squares; p values <0.05 were considered significant. Data are expressed throughout as mean ± SE.

2.4. RESULTS

2.4.1. Early and late changes in R_L after allergen:

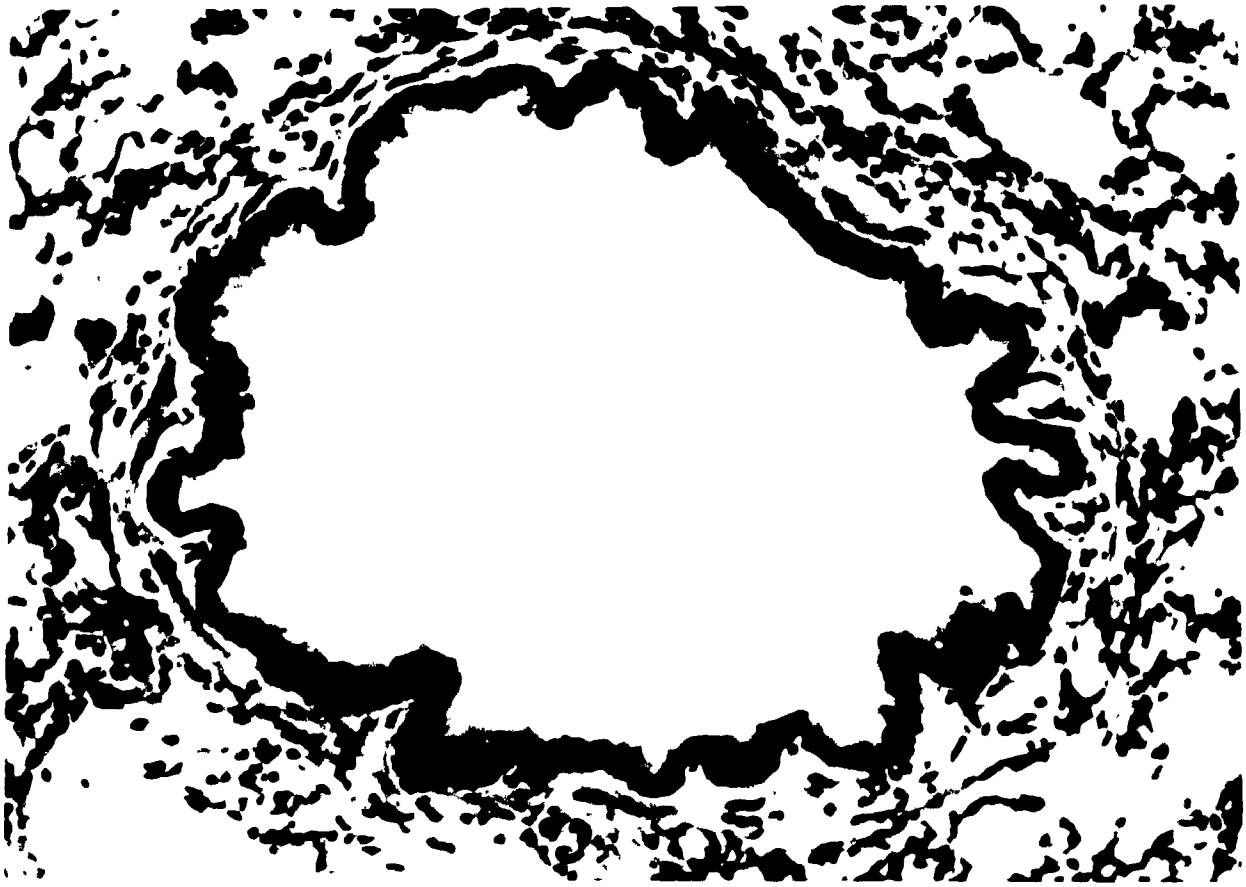
Early responses (ER) appeared in all eight test animals after OA challenge and reached a value of 327±47% of the baseline R_L (0.067 ± 0.034 $cmH_2O.ml^{-1}.s$). Six of the eight animals demonstrated LRs which reached a peak value of 0.107 ± 0.03 $cmH_2O_2ml^{-1}.s$ greater than baseline R_L ($p<0.05$). At least three elevated values of R_L were required to satisfy the a priori definition of a LR and R_L declined prior to sacrifice in some animals. Therefore, the mean R_L at the time of sacrifice was slightly lower. The remaining two tests rats did not show a LR and were excluded from analysis, along with their matched controls.

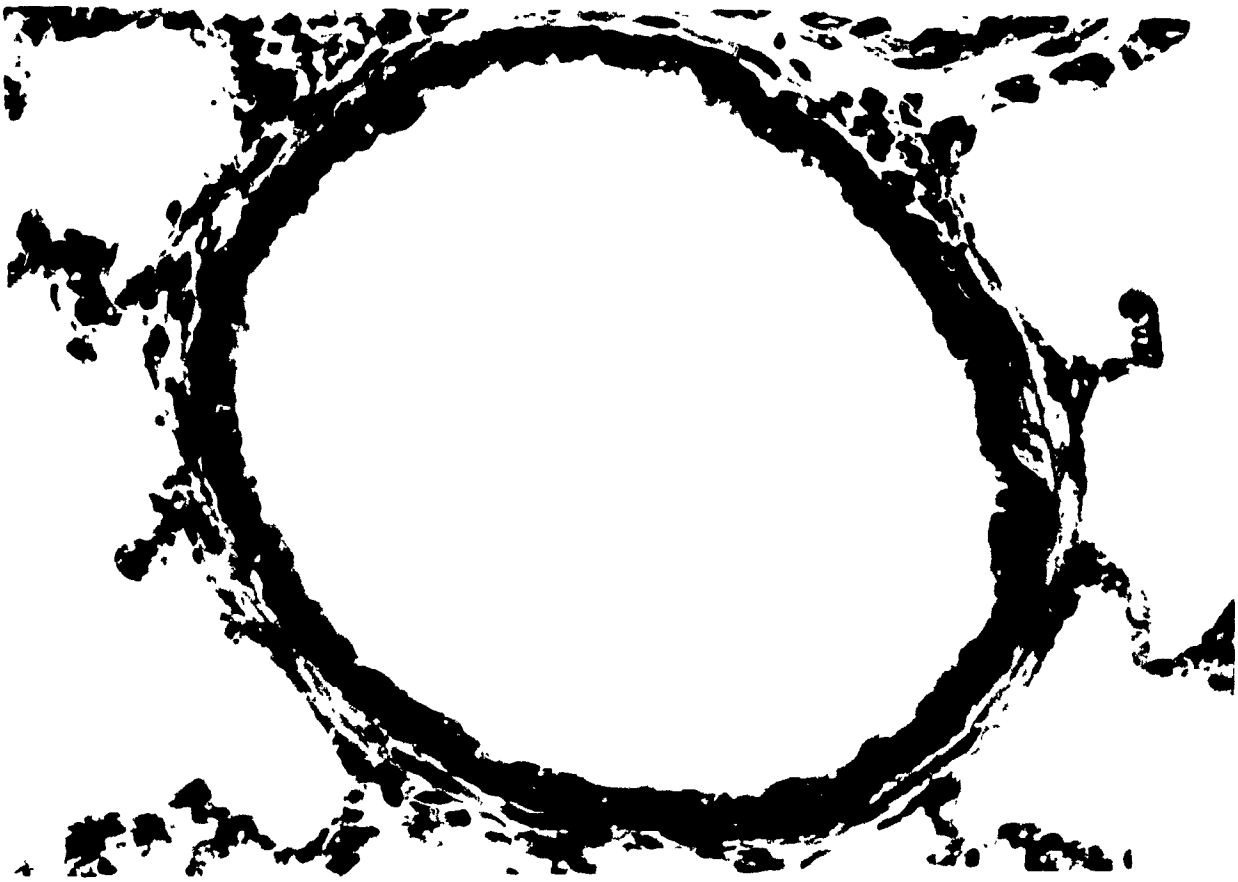
2.4.2. Light Microscopic Observations:

The degree of lung inflation was grossly similar in both the test and control animals. However, a few microscopic foci of atelectasis were evident on most sections from both test and control rats. Most airways were patent and free of secretions. Occasionally, a small amount of mucus was present on the surface of the epithelium, but it was seen in animals from both test and control groups. Mucous plugs were not observed. Alveolar spaces were clear. There was a mild cellular infiltration of the airway

Fig. 2.1a. Photomicrograph of an airway from a test rat which shows an infolding of the epithelium (HPS stain; x150).

Fig. 2.1b. Photomicrograph of an airway from a control rat that shows a smooth epithelium (HPS stain; x150).





walls in the test animals which was composed of both mononuclear and polymorphonuclear cells.

A representative photomicrograph of a larger airway from a test rat is shown in figure 2.1a. The epithelium of this airway is infolded. In contrast, the epithelium of an airway from a control rat is smooth (Figure 2.1b). The infolding of the epithelium of the airway from the test animal indicates airway narrowing.

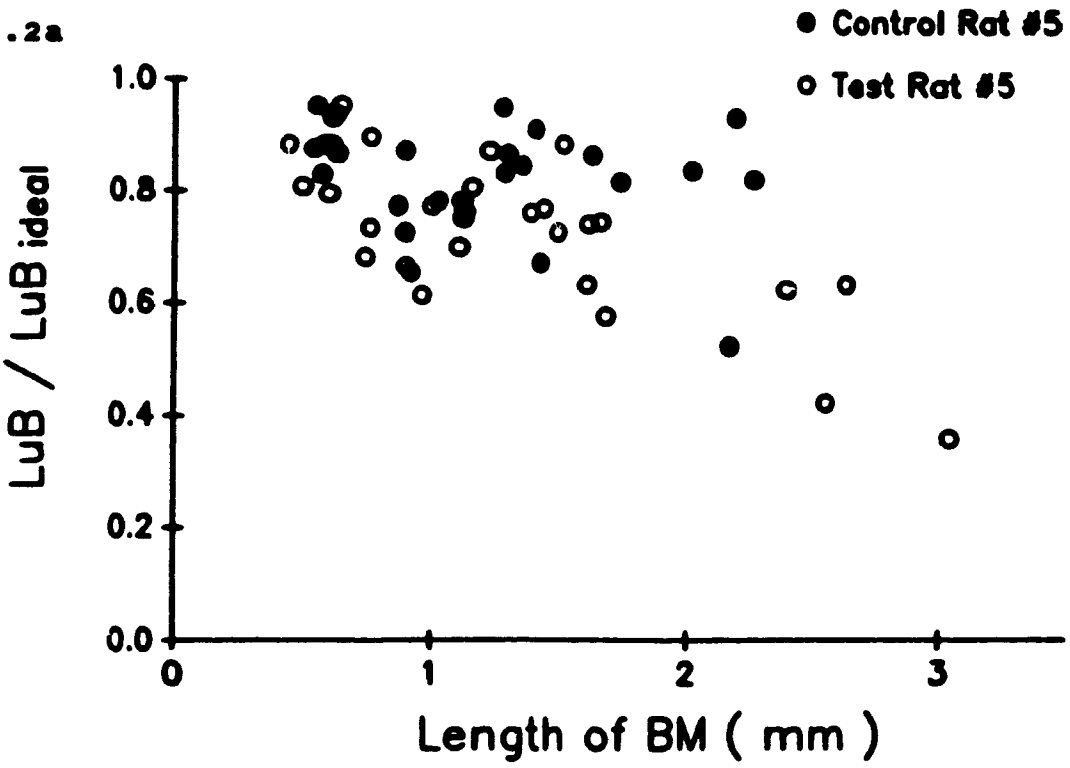
2.4.3. Morphometric Findings:

Illustrative findings for two of the test rats (Rats 5 and 6) and their matched controls are shown in figures 2.2a and 2.2b. The test rats both had narrower airways than the controls as indicated by the fact that the measured lumens (LuB) expressed as a fraction of the unconstricted ideal lumen (LuB_{ideal}) were smaller in test than in control animals. The ideal lumen of each airway was calculated from the basement membrane, assuming that the unconstricted airway was a perfect circle. There was considerable overlap in data for small airways (BM < 1.0 mm) between test and control animals so that differences were apparent only for airways larger than 1 mm.

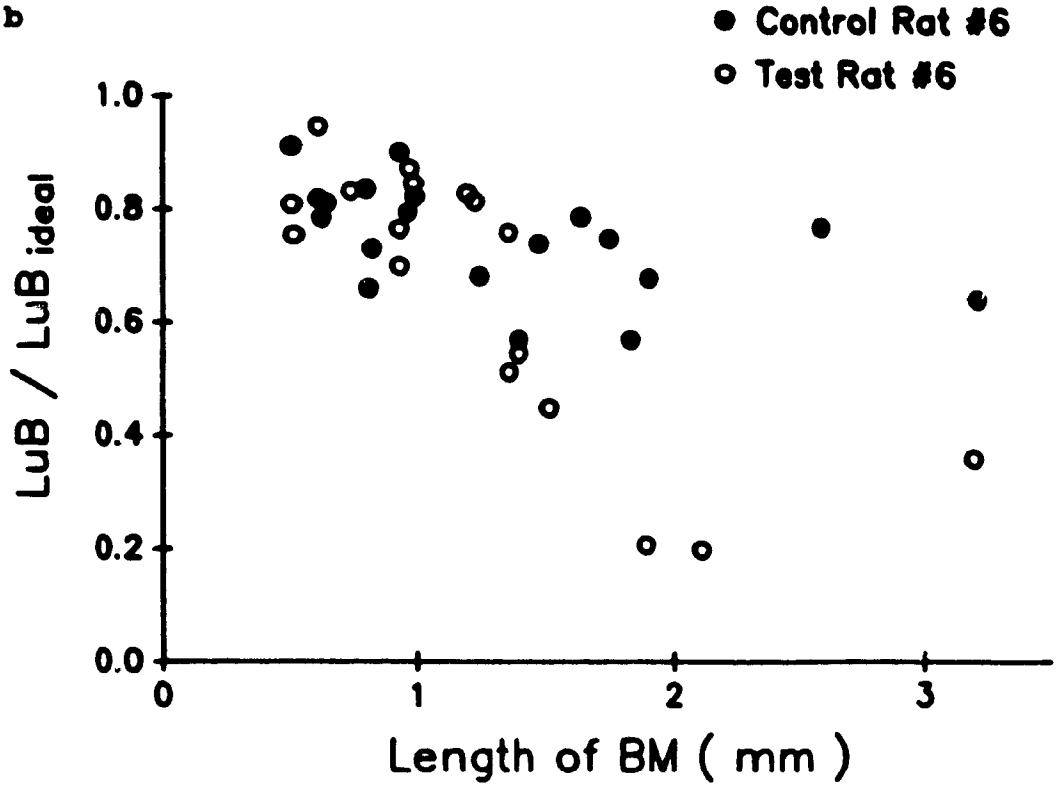
Similar to figure 2.2, the mean values of LuB for the small membranous airways (BM 0-0.99 mm) was identical for both test and control animals (figure 2.3). There was a small but non-significant difference between the LuB for medium sized airways in test and control animals. However, there was substantial narrowing of the large airways in test rats; LuB was 0.039 ± 0.006 mm^2 for test

Fig. 2.2a and 2.2b. The airway lumens (LuB) are expressed as a ratio of the unconstricted ideal lumen (LuB_{ideal}) assuming that the airway is a perfect circle. Two test (open circles) and two control rats (closed circles) are shown. There is a fall in the LuB/LuB_{ideal} with increasing BM length in the both test rats indicating narrowing of the airways as a function of size. Figure 2.2a.: Rat 5; figure 2.2b.: Rat 6.

2.2a


Control Rat #5
Test Rat #5
LuB / LuB ideal
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
Length of BM (mm)

2.2b


Control Rat #6
Test Rat #6
LuB / LuB ideal
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
Length of BM (mm)

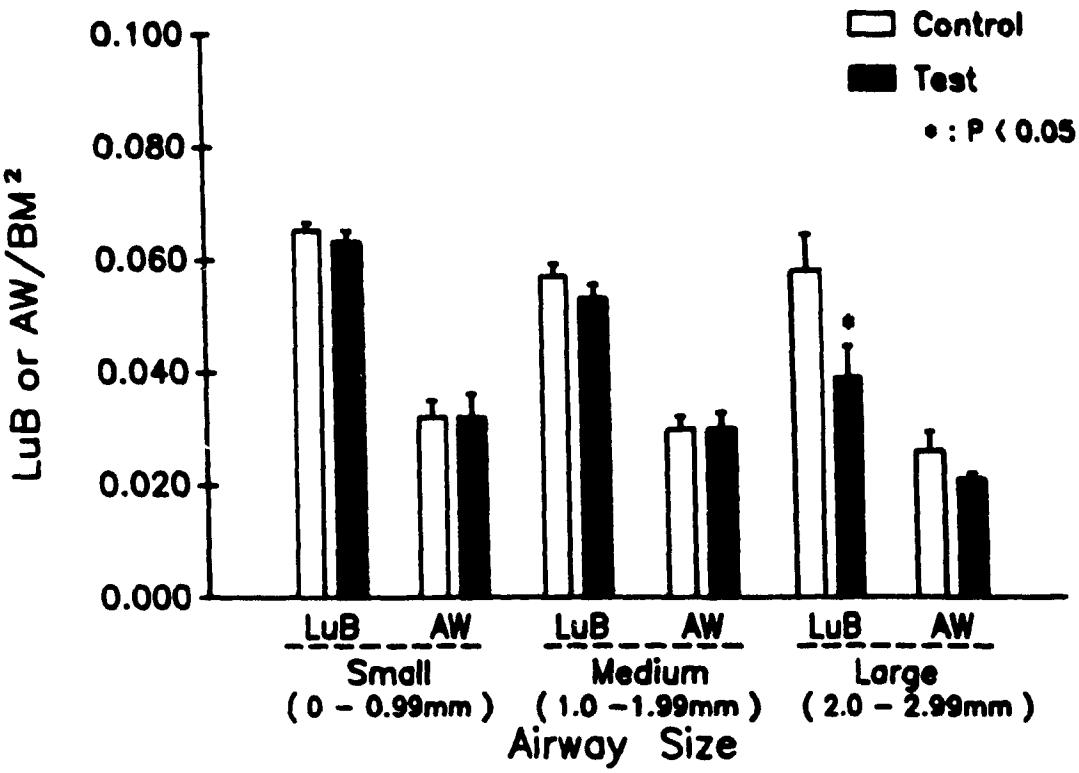


Fig.2.3. LuB and AW of small, medium, and large sized airways of OA-challenged test rats (closed bars) and their matched control rats (open bars). A significant decrease in LuB appeared only in large membranous airways of the test rats ($p<0.05$).

rats compared to 0.058±0.006 mm^2 in controls ($p<0.05$). There was no difference in the thickness of any category of airway as measured by airway wall area (AW) (figure 2.3). The area of the epithelium did not differ between test and control animals for any category of airway (data not shown).

Even though LuB was less in the large airways of the test animals, there was no correlation between the increase in R_L during the LR and the narrowing of the lumen of these airways.

Test and control animals did not differ in the degree of lung atelectasis. The percentage of the cross sectional area of the section of the lung from each animal (including tissue and airspace) occupied by lung tissue ranged from 41% to 64%. There was virtually complete overlap between groups, indicating that comparable degrees of atelectasis were present in both groups (figure 2.4).

Allergen challenge did not elicit significant mucous secretion as indicated by the finding that the percentage of mucus-positive airways was similar in both test (22.3 ± 9.2%) and control lungs (31.4 ± 6.7%) (figure 2.5).

2.4.4. <u>Mast cell distribution</u>:

There was a striking gradient in the number of mast cells from small peripheral airways to the large central airways (figure 2.6). The number of mast cells was small in the peripheral airways (BM < 1 mm), averaging approximately 1.4 mast cells/mm of basement membrane. In contrast, there were 6.2 mast cells/mm of basement membrane in the large membranous airways (BM 2-2.99 mm).

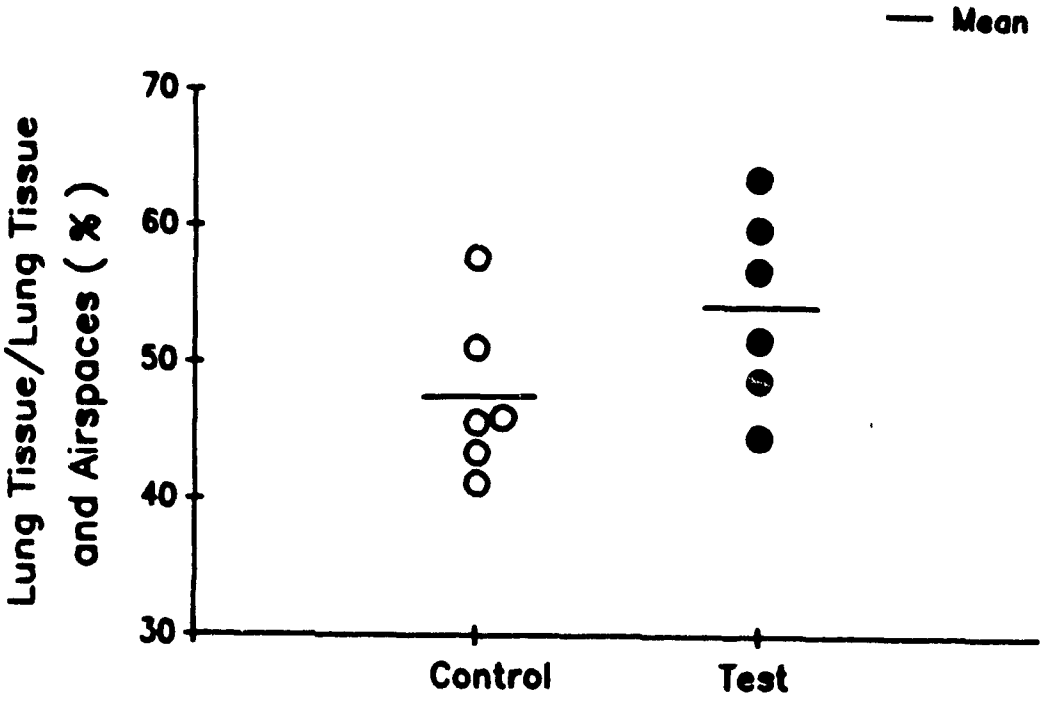


Fig. 2.4. The percentage of the area of lung parenchyma (including lung tissue and airspace) occupied by the lung tissue in each animal ranged from 41% to 64%. There was no difference between test animals (closed circles) and controls (open circles). The horizontal lines indicate the group means.

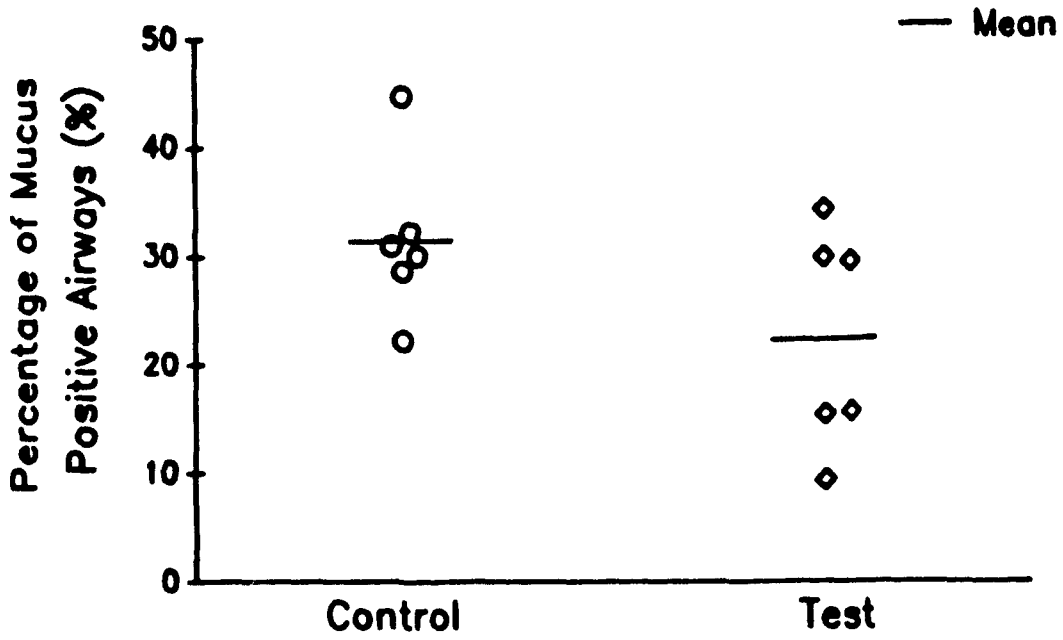


Fig.2.5. The percentages of mucus-positive airways in the test (open diamonds) and control (open circles) rats were not significantly different. The horizontal lines indicate the group means.

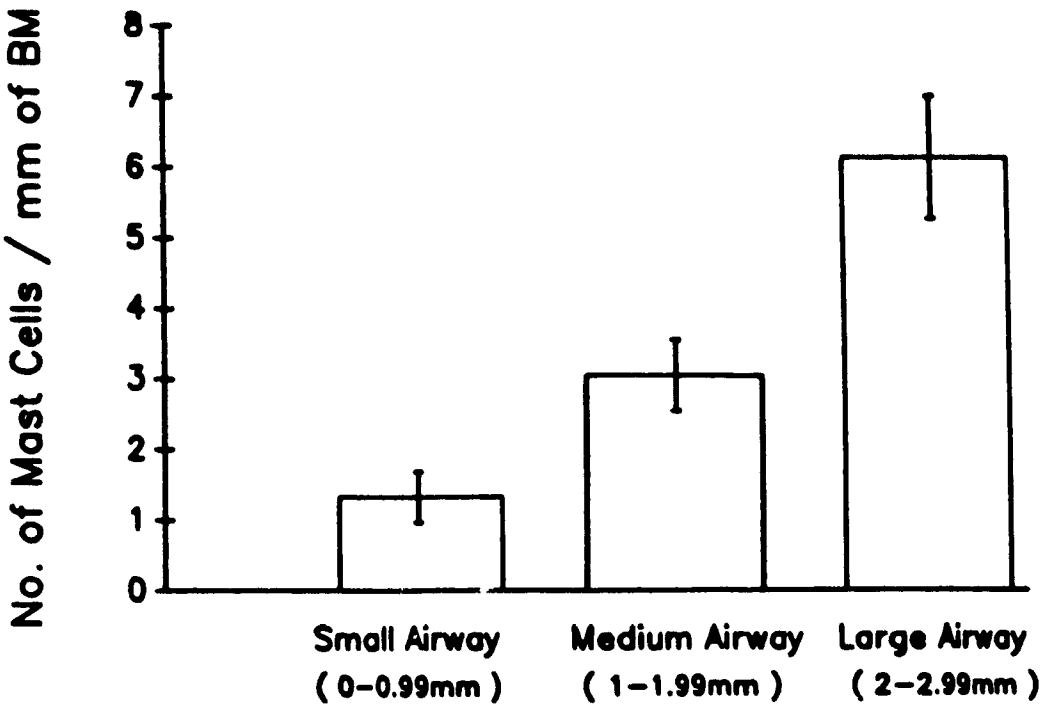


Fig.2.6. The number of mast cells is expressed per unit length of basement membrane and shows a marked increase as a function of airway size.

2.5. DISCUSSION

During the late increase in pulmonary resistance following allergen challenge, the airways of the rat (Blythe et al. 1986), rabbit (Marsh et al. 1985) and guinea pig (Hutson et al. 1988b) demonstrate an inflammatory process with a mixed polymcrphonuclear and mononuclear cell infiltration. An increase in inflammatory cells has also been found in bronchoalveolar lavage fluid during the LR in sheep (Abraham et al. 1988) and human subjects (de Monchy et al. 1985; Wardlaw et al. 1988). The association between the LR and airway inflammation led us to postulate that airway wall thickening caused principally by edema might account for a major portion of the airway narrowing. Contrary to expectations, the airways of the allergen-challenged rats were not measurably altered in thickness, despite the presence of inflammatory cells within the airway walls. Because the airways of experimental animals were not thicker than those of controls the narrowing of the airway lumen and presumably the LR must have been caused by airway smooth muscle contraction.

To correct for the differences in airway dimensions related to the size of the airways, we normalized morphometric measurements for the length of the basement membrane. This approach has been previously used for the calculation of the airway lumen and airway size (James et al. 1988). The major finding was that the lumen of the large membranous airways of antigen challenged animals was smaller than in controls (figures 2.2 and 2.3). It is probable, therefore, that the LR was caused by bronchospasm involving large

airways because airway narrowing was not caused by airway wall thickening. In fact, the airway wall area was slightly less in test animals although the difference was not quite statistically significant. James and coworkers (1988) also observed a reduction in airway wall area in carbachol-constricted porcine airways, which they suggested might be caused by the displacement of blood and lymph from the airway.

Even though we found no thickening of the airway wall during the late response in the rat, our data are not at variance with descriptions of the histopathology of the late phase response in the rat and other animal species. Blythe and coworkers (1986) found "no marked differences in the peribronchial or alveolar histopathologic findings between control and passively-sensitized, antigen challenged rats." However, no reference was made to the presence or absence of airway wall thickening. Airway infiltration has been reported for the guinea-pig with eosinophils and neutrophil polymorphonuclear leukocytes within 6 hours of ovalbumin challenge (Hutson et al. 1988a). In this report also, no reference was made to the thickness of the airway wall. Iijima and colleagues (1987), who used a rapid freezing technique with liquid nitrogen to examine the airways of allergen-challenged guinea pigs found narrowing of the airways, which they attributed jointly to mucus, edema and bronchial smooth muscle spasm. Perhaps the differences between the rat and guinea pig relate to differences in the intensity of the inflammatory response following antigen challenge.

There are two possible reasons to suspect that LR should affect large airways more than small. Sertl and coworkers (1988) have recently shown that the microvascular leak after intravenous administration of antigen to sensitized Sprague- Dawley rats occurs in the large airways and corresponds to the predominantly central distribution of mast cells in these animals. An earlier and somewhat more detailed study of the distribution of pulmonary mast cells in Lewis rats also showed that the density of mast cells was greatest in trachea (Goto et al. 1984). Our results confirm the striking gradient of mast cell density from small to large airways in the Brown Norway rat (Figure 2.6). It is also probable that much of the inhaled antigen was deposited in larger airways because most of the aerosol generated by the Hudson nebulizer is contained in large particles (>10 u) despite a small mass median diameter (< 2 u). This phenomenon should favour bronchoconstriction in the larger airways.

The lack of a relationship between the extent of large airway narrowing and the value of the pulmonary resistance at the time of sacrifice during the late response is not surprising. Some animals had started to recover from the late response at the time they were killed, and the degree of airway narrowing may well have been somewhat less than had been present at the time of the last measurements of pulmonary resistance. Perhaps of more importance is the fact that pulmonary resistance is at best only an approximate measure of airway calibre since it comprises not only the changes in transpulmonary pressure related to airway narrowing

but also changes caused by tissue viscance and ventilation-perfusion inhomogeneities. The probability of finding a correlation between morphometry and lung function should be greatly enhanced by using a more direct measure of airway calibre.

Other changes that potentially could have contributed to the LR were atelectasis and secretion of mucus. Both were seen to some extent but there were no significant differences between control and test animals. Although mucous plugs were described in guinea pigs at 72 hours after allergen challenge, they were not present at 6 hours after challenge (Hutson et al. 1988a), approximately the time at which we killed our animals. The paucity of mucus may reflect the fact that mucous glands are not prominent in the rat. Atelectasis is not a surprising finding because of the expected loss of gas volume caused by the freezing of the lungs with liquid nitrogen while keeping the trachea clamped.

The mediators that account for most of the early response to allergen challenge in the rat are serotonin (Piechuta et al. 1987; Church 1975) and the leukotrienes (Piechuta et al. 1987; Dorsch et al. 1983). Methysergide, peptido-leukotriene antagonists and a 5-lipoxygenase inhibitor significantly attenuate the response (Piechuta et al. 1987). The mediators that account for the LR are less well-characterized. A secondary rise in histamine has been demonstrated during human nasal LR, suggesting that its equivalent in the rat, serotonin, also may be involved. Thromboxane A_2 has been implicated in cutaneous LR (Dorsch et al. 1983) but its role in LR in other tissues is unknown. The LR is inhibited in sheep (

Lanes et al. 1986) and the Brown-Norway rat (Sapienza et al. 1990) by LTD_4 antagonists, suggesting an important role for 5-lipoxygenase products of arachidonic acid metabolism. Indeed, exogenously administered LTD_4 can mimic the LR in the sheep (Abraham et al. 1985). The findings of the present study suggest that it is the bronchoconstrictive property of LTD_4 that mediates the LR.

We conclude that the LR in the Brown-Norway rat is caused by airway smooth muscle constriction in the large airways. The site of airway narrowing corresponds to the distribution of mast cells, which are in greater density in large than small airways. Despite cellular infiltration, the airway wall is not measurably thickened. In this animal, structural changes do not appear to contribute to the LR either directly or by amplifying the effects of airway smooth muscle shortening during the LR. Whether the mechanism of the LR is the same in other species, including man, requires further study.

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CHAPTER III

MORPHOMETRIC CHANGES DURING THE EARLY AIRWAY RESPONSE TO ALLERGEN CHALLENGE IN THE RAT

3.1. ABSTRACT

The purpose of this study was to determine the relative contributions of airway wall edema and smooth muscle contraction to the early response (ER) of allergic bronchoconstriction. Nineteen male Brown Norway rats, 6 to 7 weeks old, were actively sensitized with ovalbumin (OA). Two weeks later, anesthetized rats were challenged with either OA (n=11) or saline (n=8). Pulmonary resistance (R_L) was measured at one minute intervals until it increased to 150% of the baseline, defined as a significant ER, or 15 minutes elapsed. Eight OA challenged test rats with a significant ER and 8 saline challenged control rats were used for morphometric studies. The lungs were quick-frozen with liquid nitrogen and processed with freeze substitution. Five micron thick slices from sagittal sections of the lungs were stained with hematoxylin and eosin. The airway lumen subtended by the epithelial basement membrane (LuB) and cross section airway wall area of all airways were measured by camera lucida and digitization. Airway narrowing was quantitated as the ratio of LuB over ideal airway lumen (LuB_{ideal}) assuming a circular airway in the unconstricted state. The median LuB/LuB_{ideal} was 0.714 for the test rats, significantly less than that for the control rats (0.81; $p < 0.01$). The difference in LuB/LuB_{ideal} between the test and control rats increased progressively from small to large airways. Airway wall thickness was not significantly different between the two groups. We conclude that in the Brown Norway rats, the ER to inhalational challenge with OA is due to smooth muscle contraction

rather than airway wall edema. The large airways are the predominant site of airway obstruction

3.2. INTRODUCTION

The early response (ER) to allergen inhalational challenge of sensitized subjects appears within minutes of the allergen exposure and resolves spontaneously within 1 to 3 hours (O'Byrne et al. 1987). The ER is not only a feature of human asthma, but also occurs in sheep (Abraham and Perruchoud 1986, 1989), guinea pig (Hutson et al. 1988), rabbit (Behrens et al. 1987), monkey (Richards et al. 1983, Patterson et al. 1983, Hamel 1986), dogs (Peters et al. 1982; Mapp et al. 1985, Becker et al. 1989) and rat (Eidelman et al. 1988) following allergen inhalational challenge. It is associated with IgE-mediated mast cell activation (O'Byrne et al. 1987; Keyzer et al. 1984) and bronchospasm (Kay 1986). The reversibility of the ER by beta- agonists has been frequently interpreted as indicating that it is mainly caused by airway smooth contraction (Booij-Noord et al. 1971, 1972). However, airway narrowing, hyperemia and edema have been observed by bronchoscopy after local challenge of allergic asthmatic patients with allergen (Metzger et al. 1987). Thirty minutes after inhalational challenge of allergic rabbits, edema and vasodilation can be observed histologically in large airways (Behrens et al. 1987).

Even though published data suggest that edema as well as smooth muscle constriction may be involved in the pathogenesis of

airway narrowing during the early response, quantitation of the contributions of the different factors involved in the airway narrowing does not appear to have been attempted. One of the difficulties inherent in such a study is that the ER is short, making it hard to demonstrate the pathological changes in vivo using conventional methods of fixation. To overcome this difficulty, a quick freeze technique was used for the detection of in vivo changes during the ER. As soon as the ER developed following allergen challenge of sensitized Brown Norway rats, the lungs were rapidly frozen using liquid nitrogen. Morphometric measurements were performed on lungs from test and control rats whose lungs were frozen at identical time points. We found that the allergen induced ER was mainly due to airway smooth muscle contraction and that the dominant site of airway narrowing was the large airways.

3.3. MATERIALS AND METHODS

3.3.1. Animals and sensitization:

Nineteen male Brown Norway rats, 6 to 7 weeks old and ranging in weight from 210 to 290 g were obtained from Charles River Inc. (St. Constant, Quebec, Canada). The animals were actively sensitized with a single subcutaneous injection of a sterile suspension of 1 mg ovalbumin (OA) and 200 mg of aluminum hydroxide in 1 ml of 0.9% saline. Simultaneously, 1 ml of Bordetella pertussis vaccine containing 6 x 10^9 heat-killed organisms was given intraperitoneally as an adjuvant (Eidelman et

al. 1988).

3.3.2. Measurement of Early Responses:

Fourteen to eighteen days after sensitization, rats were anesthetized with urethane (1 gm/kg, intraperitoneally). A tracheostomy was performed using an 11 cm length of polyethylene tubing (PE 240). The animals were mechanically ventilated (Harvard Apparatus, Model 680) via the tracheal catheter with a tidal volume of 1.2 ml and a frequency of 90 breaths/minute. A positive end-expiratory pressure of 2 cm H_2O was applied. To facilitate removal of the lungs at the end of the experiment, the abdominal cavity was opened by a midline incision and the thorax was opened through the diaphragm. A heating lamp was used to maintain a constant body temperature.

The rat was placed in a tightly closed constant volume body plethysmograph for the physiological measurements. Volume was obtained by measuring changes in pressure inside the box using a differential pressure transducer (Validyne MP-45; $\pm$5cm H_2O). Airflow was obtained by differentiation of volume. The pressure inside the box was restored to atmospheric pressure periodically. Tracheal pressure was measured through a sideport in the tracheostomy tube using a differential pressure transducer (Validyne MP-45; $\pm$100cm H_2O).

Aerosols were delivered by ultrasonic nebulizer (DeVilbiss, Model 100HC) into the intake port of the ventilator. An airflow of 11 ml/s was used with a nebulizer output of 0.18 ml/min. Each

animal was challenged with aerosolized saline for 5 minutes and a measurement of pulmonary resistance was taken, which was considered as baseline. Test animals were then challenged with aerosolized OA for 5 minutes and pulmonary resistance was then measured every minute for a maximum of a further 10 minutes. Each control rat was challenged with aerosolized saline and killed at the same time point as its matched test rat.

Experimental data were conditioned by anti-aliasing filters and sampled at 200 Hz with an analog to-digital board (DT2801-A, Data Translation, Marlboro, MA) installed in a microcomputer (Deskpro 286, Compaq, Houston, TX). Data were collected for two 10 second periods. Lung resistance was calculated using multiple linear regression by obtaining the best fit for the equation: $P_{tr}=\dot{V}R_L+VE_L+K$. where P_{tr} is tracheal pressure, $\dot{V}$ is flow, V is volume, R_L is pulmonary resistance, E_L is elastance and K is a constant (Bates et al. 1989).

3.3.3. Tissue Preparation:

After the final measurement of R_L, the endotracheal tube was clamped at end-expiration and the animal was removed from the box. The endotracheal tube was re-connected through a T-piece to a constant flow of gas at a pressure of 4 cm H_2O. Liquid nitrogen was poured into the thoracic cavity. Ten minutes later, the lungs were removed and then fixed in Carnoy's solution at -80°C overnight. Carnoy's solution was prepared by mixing ethanol (E; 60%), chloroform (C; 30%) and acetic acid (A; 10%). Three

modified Carnoy's solutions (MC) were made as follows: MC-1 (E : C : A = 70% : 22.5% : 7.5%); MC-2 (E : C : A = 80% : 15% : 5%) and MC-3 (E: C : A = 90% : 7.5% : 2.5%). After fixation, the lung tissue was immersed for consecutive one hour periods in MC-1, MC-2, MC-3 and finally 100% ethanol at -20°C. Ethanol at -20°C was replaced with ethanol at 4°C overnight.

After the above procedure, the lungs were maintained in ethanol at room temperature for 2 hr until sagittal blocks, about 1 mm from hilum of the lungs, were cut. The blocks were embedded in paraffin and 5 micron thick sections were cut and stained with hematoxylin and eosin.

3.3.4. Morphologic and Morphometric Studies:

Sections from both the left and right lungs were examined by light microscopy. For each airway, the airway structure was traced on paper using a drawing tube attachment (Leitz, Germany) and then was measured using a commercial digitizing program (Sigma Scan, Jandel Scientific, Corte Madera, CA). Airways whose ratio of maximal to minimal internal diameter was equal to or larger than 2 were considered to be cut obliquely and not measured. All other airways present on the slides from both lungs (mean: 18 airways/per animal; range: 11 to 29 airways) were measured. The following measurements were made: 1) airway lumen defined by the basement membrane of epithelium (LuB), 2) the length of the airway basement membrane (BM), and 3) the airway defined by the outer border of the airway wall (Ae). The area of the airway wall

(AW) was calculated (AW = Ae - LuB).

3.3.5. Data Analysis:

A significant early airway response was defined as an increase in R_L to a value of at least 150% of the baseline. This value corresponds approximately to two standard deviations above the mean of sequential measurements of resistance made over a similar time period after a saline challenge only. Only animals in the test group that showed such a response were retained for further analysis.

Morphometric measurements were made by a single observer blinded to the group status. A sample of ten airways were measured by each of two observers independently and the correlation of measurements among and within observers was excellent ($r^2 > 0.97$). Since extra-large membranous and cartilaginous airways (BM > 4.0 mm) were not always present in all of the lung sections, these airways were excluded from analysis. 136 airways were analyzed from 8 test rats and 146 airways from 8 matched controls. These airways were divided into three groups based on the length of the basement membrane: small (BM; 0-0.99 mm), medium (BM: 1-1.99 mm) and large (BM: 2- 3.99 mm; Du et al. 1991). Cumulative frequency distributions of LuB/LuB_{xdcal} were constructed to evaluate the degree of airway narrowing, where LuB_{xdcal} is the lumen calculated from the BM length, assuming that the unconstricted airway is a perfect circle. The area of each airway wall was standardized by dividing by BM^2 to adjust for differences in airway size.

The data are expressed throughout as the mean±SE. The morphometric data between test and control rats were analyzed by unpaired t-tests. A Wilcoxon signed rank test was used to compare the baseline R_L and the peak value of R_L after challenges ($R_L$max) in the test and control groups. Comparisons of the baseline R_L and $R_L$max between the two groups were made by Mann- Whitney U test. The significance of differences between cumulative frequency distributions was tested by the Kolmogorov Smirnoff test. P values < 0.05 were considered significant.

3.4. RESULTS

3.4.1. Early Response:

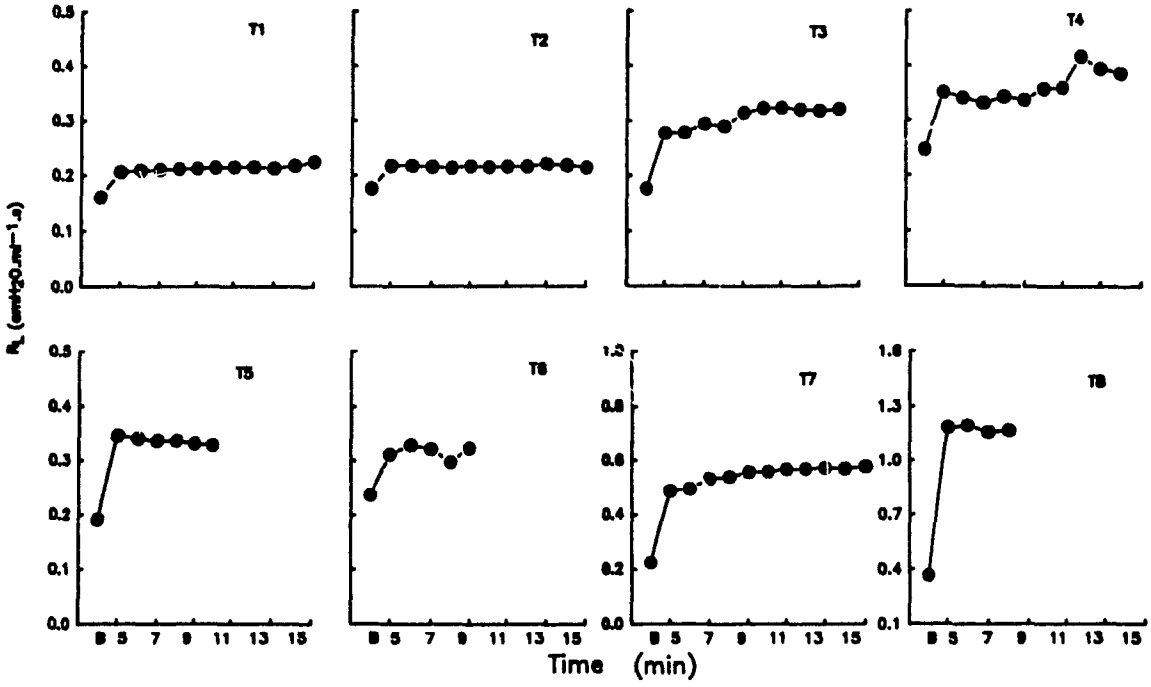
The baseline values of R_L were similar in both groups (control = 0.239±0.022 : test = 0.242±0.024 cm H_2O/ml/s.) An early response was present in 8 out of 11 test rats after OA challenge and R_L changed from the baseline to a peak value of 0.453±0.113 ($p < 0.02$), an increase of 173±22% from the baseline R_L. However, after saline challenge the R_L did not change significantly from the baseline (0.235±0.028; $p > 0.05$). The data for individual test and control animals are shown in figures 3.1a and 3.1b.

3.4.2. Morphological Findings:

On light microscopy, the airway wall generally appeared normal. A slight inflammatory cell infiltration was observed in the walls of some airways from OA challenged rats, but not in the saline challenged controls. Both polymorphonuclear and mononuclear

Fig. 3.1. Changes in the pulmonary resistance (R_L) from baseline (B) not prim to OA challenge and the subsequent period of the early response (ER) in each individual test (T; Fig. 3.1a.) and time matched control rats (C; Fig. 3.1b.). In test rats, R_L increases significantly following OA compared with saline challenged controls, indicating the presence of ER.

3.1a.


T1
T2
T3
T4
T5
T6
T7
T8
Time (min)

3.1b.

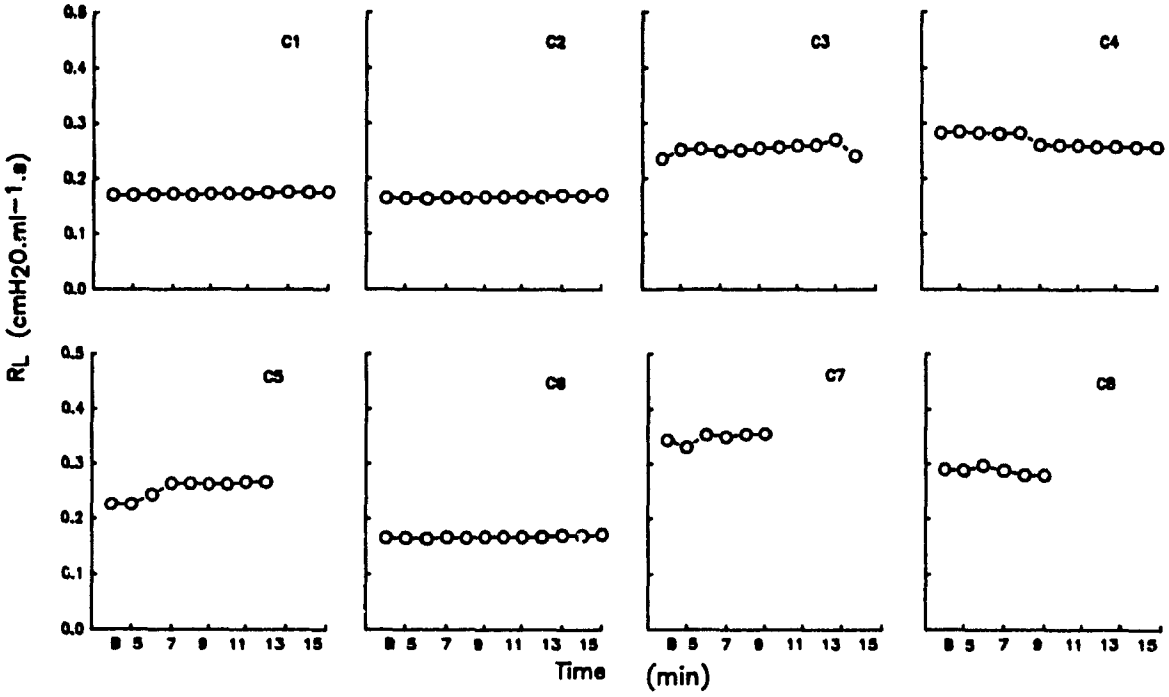
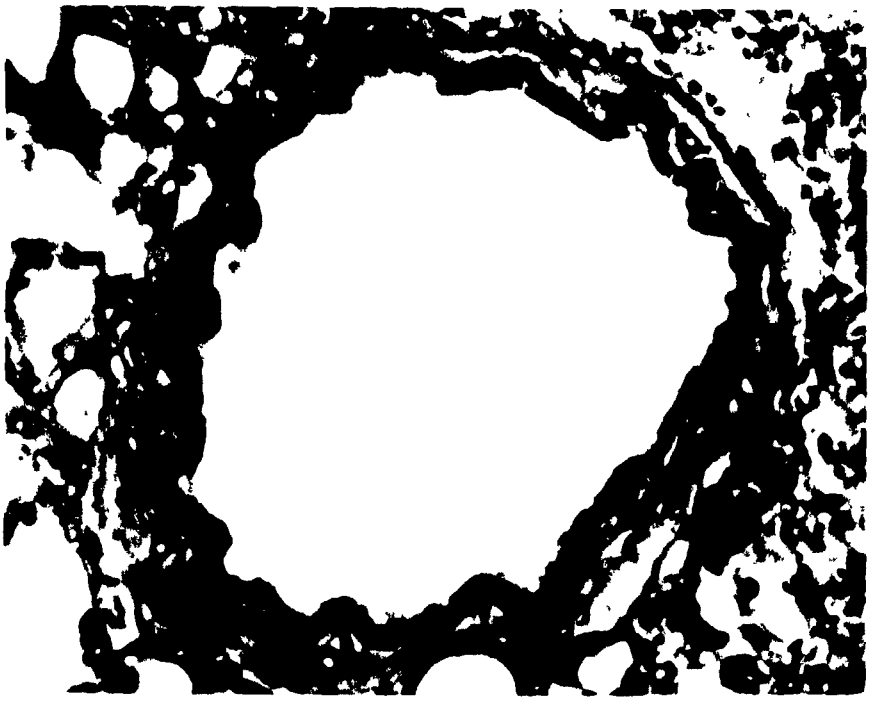

C1
C2
C3
C4
C5
C6
C7
C8
RL (cmH2O.ml−1.s)
Time (min)

Fig. 3.2. Two representative airways from an allergen challenged test (3.2a) and a saline challenged control rat (3.2b). The epithelia infolding in the airway from the test rat indicates airway narrowing. In contrast, the airway from a control rat appears normal. x140.

3.2a



3.2b

cells were present. Basement membrane undulation, indicating narrowing of the airway lumen, was present and very obvious in some airways, especially in the test group (Fig. 3.2a and 3.2b).

Although a small amount of mucus was present on the surface of the epithelium, mucus plugs were not observed. In both groups, most alveolar spaces were well inflated and clear. Occasionally, a small amount of proteinaceous material was found in some of the air spaces of both test and control animals.

3.4.3. Morphometric Findings:

The distributions of airway size for control and test animals were similar as demonstrated by the cumulative frequency distributions shown in figure 3.3. The proportion of airways in each size category as a fraction of the total airways was almost identical in both groups.

The degree of bronchoconstriction for all airways in different categories of airway size is shown in the form of cumulative frequency distributions in figures 3.4 and 3.5. The ratio of LuB/LuB_{ideal} was significantly smaller for the test than in control airways; the is demonstrated by the leftward shift of the the cumulative frequency distribution of LuB/LuB_{ideal} in test animals compared to controls ($p < 0.01$; figure 3.4). A significant reduction in airway lumen in test animals was present for airways of all sizes (figures 3.5a, 3.5b and 3.5c). However, the maximal difference in frequency distributions between test and control animals increased progressively from the small to the large airways

(table 3.1). The substantial heterogeneity in the degree of airway narrowing is also evident in the frequency distributions. The dispersion of airway lumen sizes increased as a function of airway size.

Airway wall thickness in test animals was slightly, but not significantly, greater in small and large airways than airways of the controls ($p > 0.05$; figure 3.6).

We did not find any significant correlation between the increase in R_L in the test animals and the mean or median of LuB/LuB_{ideal} of either all airways or the airways considered according to different categories of size.

3.5. DISCUSSION

We found a progressive airway narrowing from small to large airways in the actively sensitized Brown Norway rat following allergen challenge. The airway narrowing appeared to be caused by airway smooth muscle contraction because the airway walls in OA challenged rats were not significantly thicker than those of the controls. There was little mucus on the surface of the epithelium in both test and control animals. Therefore, neither airway wall edema nor mucous secretions appear to be causative factors in airway narrowing during the early response in the rat.

The most striking morphological difference between the airways of test and control animals was infolding of the epithelial basement membrane, indicating a reduction of the cross sectional area of the lumen. This impression was confirmed by the

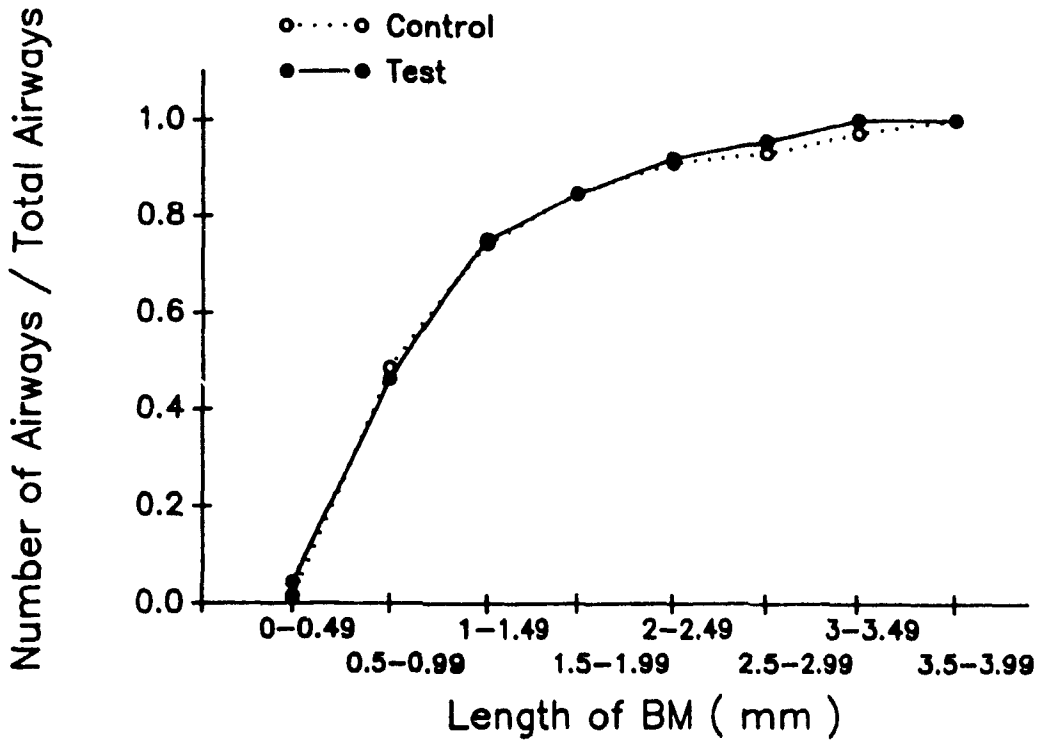


Fig. 3.3. Cumulative frequency distribution of airway size of test and controls. Each airway was sized by the length of the basement membrane (BM). The proportion of airways in each size category is not different between the test and control animals.

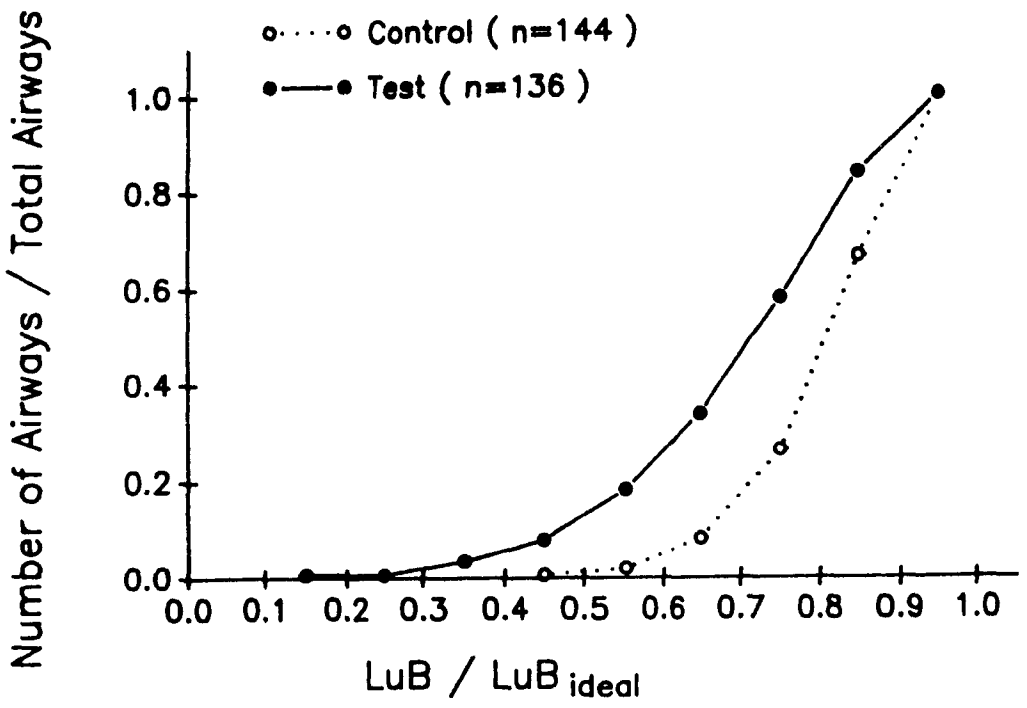
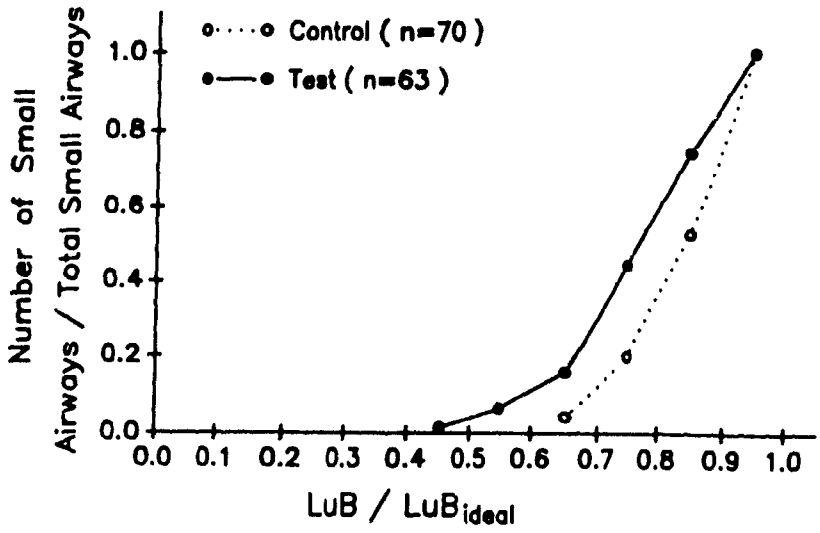


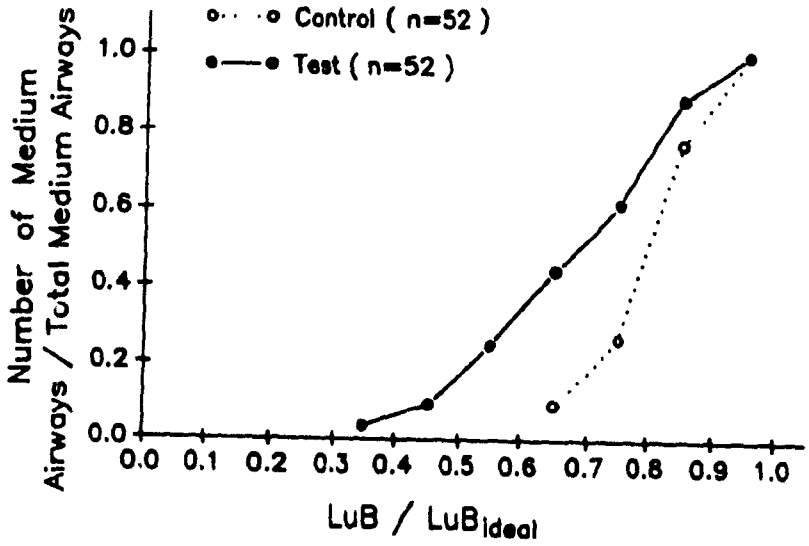
Fig.3.4. Degree of airway narrowing for all airways is shown in the form of cumulative frequency distribution. For each airway, the area of the airway lumen described by the basement membrane (LuB) was standardized to the ideal area (LuB_{ideal}; see methods). The distribution of LuB/LuB_{ideal} was shifted to the left in the test rats and different significantly to the controls, indicating that the airway narrowing was present following the allergen inhalation.

Fig. 3.5. The degree of airway narrowing for small (3.5a), medium (3.5b) and large (3.5c) sized airways is shown in the same format as in figure 3.4. For each airway, the area of airway lumen (LuB) was normalized by the ideal area (LuB_{ideal}). Airway narrowing was significantly present in all sized airways ($p < 0.01$). However, the difference between the distributions in test and control rats increased progressively from small to large airways. The degree of airway narrowing was greatest in the large airways and least in the small airways.

3.5a


Control (n=70)
Test (n=63)
Number of Small Airways / Total Small Airways
1.0
0.8
0.6
0.4
0.2
0.0
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
LuB / LuB ideal

3.5b


Control (n=52)
Test (n=52)
Number of Medium Airways / Total Medium Airways
1.0
0.8
0.6
0.4
0.2
0.0
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
LuB / LuB ideal

3.5c

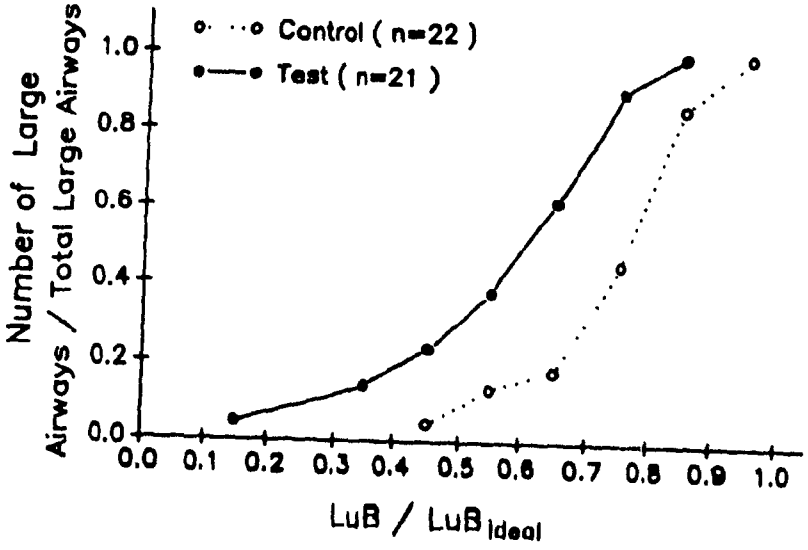

Control (n=22)
Test (n=21)
Number of Large Airways / Total Large Airways
1.0
0.8
0.6
0.4
0.2
0.0
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
LuB / LuB ideal

Table 3.1.

Medians and Maximal Differences of Cumulative Frequency Distributions of luB/LuB_{ideal} for Test and Control Rats

	Median in Control Rats(C)	Median in Test Rats(T)	Difference between C&T	Maximal Difference between C&T
Small Sized Airways	0.84	0.77	0.07*	0.24*
Medium Sized Airways	0.8	0.68	0.12*	0.35*
Large Sized Airways	0.76	0.6	0.16*	0.45*
Total Airways	0.714	0.81	0.096*	0.32*

* $p < 0.01$, when compare with control group.

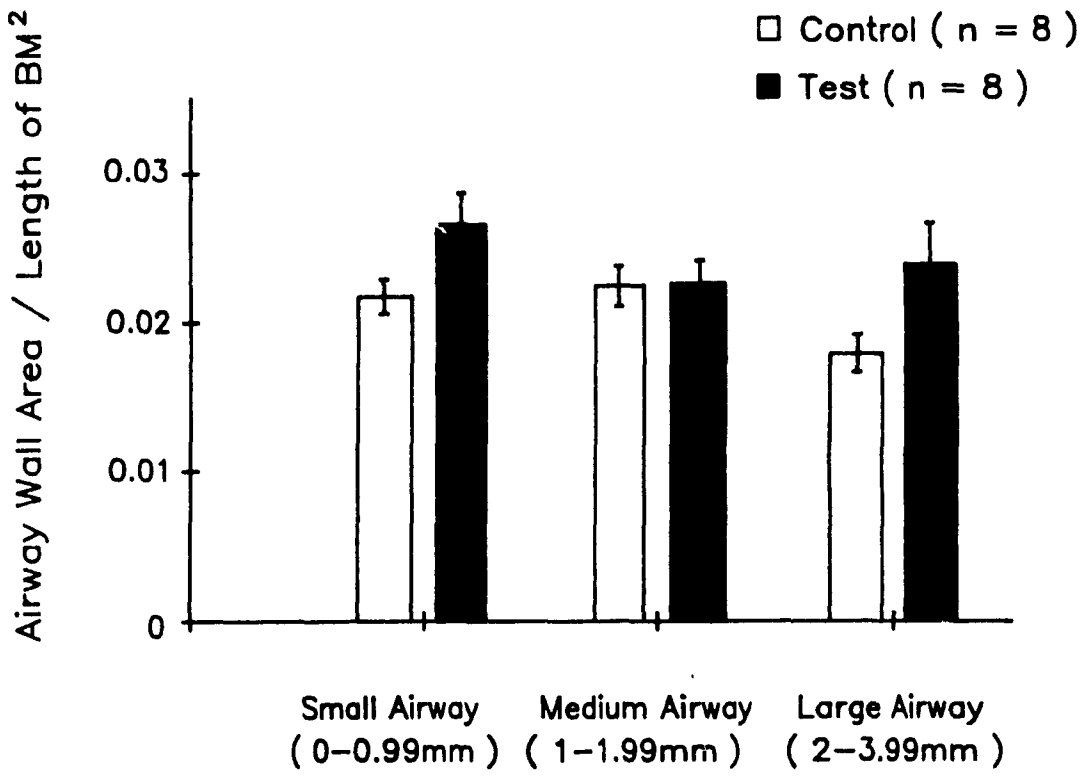


Fig. 3.6. Comparison of airway wall areas for test and control rats. The airway wall area of each airway was standardized for size dividing by the square of basement length (BM^2). The results are presented as mean±SE. There was no significant difference in the airway wall area between OA challenged test (filled bars) and saline challenged control rats (open bars).

morphometric finding of smaller airway lumens in test rats compared to controls. The reversibility of the early airway response suggests that airway smooth muscle contraction is an important component of the response (Kay 1986). Infolding of the epithelium is expected when smooth muscle shortens because of the incompressibility of the cells and basement membrane.

Although airway narrowing was present in all sized airways, the difference in LuB/LuB_{ideal} between test and control rats increased progressively from small to large intrapulmonary airways. At least two factors may have led to a greater response in large than small airways. Delivery of allergen by aerosol leads to preferential central deposition and the density of mast cells in the airway wall of the Brown Norway rat increases progressively from small to large airways (Du et al. 1991). A similar distribution of mast cells has been observed in Sprague- Dawley (Sertl et al. 1988) and Lewis rats (Goto 1984). Sertl et al. (1988) reported a predominantly central location of microvascular leak even after intravenous antigen administration to the rats, a finding which supports the notion that the spatial distribution of mast cells may account for the location of airway narrowing during the ER. Interestingly, we also found that airway narrowing during the late response was greatest in the large airways of allergen challenged BN rats (Du et al. 1991). However, airway narrowing is somewhat more extensive during the early response.

We did not find any correlation between increased pulmonary resistance and morphometric measurements of airway narrowing. The

techniques used for this study were chosen so as to maintain all of pathological changes in the ER as close to their in vivo conditions as possible. A slow increase in tissue temperature and the concentration of ethanol in Carnoy's solution was used to ensure better fixation and to avoid tissue damage. Since the lung tissue was frozen within a very short period during the ER, all of the pathological alterations should have remained virtually unchanged for evaluation under the microscope. It is possible that lack of correlation may relate to the difference in timing between the last measurement of pulmonary resistance and the freezing of the lungs. Perhaps more likely is the fact that pulmonary resistance is an indirect measure of change in airway caliber. Ventilation inhomogeneities and tissue viscance will both influence measurement of pulmonary resistance. Tissue viscance contributes substantially to pulmonary resistance in the mechanical ventilated dog and has been reported to increase with inhalation of histamine (Ludwig et al. 1989). Whether tissue viscance changes after allergen challenge is not known, but it is potentially a confounding factor that may mask relationships between airway narrowing and airway resistance.

Airway wall thickening and mucus could also be involved in the reduction of airway lumen during the early response. Airway wall edema, resulting from inflammatory cell infiltration and vascular leakage, might increase the thickness of the airway wall inside the layer of smooth muscle. Any increase in tissue mass in the luminal side of the airway smooth muscle would be expected to amplify the

effects of smooth muscle shortening (Moreno et al. 1986; Benson 1975). If edema were to developed peribronchially, it is possible that it might interfere with the normal coupling of the parenchyma to the airway and permit excessive smooth muscle shortening (Macklem 1985). Contrary to previous suggestions that bronchial wall edema was an important component of the early response (Metzger et al. 1987; Behrens et al. 1987), there was neither morphologic nor morphometric evidence in support of airway wall thickening. At least in the BN rats, airway wall edema is not an important factor in airway narrowing during the ER.

We conclude that in BN rat model, the allergen induced ER is mainly caused by airway smooth muscle contraction rather than edema or mucus secretion. In this model, the airway narrowing progressively increases from small to the large airways. Which in turn, may in part be related to the distribution of mast cells in the airways of the Brown Norway rat. The extent to which these mechanisms of early response are present in human subjects requires further study.

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CHAPTER IV

MICROVASCULAR LEAKAGE IN THE AIRWAY WALL DURING ALLERGEN INDUCED EARLY AND LATE RESPONSES IN RATS

4.1. ABSTRACT

The purpose of this study was to quantitate the extent of microvascular leakage to airway narrowing during the early (ER) and late (LR) bronchoconstriction following inhalational challenge with allergen of the sensitized Brown Norway (BN) rat. To do this, 28 BN rats, 7 to 8 weeks old, were actively sensitized with ovalbumin (OA). Fourteen to 18 days after sensitization, the rats were either challenged with aerosolized OA or saline. The animals were separated into two groups for the study of either OA induced ER (n = 13) or LR (n = 15). Pulmonary resistance (R_L) was used to measure the airway response and intravenous administration of Evans blue (30 mg/kg) was used for the measurement of microvascular leakage. Evans blue was injected either into the test rats or time-matched controls of ER group at 3 minutes after inhalational challenge for the ER or at 45 minutes after the onset of the LR. In order to compare leakage after allergen challenge to that with serotonin (5-HT), a known inducer of pulmonary microvascular leakage, 6 sensitized rats were injected with Evans blue and then challenged with aerosolized 5-HT. Rats were killed 8 minutes after injection of Evans blue. In the ER group, 6 of 7 test rats challenged with OA had an ER; R_L of the animals increased from a baseline of 0.148 ± 0.012 to a peak value of 0.351 ± 0.04 ($n=7$; $p<0.01$); R_L of saline challenged controls did not change (n=6). In the LR group, 6 of 8 rats showed a significant LR after OA inhalation; R_L changed from baseline of

0.174±0.017 to a peak value of 0.479±0.129 (n=8; p<0.02), whereas R_L of saline challenged controls was not changed significantly (n=7; p>0.2). After 5-HT challenge, R_L increased from a baseline of 0.159±0.018 to a peak value of 0.636±0.181 (p<0.03). After sacrifice, the airways were divided into main bronchi, central and peripheral intrapulmonary airways. Evans blue dye was extracted with 100% formamide at 40°C for 24 hours and then the concentration determined by light absorbance at 620 nm wavelength. The tissues from ER group and 5-HT challenged rats were dried at 60°C for 72 hours and then weighed. The results from either increased amounts of Evans blue or increased wet/dry weight ratio demonstrated that vascular leakage was present in all of the airways of 5-HT challenged rats but not OA challenged rats during either ER or LR. There was no correlation between the concentration of Evans blue and the changes in R_L in any of the test animal groups. These data indicate that vascular leakage is not significantly increased during allergen induced ER and LR in BN rats.

4.2. INTRODUCTION

Following allergen inhalation, two phases of airway narrowing can be detected in sensitized subjects, namely the early (ER) and late responses (LR). The ER is generally considered to be caused mainly by airway smooth muscle spasm (Kay 1986). In addition to airway smooth muscle constriction, airway inflammation, edema and mucous hypersecretion are thought to contribute importantly to the LR (O'Byrne et al. 1987).

Plasma leakage is an important cause of airway wall thickening, inflammatory cell infiltration, epithelial damage and mucus hypersecretion (Persson 1988). Several of the putative mediators of asthma when derived from various inflammatory cells may cause plasma exudation and inflammatory cell infiltration, including PAF (Humphrey et al. 1982; Pirotzky et al. 1984), leukotriene B_4 (Ford-Hutchinson et al. 1980; Nagy et al. 1982; Palmer et al. 1980) and histamine (Propst et al. 1978; Raphael et al. 1986).

Using morphometric techniques, we have demonstrated previously that airway wall thickening does not appear to contribute to either the allergen induced ER or LR in sensitized Brown Norway rats (Du et al. 1991a and 1991b). However, the sensitivity of this experimental approach for the detection of airway edema is unknown. For example, if the compliance of the extravascular space within the airway wall were low, it is possible that significant microvascular leakage could occur without a substantial accumulation of edema fluid. The purpose of the present study was to quantify vascular leakage during the early and late responses. To do this, we used the Evans blue technique administered during the early and late responses after challenge of actively sensitized rats with ovalbumin. Serotonin induced bronchoconstriction was produced following a time course similar to the early response after allergen challenge and the associated microvascular leakage was measured. This served as a positive control for allergen.

4.3. MATERIALS AND METHODS

4.3.1. Animals and sensitisation:

Thirty-two pathogen-free Brown Norway rats, 7 to 8 weeks old, ranging in body weight from 190 to 295 g, were obtained either from Charles River (St. Constant, Quebec, Canada) or from Bantin & Kingman (Fremont, California, USA). The rats from Charles River were used for the study of LR, whereas the animals from Bantin & Kingman were used for the study of ER and for the challenge with serotonin. Rats were housed in a conventional rat colony at the Meakins-Christie laboratories.

All of the animals were actively sensitized with a single subcutaneous injection of 1 ml of a sterile suspension of 1 mg ovalbumin (OA) and 200 mg of aluminium hydroxide in 0.9% saline. At the same time, 1 ml of Bordetella pertussis vaccine containing 6 x 10^9 heat-killed bacilli was given intraperitoneally as an adjuvant.

4.3.2. Measurement of airway responses:

Fourteen to eighteen days after sensitization, the rats were anesthetized with urethane (1.1 g. kg^{-1} intraperitoneally). Blind orotracheal intubation was performed using a 6 cm length of polyethylene tubing (PE 240). Animals were placed on a heating blanket and rectal temperature was monitored with an electronic thermometer throughout the experiment. The lateral decubitus posture was alternated every hour. Airway responses were determined from measurements of pulmonary resistance (R_L), using methods that have been described previously in detail (Eidelman et

al. 1988). Briefly, the end of the endotracheal tube was inserted into a small Plexiglas^{R} box (volume: 265ml) for the delivery of aerosols and for the measurement of airflow. A Fleisch no. 0 pneumotachograph coupled to a differential pressure transducer (MP 45±2cm H_2O; Validyne Corp., Northridge, CA) was attached to another end of the box. Changes in esophageal pressure were measured using a saline-filled catheter and differential pressure transducer (Sanborn 267 BC; Hewlett- Packard, Waltham, MA). Another port of the transducer was connected to the box. The esophageal catheter consisted of polyethylene tube (PE 240) 20 cm long attached to a shorter length tubing (6 cm; PE-100) that was advanced into the esophagus of the rats until a clear cardiac artifact was discernible. Transpulmonary pressure (Ptp) was computed as the difference between esophageal and box pressure. Pulmonary resistance was calculated using multiple linear regression by obtaining the best fit for the equation: $P_{tr}=\dot{V}R_L+VE_L+K$. where P_{tr} is tracheal pressure, $\dot{V}$ is flow, V is volume, R_L is pulmonary resistance, E_L is elastance and K is a constant (Bates et al. 1989). The resistance of the endotracheal tube was measured as 0.11 cm H_2O/ml/s. R_L is reported after subtraction of tube resistance.

Aerosol was generated using a Hudson nebulizer with an output of 0.18 ml/min connected to one side port of the box. During the aerosolization, the pneumotachograph was occluded and airflow was diverted through a second side port. The box was flushed with a stream of fresh air between each measurement in order to prevent

the accumulation of CO_2.

4.3.3. Measurement of Microvascular Leakage:

The technique of Evans et al. (1988) was used with a slight modification to evaluate microvascular permeability. Briefly, Evans blue dye was diluted in saline at a concentration of 30 mg /ml and then filtered through a 5 um Millipore filter. The external jugular veins were exposed and Evans blue dye (30 mg/kg) was injected into one of them. Five minutes after injection, the thorax was opened and a blunt-end 18-gauge needle was passed through the left ventricle into the aorta. Since in sensitized guinea pigs, the quantity of Evans blue in the airways remained constant between 5 to 10 minutes after the dye injection and intravenous allergen challenge (Evans et al. 1988), we chose to open the right atrium to evacuate the intravascular Evans blue and to perfuse the animal with 100 ml of 1% paraformaldehyde in saline at 130 cm H_2O at 8 min after Evans blue injection. After perfusion, the lungs and trachea were excised and the trachea and main bronchi were separated from intrapulmonary airways which were stripped of parenchyma and divided into " central " (about 3 mm from main bronchi) and " peripheral" components. Since the animals were orotracheally intubated, the measurement of vascular leakage in the trachea was not considered. Wet weights of other tissues were measured. Evans blue dye was extracted in 4 ml of 100% formamide at 40°C for 24 hours. The concentration of Evans blue was evaluated by measuring light absorbance at 620 nm wavelength using a

spectrophotometer (Model 710; Bausch & Lomb Inc., Rochester, NY) . The results are presented as ng dye/mg of wet tissue. After Evans blue extraction, the tissues from animals which were used in the study of ER or challenged with 5-HT were dried at 60°C for 72 hr. The dry tissues were weighed and the wet/dry ratio of the tissue was calculated.

4.3.4. Protocols:

Microvascular leakage after inhalational challenge with 5- HT: In order to test the sensitivity of the technique for the measurement of vascular leakage in the Brown Norway rat, six sensitized rats were challenged with aerosolized 5-HT as a form of positive control. After exposure of the jugular veins, baseline R_L was measured and Evans blue was injected. Two concentrations of 5-HT were aerosolized using a Hudson nebulizer. Each aerosolization was given for one minute and the peak value of R_L was measured. The concentrations of 5-HT were chosen to induce a change in R_L of similar magnitude to the ER after allergen. Five minutes after Evans blue injection, the chest cavity was opened. The paraformaldehyde perfusion was started at 8 minutes after Evans blue injection.

Microvascular leakage during the ER: The jugular veins were exposed. Baseline R_L was then measured. The rats were challenged for 5 min with either OA (5% W/V in saline; test rats) or saline (control rats). The animals with ERs were matched with saline challenged controls. The rat which did not show an ER was not

matched with a control. The R_L was measured at one minute intervals for 3 min. Then Evans blue was injected over a one to two min period into the jugular vein and R_L was measured each minute for a further 5 minutes. The chest cavity was opened and perfusion with formaldehyde was started at 8 minutes after Evans blue injection.

Microvascular leakage during the LR: Saline aerosols were delivered for 5 min using a Hudson nebulizer. The measurements of R_L after saline inhalation served as a baseline for evaluating airway responses. After measurement of baseline, a test rat was challenged with OA aerosol (5% W/V) for 5 min. If the animal showed a LR, a paired rat was challenged with saline and served as a control. R_L was measured at 5, 10 and 15 minutes after OA challenge and subsequently at every 15 minute until the end of the experiment. When a LR was identified in the test animal, both the test animal and matched control were injected with Evans blue. Six rats with LR were matched with control rats. An additional saline control was arbitrarily time matched to one of the 6 animals with LRs to provide 7 controls. The 2 animals without LRs were not paired with a control.

4.3.5. Data Analysis:

A significant early airway response was defined as an increase in R_L to at least 150% of the baseline value within 15 min of the start of allergen challenge.

A late airway response was considered as an increase in R_L to at least 150% of the post-saline value for a minimum period of 45

min between 4 and 8 hours after OA challenge. The peak value of R_L for allergen challenged tests or saline challenged controls was the maximum value of R_L during the 45 min period corresponding to the LR.

The data are expressed throughout as the mean±SE. Comparisons of means between test and control groups were made by the Student t-test for unpaired data. Paired t-tests were used to compare the baseline and peak values of R_L after challenges. However, the peak values of the late response and peak value after 5-HT inhalation were not normally distributed and a Wilcoxon signed ranks test was used to compare the baseline R_L and the peak values in these two groups. Comparisons of the peak values during the period of the late response after either OA or saline challenge were made by the Mann-Whitney U test.

Correlation between vascular leakage and increased pulmonary resistance was tested by linear regression analysis using the method of least squares. $P < 0.05$ was considered significant.

4.4. RESULTS

4.4.1. Changes in pulmonary resistance after inhalational challenges:

Seven test and 6 control rats were used for the study of vascular permeability during the ER. The baseline values of R_L were similar in both groups (control = 0.163±0.011 : test = 0.148±0.012 cm H_2O/ml/s.; $p > 0.1$). An early response was found in 6 out of 7

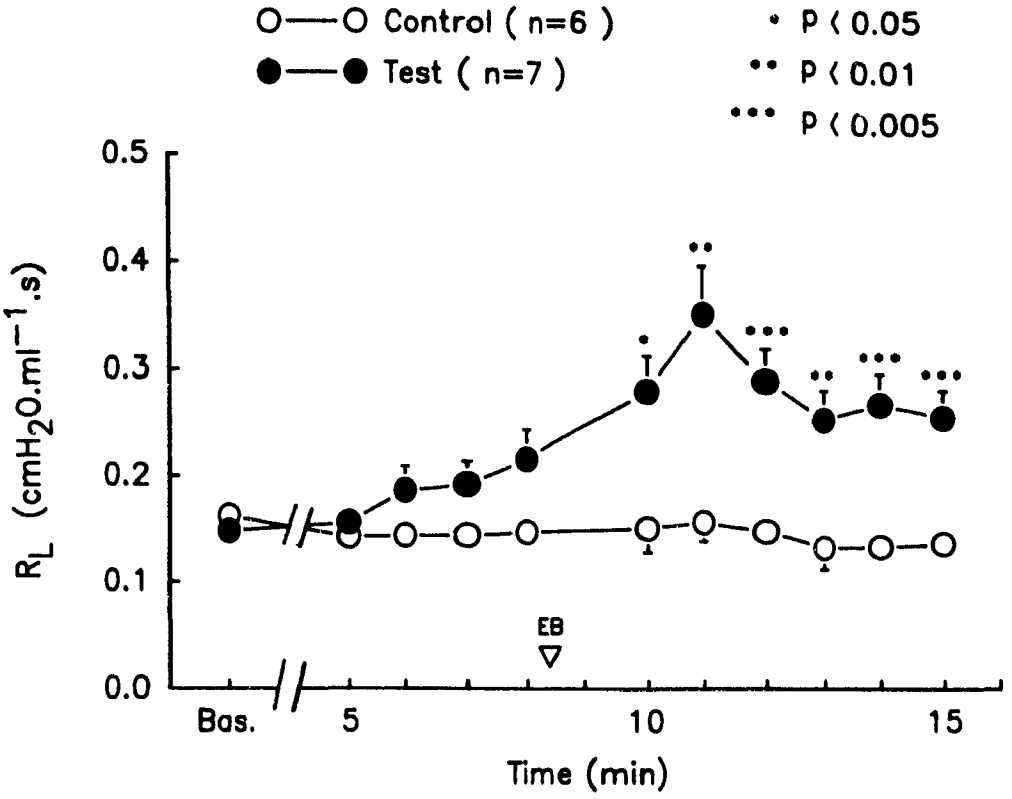


Fig. 4.1. Changes in the pulmonary resistance (R_L) during the OA induced early response (ER). In test rats, R_L was significantly increased following OA inhalation compared with that in saline challenged controls, indicating the presence of ER. Bas = Baseline value. The arrow indicates the time of administration of Evans blue (EB) through the external jugular vein. The vertical bars indicate one standard error of the mean.

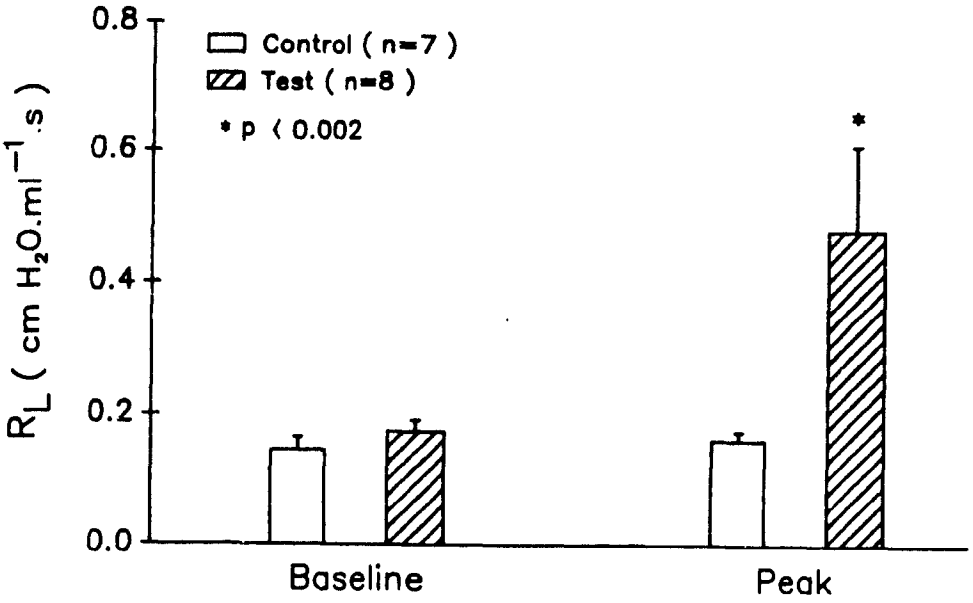


Fig.4.2. Comparison of the baseline value of pulmonary resistance (R_L) and peak value of R_L during the late response (LR) between the tests and controls. The baseline R_L was similar in both both test and control rats. The peak value of R_L for the tests and controls was the maximum value of R_L during the 45 minutes period corresponding to the LR. The peak value of R_L during the OA induced LR in the test rats was significantly greater than in controls. The vertical bars indicate one standard error of the mean.

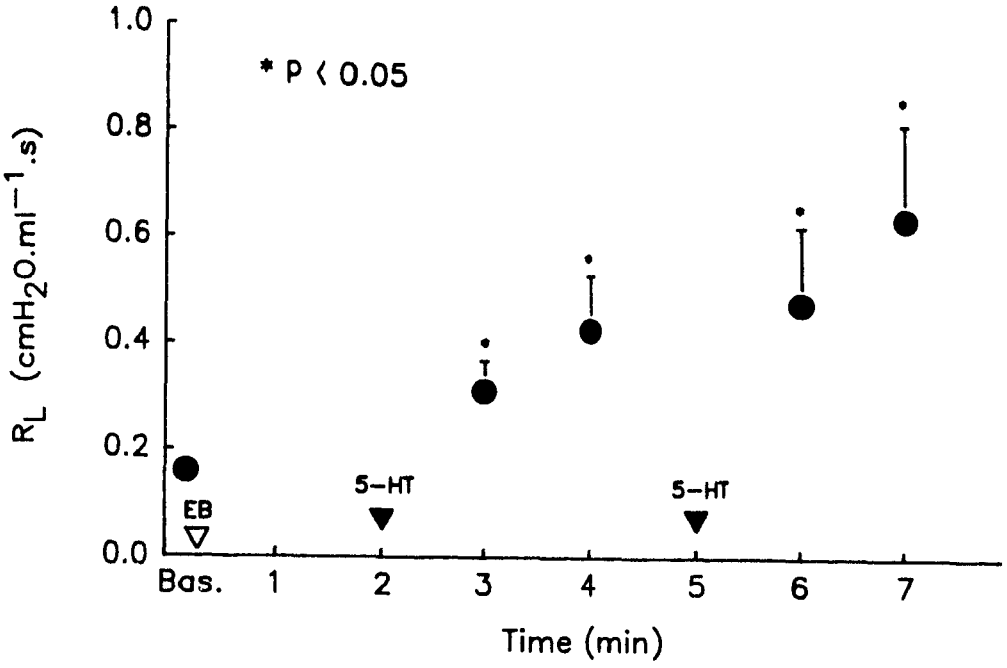


Fig.4.3. Comparison of baseline values of pulmonary resistance (R_L) and peak value of R_L after 5-HT challenge. Arrows indicate time of administration of Evans blue (EB) through the external jugular vein (open arrow) and 5-HT by aerosol (filled arrows). 5-HT induced a significant increase of R_L compared with the baseline value (Bas). The vertical bars indicate one standard error of the mean.

test rats following OA challenge; the peak value of R_L was 0.351±0.04 (n=7; $p < 0.01$). In time matched control rats, the R_L changed to 0.157±0.018 which was not a significant change ($p > 0.1$; figure 1).

The baseline values of R_L were similar in both groups of LR animals (control = 0.146±0.02 : test = 0.174±0.017 $cmH_2O/ml/s.$; $p > 0.4$). A late response was present in 6 out of 8 test rats after OA challenge; R_L increased to a peak value of 0.479±0.129 (n=8; $p < 0.02$). In time matched control rats, the R_L increased to 0.162±0.014 which was not significant ($p > 0.2$). The peak values of R_L between the two groups were significantly different ($p < 0.002$; figure. 2).

Six sensitized rats were challenged with aerosolized 5-HT. The baseline value of R_L in 5-HT challenged rats was 0.159±0.018. After 5-HT inhalation, the R_L increased to 0.636±0.181. The increase was significant compared to the baseline ($p < 0.03$; figure 3).

4.4.2. Microvascular Leakage during the early and late responses:

The quantity of Evans blue dye in the airway tree was slightly greater in the test rats during the ER. However, the difference was not significant (table 1; figures 4a, 4b and 4c). During the late response, there was also no significant increase in the quantity of Evans blue dye in the tissues of test animals compared to paired controls (table 1; figures 4a, 4b and 4c).

Compared with the control rats from the ER group,

Table 4.1.

Quantity of Evans Blue in the Wet Tissue (ng/mg)

Duration	ER		LR		5-HT induced
Groups	Test (n=7)	Control (n=6)	Test (n=8)	Control (n=7)	Responses (n=6)
Tissues					
M-Bron.#	37.9±8.2	19.3±4.3	21.7±4.9	32.8±12.8	90.7±20*&**
C-AW.#	27.6±6.8	24.6±4.6	39.4±8.7	26.4±7.8	42.6±3.9*
P-AW.#	30.4±6.8	27.8±5.9	35.9±3.5	29.9±6.1	40.5±5.8

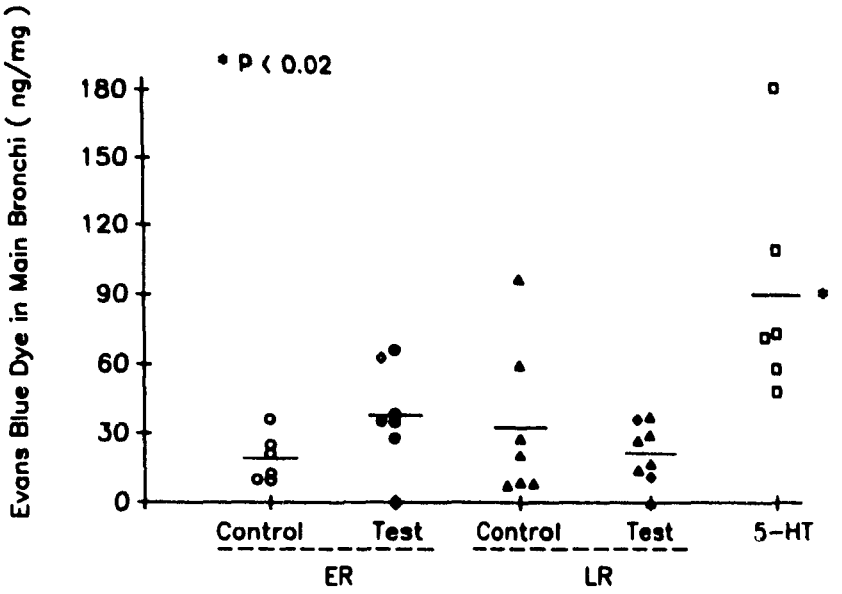
#. M-Bron. = Main Bronchi; C-AW. = Central Airways;
P-AW. = Peripheral Airways.

*. $p < 0.02$, when compared with the control rats in the ER group.

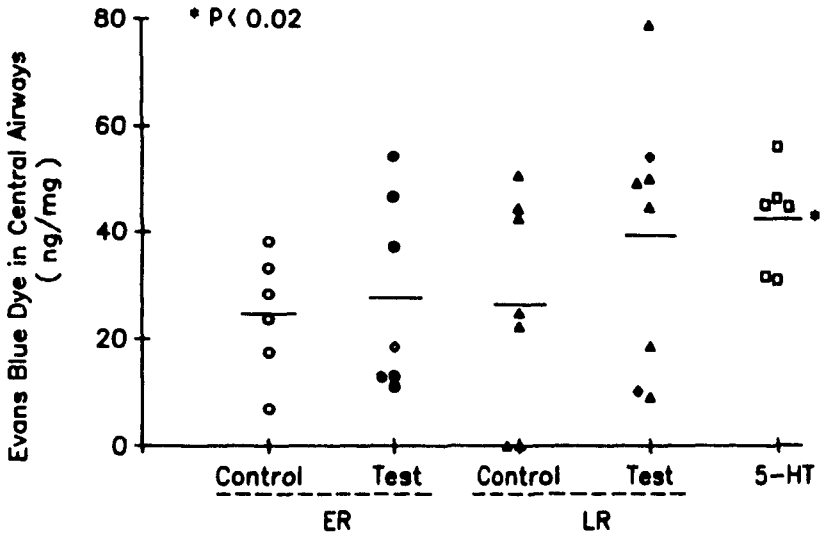
**. $p < 0.05$ when compared with the test rats in the ER group.

Fig.4.4. Comparison of the quantity of Evans blue (EB) in the tissues from 5-HT challenged rats (open squares), controls during the ER (open circles) or LR (open triangles), the OA tests in ER (filled circles) or LR (filled triangles) groups. The open diamonds are the test rats, which did not show an ER or LR. The horizontal bars indicate the mean values for each group. In 5-HT challenged rats, EB was significantly greater in the main bronchi than that of both tests and controls of ER group (4a); EB was significantly greater in the central airways than the controls of ER group (4b), indicating vascular leakage in the main bronchi and central airways of 5-HT challenged rats. EB in 5-HT challenged rats did not differ significantly from ER controls and tests in the peripheral airways (4c). There was no difference in the quantity of EB between the OA test and controls of ER or LR groups in any sized airways.

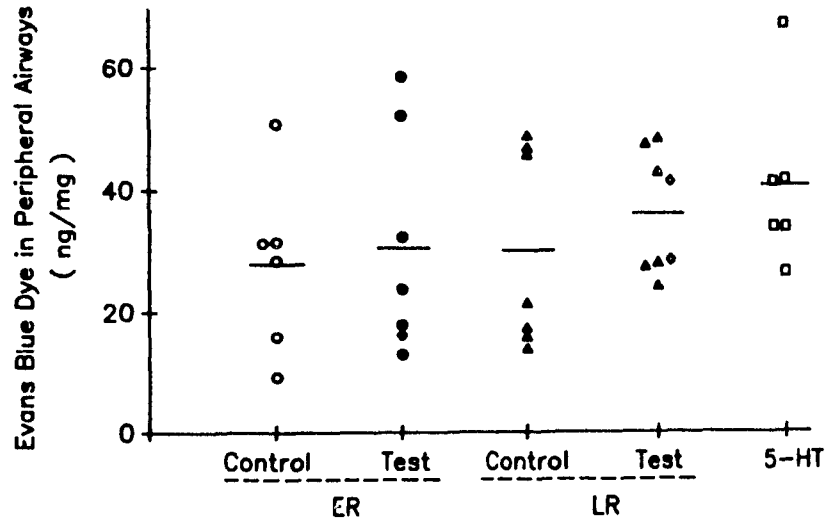
4.4a.


* P < 0.02
Evans Blue Dye in Main Bronchi (ng/mg)
180
150
120
90
60
30
0
Control
Test
Control
Test
5-HT
ER
LR

4.4b.


* P< 0.02
Evans Blue Dye in Central Airways
(ng/mg)
80
60
40
20
0
Control
Test
Control
Test
5-HT
ER
LR

4.4c.


Evans Blue Dye in Peripheral Airways
(ng/mg)
60
40
20
0
Control
Test
Control
Test
5-HT
ER
LR

inhalational challenge with serotonin caused a significant increase in microvascular leakage in the main bronchi and central airways, but not in the peripheral airways (table 1; figures 4a, 4b and 4c). Using wet/dry weight ratio, the degree of fluid leakage was significantly increased in both central and peripheral airways after inhalational challenge of serotonin. However, wet/dry weight ratio did not show a significant difference in the main bronchi between serotonin challenged rats and saline challenged rats (table 2 and figure 5).

There was no correlation between the quantity of Evans blue and changes in pulmonary resistance from baseline in all animal groups.

4.5. DISCUSSION

The results of this study demonstrated that there was no statistically significant increase in the quantity of Evans blue in the intrapulmonary airways in OA challenged rats compared to controls during both the ER and LR. This suggests that significant microvascular leakage was not present during the allergen induced ER and LR. In contrast, increases in pulmonary resistance of comparable magnitude induced by 5-HT were associated with a measurable increase in leak of Evans blue.

We have previously shown that both ER and LR can be induced by inhalational challenge with allergen in sensitized Brown Norway rats (Eidelman et al. 1988). Using a quick freezing technique to preserve the lung structure, we found that area of the airway wall

Table 4.2.

Wet / Dry Ratio of the Tissues after OA or Serotonin (5-HT) Challenge

Tissues#	Test (n=7)	Control (n=6)	5-HT (n=6)
M-Bron.#	4.067±0.15	3.771±0.191	4.214±0.248
C-AW.#	3.907±0.434	4.105±0.149	4.618±0.139*
P-AW.#	4.778±0.146	4.407±0.167	5.0396±0.157*

#. Tissues from either OA or saline challenged rats were in the ER group. M-Bron.= Main Bronchi; C-AW.= Central Airways; P-AW.= Peripheral Airways.

*. $p < 0.05$, when compared with saline challenged rats in the ER group.

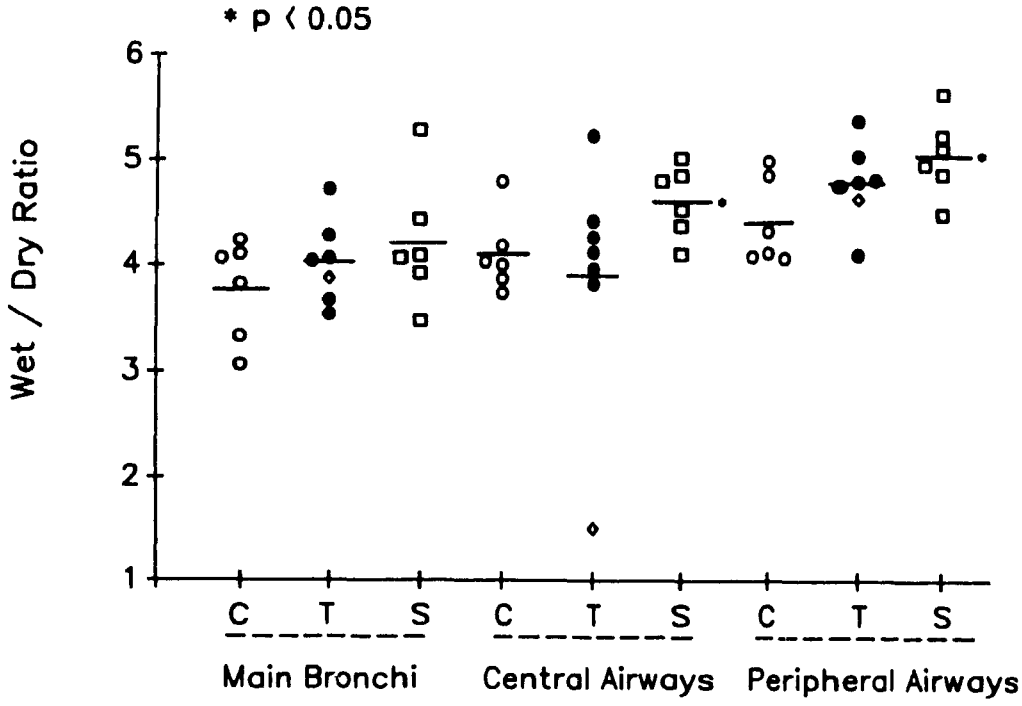


Fig. 4.5. Comparison of wet/dry weight ratio of the tissues from 5-HT challenged rats (S; open squares), and control (C; open circles) and test (T; filled circles) in the ER group. The open diamond represents a test rat, which did not show an ER. The horizontal bars indicate the mean values for each group. The results showed that wet/dry weight ratio was significantly greater in the central and peripheral airways of 5-HT challenged rats than the controls of ER group. However, there was no difference in the wet/dry weight ratio between the OA test and controls in ER group.

was not significantly increased during ER and LR (Du et al. 1991a and 1991b). This suggested that in the rat, airway wall thickening did not contribute to the airway narrowing of either ER or LR. Because of the uncertain sensitivity of morphometric techniques for the detection of airway edema, we felt that it was appropriate to investigate microvascular leakage using a different approach. The results of the present study confirm that airway vascular leakage is not significantly altered, consistent with our previous conclusions. These result are somewhat at variance with other published observations. In allergic asthmatic patients, airway hyperemia and edema has also been observed by bronchoscopy within a short period after local allergen challenge (Metzger et al. 1987). Furthermore, after allergen inhalation, edema and vessel dilation can be observed in the airways of allergic rabbits during the periods of ER and LR (Behrens et al. 1987). It is possible that these difference are related to the intensity of the allergic response, which may have been greater in these studies. At least as reflected in pulmonary resistance, the allergic response of the rat to aerosol challenge is relatively modest.

The intravenous injection of Evans blue was the technique applied in this study for the detection of vascular leakage during the ER and LR in sensitized Brown Norway rats. Both Evans blue and radio-labelled albumin are substances commonly used to detect vascular leakage (Chung et al. 1990; Brokaw and McDonald 1987). In both the skin and airways, it has been demonstrated that estimates of vascular permeability using the Evans blue technique

are well correlated with leak of radio- labelled albumin (Udaka et al. 1970; Rogers et al 1989). Using this method, an increase of Evans blue in the airways of the guinea pig can be induced by intravenous administration of both PAF and histamine (Evans et al. 1987).

Since serotonin has been described as a potent chemical mediator for the induction of pulmonary microvascular leakage in the rat (O'Donnell et al. 1987) and dog (Kamiya et al. 1987), we used 5-HT as a positive control to validate the technique. As previously reported, 5-HT inhalation resulted in a significant increase of either Evans blue dye or wet/dry weight ratio in all of the airways (figures 4 and 5; tables 1 and 2). However, there was substantial variability in values of Evans blue detected in the tissues of saline challenged animals, ranging from as little as 9.2 to 50.8 ng/mg in the peripheral airways during the period corresponding to the ER. This variability seriously limits the value of this technique for the detection of small changes.

The wet/dry weight ratio of the tissues from animals either during early response or after serotonin inhalation were also examined. The results were similar to those of the Evans blue in that there was no evidence of increased extravascular water after antigen challenge. However, the wet/dry weight ratio was significantly different in the smaller intraparenchymal airways but not in the main bronchi after 5-HT, whereas the Evans blue accumulation was greater in the main bronchi but not in the smaller intraparenchymal airways. One explanation might be due to the

anatomical difference between intrapulmonary airways and main bronchi, because the main bronchi contains more cartilage than the intrapulmonary airways and the proportion of liquid in the peripheral airways is greater than in other part of the airways. The sensitivity of wet/dry weight ratio as a measure of vascular leakage appears to be controversial (Jeffries et al. 1981; Persson et al. 1986).

Small changes in airway wall thickness may greatly influence airway closure (James et al. 1989; Moreno et al. 1986) and thus even slight microvascular leakage might provoke large increases in pulmonary resistance. However, we did not find a correlation between the amount of Evans blue and pulmonary resistance within any of the test groups. The absence of microvascular leakage together with our previous finding of a lack of measurable changes in airway wall thickness during allergen induced ER and LR (Du et al. 1991a and 1991b) further support the conclusion that, at least in the Brown Norway rat, both the ER and LR are due principally to airway smooth muscle contraction.

As reported by Sertl and coworkers (1988), in Sprague-Dawley rats passively sensitized by murine monoclonal IgE-anti-dinitrophenyl (DNP), vascular leakage can be induced by intravenous challenge of mouse serum albumin conjugated DNP. Compared with the control animals, radio-labeled albumin in trachea and hilum was increased approximately 300% and 100%, respectively. The difference between their results and ours may be due to either the method of allergen challenge or strain difference. Since it has

been reported that genetic factors can influence the nonspecific bronchial reactivity in different inbred rat strains (Pauwels et al. 1985), the degree of vascular leakage might also vary between different rat strains. In contrast, it has also been shown that intravenous administration of allergen can cause a significant increase in vascular permeability in the airways of sensitized guinea pigs (Evans et al. 1988). The difference between reported responses in the BN rat and guinea pig may also be due to either the method of allergen challenge or to species difference. So far, to our knowledge, the difference between intravenous and inhalational allergen challenge induced vascular leakage has not been compared. Possibly, the presence of allergen in the blood vessel may directly effect the endothelial cell and cause more severe vascular leakage than by inhalation allergen challenge. In guinea pig, it has been suggested that PAF receptors responsible for increased vascular permeability are situated on vascular endothelial cells (Evans et al. 1987), but these receptors have not yet been accurately localized.

In conclusion, we have demonstrated that following allergen challenge of sensitized BN rats, significant vascular leakage can not be detected in the airway wall and therefore it is not an important contributing factor to the airway narrowing of early and late responses. In this animal, airway smooth muscle contraction accounts for the changes in pulmonary resistance during during both early and late airway responses following the inhalational challenge of allergen.

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CHAPTER V

STRUCTURAL CHANGES IN THE AIRWAYS OF SENSITIZED BROWN NORWAY RATS FOLLOWING REPEATED ANTIGEN CHALLENGES

5.1. ABSTRACT

The purpose of this study was to quantitate the structural changes in the airways of sensitized rats after repeated challenge with aerosolized antigen and to examine the relationship between these changes and alterations in responsiveness to methacholine (MCh). We studied 28 Brown Norway rats that were actively sensitized to ovalbumin (OA). Responsiveness to aerosolized MCh was quantitated as the concentration of MCh required to double pulmonary resistance ($EC_{200}R_L$). The $EC_{200}R_L$ was determined before and 1 and 5 days after 3 inhalational challenges with OA (n=17) or saline (n=11) at 5 day intervals (on days 14, 19 and 24 after sensitization). Responsiveness to MCh increased after OA; $EC_{200}R_L$ fell from 1.71 mg/ml to 0.71 mg/ml at 1 day ($p<0.01$) and 0.87 mg/ml at 5 days ($p<0.02$) after OA, but did not change after saline challenge. Formalin-fixed lungs from a sample of OA challenged (n=12) and saline challenged (n=6) animals were paraffin-embedded and 5 micron sections were stained with hematoxylin-phloxine-saffron. Cross-sectional areas of the airway wall and smooth muscle (ASM) were determined for all intra-pulmonary membranous airways. There was an approximately two-fold increase in the quantity of airway smooth muscle in airways of OA challenged animals compared to saline challenged controls. Airway wall area did not change significantly. There was a correlation ($r=0.618$, $p<0.05$) between the quantity of ASM in large airways (basement membrane length: 2.00 to 2.99 mm) and change in responsiveness to MCh.

5.2. INTRODUCTION

After specific antigen exposure, sensitized subjects frequently experience an increase in airway responsiveness to methacholine or histamine (Cartier et al. 1982; Durham et al. 1988; Mussaffi et al. 1986) that may remain for several days. The mechanism of the increase in responsiveness is unknown but may be linked to airways inflammation (Durham et al. 1988). This possibility is suggested by the association between the development of airways hyperresponsiveness and the presence of late responses to provocation testing with inhaled antigen (Cartier et al. 1982; Mussaffi et al. 1986; Cockcroft et al. 1977); late responses are associated with the appearance of inflammatory cells in the bronchoalveolar lavage (De Monchy et al. 1985) and an inflammatory response in the airways of animals (Chung et al. 1985; Abraham et al. 1988; Marsh et al. 1985; Hutson et al. 1988; Iijima et al. 1987).

Structural changes have been described in the airways of asthmatic subjects that might account for some of the hyperresponsiveness of asthma. James et al. (1989) have described wall thickening in the airways of asthmatic subjects that theoretically could contribute substantially to exaggerated airway narrowing, without invoking any alteration in the properties of airway smooth muscle. An increase in the quantity of airway smooth muscle has also been demonstrated in asthmatic airways (Heard and Hossain 1973; Kleinerman and Adelson 1987). However, the

relationship of either airway wall thickening or increase in muscle to hyperresponsiveness is uncertain because both of these changes are conceivably consequences of longstanding asthma and are not necessarily linked to hyperresponsiveness. Recently we have reported that the Brown Norway rat develops airway hyperresponsiveness following active sensitization and repeated inhalational challenges with ovalbumin (Bellofiore and Martin 1988). The present study was designed to examine the airways of this same rat strain following multiple antigen challenges for changes in thickness and smooth muscle and to see if such changes might account for airways hyperresponsiveness induced in this manner.

5.3. MATERIALS AND METHODS

5.3.1. Animals and sensitization:

Twenty-eight Brown Norway rats (16 male and 12 female), 7-8 weeks old and 130 to 240 g in body weight were actively sensitized by a single subcutaneous injection of 1 mg of ovalbumin (OA) and 200 mg of aluminum hydroxide. Simultaneously, 1 ml of Bordetella pertussis vaccine, containing 6 x 10^9 heat-killed organisms, was given intraperitoneally as an adjuvant.

5.3.2. Protocol for challenges:

In order to evaluate the baseline airway responsiveness prior to exposure to aerosolized antigen, 17 rats underwent methacholine (MCh) challenge at 10 days after sensitization. Subsequently

they received three OA challenges at 5 day intervals (on days 14, 19 and 24 after sensitization). Two more MCh challenges (on days 25 and 29 after sensitization) were performed to estimate airway responsiveness at 24 hours and 5 days after the final OA exposure. A group of 11 rats that were used as control were challenged with MCh initially and again after three challenges with aerosolized saline. Saline challenges were performed at 5 day intervals following an identical time course to the 17 test animals.

5.3.3. Animal preparation and lung mechanics:

Rats were first sedated with xylazine (7 mg/kg i.p.) and 15 min later, anesthetized with pentobarbital sodium (30 mg/kg i.p.). Blind oro-tracheal intubation was performed using a 6 cm length of polyethylene tubing (PE 240). The tip of the tracheal tube was inserted into a small Plexiglas box (265 ml in volume). Airflow was measured by a Fleisch no. 0 pneumotachograph coupled to a differential transducer (MP-45$\pm$2 cmH_2O; Validyne Corp., Northridge, CA) which was attached to the box.

Changes in esophageal pressure (Pes) were measured using a saline-filled catheter that was placed in the lower third of the esophagus and connected to one port of a differential pressure transducer (Sanborn 267 BC; Hewlett-Packard, Waltham, MA). The other port of the transducer was connected to the box. Transpulmonary pressure (Ptp) was obtained from the difference between Pes and box pressure. Pulmonary responses were evaluated from pulmonary resistance (R_L) which was obtained from flow, Ptp

and the integrated flow signal by fitting the equation of motion of the lung using multiple linear regression as previously described (Bates et al. 1989). In the intervals between measurements, the box was flushed with air to prevent accumulation of carbon dioxide.

5.3.4. Methacholine inhalational challenge:

Aerosols were generated by a Hudson nebulizer connected to a side port of the box and driven by compressed air to produce an output of 0.18 ml/min. Aerosols of saline or MCh were inhaled by the spontaneously breathing animals over a 30 second period. After a baseline measurement of R_L, an aerosol of saline was administered followed by MCh in progressively doubling concentrations. The concentration of MCh was increased from 0.0625 mg/ml to a concentration that was sufficient to cause at least a 200% increase of the baseline value of R_L. For a period of approximately one minute following each administration the Ptp was inspected until it reached a new stable value. This method permitted the identification of the peak response to each challenge. On the final challenge with MCh the concentration of MCh was increased until a maximal or plateau response was obtained for 6 of the saline challenged animals and 7 of the OA challenged animals.

5.3.5. Ovalbumin inhalational challenge:

Challenge with ovalbumin was performed using the same apparatus as for MCh. Ovalbumin (OA) was diluted in saline immediately before each experiment (5% W/V). After a baseline

measurement of R_L, saline was inhaled for 5 minutes and the value of R_L immediately afterwards served as control. Then rats inhaled an aerosol of OA for 5 minutes and measurements of R_L were taken at 5, 10, 15, and 30 minutes after exposure. The control group received an inhalational challenge of saline only for 5 min.

5.3.6. <u>Tissue preparation, histology and morphometric studies</u>:

Twelve rats that received OA challenges and 6 saline challenged controls were selected for morphometric studies. Among the test animals we selected 6 whose concentration-response curves to MCh by inspection showed no increase in responsiveness after antigen challenge and 6 that demonstrated the largest changes in responsiveness. The saline controls were chosen randomly. Immediately following the last MCh challenge, the rats were exsanguinated by cardiac puncture and the lungs were removed for histological studies. Lungs were fixed with intrabronchial perfusion of 10% neutral formalin at a constant pressure of 25 cmH_2O and at room temperature for a period of 48 hours.

Morphometry was performed on 5 micron thick paraffin embedded sections (Microtome model 820: American Optical Corporation, New York, NY) taken from the midsagittal regions of each lung. The sections were stained with hematoxylin-phloxine- saffron (HPS). Measurements of the airway were performed by projecting the image of the airway onto paper using either photographic slides for smaller airways (magnification 250 - 400X) or direct projection of the image from the glass slides for larger airways (

magnification 62.5X) using a microprojector (Zeiss, W. Germany). The outlines of the airway structures of interest were traced on paper and subsequently measured using a computer-controlled digitizing board (Jandel Scientific, Corte Madera, CA) and commercial software (Sigma Scan, Jandel Scientific, Corte Madera, CA).

Airways were analyzed consecutively on slides from left mid-sagittal sections until a representative sample was obtained. In cases where the number of large airways was very small the right mid-sagittal section was also analyzed. Airways whose ratios of maximum internal diameter to minimum internal diameter was equal to or larger than 2 were considered to be cut tangentially and were not measured. The internal diameters were measured from basement membrane of one side of the airway to the basement membrane on the opposite side. All airways were evaluated for the following morphometric dimensions: 1) internal airway lumen defined by both the inner border of the epithelium and the basement membrane of the epithelium (LuB); 2) length of airway basement membrane (BM); 3) external area of the airway defined by the outer boundary of the airway wall (Ae); 4) area of airway smooth muscle. The area of the airway wall (Aw) was calculated as the difference between Ae and LuB.

5.3.7. Data analysis:

We defined a significant early response (ER) to OA as one in which the value of R_L reached at least 150% of the baseline

measurement within 30 minutes after challenge. The magnitude of the ER was calculated from the highest value of R_L within the 30 minutes following challenge. Airway responsiveness to methacholine was defined as the concentration of methacholine that was required to double R_L ($EC_{200}R_L$) which was determined by interpolation from the concentration-response curve. We also calculated the $R_L$max which was the plateau response.

Airways were grouped into three categories based on the length of the basement membrane: small (BM: 0-0.99 mm), medium (BM: 1.0-1.99 mm) and large airways (BM: 2.0-2.99 mm). Since large sized membranous airways (BM > 3.0 mm) were not always present in all of the lung sections, the occasional airway that had a BM in excess of 3 mm was excluded from analysis. Data for individual airways were standardized for airway size by dividing each area by the square of the BM length. All morphometric measurements were performed by an observer (T. D.) blinded to the group status. The intra-observer and inter-observer variability were tested on a sample of 12 airways and the correlations between all results were highly statistically significant ($r^2>0.93$).

Comparisons of the $EC_{200}R_L$ for MCh were made using paired or unpaired t-tests, as appropriate, on logarithmically transformed data. Mean values for the magnitude of the ER were compared by paired t-test. Group means for baseline R_L, $R_L$max and data for morphometric measurements between saline challenged rats and OA treated rats were analyzed by unpaired t-test. Linear regressions were performed using the method of least squares. A p value less

than 0.05 was accepted as significant. Data are expressed as mean ± SEM throughout.

5.4. RESULTS

5.4.1. Measurement of Responsiveness to MCh:

Baseline values of R_L before each MCh challenge were similar in both saline challenged control and OA challenged test rats (Table 5.1). Likewise, no significant difference was found in the baseline $EC_{200}R_L$ to MCh between control (1.59 mg/ml) and test (1.72 mg/ml) groups.

All of the test animals responded with a significant ER to at least one of the 3 OA challenges. The prevalence of significant ERs (R_L > 150% of the post saline value) was 82%, 71% and 76% after the 1st, 2nd and 3rd OA challenges, respectively. Furthermore, there was no evidence of tolerance associated with multiple challenges. The magnitude of the ER was 260±30 (% of the baseline value) after the first, 298±82 after the second and 225±17 after the third challenge.

MCh responsiveness increased significantly from the baseline value at 24 hours after the last OA (Figure 5.1). The $EC_{200}R_L$ for MCh was 0.71 mg/ml at 24 hours after the last OA exposure, significantly lower than the first MCh challenge (1.72 mg/ml; $p < 0.01$). The $EC_{200}R_L$ was still significantly reduced on the third MCh challenge (0.87 mg/ml; $p < 0.02$) at 5 days after the last exposure to OA. In contrast there was no significant change in the $EC_{200}R_L$ following repeated saline challenges; the $EC_{200}R_L$ was

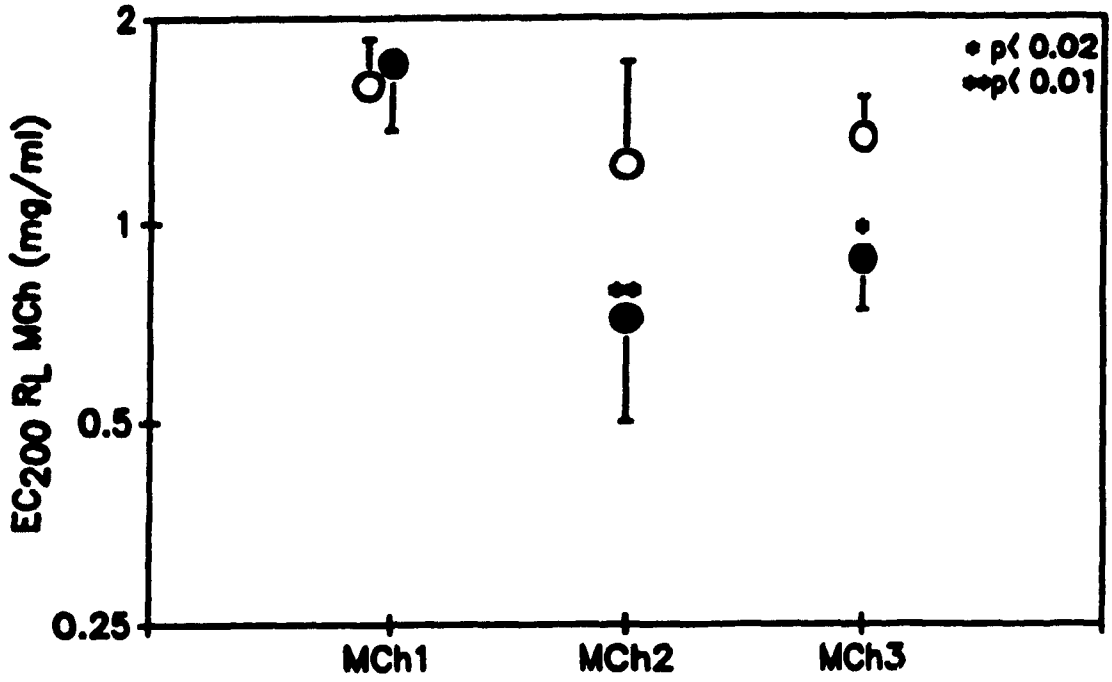


Fig.5.1. Airway responsiveness to methacholine ($EC_{200}R_L$) was significantly greater at 24 hrs (MCh 2) and also at 5 days (MCh 3) following the 3rd OA challenge. In contrast, control animals did not show any significant change in $EC_{200}R_L$ over a comparable time period. The vertical bars indicate one standard error of the mean.

1.33 mg/ml at 24 hours after the third saline challenge and was not significantly different from the baseline $EC_{200}R_L$ for MCh of 1.59 mg/ml.

5.4.2. Morphometric measurements:

Photomicrographs of illustrative airways from a control and test animal are shown in Figure 5.2. The normal airway shows a thin strip of smooth muscle which is larger in the airway from the test animal. The epithelium is intact in both airways. There is a mild peribronchial mononuclear and polymorphonuclear cell infiltrate in the airway from the test animal.

The average number of airways analyzed per rat ranged from 8-19 (mean 13.7). There was a substantial increase in the quantity of airway smooth muscle in all categories of airway (Figure 5.3a). The magnitude of the increase, approximately 2-fold, was similar among different sized airways. There were no statistically significant changes in either the area of the epithelium or of the total airway wall between control and test animals (Figures 5.3b and 5.3c).

There was no correlation between the quantity of airway smooth muscle overall and the change in responsiveness to MCh. The quantity of ASM was determined for each animal as the average ASM for all airways after standardization for size. The change in responsiveness was calculated as the difference between the values in logarithms of $EC_{200}R_L$ from the 1st and 3rd challenges with MCh. There was also no correlation between the $R_L$max measured on the 3rd

TABLE 5.1.
Baseline R_L (cm $H_2O.ml^{-1}.s$)

	MCh 1	MCh 2	MCh 3
Control (n=11)	0.12 ± 0.04	0.095 ± 0.019	0.11 ±0.019
Test (n=17)	0.11 ± 0.025	0.097 ± 0.022	0.094 ±0.016

Fig. 5.2. Illustrative airway from a control animal (a) and test animal (b). There is an increase in smooth muscle and a mild inflammatory infiltrate in the airway wall of the test animal. x200.

5.2a.

5.2b.

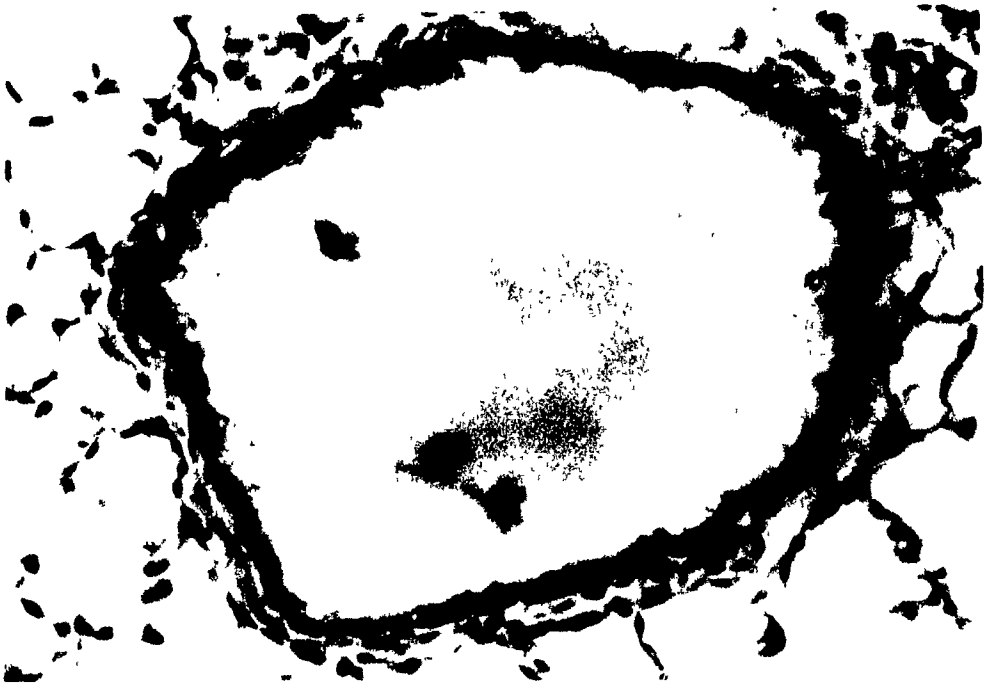
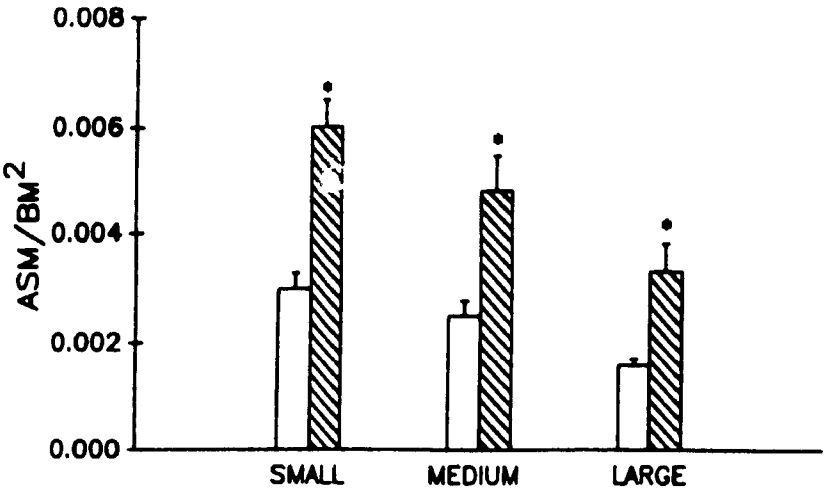
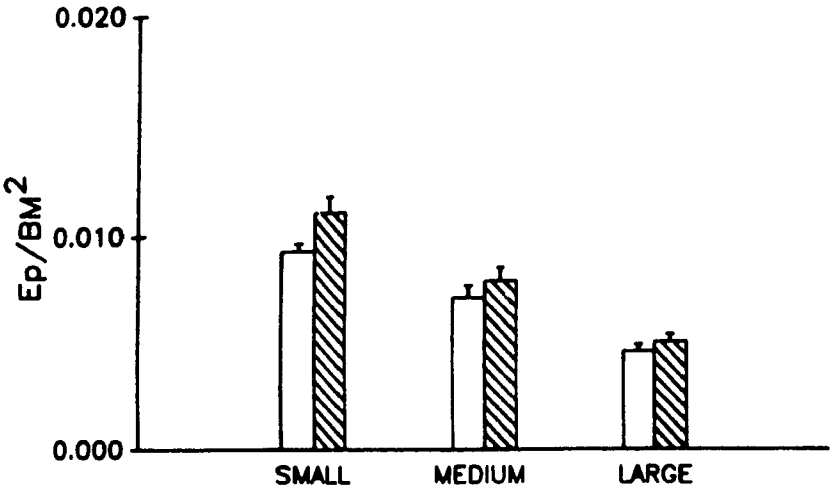


Fig. 5.3. a) Airway smooth muscle (ASM) standardized to the square of the basement membrane (BM^2) was greater in all categories of airway from test animals than controls; **b)** The area of epithelium was similar in the two groups; **c)** The airway wall area was not significantly greater in test animals than controls. Hatched bars indicate test animals and open bars the controls in all panels. The vertical bars indicate one standard error of the mean.

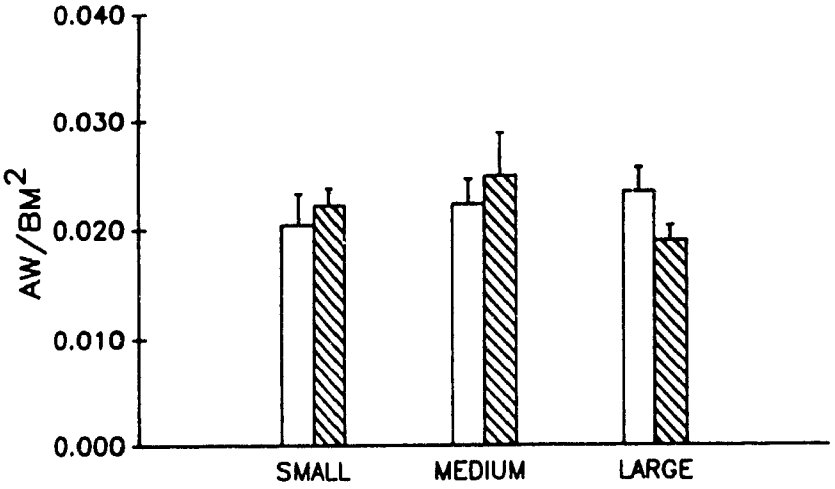
5.3a.


0.008
0.006
0.004
0.002
0.000
ASM/BM2
SMALL
MEDIUM
LARGE

5.3b.


0.020
0.010
0.000
Ep/BM2
SMALL
MEDIUM
LARGE

5.3c.


0.040
0.030
0.020
0.010
0.000
AW/BM2
SMALL
MEDIUM
LARGE

challenge with MCh and the quantity of ASM (Figure 5.4).

The relationship between change in responsiveness to MCh and airway smooth muscle in different sized airways is shown in Figures 5.5a, 5.5b, and 5.5c. The correlation between log $EC_{200}R_L$ and airway smooth muscle improved progressively from the small airways (n=0.241) to the large airways (n=0.6177). The correlation coefficient for the medium sized airways (n=0.3399) was intermediate between the small and large airways. The correlation reached statistical significance for the large airways. However, when the data for the animal with the most extreme quantity of smooth muscle (indicated by an asterisk) were eliminated the correlation coefficient fell to 0.4222 and was no longer statistically significant.

5.5. DISCUSSION

The results of this study show that three exposures to inhaled antigen at five day intervals cause a small but significant increase in responsiveness to MCh in the actively sensitized Brown Norway rat. There is a striking increase in the quantity of airway smooth muscle in all intrapulmonary membranous airways. There appears to be a relationship between the quantity of airway smooth muscle in the large airways and the observed changes in responsiveness to MCh.

We have previously demonstrated that the repeated administration of antigen by aerosol to sensitized Brown Norway rats leads to an increase in responsiveness to MCh (Bellofiore and Martin 1988). Following a similar protocol our present study

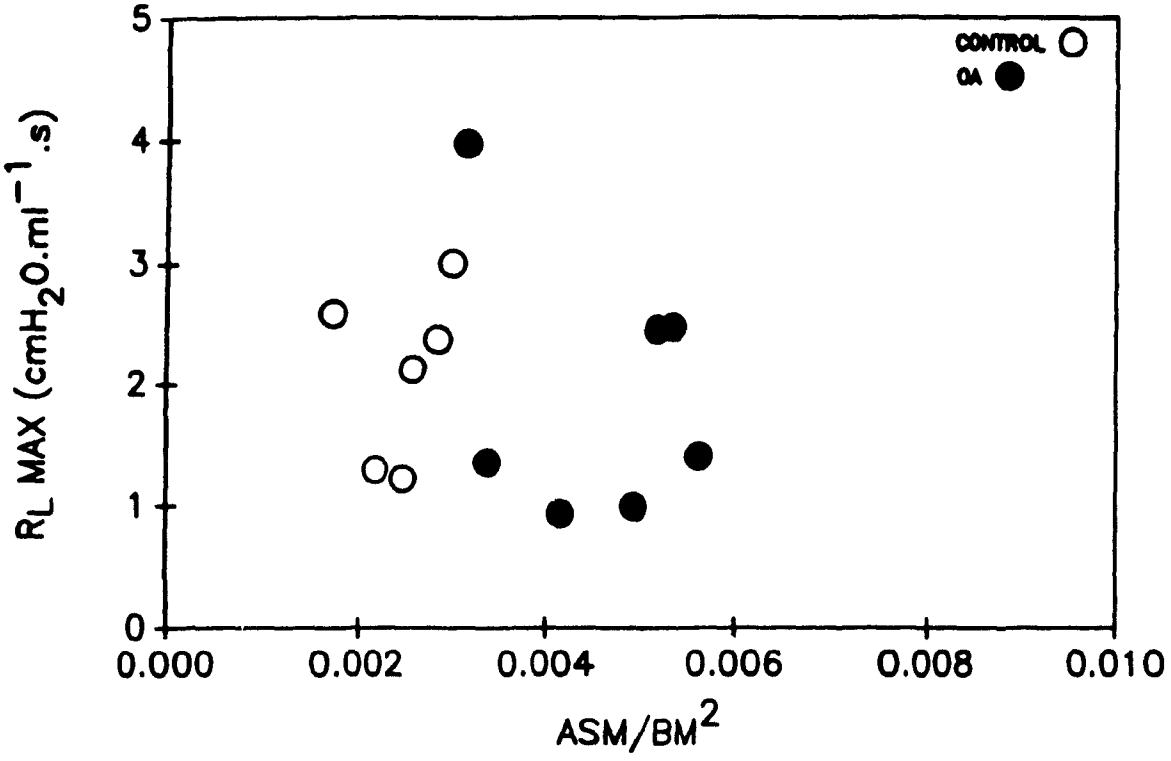
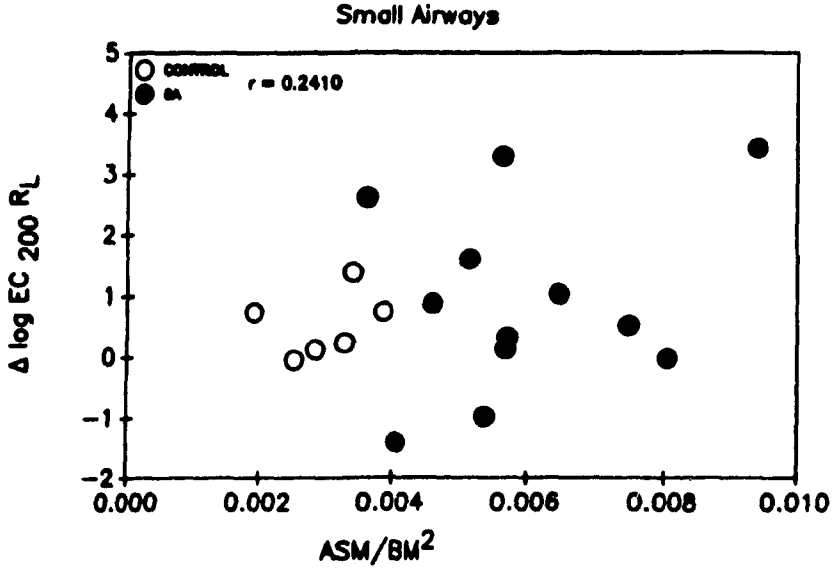


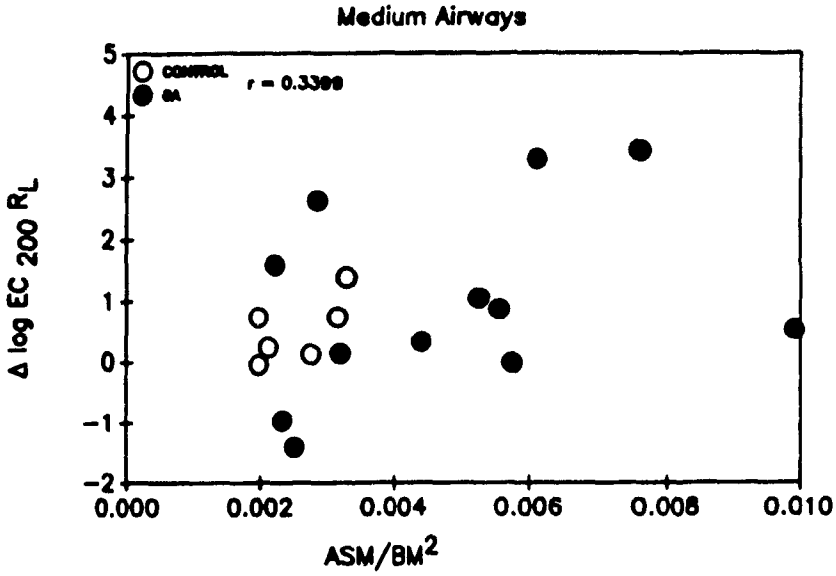
Fig.5.4. Despite a greater quantity of airway smooth muscle in OA challenged animals there was no difference in the maximal increase in R_L that could be induced by MCh ($R_L$max) between groups.

Fig.5.5.a) There was no statistically significant correlation (r=0.241) between the quantity of ASM in small airways (BM < 0.99 mm) and the change in airway responsiveness (log $EC_{200}R_L$). **b)** There was a slightly stronger relationship between log $EC_{200}R_L$ and ASM in medium airways (BM 1.0-1.99 mm; r=0.3399) and **c)** in large airways (BM 2.00-2.99 mm; r=.6177; $p<0.05$). In the latter group the correlation fell to r=0.4222 when the data point indicated by an asterisk was excluded.

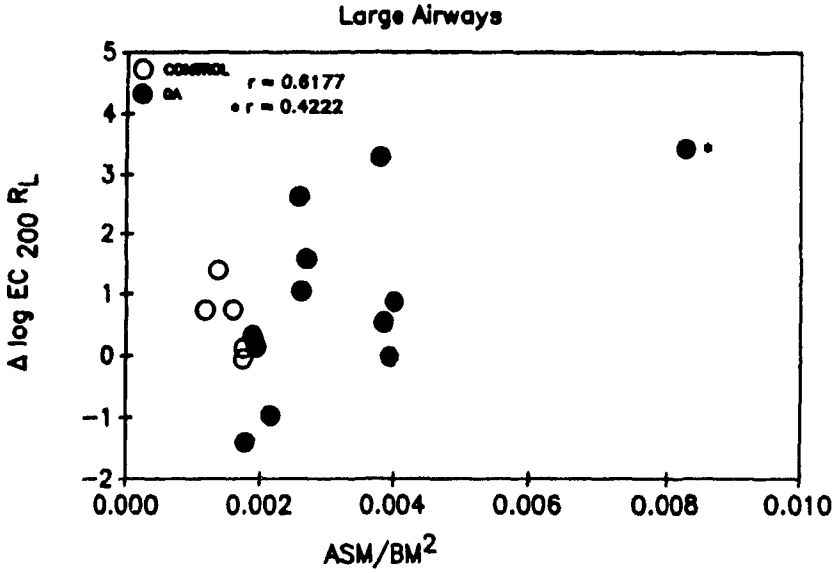
5.5a.


Small Airways
CONTROL
OA
r = 0.2410
Δ log EC 200 R_L
ASM/BM^2
5
4
3
2
1
0
−1
−2
0.000
0.002
0.004
0.006
0.008
0.010

5.5b.


Medium Airways
CONTROL
OA
r = 0.3399
Δ log EC 200 R_L
ASM/BM^2
5
4
3
2
1
0
−1
−2
0.000
0.002
0.004
0.006
0.008
0.010

5.5c.


Large Airways
CONTROL
OA
r = 0.6177
• r = 0.4222
Δ log EC 200 R_L
ASM/BM^2
5
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−1
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0.000
0.002
0.004
0.006
0.008
0.010

confirms these findings. Indeed, the magnitude of the change in responsiveness, albeit small, is very similar to our previous report. However, not all animals demonstrated an increase in responsiveness to MCh. Whereas Bellofiore et al. (1988) found that a failure to develop an early response to antigen seemed to account for the absence of changes in responsiveness to MCh, no sensitized animal in the present study consistently failed to show an early response to antigen provocation. Other investigators have found a closer relationship between the development of a late response to antigen challenge and hyperresponsiveness (Cartier et al. 1982; Mussaffi et al. 1986; Cockcroft et al. 1977). Therefore, it is possible that animals in the present study which failed to change responsiveness to MCh may not have had late responses to antigen because the prevalence of such reactions is only 60 to 70% in the Brown Norway rat (Eidelman et al. 1988). Unfortunately, the current study was not designed to determine whether late responses occurred.

The work of Moreno and colleagues (Moreno et al. 1986) has provided a strong theoretical basis for considering mechanical factors as potential causes of airways hyperresponsiveness. Although there are a substantial number of potential explanations for changes in airway responsiveness following antigen provocation, thickening of the airway wall and an increase in airway smooth muscle are both attractive candidates because both changes are observed in the airways of asthmatic subjects (James et al. 1989; Heard and Hossain 1973). The changes in the airway wall of the

rats following antigen provocation are similar to the reported changes in asthmatic airways (James et al. 1989) in that the quantity of airway smooth muscle increased but differed in that there was no change in the thickness of the airway wall overall. This apparent discrepancy probably relates to the fact that smooth muscle comprises only between 10 and 20% of the total airway wall area. Presumably significant airway wall thickening would have been recorded had the increases in ASM been greater. Consistent with the absence of significant airway wall thickening is the lack of any change in the baseline airway caliber, at least as reflected in the pulmonary resistance (Table 5.1).

There was no relationship between the quantity of airway smooth muscle overall and change in responsiveness. However, narrowing of the large airways is likely to be a more important determinant of changes in pulmonary resistance after methacholine because of preferential deposition of MCh in central airways related to aerosolization as well as the fact that large airways contribute more to resistance measurements than do small airways. Consistent with this argument, there was a progressive increase in the correlation coefficient for the relationship between smooth muscle and responsiveness when we examined small, medium and large airways separately (Figure 5.5). Indeed, the correlation reached statistical significance for the large airways. Because in the present study we did not analyze the largest airways, namely cartilaginous ones, it is conceivable that the relationship between airway smooth muscle in cartilaginous airways and responsiveness

might have been even stronger.

We were surprised that there was no difference in the maximal degree of bronchoconstriction between antigen challenged and control animals. Recent experiments are consistent with the hypothesis that the $R_L$max is determined by the balance of forces between the contracting smooth muscle and the load to shortening imposed by the elastic properties of the parenchyma (Ding et al. 1987; Bellofiore et al. 1989). Changing lung volume (Ding et al. 1987) or altering lung recoil by the induction of experimental emphysema (Bellofiore et al. 1989) can substantially alter the $R_L$max. Therefore we expected that the $R_L$max would also be affected by the quantity of airway smooth muscle. We do not, of course, know if the contractile properties of the smooth muscle in antigen challenged animals are similar to normal muscle.

Several studies have failed to find a relationship between airway responsiveness of subjects with chronic obstructive lung disease in vivo and the responses of isolated airway smooth muscle to pharmacologic agonists (Armour et al. 1984a; 1984b). Likewise, there was no relationship between responsiveness in vivo and the quantity of airway smooth muscle determined by morphometry. However, these studies were performed on airways taken from lung tissue resected surgically. Therefore it is possible that airway samples may not have been representative in that measurements of responsiveness may have been influenced by the presence of coexisting chronic obstructive lung disease.

The mechanism of the change in airway smooth muscle is at

present unknown but it seems plausible to hypothesize that growth factors are released following antigen challenge which stimulate hypertrophy and/or hyperplasia of muscle. The macrophage is one source of growth factors that is potentially involved in the process. Both interleukin-1 and platelet derived growth factor are mitogenic for smooth muscle cells (Raines et al. 1989). The presence of low affinity IgE receptors on the macrophage (Thorel et al. 1988) raises the possibility that activation of this cell by antigen challenge may lead to the synthesis and release of such factors. Interestingly, leukotriene C4 has been shown to stimulate the release of fibroblast growth factor from macrophages and the peptide leukotrienes have been clearly implicated in both the early and late responses to antigen challenge in the rat (Foster et al. 1988; Sapienza et al. 1990). Direct mitogenic effects of histamine have been demonstrated using primary canine tracheal smooth muscle cultures (Panettieri et al. 1990). Such effects may also be important in the rat, particularly if peptide leukotrienes and serotonin have similar properties.

Studies of the airways histology in asthmatics have reported denudation of the epithelium (Panettieri et al. 1990; Laitinen et al. 1989). Epithelial damage has the potential to cause changes in responsiveness because of loss of the physical barrier to bronchoconstrictive agonists when inhaled as well as the loss of epithelial derived relaxant factors (Jeffery et al. 1989; Butler et al. 1987; Barnes et al. 1985; Flavahan et al. 1985; Hay et al. 1986; Goldie et al. 1987). The epithelium seems unlikely to be

implicated in the changes observed in the current study because we did not find any evidence of damage to it. The cross-sectional area of the epithelium was unchanged nor was there any gross evidence of epithelial damage on light microscopy.

We conclude that one of the histologic features of the asthmatic airway, namely increase in smooth muscle, can be mimicked in the actively sensitized and antigen challenged Brown Norway rat. Our results suggest that there is a relationship between changes in airway responsiveness to methacholine and alterations in muscle in large airways. This model may be a useful way to explore the factors involved in the induction of airway smooth muscle following allergic airway responses.

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CHAPTER VI

GENERAL CONCLUSIONS

The major features of allergic asthma are allergen induced early and late responses, airway hyperresponsiveness and airway inflammation (Kay 1986). All of the features are manifested clinically by airway narrowing. This thesis provides evidence that pertains to the mechanisms of airway narrowing in a rat model of bronchoconstriction.

Theoretically, airway narrowing can be caused by airway smooth muscle shortening, airway wall thickening and mucus secretion. However, the mechanical factors which contribute to allergen induced early and late responses and the site of airway narrowing during these responses have never been clearly described perhaps because of technique difficulties. Using a modified quick freezing technique, I demonstrated that the airway narrowing during the early response is attributable to airway smooth muscle constriction but not to airway wall thickening and mucus. The degree of airway narrowing during the early response is greater in the large airways and smaller in small airways. The late response is also caused mainly by airway smooth muscle shortening and appears to be limited to the large airways.

Based on the known biological functions of the mast cell, including its high affinity IgE receptors and the capacity to release mediators when activated, this cell is considered as a primary trigger for the allergic responses. In this thesis, data is presented showing that the density of mast cells progressively increases from small to large intrapulmonary airways. The mast cell density in the airway tree is closely correlated with the

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Based on the results of my studies, the following information can be added to the literature:

1). In sensitized Brown Norway rats, allergen induced early and late responses are caused by airway smooth constriction.

2). Neither airway thickness nor mucous secretions are important contributing factors to the airway narrowing of allergen induced early and late responses in the sensitized Brown Norway rat.

3). Allergen induced airway narrowing during the early response affects the entire airway tree of sensitized rats. The degree of airway narrowing is progressively increases from small to large intrapulmonary airways.

4). The predominant site of airway narrowing during the allergen induced late response is the large intrapulmonary airways.

5). The density of mast cells progressively decreases from large to small intrapulmonary airways of normal Brown Norway rats.

6). The distribution of mast cells in the airway tree is closely related to the location of airway narrowing during the allergen induced early and late responses, suggesting that the mast cell may be the primary source of the bronchoactive mediators of the late as well as the early responses.

7). During the periods of allergen induced early or late responses, the microvascular permeability was not significantly increased in sensitized Brown Norway rats, indicating that vascular leakage does not contribute to allergen induced early and late responses.

8). An inflammatory cell infiltration of the airway wall is more closely associated with the late response than the early response after inhalational allergen challenged of the rats.

9). Following repeated allergen challenges, airway responsiveness to methacholine is increased in sensitized Brown Norway rats. The increased airway responsiveness is related to the increased quantity of airway smooth muscle, but not to airway wall thickening.

10). Repeated allergen inhalation may possibly induce either hypertrophy or hyperplasia of airway smooth muscle in the sensitized rat. This observation suggests that the rat may be a suitable model for the study of change in airway smooth muscle following chronic allergen inhalation.

11). Rapid freezing with liquid nitrogen may be a useful technique for further studies of the the mechanisms of airway narrowing in vivo.

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