THE ROLE OF SMOOTH MUSCLE IN DETERMINING HUMAN NON-SPECIFIC BRONCHIAL RESPONSIVENESS

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Page Contents

INDEX OF TABLES AND FIGURES

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PUBLICATIONS

Publications and presentations based on the work contained in this thesis.

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   Comparison of in vivo and in vitro smooth muscle sensitivity to methacholine in man.
   Thorax 1984; 39: 837-843

2. Roberts J.A., Rodger I.W., Thomson N.C.
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Thorax 1986; 41: 734
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ABBREVIATIONS

The following abbreviations have been used in this thesis -

CAO	Chronic airflow obstruction
Ca^{2+}	calcium ion
VOC	voltage operated calcium channel
FEV_1	forced expiratory volume in 1 second
Raw	airways resistance
TGV	Thoracic gas volume
sGaw	specific airways conductance (= Raw^{-1} x TGV^{-1})
TLC	total lung capacity
$V_x(p)$	partial expiratory flow rate at x% of TLC
$V_x(c)$	complete expiratory flow rate at x% of TLC
PC	Provocation concentration
NSBR	Non specific bronchial responsiveness
PG	Prostaglandin
LT	leukotriene
EC_x	measure of <u>in vitro</u> sensitivity being the concentration of agonist producing x% of maximum response obtained for that tissue
NANC	Non-Adrenergic, non cholinergic nervous system
SRS-A	Slow reacting substance of anaphylaxis
mg	milligrams
ml	millilitres
G	grammes
L	litres

SUMMARY

This project tested the hypothesis that variation in non specific bronchial responsiveness between individuals could be explained by differences in the sensitivity of the smooth muscle present in the airway. Non-specific bronchial responsiveness (NSBR) is closely associated with asthma and chronic airflow obstruction. The approach used was to measure the NSBR of patients due to undergo thoracic surgery, and compare this with the *in vitro* sensitivity of bronchial strips obtained from thoracotomy specimens.

In vivo measurements

The first part of the project was devoted to developing methods and protocols to measure changes in airway calibre so that responsiveness could be measured. FEV_1 is a reliable measure of airway calibre, but reflects overall airway function. As the *in vitro* measurements were made on larger airways, changes in specific airways conductance (sGAW) were used because they reflect changes in the larger airways. The measurement of sGaw by hand was time consuming and the measurement had large co-efficient of variation. To overcome these problems an automated system for the measurement of sGaw was developed.

In vivo versus **in vitro**

The first agonist used to compare *in vivo* responsiveness and *in vitro* smooth muscle sensitivity was the cholinergic agonist methacholine. Patients were assessed for evidence of baseline airflow obstruction, chronic bronchitis and atopy. One patient's results were highly suggestive of asthma, but this diagnosis had not been confirmed before his presentation for thoracotomy. Both *in vivo* responsiveness and *in vitro* sensitivity varied widely, but there was no correlation between the results. The probable asthmatic patient was most responsive *in vivo* but did not have increased sensitivity *in vitro*.

Methacholine acts by inhibiting the cholinergic system, rather than/

than as a direct receptor agonist. A direct smooth muscle stimulator histamine was next tested. A group of 'non-smoking' normal subjects, and a group of asthmatic patients both in the same age range as the thoracic surgical patients were assessed by the in vivo protocol with histamine.

There was complete separation of the two groups as assessed by $PC_{20}FEV_1$ and only slight overlap when assessed by PC_{35} sGaw and $PC_{30}\dot{V}_{30}(p)$.

Using histamine as agonist to compare in vivo responsiveness and in vitro sensitivity did not demonstrate a relationship. As histamine may act both directly on a specific smooth muscle receptor and indirectly (via vagal reflexes) another group of patients were pretreated with atropine to reduce the effect of in vivo vagal reflexes, which would not be present in the denervated in vitro preparations. Atropine modified in vivo responsiveness, but did not improve the relationship between in vivo responsiveness and in vitro sensitivity. An asthmatic patient in this group was the most sensitive by in vivo criteria but his smooth muscle was not more sensitive in vitro.

As an adjunct to the main thrust of the project the ultrastructure of airway smooth muscle was examined. Electron micrographs of bronchial samples which demonstrate the major structural features of human bronchial smooth muscle are included.

The leukotrienes (formerly known as slow reacting substance of anaphylaxis) are released in acute bronchoconstriction, cause a slow onset, prolonged smooth muscle contraction, similar to that occurring in acute asthma, and alter in vitro smooth muscle sensitivity in animals models of asthma. A series of experiments was embarked upon to investigate the importance of leukotriene D_4 in airway responsiveness.

The first investigation looked at LTD_4 induced bronchoconstriction in normal volunteers and the effect of the calcium blocker verapamil on this leukotriene produced bronchoconstriction/

bronchoconstriction at doses of <50 ug/ml. Bronchconstriction was inhibited by verapamil, indicating that LTD_4 was acting, in part, via voltage dependent calcium channels. The lack of effect of verapamil in the *in vitro* system suggests that LTD_4 must be acting both by a direct action on smooth muscle receptors, and by an indirect verapamil sensitive pathway *in vivo*.

The second study looked at LTD_4 induced bronchconstriction in asthmatic patients, and the effect of verapamil and sodium cromoglycate on this. The asthmatic subjects were more responsive to LTD_4 by a factor of 10, but in contrast with the normal subjects verapamil did not inhibit LTD_4 induced bronchoconstriction. Possible reasons for this difference are discussed.

LTD_4 was used as the agonist in the *in vivo* responsiveness and *in vitro* sensitivity protocol. There was no relationship between *in vivo* responsiveness and *in vitro* sensitivity. In this study the amount of smooth muscle present in the bronchial strips was measured. The quantity of smooth muscle present correlated significantly with the maximum tension produced *in vitro* but neither muscle quantity nor tension generated per unit mass of muscle was related to *in vivo* responsiveness. This result suggests that smooth muscle hypertrophy may contribute to NSBR.

Using 3 bronchconstrictor agents no relation was found between *in vivo* NSBR and *in vitro* smooth muscle. Two asthmatic patients were responsive by *in vivo* criteria, but smooth muscle obtained from them did not have increased sensitivity.

The final chapter of the thesis examines whether passive sensitisation of muscle, to render it atopic as in allergic asthma, alters *in vitro* sensitivity to histamine. Neither sensitisation per se, nor sensitisation followed by specific allergen challenge altered *in vitro* sensitivity of human airway smooth muscle.

In conclusion, airway smooth muscle sensitivity is not the sole/

sole determinant of NSBR. Smooth muscle hypertrophy did not relate to *in vivo* responsiveness, although this result was obtained in patients with C.A.O. who may have different mechanisms for NSBR. Increased NSBR is due to a complex interaction between smooth muscle, neural and humoral factors.

CHAPTER I

INTRODUCTION

1. INTRODUCTION

1.1.1. Mechanisms of airflow obstruction

Airflow obstruction is a common respiratory condition and is a major cause of morbidity and mortality in the community (Black and Pole 1975) (WHO - 1977). The condition can be divided into two sub groups - asthma and chronic airflow obstruction. Asthma is a disease characterised by wide variations over a short period of time in resistance to flow in intra-pulmonary airways (Scadding 1982). Chronic airflow obstruction (CAO) (also referred to as chronic obstructive airways disease or chronic obstructive pulmonary disease) is characterised by increased resistance to flow in the intra pulmonary airways which is relatively fixed, i.e. does not vary by >15% (Thurlbeck 1977).

Asthma divides into two subgroups. The first is closely associated with atopy. It tends to occur in children and often resolves before adulthood. Asthma occurring de novo in adults is less clearly characterised. It is not related to atopy and tends to be less responsive to treatment (Turner-Warwick 1971).

Chronic airflow obstruction shares some of the characteristics of adult onset asthma, but differs in that there is little or no reversibility in the degree of airflow obstruction. It is often co-incident with chronic bronchitis and usually occurs in cigarette smokers (Higgins 1957, Fletcher et al 1976).

To reduce the prevalence and improve the treatment of asthma and CAO, it is important to understand the pathophysiology of airflow obstruction. There are three major determinants of airflow obstruction.

1.1.2 Mucous Secretion

Mucous secretion is increased in asthma (Florey 1962) and CAO when associated with chronic bronchitis. In patients dying of an acute asthmatic attack the characteristic findings at post mortem/

mortem are increased mucous gland and goblet cell numbers with plugging of the peripheral bronchi by mucous (Spenser 1971). The increase in intra-luminal mucous may be related in part to a failure of clearance mechanisms (Warner et al 1975).

1.1.3 Mucosal Inflammation

The superficial layers of the epithelium may be shed in severe cases of asthma (Reid 1954, Dunill 1960) and is incomplete, even in mild asthma (Laitinen et al 1985). Inflammatory cells invade the whole thickness of the bronchial wall. The majority of these cells are eosinophils (Lowell 1967) although plasma cells, polymorphs and lymphocytes are also present. Epithelial cells are joined by tight junctions at points of membrane to membrane contact. Electron microscope studies suggest that these are normally impermeable to large molecules (Hogg et al 1979) but antigen challenge in asthma, or cigarette smoke in CAO (Boucher et al 1980) may cause leakiness of these junctions allowing penetration of the mucosa by inflammatory mediators which can then act on airway smooth muscle.

1.1.4 Airway smooth muscle

Hypertrophy/hyperplasia of airway smooth muscle is an important feature of asthma (Spenser 1977) and CAO (Hossain and Heard 1970). Whether this is a prime cause of airways narrowing in asthma or CAO, or is a secondary response to another determining stimulus, has not been established.

However smooth muscle contraction is a major factor in the acute asthmatic response. The reversal of acute bronchospasm by B_2 adrenoceptor agonists occurs too rapidly for clearance of secretions or oedema to have occurred.

The reasons for increased responses of muscle in airflow obstruction/

obstruction are unknown. They may be due to muscle hypertrophy, to altered physiological and/or pharmacological responses of muscle, or to differences in the neurohumeral regulation of the smooth muscle. Alternatively, other factors, not directly related to smooth muscle could be important, e.g. differences in the distribution or penetration of bronchoconstrictor agonists to their site of action (see 1.1.2)

1.2 AIRWAY SMOOTH MUSCLE

1.2.1 Anatomy

The arrangement of muscles in airway is relevant to an understanding of airflow obstruction. Within the walls of the trachea and main bronchi smooth muscle is arranged as circular bands in bundles attached to the two limbs of C shaped cartilege. The muscle lies posteriorly. There are also smaller longitudinal bands of muscle. Contraction of muscle in this region draws the plates of cartilege together but does not obliterate the airway lumen.

Where the main bronchi enter the lung, the cartilege plates become smaller and are distributed around the entire circumference of the airway. Muscle bundles are arranged around the circumference of the airway. Muscle does not form a compact coat but branches and connects to form a geodesic network so that the arrangement of muscle is more spiral than circular (Miller 1947). Within the lung, the contraction of muscle has a sphincteric action which may completely occlude the airway lumen.

Bronchomotor tone is present in normal subjects (Vincent 1970) and may have the effect of improving airway stability and preventing collapse during dynamic expiratory effort. Excess bronchomotor tone produces airway narrowing (bronchoconstriction).

1.2.2 Ultrastructure

Under the light microscope airway smooth muscle comprises fusiform cells approximately 1 mm. in length with a diameter of 3 um. The nucleus is cigar shaped and lies centrally within the cell. The long axes of cells within a muscle are parallel. Mast cells are found in close proximity to bundles of smooth muscle. Thick, intermediate and thin filaments are abundant, and microtubules are present (Stephens et al 1980). Nerve axons run parallel to smooth muscle bundles. Nerve bundles are present at all levels (trachea to 7th order bronchi), but are more frequent in the smaller bronchi (Daniel et al 1986).

Frequent connections exist between cells, often of the gap junction type. This form of connection implies electrical coupling of the cells, such as has been demonstrated in cardiac muscle (Richardson and Ferguson 1979). The presence of significant numbers of gap junctions will cause a spread of a contractile stimulus from cell to cell producing an increased response to that stimulus. This form of neuro-muscular arrangement is known as myogenic (Burnstock 1970).

In more exacting examination of human smooth muscle Daniel et al (1986) found moderate numbers of gap junctions 2.7/100 cells, in larger order airway ($\geq$ 2nd order bronchi) but found fewer gap junctions in 4th - 7th order bronchi. However numerous small cell to cell contacts were present, but the authors were unable to confirm that these were functional as cell-to cell electrical connections.

1.2.3 Smooth Muscle Contraction

Smooth muscle contraction is initiated at the cellular level by cell membrane depolarisation. In its resting state the cell membrane is largely impermeable to calcium ions (Ca^{2+}) (Van Breenan et al 1975). When the membrane depolarises, a large increase in the permeability of the membrane to Ca^{2+} and hence an influx/

influx of Ca^{2+} occurs (Bolton 1979). An alternative source of intracellular calcium is the release of Ca^{2+} from intracellular stores (Evans et al 1958, Bolton 1979). The relative importance of these two sources has not been defined for human airway smooth muscle.

Extracellular calcium enters the cell through at least two distinct channels - a receptor operated channel (ROC) and a voltage dependant channel (VOC). VOC can be anatagonised by various "calcium blocking drugs".

Once inside the cell the Ca^{2+} complexes with calmodulin which has 4 Ca^{2+} binding sites. Once two or more of these sites are occupied, the calmodulin changes conformation and may then activate myosin light chain kinase. Myosin is phosphorylated and may then interdigitate with actin and then, by the action of actin-myosin ATPase, produces muscle fibre shortening and hence smooth muscle contraction.

1.3 FACTORS INCREASING SMOOTH MUSCLE RESPONSES

Increase in the contractility of smooth muscle could be due to three separate mechanisms. The muscle cell could produce a greater shortening for a given stimulus, there could be more muscle which would therefore produce a stronger contraction, or more muscle cells could be recruited by a single stimulus. These mechanisms are discussed below.

i. Increased response. Changes in the calcium channel permeability could affect smooth muscle responses. Either a 'leaky' channel or a channel allowing a greater number of calcium ions through would have the effect of producing an increase in smooth muscle contraction. If this mechanism involved the VOC then specific blockers of this channel might alter smooth muscle responses.

Differences in calcium handling could also explain increases in smooth muscle responses. If the smooth muscle was in a state of/

of partial depolarisation so that it were reactive to low concentrations of various agonists the airway would appear hyper-responsive (Middleton 1983). In support of this theory in vitro smooth muscle which is partially depolarised is hyper-reactive to various agonists (Fleming 1980). However there is no evidence that smooth muscle in asthmatic airway is partially depolarised. Indeed Souhadra et al(1981) have demonstrated increased depolarisation in the airway of sensitised guinea-pig. Other factors increasing availability of Ca^{2+} to intracellular mechanisms such as increased release or reduced sequestration from intracellular stores or decreased efflux of Ca^{2+} from the cell could increase cellular responses to stimuli (Rodger 1985).

ii. An increase in the absolute amount of smooth muscle would generate more tension, thereby increasing the apparent response of the airway to a stimulus. Hypertrophy and hyperplasia of smooth muscle occurs in asthma (Huber and Koesler 1983, Dunill 1960, Takizawa et al 1971) and chronic bronchitis (Hossain and Heard 1970). Increased thickness of vascular wall has been shown to increase vascular reactivity in rats (Foklow 1978). The dose response curve to norepinephrine had a steeper slope and a greater maximum response in hypertensive than normotensive animals. However, an increased quantity of smooth muscle could not explain the transient increase in airway responsiveness that occurs in normal human subjects during viral infections (Empey et al 1976) or after exposure to irritant chemicals (Goldie et al 1978, Orehek et al 1976). Thus it is possible that the smooth muscle hypertrophy observed in asthma may be an effect of increased responsiveness, rather than a primary cause of same.

iii. Increased contractility of smooth muscle could be an intrinsic property of the muscle cell, or could be related to modifying factors acting on the airway (discussed later).

Properties of the muscle which could alter contractility conclude an increase in the cell-to-cell connections (gap junctions) between/

between smooth muscle cells. These would allow action potentials to spread between cells thereby increasing the response to a depolarisation of a single muscle cell. (Perechia 1974).

Smooth muscle may be divided into two sub types distinguished by different control mechanisms. These have been designated myogenic or neurogenic (Burnstock 1972).

Myogenic muscle has numerous cell-to-cell connections and few efferent nerves. Muscle bundles therefore respond as a unit. Single stimuli will produce a larger response and spontaneous activity is more likely. An example of this type of muscle is that found in the intestine.

Neurogenic or 'multi-unit' systems have few inter-cellular connections and a rich nerve supply. This allows finer control of response. Neurogenic mechanisms are present in larger blood vessels and the pupillary muscles of the eye.

There are few studies of the ultrastructure of human airway smooth muscle. Daniel et al (1980) showed cell-to-cell connections (gap junctions) were present in human bronchial and tracheal airway.

More recent studies have shown more gap junctions in the proximal, and less in the distal airway (Daniel et al 1986).

Increased responsiveness of airway could be explained by increased cell-to-cell coupling due to increased numbers of gap junctions. An example of this mechanism occurs in rat myometrium at the time of parturition (Garfield et al 1978). Gap junction numbers increase dramatically around the time of parturition, thereby facilitating the synchronised muscle contractions of labour. Numbers decrease to normal levels within 48 hours of parturition. A mechanism such as this could explain the rapid changes in responsiveness seen in airway smooth muscle.

1.4/

1.4 MEASUREMENT OF AIRWAY CALIBRE

The resistance of the airways depends on the driving pressure, i.e. the difference between alveolar pressure and mouth pressure, and the flow rate analagous to Ohms law of electricity, where Resistance = Voltage/current.

1.4.1. In routine clinical use, the commonest index of airway resistance is the volume of air able to be exhaled in 1 second the forced expiratory volume in one second (FEV_1). The FEV_1 typically involves 70% or more of the expiratory flow volume curve and so is an index of the integrated value of a large part of the curve. The test is simple for both subject and operator. FEV_1 reflects a flow-limiting segment of the airway. Changes distal to this rate limiting area do not affect readings and are therefore not detected by this measurement. In normal subjects most of the airway resistance is in central airways so FEV_1 will not detect changes distal to these airways. Furthermore FEV_1 is, to an extent, effort dependent.

1.4.2. Airways resistance (Raw)

Airway resistance may be assessed using the body plethysmograph which measures mouth pressure and flow directly, and alveolar pressure by an indirect method using volume changes and Boyle's Law.

Airways resistance is a sensitive measure of bronchial calibre as the resistance of a tube is inversely proportional to the fourth power of its radius. Because the surface area of the airways increases towards the periphery, most of the resistance of human airway is located in the central intrathoracic airways and the extrathoracic airways. By measuring Raw during a panting manoeuvre which causes abduction of the vocal cords, a sensitive index of large airway changes is obtained. As airway calibre is to a degree dependent on lung volume, it is usual to correct values/

values obtained for the thoracic gas volume (TGV) producing the airways specific conductance $sGaw = \frac{1}{(Raw \times TGV)}$.

1.4.3 Partial expiratory flow rates

Partial expiratory flow rates were measured as a sensitive index of changes in airway calibre. They were obtained by measuring the flow rates towards the end of expiration. As full inspiration may produce a transient bronchodilation (Nadel and Tierney 1961) assessment of the expiratory flow rate is best made without the subject inhaling to TLC. The total manoeuvre is a complete exhalation from tidal volume, followed by inhalation to TLC and then a full exhalation. If these manoeuvres are recorded as a flow against volume tracing TLC and a flow rate at a chosen percentage of TLC (eg 30%) can be measured.

1.4.4 Concentration-response curves

Non-specific bronchial responsiveness is usually assessed using methacholine or histamine as the bronchoconstrictor agonist. Increasing doses (usually doubling) of the agonist are inhaled through a nebuliser for a specified period. The output characteristics of the nebuliser must be measured and, ideally the same nebuliser used for a series of measurements where comparisons of responses are measured. The change in airway calibre is measured and a log concentration-response curve is constructed. From this an end point fall in airway calibre is selected and the concentration of agonist producing this fall is recorded. For FEV_1, the provocation concentrations producing a 20% fall (the $PC_{20}FEV_1$) is used as an index of airways responsiveness. A wide experience with this measurement for both methacholine and histamine has accummulated in the literature and generally accepted normal ranges are available. A $PC_{20}FEV_1$ methacholine/

methacholine or histamine of <8 mg/ml indicates increased airway responsiveness and a $PC_{20}FEV_1$>16mg/ml is considered normal (Hargreave et al 1985). Provocation concentrations producing falls in sGaw and partial expiratory flow rates $\dot{V}_{30}(p)$ are also used as an index of airway responsiveness. A greater percentage fall is taken as the end point with sGaw and $\dot{V}_{30}(p)$ (usually 35 or 40%) as these are more sensitive and hence more variable measurements. Experience with these latter measures is less so that normal ranges cannot be confidently stated.

1.5 NON SPECIFIC BRONCHIAL RESPONSIVENESS FIGURE 1.1

1.5.1. Introduction

Increased nonspecific bronchial responsiveness (NSBR) is characteristic of asthma (Curry 1946, Cockcroft et al 1977) and also occurs in chronic airflow obstruction (Klein and Salvaggio 1966, Laitinen 1974). Patients with NSBR have an increased sensitivity to a wide range of unrelated physical and chemical stimuli.

Historical perspective

In 1929 Weiss et al demonstrated that intravenous histamine precipitated bronchospasm in patients 'prone to bronchial or cardiac asthma', and subsequently demonstrated that this change did not occur in normal subjects (Weiss et al 1932). Since this initial observation differences in responses between normal subjects and asthmatic patients have been described to methacholine (Curry 1947) prostaglandin $F_{2\alpha}$, (Mathé et al 1973) leukotriene D_4 (Barnes et al 1986) and also to physical stimuli such as exercise (McNeill et al 1966), hyperventilation (Simonsson et al 1967) and cold air inhalation (Wells et al 1960).

This increased responsiveness occurs to such a wide range of unrelated/

unrelated stimuli. It suggests that airway smooth muscle is producing a greater contraction in asthmatic patients, i.e. that the airway is hyper-responsive and that the cause of the increased responsiveness must be due to post receptor differences in the airway smooth muscle. The first possibility is that the muscle itself is more sensitive for reasons discussed in section 1.3. Other possible differences are altered nerve supply, or the presence of chemical factors which modify airway function.

Increased bronchial responsiveness is closely associated with asthma and some authorities suggest it is a prerequisite (Hargreave et al 1981). Some authors who have induced hyper-responsiveness by allergen challenge have suggested that it is the cause of asthma (Cockcroft et al 1979, Permutt et al 1977, Stephens et al 1980) although this has been disputed (Stanescu and Frans 1982). It seems likely that clinical asthma is an interaction between increased bronchial responsiveness and a broncho-constrictor stimulus.

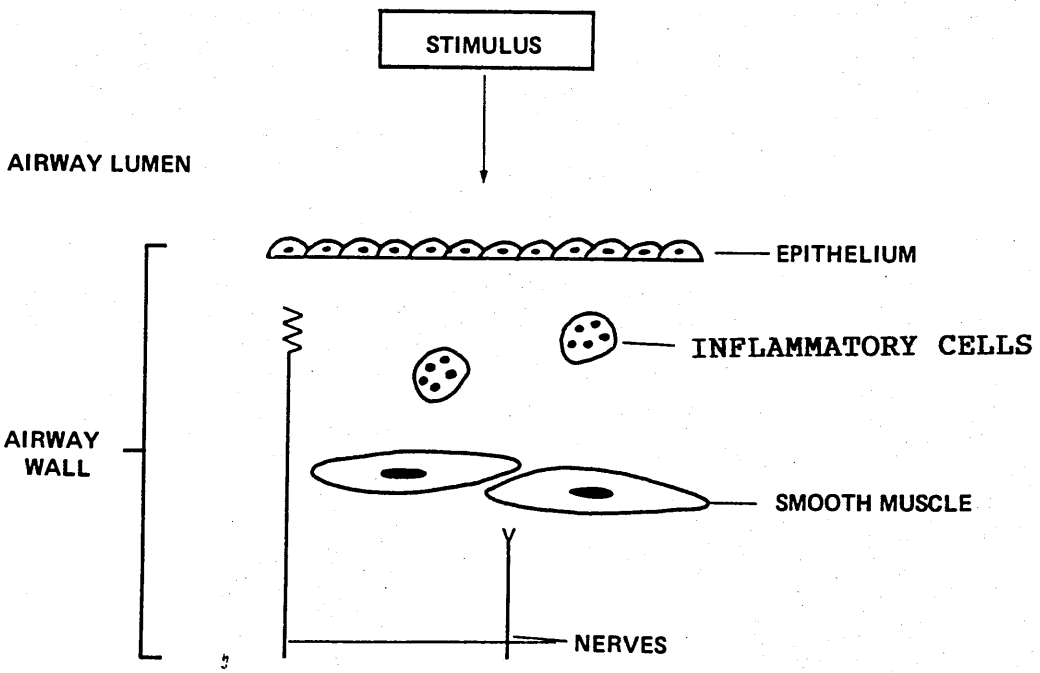
Although less well defined, increased bronchial responsiveness has also been found in patients with chronic airflow obstruction (Orie et al 1961, Barter and Campbell 1976) and also in patients who smoke cigarettes (Mullen et al 1984). It is not clear whether the aetiology of bronchial responsiveness in chronic airflow obstruction is different (Du Toit et al 1986), e.g. it has been suggested that starting airway calibre is important (Orehek and Gayrard 1976) although it does not fully explain changes in airway responses (Mullen et al 1984).

I.5.2 Neural mechanisms

The role of neural influences on the airway has been extensively studied (reviewed by Boushey et al 1980, Barnes 1986). The presence of nerve supply to the human lung has been known for 3 centuries. The presence of nerves in the airway was first described by Willis who also suggested a connection with asthma/

FIGURE 1.1

FACTORS INFLUENCING AIRWAY RESPONSIVENESS



asthma (Willis 1679). Animal models in which vagal stimulation produced airway spasm similar to that in human asthma have been produced (Dixon and Brodie 1903). Airway innervation has been extensively studied using numerous animal models: cat (Silva and Ross 1974) domestic chicken (McLelland 1973), mouse (Honjin 1956) and rat (Zussman 1966), rabbit (Mann 1971), guinea pig (Coburn and Tomita 1973) and non human primates (El-Bermann and Grant 1975).

The message which arises from this research is that nerve supply is present in all species investigated but that there are large inter-species differences (Richardson 1979) so that extrapolation to human airway is of dubious merit. The following review therefore refers only to studies of human airway unless stated.

Parasympathetic supply

The parasympathetic nerve supply to human airway is carried via the vagus nerve. Parasympathetic agonists produce bronchoconstriction (Alexander and Paddock 1921) and antagonists produce bronchodilation and are used as inhaled therapy for asthma (Ward et al 1981). It has been hypothesised that parasympathetic reflex activity could be exaggerated in asthma (Simonsson et al 1967). However, although some studies have reported significant reductions in bronchial responsiveness to histamine after atropine (Holtzman et al 1980), prostaglandins (Alanko and Poppius 1974) and exercise (Sheppherd et al 1982) others have not (Itkin and Anand 1970, Casterline et al 1976). Furthermore, there is no evidence for increased vagal tone in asthma (Barnes 1986).

Thus, although vagal reflexes may have a minor role in the control of airway responsiveness it is unlikely that they are a major determining factor.

Sympathetic nervous system

The sympathetic nerve supply to the airway arises from upper thoracic preganglionic fibres that end in the extra pulmonary stellate ganglion. Sympathetic nerve supply to human airway is sparse and greatly outnumbered by cholinergic (Laitinen et al 1985). Electric field stimulation is unaffected by pretreatment with propranolol suggesting that there is no direct sympathetic innervation of airway smooth muscle (Doidge and Satchell 1982, Davis et al 1982).

However, human airway smooth muscle relaxes when exposed to B-agonists in vitro (Davis et al 1982, Goldie et al 1982) so circulating catecholamines may have an influence on airway responses to bronchoconstrictors. Catecholamine levels in asthma are within the normal range, but infusions of adrenaline, but not noradrenaline, which produce physiological blood levels cause bronchodilation in asthmatic (Berkin et al 1984) and normal subjects (Berkin et al 1983) suggesting normal B receptor sensitivities and lack of functional α receptors on human airway smooth muscle.

(Barnes et al 1980) found that the clinical variation in airway function parallels circulating adrenaline concentrations suggesting that adrenaline may be influencing airway calibre, although a direct effect on smooth muscle was unlikely at the low levels of adrenaline measured. This relationship could be caused by effects on mast cells stabilisation. Furthermore, studies have demonstrated a blunted increase in plasma adrenaline in asthmatic patients after exercise (Barnes et al 1981, Warren et al 1982) but not in acute severe asthma warranting hospital admission (Ind et al 1985). However, although the B adrenergic system may have a role in control of airway function it does not appear to have any relationship with bronchial hyper-responsiveness.

Other/

Other neural mechanisms

A third autonomic nervous system is present in the airway. It is neither cholinergic nor adrenergic, and when stimulated produces relaxation (Bulbring and Tomita 1967). It has been demonstrated *in vitro* in man. (Doidge and Satchell 1982, Davis et al 1982, Richardson and Beland 1976). Unfortunately, the transmitter used in this system is unknown. This limits *in vivo* investigation in man but animal work has demonstrated an *in vivo* bronchochodilator effect in cats after cholinergic and adrenergic blockade by electrical stimulation of the vagus nerve (Irvin et al 1980). If this system, designated the non adrenergic, non-cholinergic (NANC) system were having a significant regulatory role in normal airway, impaired function of the NANC pathway could explain increased bronchial reactivity in asthma. However, until the pathway is better characterised, its transmitter substances clarified, and specific antagonists developed, the role of NANC in the determination of the level of bronchial responsiveness will remain speculative.

1.5.3 Mediators

Bronchoconstriction is due to a combination of smooth muscle contraction, oedema of the bronchial mucosa and mucous hypersecretion (Hogg et al 1977). Asthma in young subjects (< 20 years) is usually associated with atopy to a variety of allergies (Brown et al 1979). Exposure of susceptible subjects to allergen produces an immediate bronchoconstriction reaching maximum 10 - 15 minutes after challenge. This is often followed by a 'late reaction' 3 - 24 hours later. This late response is associated with a rise in non-specific bronchial responsiveness (Cartier et al 1982).

The early reaction is thought to be due to IgE - antibody complexes forming on the mast cell surface inducing secretion of mediators in the airways (Pepys 1967). Various mediators are released/

released. The first discovered and most studied is histamine (Atkins et al 1980, Holgate 1985). Histamine is present in high concentrations in the membrane bound granules of mast cells and is released from the mast cell following antigen challenge or exercise. It produces immediate bronchoconstriction but is not associated with the late response or increased bronchial hyper-responsiveness. The older anti-histamines do not block the immediate response after allergen (Eiser et al 1978) or exercise (Thomson 1980) challenge in susceptible patients, although recent work with non sedative H_1 antagonists has demonstrated a significant inhibitory effect on acute bronchoconstriction (Rafferty et al 1987).

Prostanoids

Mast cell degranulation releases biologically significant levels of prostaglandin D_2 (Lewis et al 1981) and thromboxane A_2 (Lewis et al 1981). These are generated from cell membrane arachidonic acid via the cyclooxygenase enzyme. Mast cells produce mainly PGD_2 (Holgate et al 1984). There is an eighty fold increase in PGD_2 after in vitro anaphylaxis of human lung fragments (Schulmann et al 1981). PGD_2 concentrations in bronchial lavage fluid increased 150 fold following specific allergen challenge in asthmatic subjects (Murray et al 1986). PGD_2 causes bronchconstriction in normal and asthmatic subjects with asthmatic subjects being more sensitive (Hardy et al 1984). If prostaglandins were a major mediator in the active asthmatic response, non steroidal anti-inflammatory drugs such as aspirin or indomethacin should modify the acute bronchoconstriction observed. Although this has been shown to be the case in some patients (Kordanzky et al 1978) in the majority they have little effect (Smith and Dunlop 1975, Fish et al 1981). Using the thromboxane synthetase inhibitor OKy-046 Fujimara et al (1986) found a reduced bronchial responsiveness to acetylcholine in asthmatic/

asthmatic subjects. In a small sub group of asthmatic patients NSAIDS may stimulate bronchoconstriction (Szczeklik et al 1975). This has been suggested to be due to diversion of arachidonic acid metabolism to the lipoxygenase pathway to produce leukotrienes (Walker 1972), although an alternative explanation would be that the inhibition affects preferentially the bronchodilater prostaglandins (Szczeklic et al 1975).

More important than an immediate bronchoconstrictive response is the ability to modify airway responses to other bronchoconstrictors thereby producing hyper-responsiveness. Recent work with PGD_2 has shown a small but statistically significant increase in non-specific responsiveness of asthmatic patient following PGD_2 inhalation (Fuller et al 1986). The duration of this effect was not assessed. However, Hardy et al (1986) were unable to demonstrate a pharmacological interaction between PGD_2 and histamine in asthmatic subjects. Thus it seems unlikely that PGD_2 is having a clinically significant effect on bronchial responsiveness in asthma.

Ecosanoids

Slow reacting substance of anaphylaxis (SRS-A) has been recognised as a mast cell product which produces bronchoconstriction since the 1940's (Kellaway and Trethewie 1940). Because of its slow onset and prolonged effect it was called slow reacting substance of anaphylaxis. During the 1950's evidence for the role of SRS-A in hypersensitivity reactions accumulated (Brocklehurst 1962). It was not until the late 70's that it was discovered that SRS-A was a product of lypoxygenase metabolism of arachidonic acid (Samuelsson et al 1979).

SRS-A is a mixture of several lipoxygenase metabolisms designated leukotriene B_4, D_4 and E_4. Leukotriene C_4 (LTC_4) and leukotriene D_4 (LTD_4) account for the major biological activity from human lung (Lewis et al 1981).

Since/

Since its discovery and synthesis LTD_4 has been shown to be a potent bronchoconstrictor in human airway, both normal (Kern et al 1985, Barnes et al 1986, Smith et al 1985) and asthmatic (Smith et al 1985, Dahlen 1983). Not only are the leukotrienes the most potent bronchoconstrictors discovered, but the bronchoconstriction produced is of slow onset and lasts 1 - 2 hours which is more akin to the bronchconstriction occurring in acute asthma than that produced by other mediators.

In vitro studies have shown that LTD_4 can increase guinea-pig smooth muscle sensitivity to histamine and acetylcholine (Creese and Bach 1983). LTD_4 is released by in vitro antigen challenge of lung tissue obtained from asthmatic patients (Dahlen et al 1983). All these results are consistent with an important role for leukotrienes in asthma. However, the importance of leukotrienes in asthma is not yet defined.

Studies examining the effect of leukotriene antagonists are necessary to define the role of leukotrienes in clinical bronchoconstriction. Piriprost, a lipoxygenase inhibitor had no effect on allergen nor exercise induced bronchoconstriction when given by the inhaled route (Mann et al 1987). An LTD_4 antagonist, L649,923, did not alter allergen induced bronchoconstriction when taken orally (Britton et al 1987). However, these studies did not assess the potency of the inhibitor under the experimental conditions used, and in both cases, side effects were a problem. L649,923 has been shown to produce a 4 fold shift of the LTD_4 dose-response curve to the right (Barnes et al 1987). More potent antagonists are needed to fully assess the role of leukotrienes in determining bronchial responsiveness.

1.5.4 Other Factors in increased responsiveness

The mucosal layer in normal airway is complete (Laitinen et al/

al 1985). In patients who die during an acute attack, the mucosa is extensively damaged (Filley et al 1985) and even in mildly asthmatic patients the mucosa has areas of damage (Laitinen et al 1985). Chronic cigarette smoking increases the permeability of airway mucosa (Kennedy et al 1984). These facts have led to the hypothesis that bronchconstricting stimuli have better access to airway muscle when the mucosa is damaged, and that the lack of mucosal barrier may play a part in determining increased airway responsiveness. Further work is required to test this hypothesis.

1.6 AIRWAY SMOOTH MUSCLE PHARMACOLOGY

Smooth muscle pharmacology has been extensively investigated. Gut wall has been a useful source of smooth muscle preparations. Airway has been less studied because of marked variation in the characteristics of airway smooth muscle responses between species and between different ages of animal. No model demonstrating characteristics similar to human airway has been developed. The use of *in vitro* preparations has been the only method of studying human airway smooth muscle. *In vitro* preparations have the advantage of simplicity. Neural influences and reflexes are absent, and conditions such as temperature, pH, pO_2, pCO_2, drug concentrations and exposure time can be closely controlled and/or monitored.

Furthermore, passive forces on the muscle are also a source of variation, and these many also be determined by the experimenter. Drug access to smooth muscle receptors is also much less of a problem compared with the *in vivo* approach.

The importance of temperature in airway responses has been suggested by McFadden and Ingram (1982) who have proposed that airway cooling is a major cause of bronchconstriction in exercise induced asthma. *In vitro* human airway preparations produce a lower maximum contraction and have a reduced sensitivity to histamine when tested at $20^{o}C$/

20°C as compared to 37°C (Black et al 1984). In experimental animals, *in vivo* increases in inhaled pCO_2 produce bronchoconstriction (Loofbourrow et al 1957) but this has not been consistently found in human subjects (Butler et al 1960) and, using rat trachea, Twort and Cameron (1986) demonstrated that responses to changes in pCO_2 *in vitro* occurred and that these appeared to be due to pH dependent changes in Ca^{2+} uptake by smooth muscle. Thus it is important to control pCO_2 and temperature when using *in vitro* preparations.

The reproducibility of response is also an important feature if useful *in vitro* studies are to be performed. Experiments with human airway, obtained at post mortem, have demonstrated that reproducible responses to a variety of stimuli, including acetylcholine and histamine, were obtainable for up to 30 hours after death. (Thulesius and Boe 1978).

It would appear that, if care is taken of the *in vitro* environment of human airway preparations, that investigation of smooth muscle responses, removed from the influence of neural and humoral factors, is practical.

In vitro smooth muscle may be kept in a closely controlled environment at a fixed temperature. Differences in the sensitivity of tissues are assessed by a shift in the dose response curve conventionally quantified as the concentration of agonist producing a concentration 50% of the maximum obtained although, in practice, any percentage of maximum could be chosen. The maximum contraction can also be measured. This depends on the contractility and the quantity of smooth muscle present.

1.7 IN VITRO STUDIES OF HUMAN AIRWAY

The response of airway smooth muscle *in vivo* is a complicated interaction of myogenic, neurogenic, chemical and perhaps other undiscovered factors. Investigation of bronchial strips of tissue containing smooth muscle simplifies this system and allows analysis of the/

the role of the airway smooth muscle in determining airway responsiveness. Major differences exist in the control mechanisms in the airways of different species (Richardson 1979). Thus, applying results of experiments on in vitro animal airways must be interpreted with caution when trying to understand human airway. Fortunately, it is possible to study human airway obtained when removed at thoracotomy (usually from patients with underlying bronchial carcinoma, or from post mortem specimens). Reliable supplies of human airway have been difficult to obtain and so there is a relative paucity of studies of in vitro human airway smooth muscle.

Shultz and Dale (1910) first demonstrated that passively sensitised guinea-pig airway smooth muscle contracted when exposed to specific antigen in vitro. Lung tissue obtained from human asthmatic patients will contract when exposed to antigen (Schild et al 1951, Dahlen et al 1983). Passive sensitisation using human sera from atopic patients has also been achieved (Sheard et al 1967). More recently, release of bronchoconstrictor mediators from passively sensitised human lung have been demonstrated in vitro in human tissue (Adkinson et al 1980 Davis et al 1983).

It therefore appears possible to produce a good in vitro model of airway sensitisation. However, the effect of sensitisation on the sensitivity of in vitro airway has not, to date, been assessed.

In vitro tissue will produce a contractile response to all the in vivo chemical bronchconstrictors including methacholine, histamine, prostaglandin D_2 and the leukotrienes. Thus, in vitro human airway provides a useful model for studying the role of smooth muscle in determining differences in airway responsiveness and factors which might modify this. Leukotrienes have been shown, in vitro, to produce hyper-reactivity of guinea-pig airway to acetylcholine and histamine, but only if the concentration of extra-cellular calcium was low (Creese and Bach 1983).

There are few studies of in vitro human asthmatic tissue and conflicting results exist for this. (Dahlen et al 1980) examined bronchial/

bronchial strips from two birch pollen sensitive asthmatics obtained at thoracotomy from patients with bronchial carcinoma, and found that bronchial strips were no more sensitive to histamine or leukotriene than strips from non-asthmatics. Post mortem tissue from 2 asthmatic patients who died during an acute asthmatic attack did not exhibit increased sensitivity, using histamine and carbachol as agonists (Paterson et al 1982) but tissue from an asthmatic subject who also had a carcinoid tumour had increased responsiveness to histamine, but not methacholine or leukotriene (Schnellenberg and Foster 1984). Dejongste et at (1987) found increased responses in bronchial strips obtained from an asthmatic subject. The results show in increased maximum response rather than increased smooth muscle sensitivity. Cerrina et al (1986) found no evidence of increased responses to histamine in vitro in tissue from 5 asthmatic subjects but did demonstrate reduced sensitivity to isoprotenerol of asthmatic tissue. However, the asthmatic patients were poorly characterised. This is an interesting result which requires further study. It does not fit in with the observation that B blockade does not alter bronchial responsiveness (Zaid and Beal 1966). Because it is very unusual for asthmatic patients to require thoracotomy, these case reports represent all the information available on asthmatic airway responses in vitro.

1.8 AIMS OF STUDY

Increased non-specific airway responsiveness is an important feature of obstructive airways disease. Understanding the mechanisms underlying differences in airway responsiveness is an important step in the understanding of the causes of airflow obstruction and may lead to improvement in the treatment of this condition.

One possible cause of increased airway responsiveness is increases in the sensitivity of airway smooth muscle. In this thesis I will use in vitro preparations of human bronchi which removes the smooth muscle from neural and humeral effects encountered in vivo. Using this model/

model I will establish whether differences in airway responsiveness measured *in vivo* can be correlated with differences in smooth muscle sensitivity measured *in vitro*.

Initially, I will establish a reliable means of measuring airway responsiveness using several different measures of airway calibre.

I will then measure bronchial responsiveness in patients due to undergo thoracotomy, and compare this to the sensitivity of bronchial preparations obtained from these patients. Thus differences *in vivo* responsiveness will be compared to smooth muscle sensitivity. I will use several bronchoconstrictor agonists including methacholine, histamine and leukotriene D_4. The last of these will be of great interest as leukotrienes may be important in the development of airway hyperresponsiveness (Robinson and Holgate 1985).

I will also examine the ultrastructural appearances of bronchi obtained from the thoracotomy patients to assess whether there are obvious structural differences in preparations which exhibit different airway responsiveness *in vivo*, e.g.whether gap junction numbers differ.

In the second part of the study I will examine the mode of action of leukotriene D_4 *in vivo* and *in vitro* to assess the role of calcium ion influx in its production of bronchoconstriction. I will use the calcium antagonist, verapamil, to assess whether the voltage dependent calcium channel is important in leukotriene induced bronchconstriction.

The *in vitro* model will also allow assessment of factors which modify smooth muscle sensitivity. I will passively sensitise smooth muscle and assess whether sensitisation itself or subsequent specific antigen exposure alters the sensitivity of the isolated airway preparations to pharmacological agonists.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 HUMAN VOLUNTEERS

This section summarizes the groups of human volunteers who participated in these experiments. More detailed description of each group of patients is tabulated in the appropriate chapter of the thesis.

2.1.1 Normal Subjects

(a) **Surgical Controls**

Seven male and three female patients undergoing routine general surgery were studied. They were aged 46-67 (mean ± SD = 53±8). None were smokers. Three were atopic as judged by positive skin tests but none had raised levels of RAST IgE.

(b) **Normal Volunteers**

Six normal subjects were recruited. They were aged 20-36 (mean± SD = 28 ±3.6). All were non smokers and had no history of respiratory disease. None were atopic.

2.1.2 Surgical patients

Forty two patients due to undergo thoracotomy, usually for removal of a bronchial carcinoma, were recruited. Their ages ranged from 43-72 (Mean ±SD = 60 ±7). Thirty were male and twelve female. Thirteen were atopic as judged by skin testing and six had elevated levels of RAST IgE. One patient had a history of asthma since childhood and had reversible airflow obstruction. Another had a history of wheezy dyspnoea and hay fever, although a diagnosis of asthma had not been established in the past.

2.1.3 Asthmatic Patients

(a) **Volunteers**

Seven asthmatic patients were studied. They were aged 22-36 years (mean ±SD = 31 ±10). All were atopic and were non-smokers. All were taking inhaler B2 adrenoceptor agonists/

agonists by pressurised aerosols, two were taking regular inhaler steroids and two were taking sodium cromoglycate.

2.1.4 (b) **Surgical controls**

Ten asthmatic patients were studied to establish the range of values expected for asthmatic subjects for the measurements used in this thesis. They were chosen to be in the same age range as the surgical patients. Age range 44-75 (mean ±SD = 60 ±9). One smoked cigarettes and one a pipe. Four had positive skin prick tests and three had raised levels of specific IgE. All were taking regular inhaler B2 adrenoceptor agonists, seven were taking regular inhaler beclomethasone and one patient was taking sodium cromoglycate.

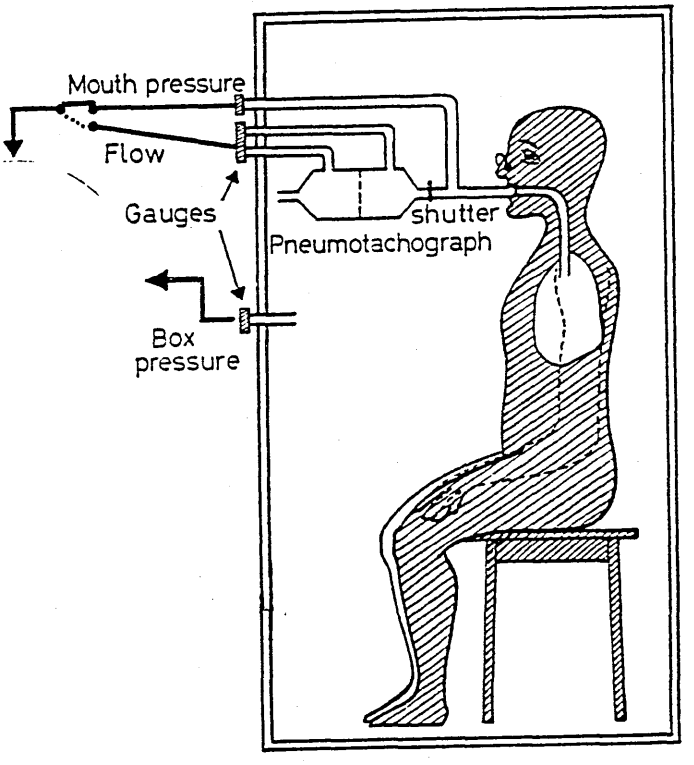
The nature of each study was explained to both patients and normal subjects. All investigations were approved by the Ethical Committee of the Western Infirmary and all patients and normal subjects gave informed consent.

2.2 EXPERIMENTAL EQUIPMENT

2.2.1 Body Plethysmograph

The constant-volume plethysmograph (Dubois et al, 1956) used in this study was manufactured by Fenyves & Gut (Basle, Switzerland). The chamber was fitted with a large window in the front wall, and a Perspex door with an electro-magnetic catch (Figure 2.1). A calibration pump for the chamber was incorporated. The volume of the plethysmograph was 690 litres. A heated air selector allowed the subject to be connected either to the outside air, the chamber or the BTPS breathing bag. Pressure changes in the plethysmograph were monitored by a capacitive differential pressure transducer (Fenyves & Gut, Basle) (sensitivity 0.001 - 0.2 kPa: frequency response flat to 50 Hz) backed off against a rigid metal container (volume 50 litres) to stabilize against thermal drift. Pressure changes/

FIGURE 2.1



Diagramatic representation of the constant volume body plethysmograph. When the subject pants, the glottis is fully open and P.mouth = P alveoli

resistance $= \Delta \frac{\text{Pressure}}{\text{flow}}$ hence airways resistance may be calculated

changes at the mouth produced by the inspiratory effort against the closed shutter were measured by a differential pressure transducer (Fenyves & Gut, Basle) sensitivity 0.5 - 5 kPa : frequency response flat to 50 Hz backed off against the interior of the box. Airflow was measured with a heated Fleisch pneumotachograph and differential pressure transducer (Fenyves & Gut, Basle) with a linear response to 14 l/sec (frequency response flat to 50 Hz). Box and mouth pressure plus airflow were plotted on an X-Y recorder (Hewlett Packard, 7041A) with a writing speed of more than 75 cm/sec and a linearity better than 0.2%. Box pressure (Pbox), mouth pressure (P mouth) and flow were calibrated before each experiment. As part of the study the measurement of flow, box pressure and mouth pressure was automated.

The electrical signal from the body plethysmograph, either airflow and box pressure, or mouth pressure with zero airflow and box pressure, depending on the mode selected. These signals were sampled with 16 bit resolution and fed to a BBC model B computer (Acorn electronics).

2.3 EXPERIMENTAL METHODS IN VITRO

2.3.1 Measurement of Raw, TGV and sGaw

The subject sat in the closed body plethysmograph until the temperature of the air, and hence the pressure inside the box, had stabilised. The pressure was maintained equal to the external pressure by means of a pressure compensation switch. The subject then put on a nose clip, fitted the box mouthpiece and panted rhythmically through the pneumotachograph at a frequency between 1 and 2 Hz. The act of panting ensured an open glottis, and the cheeks were supported by the hands to minimize the bucal component to the pressures and flows generated.

While the subject panted, the pressure compensation switch was turned off. The first mode selected was the airflow/box pressure and this was followed by the mouth pressure/box pressure mode during/

during which the subject panted against a closed shutter which closed automactically at end expiration. These two manoevres gave one determination of airways resistance (Raw), thoracic gas volume (TGV) and specific conductance (sGaw). During each period of panting, the complete sequence was carried out twice, Raw and TGV were thus measured almost simultaneously. All plethysmographic measurements were carried out by myself. The mean of the eight readings was taken as sGaw.

2.3.2 Analysis of Data

(i) **Manual Measurement**

Eight graphs of airflow against P.box and P.mouth against P.box were recorded and analysed later. Thoracic gas volume is obtained from the gradient of the P.mouth/P.box graph (Figure 2.2), which was estimated by drawing a line between the two turnover points A and B and measuring its slope, and then used in the following equation

$$TGV\ ml = K_{TGV}\ 1/grad\ (B-47) - V_{Korr}$$

Where K_{TGV} was a standardisation factor for TGV dependent on the range taken for box pressure, 1/grad is the reciprocal of P.mouth/P.box gradient, B is the barometric pressure in mm Hg. and V Korr was composed of the following values; apparatus dead space 140 ml., stomach volume with diaphram breathing 170 ml. (Bedell et al 1956)

Raw was calculated from Airflow/P.box (Figure 2.3) and P.mouth/P.box gradients using the following formula -

$$Raw = \left\{ K_{Raw} \frac{(B-47)}{TGV} \; G \right\} - 0.25$$

Where K_{Raw} was a standardisation factor for Raw dependent on range taken for flow and box pressure, B was barometeric pressure (mm Hg), G was the reciprocal of the gradient of the airflow/Pbox graph and 0.25 was a constant value taken to be the resistance of the mechanical equipment between mouthpiece and breathing bag.

(ii) **Automatic method** (Figure 2.4)

(a) The Airflow/P.box graph (Figure 2.3)

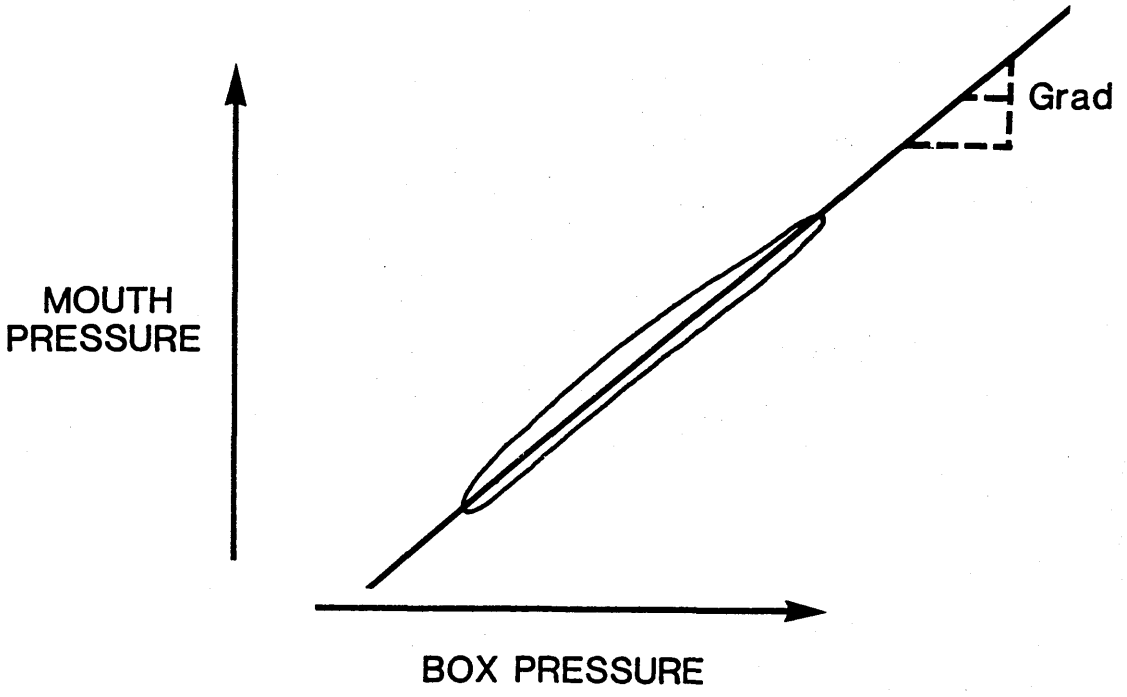
The data acquisition programmes were written in machine code and data were sampled from both output channels at rate of 1.6 K/Hz. Once the Airflow/P.box mode had been selected the flow channel was monitored continuously until the flow exceeded 0.5 l/s for at least 160 milliseconds, as this indicated that the respiratory cycle was properly underway. As soon as the flow dropped to less than 0.4 l/s data were sampled until the flow exceeded 0.5 l/s in the other direction. The sampling then ceased and recommenced when the flow was in range on the other half of the respiratory cycle. If the button was released before both sides of the cycle had been sampled, an error flag was set. An error flag was also set if too many samples had been taken while the flow was in range thus causing a memory overflow. This could be caused by the patient breathing either too quickly or with insufficient amplitude, or a combination of both.

The data were processed at a later stage, while the subject rested, before the next sampling period. The processing was performed by a compiled Basic program which identified the section of data on the inspiratory side of the cycle and evaluated the gradient with a standard least mean squares fit routine.

(b) The P.mouth/P.box Graph (Figure 2.2)

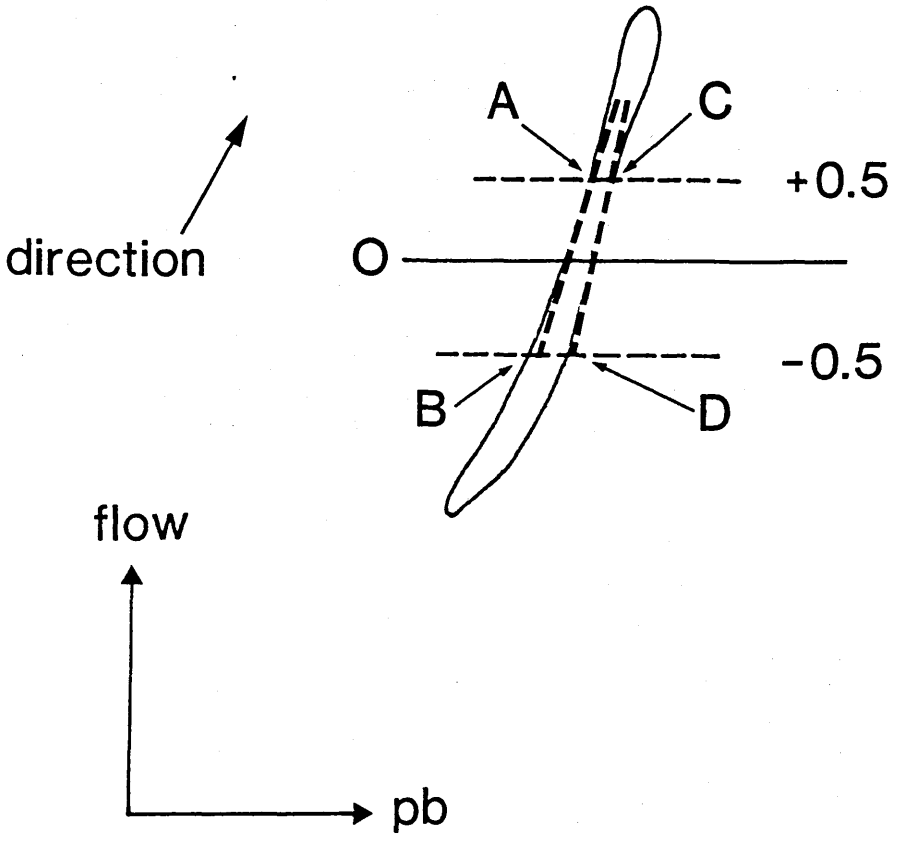
The relationship between the two variables in this case is much more linear than for the flow/box pressure measurement and it is adequate to measure the gradient at a region midway between the two extremes in mouth pressure. The mean value of mouth pressure is variable, however, and depends on the panting manoevre. Therefore, unlike the previous situation the region of interest cannot be specified/

FIGURE 2.2



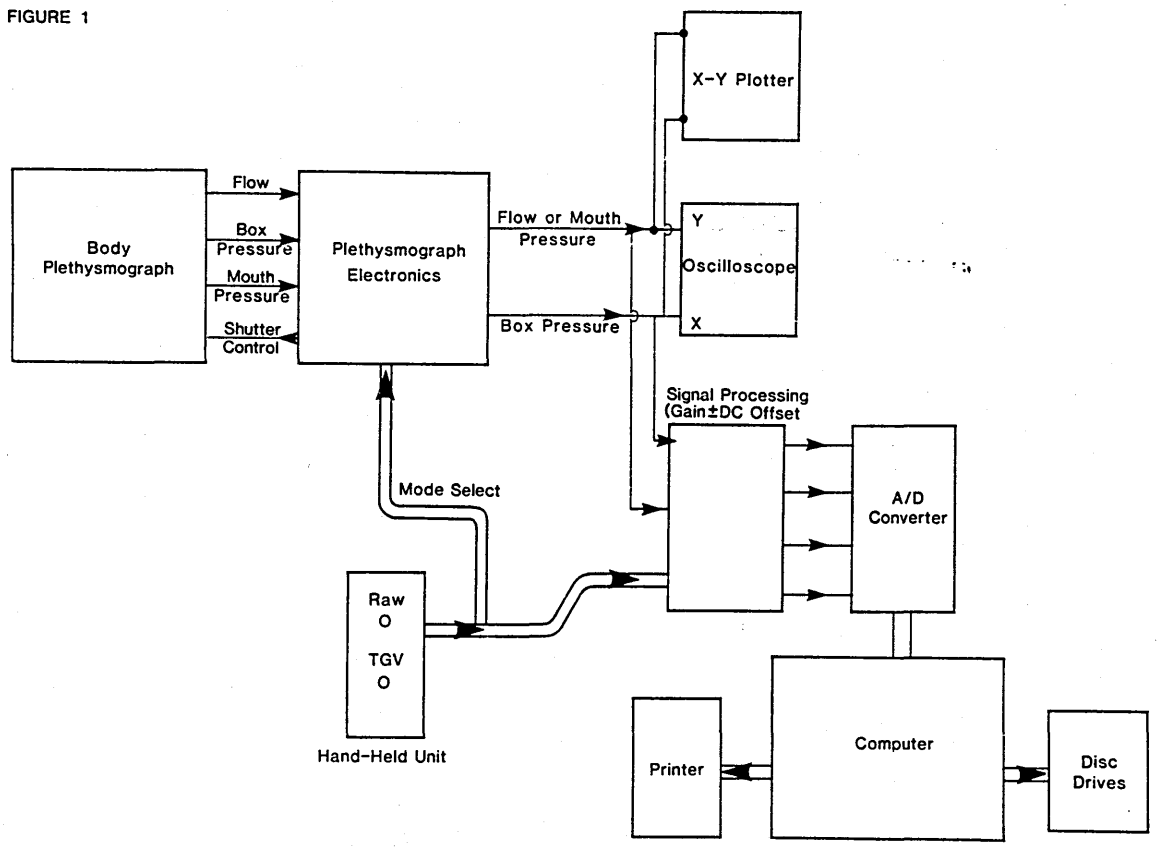
Graph of mouth pressure (P.mouth) against box pressure (P.box). A. and B are the turnover points. As the relationship between the two points is close to linear, the line joining A and B is an adequate measure of the gradient.

FIGURE 2.3



Graph of flow measured at the mouth against box pressure. Measurement of the gradient is made close to zero flow. In practice this is between -0.5 l/sec (point B) and +0.5 l/sec (point A) on the inspiratory side of the cycle and the similar region between points C and D on the expiratory half of the cycle.

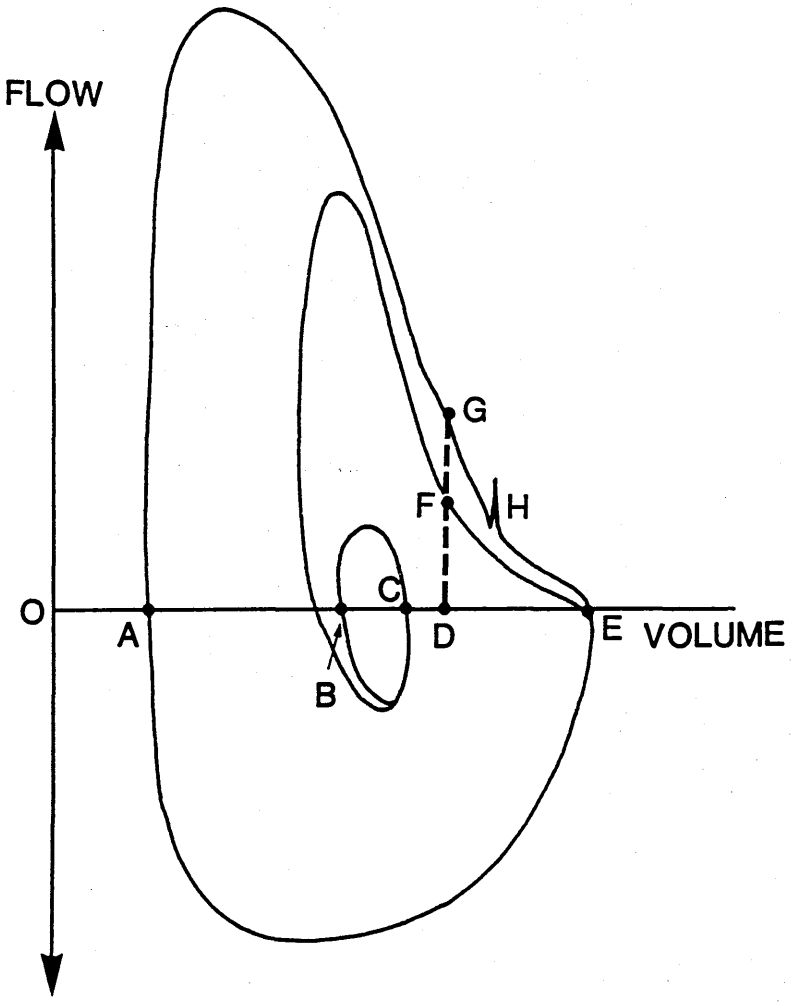
FIGURE 2.4



The computerised system for measuring Raw and TGV. The hand held unit controls the plethysmograph electronics and the computer data collection.

FIGURE 2.5

DIAGRAM OF FLOW-VOLUME TRACING



Graph of inspiratory and expiratory flow against volume

B - C		Tidal breathing
C → E		partial expiratory flow curve
E	=	RV
E → A		inspiration to TLC
A		TLC
A + E		complete expiratory flow curve
D		point on volume curve representing 30% of TLC
G	=	Flow rate at 30% of TLC on complete expiratory flow curve $\dot{V}_{30}(c)$
F	=	Flow rate at 30% of TLC on partial expiratory flow curve $\dot{V}_{30}(p)$
H		1 second timer mark allowing FEV_1 to be measured

specified until the respiratory cycle has finished. This meant that the whole cycle had to be stored in the memory, and thus to avoid memory overflow the sampling rate had to be slower. The actual sampling rate was 125 Hz and was such that two complete respiratory cycles could be stored in the memory space allocated.

During the analysis phase the data were analysed by a compiled Basic program which had identified the turnover points and hence the midpoint region in the respiratory cycle, and evaluated the gradient in this region by a standard least mean square fit process.

2.3.3 Measurement of Flow volume curves (Figure 2.5)

Flow-volume curves were recorded using the Flow-volume mode of the body plethysmograph (Fenyves and Gut, Basle, Switzerland). Partial and complete expiratory flow volume (PEFV and CEFV) curves were obtained using a heated pneumotachograph with integration of flow, and recorded on an X-Y recorder (Hewlett-Packard 7041A). The flow volume curves were obtained in the following manner. After a period of normal tidal breathing each patient expired maximally from end tidal expiratory volume to residual volume (RV) to obtain the PEFV curve. When RV was reached the patient inspired to total lung capacity (TLC) and expired maximally to RV to obtain the CEFV curve. A one second timer was incorporated into this part of the curve. From the CEFV curve the forced expiratory volume in 1 second (FEV_1), and forced vital capacity (FVC) were obtained. The volume corresponding to 35% (or 30%) of total lung capacity was obtained from the mean FVC of at least 5 baseline curves. Maximum expiratory flow rates at this lung volume were measured from the partial ($\dot{V}_{35}(p)$) and ($\dot{V}_{35}(c)$) flow volume curves for baseline and subsequent curves - that is, curves were matched at TLC in order to compare flow rates. In all studies 1 minute was allowed between each measurement of PEFR as inflation to TLC may produce temporary bronchodilation/

bronchodilation (Nadel and Tierney, 1961). The mean of 5 values was used for baseline value and the mean of two measurements used after each subsequent drug inhalation.

2.3.4 Routine Pulmonary Function Tests

Routine pulmonary function tests were carried out pre-operatively on all surgical patients. Spirometry was measured on a water sealed spirometer (Godart), static lung volumes were determined by the helium dilution technique; single breath transfer factor for carbon monoxide (Tl_{CO}) was determined by the method of Ogalvie et al (1957). Predicted normal values were taken from Cotes (1975).

2.3.5 Prick Skin tests

Prick skin testing against seven common antigens (Bencard) was carried out to assess atopic status of each subject. The allergens used were (1) control solution, (2) house dust, (3) Dermatophagoides pteronyssinus,(4) cat, (5) dog, (6) feathers, (7) mixed grass pollens and (8) Aspergillus fumigatus.

2.3.6.Inhalation tests

Inhalation tests were carried out using a modification of the method described by Cockcroft et al (1983). Aerosols were generated with the same Wrights nebuliser by air at 50 psi (345 Kpa) at a flow rate of 8l/min to give an output of 0.15 ml/min. Patients wore a nose clip and aerosols were inhaled by tidal breathing through a lose fitting facemask. Phosphate buffered saline was inhaled first followed by either methacholine 2-64 mg/ml histamine 2-64 mg/ml or leukotriene D_4 (LTD_4) 0.08-50 mcg/ml for normal subjects. For asthmatic patients methacholine 0.06 - 4 mg/ml, histamine 0.06 - 4mg/ml or LTD_4 0.0032-2 mcg/ml were given. All solutions were nebulised for 2 minutes at 10 minute intervals for methacholine and histamine and at 15 minute intervals for leukotriene.

The volume added to the nebuliser was 4 ml for methacholine and histamine and 2 ml for LTD_4 studies. It has been shown with this system that 0.3 ml of solution is nebulised each 2 minutes. The dose of drug reaching the airways was not formally measured but is likely to have been around 10% (Davies 1975).

Before inhalation the mean of 8 measurements of sGaw were recorded. Five measures of FEV_1 $\dot{V}_{30}(p)$ and $\dot{V}_{30}(c)$ were also recorded with a full minute between each measurement. The first inhalation was always that of vehicle and phosphate buffered saline. Eight sGaw and three FEV_1, $\dot{V}_{30}(p)$ and $\dot{V}_{30}(c)$ were measured. The pre and post saline values were averaged and used as the baseline from which percentage fall was calculated.

2.3.7. Calculation of responsiveness

After each inhalation study a log concentration-response curve was constructed (Figure 2.6) and the responsiveness expressed in terms of the provocation concentration (PC) of the drug in use which would produce a 10% fall in FEV_1, (PC_{10} FEV_1) or a 20% fall in FEV_1 (PC_{20} FEV_1), a 35% fall in sGaw (PC_{35} sGaw), a 30% fall in $\dot{V}_{30}(p)$ (PC_{30} $\dot{V}_{30}(p)$) or a 30% fall in $\dot{V}_{30}(c)$ (PC_{30} $\dot{V}_{30}(c)$).

If the appropriate fall was not obtained the value assigned was greater than (>) the highest concentration of the drug inhaled (e.g. >64 mg/ml).

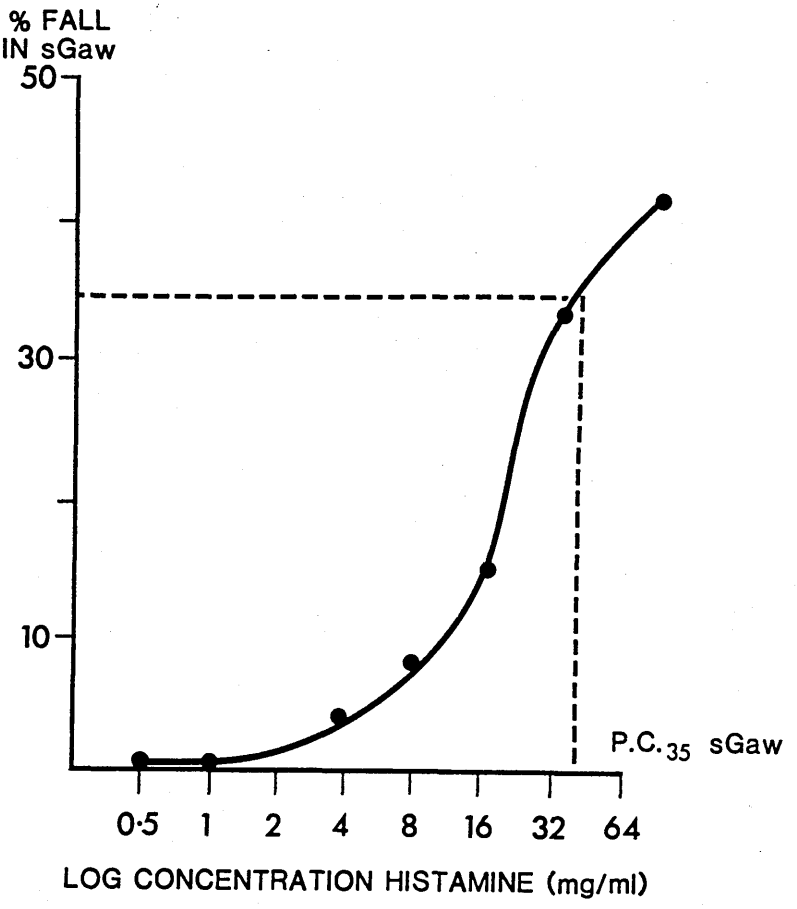
2.4 EXPERIMENTAL METHODS IN VITRO

2.4.1. Tissue collection and handling

Patients undergoing thoracotomy were premedicated with papaveritum and hyoscine. Anaesthesia was induced by either Althesin or thiopentone sodium and maintained with nitrous oxide and halothane. A variety of neuro-muscular and anaesthetic blockers were given. The trachea was intubated and the lungs ventilated artificially. Bronchial tissue was obtained immediately after removal at thoracotomy. Similar sized/

FIGURE 2.6

Concentration-response curve to histamine with derivation of PC_{35}sGAW



Graph of log concentration inhaled agonist response, as measured by percentage fall from baseline level (FEV_1 or sGaw). The concentration of agonist producing a 35% fall (PC_{35}) [or 20% fall PC_{20}] is derived and used as an index of *in vivo* responsiveness.

sized samples of second to sixth order bronchi were dissected from macroscopically normal tissue. The tissue was thoroughly oxygenated after removal and then stored overnight at 4°C in well oxygenated Krebs-Hensleit physiological saline (KHS).

Physiological Saline

KHS was prepared freshly each day. It was of the following composition (m mol litres^{-1})

Na Cl	118
K Cl	4.7
Mg SO_4	1.2
KH_2 PO_4	1.2
Ca Cl_2	2.5
Na H CO_3	25
Glucose	11.7

To ensure that Ca Cl_2 dissolved, the solution was saturated with a 95% O_2: 5% CO_2 mixture to lower the pH of the solution. If this step was not taken $Ca_2(PO_4)_3$ tended to precipitate.

2.4.2 Tension measurement

Rings of bronchi were dissected and divided to produce transverse strips. These were suspended in organ baths in KHS at 37°C and bubbled with a gaseous mixture containing 95% O_2 and 5% CO_2. An initial stretching tension of 1.5 - 2.0G (15-20 mN) was applied to each tissue which was then left for 60 minutes to equilibrate, during which time the bathing KHS was changed three times. Changes in tension were recorded using isometric force-displacement transducers (FT03C, Grass instruments, Quincy, Mass. U.S.A.) coupled to a Grass (model 7) curvilinear ink-writing polygraph. Tissues were attached to the transucers via a silk thread (6/o)

2.4.3/

FIGURE 2.7

Arrangement for <u>in vitro</u> measurements

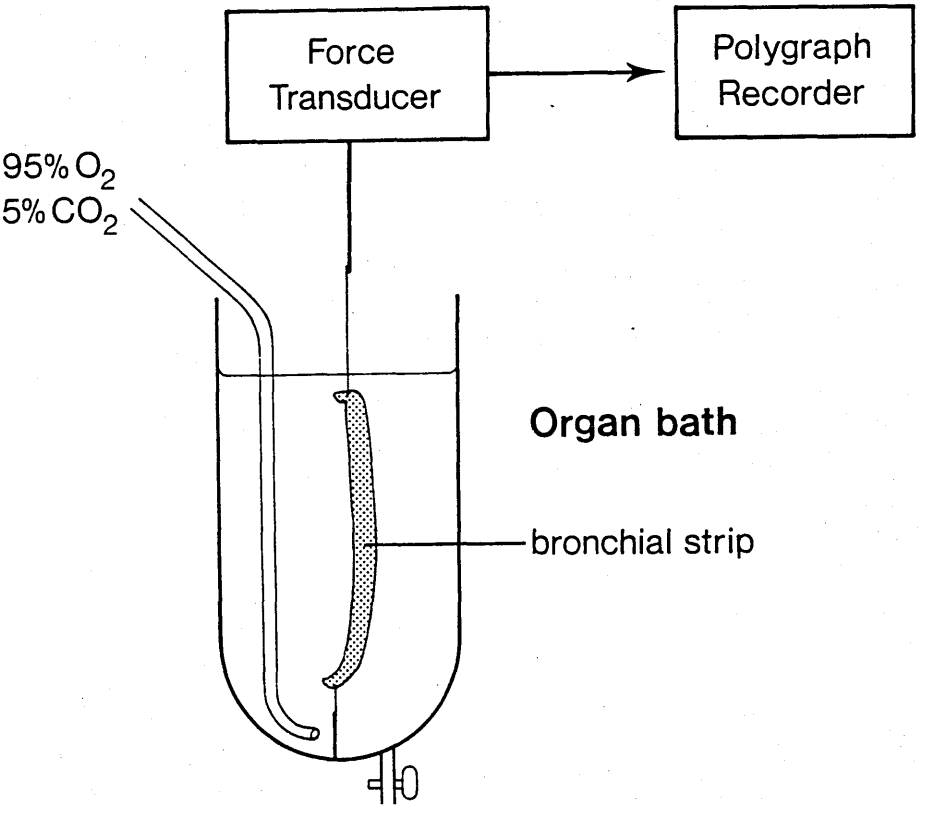


Diagram of bronchial strip suspended in organ bath attached to isometric transducer. The tone generated by the bronchial strip was recorded on a continuous polygraph trace. Agonist was added to the bath and the resultant change in tension measured from the polygraph recording.

2.4.3 Measurement of Tissue response

To assess the response of each tissue to agonist stimulation, cumulative concentration - effect curves were constructed for each agonist according to the method of Van Rossen (1963). After equilibration the tissue was exposed twice to near maximally effective concentrations of agonist at 30 min intervals to gauge the magnitude, normality and reproducibility of the contractions produced. A cumulative concentration - effect curve was then constructed by adding increasing concentrations of agonist increasing in log units (methacholine) or half log units (histamine and LTD_4) until a maximum response was reached. Each addition of agonist was made at the peak of effect produced by the preceding concentration (Figure 2.8). In all experiments the concentration of agonist producing a 50% (EC_{50}) of maximum contraction was calculated from the graphically displayed data (Figure 2.9). Additionally, the maximum tension generated was determined and expressed in g tension/mg wet weight of tissue. At least four bronchial strips from each patient were tested and mean values for EC_{50} and maximum tension generated per mg tissue determined.

In vivo responsiveness and the in vitro sensitivity of tissue obtained from the same patient were then compared.

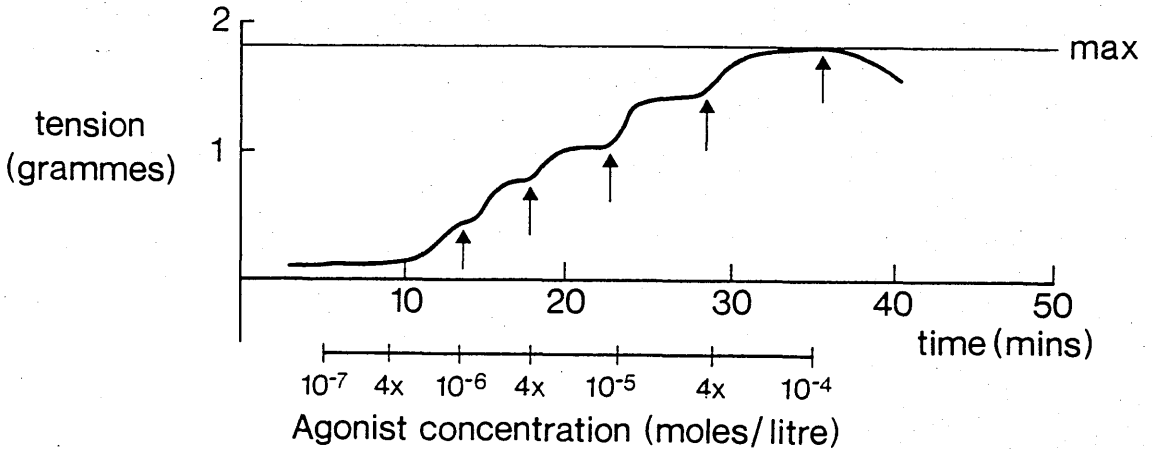
2.5 ULTRASTRUCTURAL STUDIES

Bronchial tissue was obtained immediately after removal at thoracotomy. Tissue rings were dissected from macroscopically normal tissue using a scalpel blade. The rings were stretched on a small spring and incubated in Calcium free KHS solution bubbled with 95% O_2/5% CO_2 gas mixture for ten minutes. The tissue was then fixed in freshly diluted 4% Gluteraldehyde solution.

The tissue was later embedded in Araldite. Sections of embedded tissue/

FIGURE 2.8

Polygraph tracing of tension generated against time



Tracing obtained from the force transducer attached to a bronchial strip. Increasing concentrations of agonist were added, the next concentration being added when the response to the previous concentration had plateaued.

From the tracing a concentration-response curve was drawn and EC_{50}^{0} values derived.

FIGURE 2.9

Log concentration-response curve for <u>in vitro</u> responses

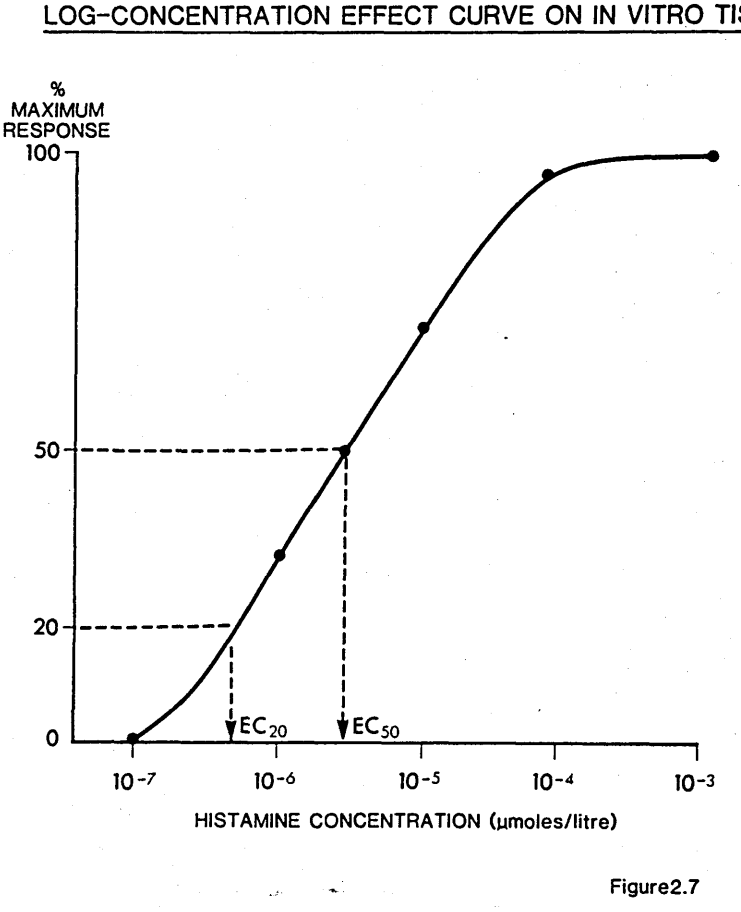


Figure2.7

Log concentration-effect curve for agonist added to organ bath.
Effect is expressed as the percentage maximum for that bronchial strip. From this the concentration producing a 50% of maximum EC_{50} or 20% of maximum EC_{20} was derived and used as an index of <u>in vitro</u> smooth muscle sensitivity.

tissue were cut on a LKB Ultratome 1. The sections were double stained with uranyl acetate and lead acetate and the ultra structure of the tissue examined under a Phillips EM 301 transmission electron microscope. Photographs of various magnifications (2,600 - 22,000) were taken for future analysis.

2.6 SMOOTH MUSCLE QUANTIFICATION

After completion of the *in vitro* concentration-effect curve, the bronchial strips were fixed in corrosive formal acid and mounted in paraffin wax. Four representative sections of each tissue were prepared and stained with Unnas Variant of Van Geesons stain (Lillie 1954). Smooth muscle was quantified using light microscopy with x 100 magnification with an incorporated 42 point eyepiece grid. For each section of bronchial strip, eight random fields were examined and the number of points overlying smooth muscle estimated. The percentage of muscle was measured for each of the four sections and an average % of muscle present in the bronchial strip derived. The percentage of smooth muscle present, the absolute amount of muscle present in strip (wet weight x % muscle) and the maximum tension per unit mass of smooth muscle was then calculated and maximum tension was compared with the percentage of smooth muscle present for each individual bronchial strip. The average values of these *in vitro* measurements for the four bronchial strips from each patient was compared with *in vivo* measurements.

2.7 DRUGS

Drug solutions used were methacholine dihydrochloride (Sigma, London) made up in solutions to a concentration of 0.06 - 64 mg/ml, histamine dihydrochloride (Sigma, London) made up of solutions of 0.06 - 64 mg/ml, and leukotriene D_4 100 mg and 10 mg ampoules diluted to 0.0032, 0.016, 0.08, 0.4, 2.0, 10.0, 50.0 mcg/ml. LTD_4 ampoules were stored at $-70^{o}C$ and diluted to appropriate concentrations immediately before use in experiments. Other drugs used were atropine sulphate 5 mg/ml (Antigen limited/

limited) sodium cromoglycate 10 mg/ml (Fisons Limited) and verapamil hydrochloride 2.5 mg/ml (Abbott).

2.8 STATISTICAL ANALYSES

In comparing *in vivo* and *in vitro* results, correlation coefficients were calculated from linear regression using the method of least squares analysis. PC results were analysed under a natural logarithm transformation.

Comparison of results after drug and placebo were analysed by analysis of variance and Students t test. Comparison of responsiveness between different groups of subjects and/or patients was made using Students unpaired t test.

The level of significance was set at $p < 0.05$.

CHAPTER 3

COMPUTERISED SYSTEM FOR THE MEASUREMENT OF SPECIFIC AIRWAYS CONDUCTANCE

COMPUTERISED SYSTEM FOR THE MEASUREMENT OF SPECIFIC AIRWAYS CONDUCTANCE

3.1 INTRODUCTION

The object of this project was to investigate the role of airway smooth muscle in determining airway responsiveness. The method to be used was to compare *in vivo* responsiveness with *in vitro* sensitivity. A reliable measure of *in vivo* airway calibre was therefore required. When FEV_1 is used, it can be difficult to obtain significant changes on bronchoconstrictor challenge in non-asthmatic subjects. Specific conductance has the advantage of being a sensitive measure of airway calibre so that relatively small changes can be detected and an index of *in vivo* responsiveness obtained. sGaw has the additional advantage of reflecting changes in the larger airways, the site from which the tissue to be tested *in vitro* is obtained. sGaw is determined using a constant volume body plethysmograph by the method of Du Bois *et al* (1956) described in the methods chapter. The measurement of specific conductance requires the drawing of two graphs mouth pressure (P.m) against box pressure (P.b) and flow ($\dot{V}$) against (P.m) on a X-Y plotter, and then estimating the slope of each graph. Estimation of the gradient of a non-linear graph introduces error and is a time consuming practice. As part of this project, the measurement of sGaw was automated. The results of PC_{35}sGaw and of baseline sGaw values obtained by the automated analysis were compared with those obtained by the traditional manual method.

3.2 METHODS

3.2.1 Equipment

A constant volume plethysmograph (Fenyves & Gut) was used for all measurements. The output signals from the apparatus were either airflow and box pressure or mouth pressure (with zero airflow) and box pressure, depending on the mode selected. These signals were sampled via an external 12 bit analogue to digital converter and fed to a BBC model B microcomputer for processing and to an X-Y plotter for future manual analysis. The mode was selected/

selected by the operator pressing one of two buttons, and signals relating to the status of these buttons were also sampled and fed to the computer.

3.2.2 Procedure

The subject was asked to pant rhythmically at 1-2 Hz. The first mode selected was airflow/box pressure and this was followed by the mouth pressure/box pressure mode. These two manoeuvres gave one determination of sGaw and during each period of panting the complete sequence was carried out twice. The data were then processed within 4-5 seconds, while the subject rested. If no errors were identified, the two values of sGaw were stored in memory. The whole procedure was repeated four times, or more if an error had occurred, giving eight values of sGaw which were stored on disc. An outline of the programming techniques used for the acquisition and analysis of the data is given in the methods chapter.

3.2.3 Clinical Evaluation

To evaluate the system over a range of values under a clinical setting, histamine bronchial provocation tests were performed on a group of human subjects. Seven subjects were tested on two occasions, at the same time of day, not more than 7 days apart (Table 3.1). All were non-smokers. Three were asthmatic patients on inhaled bronchodilator treatment. Four were considered normal with no history of atopy nor respiratory disease. Five of the seven had never previously been tested in a body plethysmograph.

Histamine inhalation tests were carried out using a modification of the method described by Hargreave et al (1981). Aerosols were generated with the same Wright nebuliser by air at 50 psi at a flow rate of 8 l/min to give an output of 0.15 ml/min. Patients wore a nose-clip and aerosols were generated by tidal breathing through a loose-fitting face mask. Buffered normal saline was inhaled first followed by doubling concentrations of phosphate/

TABLE 3.1

SUBJECT CHARACTERISTICS

SUBJECTS	Age (Years)	Sex	Height (Metres)	Baseline FEV_1 Litres	.% pred	Diagnosis	Treatments	Used body box previously
1.	21	M	1.71	3.95	99	Asthma	S, SCG	No
2.	25	F	1.61	3.37	110	Asthma	S	No
3.	35	M	1.71	3.21	89	Normal	-	Yes
4.	32	M	1.74	3.80	99	Normal	-	Yes
5.	27	F	1.54	3.12	110	Normal	-	No
6.	32	M	1.83	5.0	113	Normal	-	No
7.	54	F	1.59	1.88	84	Asthma	SCG, D	No

Treatments key S - Salbutamol

SCG - sodium cromoglycate

D - fenoterol + ipatropium bromide

phosphate buffered histamine, 2-64mg/ml for non-asthmatic patients and 0.06-4 mg/ml for asthmatics. Each inhalation was for 2 minutes at 10 minute intervals. Before the first inhalation eight sGaw determinations were recorded. From 1.5 minutes after each inhalation, responses were assessed by sGaw measurements. Inhalations were continued until the sGaw had fallen by 35% or more.

Measurements from the same panting manoeuvre were both recorded with the X-Y plotter (Hewlett-Packard 7041A) and analysed by the computerised system and, for both systems, 8 manoeuvres were analysed and the mean and standard deviation were calculated. Manual results were measured in a blinded manner after the challenge was over by me and I was unaware of the results of computer analysis. The graphs were analysed by measuring the gradient of one limb on the inspiratory part of the V/Pb graph. Using automatic and manual methods the sGaw following each concentration of histamine was estimated and the histamine concentration producing a 35% fall in sGaw (PC_{35}sGaw) was calculated by linear interpolation. The PC_{35}sGaw values obtained for each patient by these methods were used to assess reproducibility of both methods and for comparison between the automatic and the manual method.

3.2.4 Analyses

Baseline values measured by the two methods across the two days were compared by Student's paired t-test. The reproducibility of each of the two methods was assessed across the two study days by calculating correlation co-efficients from linear regression using the method of least square analysis. The level of statistical significance was set at $p < 0.05$.

3.3 RESULTS

Results were recorded for all seven subjects on two occasions.

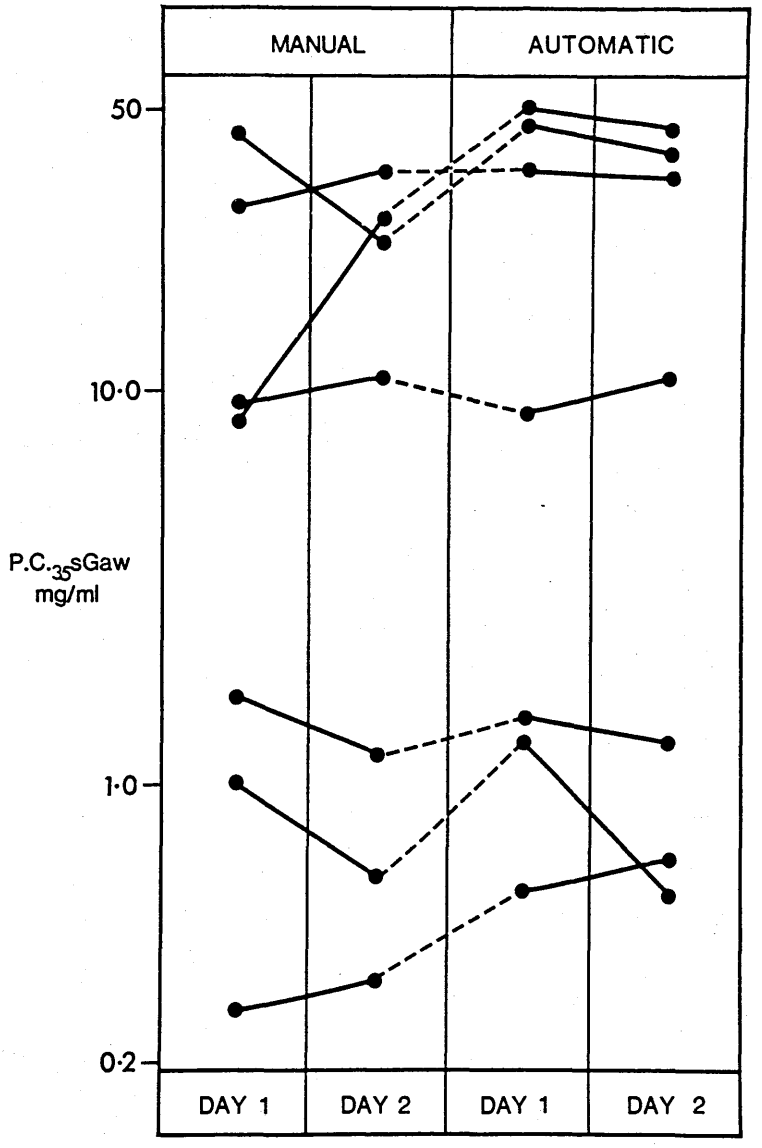
BASELINE SPECIFIC AIRWAYS CONDUCTANCE AND CO-EFFICIENT OF VARIATION

SUBJECT		MANUAL				AUTOMATIC			
		DAY 1		DAY 2		DAY 1		DAY 2	
		sGaw	C of V	sGaw	C of V	sGaw	C of V	sGaw	C of V
1	a	1.69	20.7	1.19	15.1	1.59	7.6	1.15	12.2
	b	1.53	16.3	1.34	8.9	1.55	21.9	1.12	15.2
2	a	6.73	50.5	2.24	20.1	3.25	18.5	1.27	11.0
	b	2.66	15.0	2.0	17.0	2.1	16.2	1.32	22.0
3	a	5.70	21.0	6.12	29.1	2.9	26.9	3.43	32.1
	b	5.91	32.0	7.42	34.4	3.46	23.4	4.10	35.9
4	a	2.36	20.3	2.76	51.8	2.36	33.9	1.97	19.3
	b	3.44	46.8	3.27	14.4	2.25	16.9	2.39	9.6
5	a	5.77	69.8	5.72	69.2	2.88	30.9	2.81	30.6
	b	3.82	52.4	3.47	24.8	2.35	19.2	2.46	22.4
6	a	1.66	23.5	0.94	12.8	1.32	12.9	1.03	8.5
	b	1.21	28.1	1.01	11.9	1.16	15.5	1.02	7.8
7	a	2.52	22.2	3.3	59.7	1.35	13.3	-	-
	b	2.78	32.0	4.57	66.7	1.82	24.2	1.53	10.5
			Mean = 32.2 S.E. 4.4		Mean = 31.1 S.E. 5.8		Mean = 20.1 S.E. 1.2		Mean = 18.2 S.E. 2.7

a and b are sequential measurements on the same day

FIGURE 3.1

PC_{35}sGaw values obtained on 2 days by manual and automatic method



Comparison of PC_{35}sGaw for the seven subjects on two days by the manual, and automated methods. Results are expressed on a log scale. Manual results across the two days correlated significantly ($r = 0.77$ $p < 0.05$), and automatic measurements correlated highly significantly ($r = 0.99$ $p < 0.01$).

3.3.1 Baseline sGaw

The two sets of values obtained for sGaw before drug administration on each of the two test days are presented for both the manual method and the automatic method (Table 3.2). The mean co-efficient of variation for the manual method (31.6%) was inferior to the automatic method (m = 19.2%)

3.4.2 PC_{35}sGaw Results

The relationship between the three methods of analyses was assessed by comparing the PC_{35}sGaw histamine for the seven subjects across the two days (Figure 3.1). There was no significant difference in the mean PC_{35}sGaw values obtained by the two methods of analysis. The geometric mean PC_{35}sGaw values ($\pm$ SEM) were 4.6 $\pm$ 3.9 mg/ml (one limb manual), and 6.2 $\pm$ 5.4 mg/ml (automatic).

As a measure of reproducibility, the correlation between the PC_{35}sGaw results for each subject obtained on day 1 and day 2 was investigated for each of the two methods. The correlation co-efficients were $r = 0.76$ ($p < 0.05$) for the manual method, and $r = 0.99$ ($p < 0.01$) for the automatic method.

3.5 DISCUSSION

The constant volume body plethysmograph was automated in a straight forward and low cost way. Each value of sGaw is produced within 4-5 seconds of completion of the panting manoeuvre and there is an improvement in the reproducibility of airways responsiveness as assessed by PC_{35}sGaw, where sGaw is measured from one limb of the flow-pressure loop.

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The co-efficients of variation of baseline sGaw values are comparable with values obtained in other studies and reported by Tattersfield and Keeping (1979). When the baseline values obtained by the/

the manual method are compared with the automatic method there is a significant difference, the manual analysis producing a higher result. This suggests that the second limb gradient (at the end of the inspiration) is lowering the average value. On inspection of the flow-box pressure plots it is apparent that the gradient of the second limb is lower. It is possible that this is caused by the body plethysmograph or its electronics. However, preliminary work using an oscillating pump calibrator suggest that the difference in gradients is not due to equipment or measurement technique. Another possibility is that the effect is associated with changes in the airways characteristics during the respiratory cycle.

We tested our system under conditions similar to those encountered in practice - i.e. assessing change in sGaw during a bronchial provocation challenge. When measurements of airways' responsiveness to histamine were assessed on two occasions, the results obtained by our computerised system have a higher correlation coefficient than results obtained by manual analysis and were produced at the time of measurement rather than recorded for later analysis.

The PC_{35}sGaw results obtained by the automated method show that there was a considerable difference between the two days for subject 2. This difference was also present in the manual PC_{35}sGaw analysis and in the baseline values measured by both systems, suggesting that the discrepancy between the two measurements was a genuine difference in PC_{35}sGaw.

The rapid presentation of results has several advantages. The subject is present when the results are obtained so that measurements may be repeated if analysis is, for some reason, unsatisfactory. It also means that the investigator determines the end point of the experiment when the required change in sGaw has occurred, thereby administering the minimum amount of drug necessary to obtain a result.

The high reproducibility of the automatic measurements means that smaller changes in airway responsiveness may be detected. This is particularly important in bronchoconstrictor pharmacological studies, such as those used in this thesis.

Other groups have reported an automatic system to measure airways resistance using a body plethysmograph. Lord et al (1977) developed an automated system to measure sGaw using a Hewlett-Packard 2116B computer. The gradient on the $\dot{V}$/Pb curve was calcuated by measuring the box pressure at zero flow and at 0.4 ls^{-1} on the inspiratory part of the cycle, and deducing the gradient from these two points. The method used in this thesis measures the gradient in the same region of flow. However, rather than using only the two end points all the data points are used (typically about 35), and a 'best fit' line is found by regression analysis.

The improvement gained by using such an approach is not as great as one might at first think because there is correlation between the points due to the limited system bandwith. Nevertheless there will be some improvement and because the time taken to perform such a regression analysis was not significant it is worthwhile.

The same is true in the case of the Pm/Pb curve where Lord et al (1977) use the two turnover points to calculate the gradient and our system uses regression analysis to fit a "best line" to the middle region of the curve.

Our measurement protocol differs significantly from the technique used by Lord et al (1977). Their technique was that the subject panted for about 10 cycles with no shutter (V/Pb mode) and then the shutter was introduced for 10 cycles (Pm/Pb mode) and the average gradients were then used to produce a value of sGaw. The time separation between the two modes is undesirable and panting against the shutter for 10 cycles is extremely tiring. In this method the subject has only to pant for 2 cycles before the mode is changed.

Chowienczyk et al (1981) describe a novel approach to measure the $\dot{V}$/Pb loop, in that they approximated sine waves to one cycle of the respiratory loop and deduce the value from the phase and amplitude relationships between the two signals. The processing time of this technique is about 3-4 times greater than with the system described here and could be significant in an experiment. The measurement protocol is similar to the one used in this system except that four cycles are acquired before changing mode compared with two.

Their method averages the airways resistance throughout the whole respiratory cycle whereas this technique uses the inspiratory side. Zarins and Clausen (1982) have suggested that selecting a particular region of the respiratory cycle may improve the sensitivity of the airways resistance measurement. This is an interesting possibility and the system described may be modified such that this possibility can be investigated.

An important difference between the described method and other reported systems is that the operator controls when measurements are made by using a control button to activate data collection by the computer. This allows the operator to assess whether the subject is panting properly and to allow adequate time for recovery in less robust subjects who may find the panting manoeuvre tiring. A facility is also provided for rejection of curves by pressing either of the control buttons on completion of each pair of measurements.

In summary, a computerised system has been developed which produces results more reproducible than the one limb manual measurement. As the automated method improved the precision of results, gave results at the time of experiment and was more convenient, it was used in this thesis to measure sGaw.

C H A P T E R 4

COMPARISON OF *IN VIVO* AIRWAY RESPONSIVENESS AND *IN VITRO* SMOOTH MUSCLE SENSITIVITY TO METHACHOLINE IN MAN

COMPARISON OF *IN VIVO* AIRWAY RESPONSIVENES AND *IN VITRO* SMOOTH MUSCLE SENSITIVITY TO METHACHOLINE IN MAN

4.1 INTRODUCTION

Airway responsiveness to the cholinergic agonist methacholine varies between normal subjects (Parker et al 1965) and is increased in patients with asthma (Parker et al 1965, Hargreave et al 1981). The reduction in airway calibre induced by this agonist involves the contraction of airway smooth muscle (Hawkins and Schild 1951). Thus, the difference in responsiveness to methacholine in normal and asthmatic subjects may be related to variations between individual subjects in their airway smooth muscle sensitivity to this agent. Alternatively, the variability between subjects in responsiveness may be a consequence of differences in the neural and/or humoral control of airway smooth muscle. Previous studies have found that *in vitro* airway smooth muscle sensitivity to a cholinergic agonist varies between subjects (Hawkins and Schild 1951, Brink et al 1980, Goldie et al 1982), but these investigators have not related the *in vitro* values to *in vivo* measurements of responsiveness. Therefore, the importance of airway smooth muscle sensitivity in determining *in vivo* responsiveness is unknown.

The purpose of this study was to compare *in vivo* airway responsiveness to methacholine with *in vitro* sensitivity of preparations of isolated bronchi taken from the same patient to determine whether variation in responsiveness to methacholine may be explained by differences in the sensitivity of airway smooth muscle to the drug.

4.2 METHODS

4.2.1. Patients

Ten patients scheduled to undergo lobectomy or pneumonectomy were studied (Table 4.1). Eight patients had operable bronchial carcinoma, one had a tuberculoma and one had a solitary metastasis from a renal clear cell carcinoma. All had been smokers but at the time of study two had stopped for a period of > 1 year. Four patients fulfilled the Medical Research Council's definition for chronic bronchitis (MRC 1965). One patient (No. 2) gave a history of wheezy dyspnoea and hay fever. Five were atopic as indicated by > 2 mm wheal response to prick skin testing, although only three patients had raised specific IgE levels. Four patients were on regular drug treatment prior to surgery.

4.2.2 Experimental procedure

Patients were seen on the day of admission to hospital, usually 2 days before surgery. They answered a questionnaire modified from the M.R.C. Respiratory questionnaire (Appendix 1) and prick skin tests were carried out. The patients were then trained to use the body plethysmograph. Baseline values were obtained and then increasing concentrations of methacholine were inhaled (2 - 64 mg/ml)until a > 20% fall in FEV_1 was recorded.

The wheezy subject did not take Phyllocontin tablets for 24 hours and his salbutamol inhaler for 72 hours before testing.

In vitro measurements were made the day after surgical removal of tissue. After equilibration, increasing concentrations of methacholine ($1 \times 10^{-9} - 1 \times 10^{-3}$) were added to the organ bath at the peak of the previous response. A minimum of four tissues from each patient was used. From the results obtained a log concentration-response curve was drawn and the concentration of drug producing a 50% of maximum response (EC_{50}) measured and the mean of four values obtained for the subject used as an index of *in vitro* sensitivity. The concentration producing a 20% of maximum response/

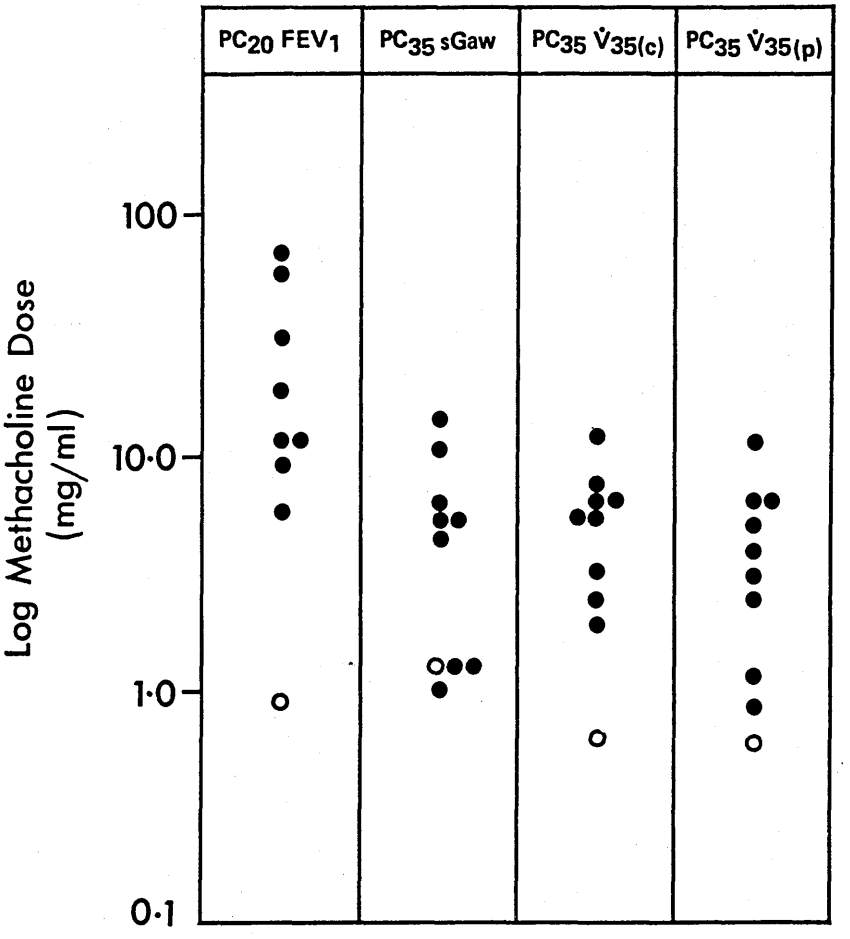
TABLE 4.1 Patient characteristics (Methacholine in vivo vs in vitro)

Patient No.	Age (yr)	Sex	Height (cm)	FEV_1 (L)	FEV_1 (%pred)	VC (%pred)	RV (% pred)	T_{LCO} (% pred)	Atopic Status	Current Smokers	Diagnosis: Surgical	Diagnosis: Other	Medication
1	50	M	170	3.92	123	122	95	61	+	+	s	Chronic bronchitis HPOA	Indomethacin Dextropropoxyphene Paracetamol
2	43	M	177	1.67	45	64	108	68	+	+	s	Chronic bronchitis Wheezy dyspnoea	Salbutamol(inhaled) Aminophylline(oral) Temazepam Levorphan Metaclopramide
3	49	F	155	2.18	96	95	148	77	-	+	l	Peripheral vascular disease	Inositol
4	63	M	178	2.91	92	98	115	75	+	+	s	-	-
5	64	M	169	2.85	106	133	135	55	+	+	a	Chronic bronchitis	-
6	66	F	165	1.64	81	69	100	106	+	+	c	Hypertension	Chlorthalidone
7	61	F	153	1.55	81	97	140	115	-	-	l	-	-
8	62	M	173	2.32	81	88	102	114	-	+	s	-	-
9	56	M	180	2.76	79	94	118	79	-	+	s	Chronic bronchitis	-
10	69	M	168	2.82	110	97	60	141	-	-	t	-	-

Surgical diagnosis
s - squamous cell carcinoma
a - adenocarcinoma
t - tuberculosis
l - large cell carcinoma
c - clear cell carconoma

FIGURE 4.1

Range of responsiveness to methacholine



Airway responsiveness shown on a log scale of ten patients to inhaled mechacholine, one of whom (O) had symptoms of wheezy dyspnoea. Results are expressed as the provocation concentration (PC) causing a decrease in FEV_1 of 20% ($PC_{20}FEV_1$), a 35% fall in sGaw (PC_{35}sGaw) and the maximal expiratory flow at 35% of vital capacity, measured from the complete $\dot{V}_{35}(c)$ and partial $\dot{V}_{35}(p)$ flow volume curves $PC_{35}\dot{V}_{35}(c)$ and $PC_{35}\dot{V}_{35}(p)$

response (EC_{20}) was also calculated. In addition the maximum tension generated and wet weight of each tissue were recorded.

In preliminary experiments, no significant difference was found between the sensitivity of bronchial strips taken from second and sixth order bronchi.

In vivo and in vitro results were compared using correlation coefficients calculated from linear regression using the method of least squares analysis. The level of significance was set at $p < 0.05$.

4.3 RESULTS

4.3.1 In vivo

Airway responsiveness to methacholine varied between individual patients (Figure 4.1). There were significant correlations ($P< 0.05$) between PC_{20} FEV_1 and $PC_{35}\dot{V}_{35}(c)$ ($r= 0.64$), PC_{35}sGaw and $PC_{35}\dot{V}_{35}(p)$ ($r = 0.65$), and $PC_{35}\dot{V}_{35}(p)$ and $PC_{35}\dot{V}_{35}(c)$ ($r = 0.77$). Baseline FEV_1 (expressed as a percentage of predicted) correlated with $PC_{35}\dot{V}_{35}(p)$ ($r= 0.71$; $p < 0.05$) but was not significantly related to the other measurements of in vivo responsiveness [$PC_{20}FEV_1$ ($r= 0.59$). PC_{35}sGaw ($r= 0.32$); $PC_{35}\dot{V}_{35}(c)$ ($r= 0.53$)]. There was no significant correlation between baseline sGaw (absolute value) and measurements of in vitro sensitivity.

4.3.2 In vitro

The sensitivity to methacholine of the bronchial strips from individual patients was highly reproducible ($r = 0.87$). There was no difference in sensitivity between second order, 4th order, and 6th order bronchi (Table 4.2). For all subsequent analysis, the results for all bronchial strips from an individual patient were combined. In contrast there were marked differences in the sensitivity to methacholine of bronchial strips prepared from different patients as indicated by the range of EC_{20} and EC_{50} values obtained, Table 4.2. The mean EC_{20} value ($\pm$SEM) from all preparations/

preparations was 4.1 ± 1.2 x 10^{-7}M (range 2.9 x 10^{-8}M to 9.3 x 10^{-7}M) and the mean EC_{50} value was 3.9 ± 1.0 x 10^{-6}M (range 4.1 x 10^{-7}M to 8.1 x 10^{-6}M). The mean maximum tension generated was 1.44 ± 0.29 g(range 0.37 g to 3.59 g).

4.3.3 Comparison of in vivo and in vitro responsiveness to methacholine

There were no significant correlations between any of the measurements of in vivo responsiveness and in vitro smooth muscle sensitivity to methacholine. The lack of relationship is illustrated for $PC_{20}FEV_1$ against EC_{50} (Figure 4.2), for PC_{35}sGaw against EC_{50} (Figure 4.3) and for $PC_{35}\dot{V}_{35}$(c) and $PC_{35}\dot{V}_{35}$(p) against EC_{50} (Figure 4.4 (a) and (b)). Furthermore, there were no significant relationships between in vivo measurements and maximum tension generated.

The patient who had a history of wheezy dyspnoea and the lowest $PC_{20}FEV_1$ (No.2) did not exhibit increased in vitro sensitivity to methacholine (EC_{50} 4.0 x $10.^{-6}$M) nor was the maximum tension generated increased (1.41 g) Figure 4.4.

TABLE 4.3 Maximum tension (g) produced by **in vitro** bronchial strips

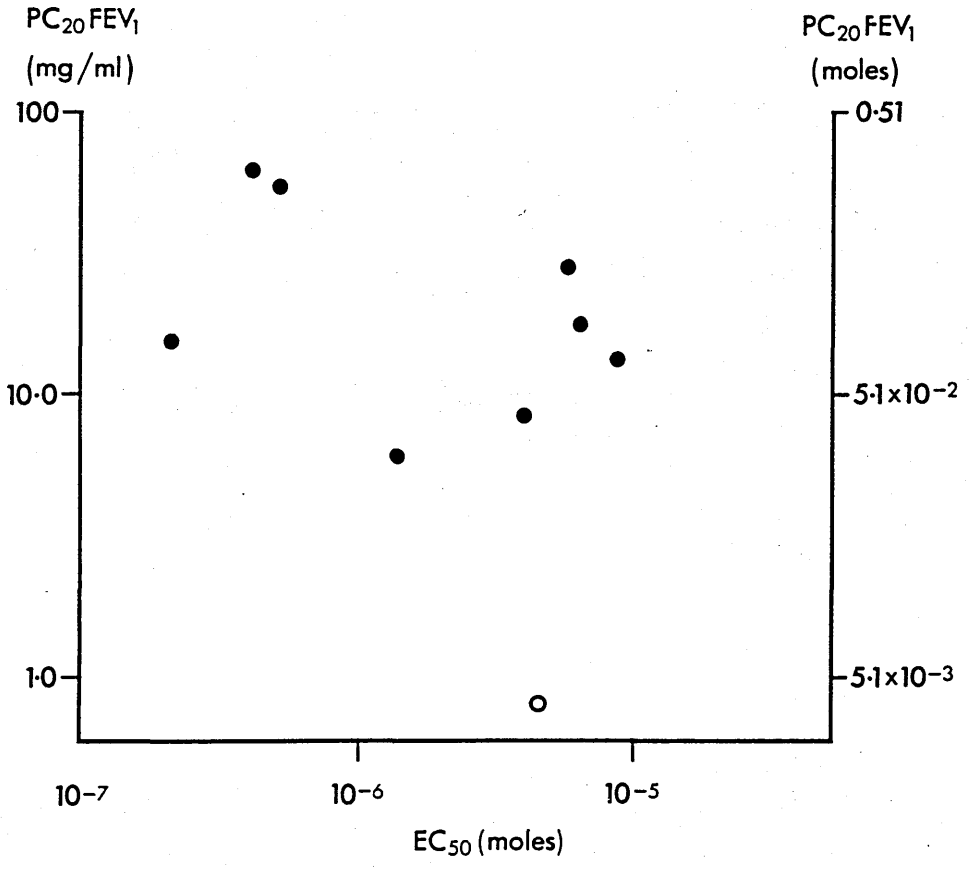
Patient	Tissue Sample	Maximum Tension (mg)	Wet Weight/ (g)	Max.Tension/ Wet Weight	Mean Max.T/Weight
4	1	2250	190	11.8	16.2
	2	1850	70	26.4	
	3	2300	222	10.4	
5	1	1950	142	13.7	18.0
	2	900	33	27.3	
	3	1500	109	27.3	
	4	2050	570	3.6	
6	1	500	-		
	2	500	-		
	3	1000	-		
	4	500	-		
7	1	1000	227	4.4	10.6
	2	950	90	10.6	
	3	900	95	9.5	
	4	950	38	25	
	5	850	235	3.6	
8	1	3800	220	17.3	17.1
	2	3400	277	12.3	
	3	8300	205	40.5	
	4	1500	315	4.8	
	5	950	89	10.7	
9	1	800	112	7.1	8.7
	2	1250	112	11.2	
	3	950	121	7.9	
10	1	2500	184	13.6	13.4
	2	1200	243	4.9	
	3	2700	145	18.6	
	4	1250	60	20.8	
	5	950	102	9.3	

TABLE 4.5 in vitro methacholine challenge results comparing 2nd, 4th and 6th order bronchi

	Order of No. bronchi	mean (EC_{50} x 10^{-6}) 2°	4°	6°	overall mean m
JA	1	1.15	0.64	0.64	0.87
DMcK	2	5.6	17.6	-	9.6
MW	3	-	-	0.27	0.27
WT	4	21.3	13.3	-	15.0
AF	5	9.25	42.7	-	26.0
MK	6	1.31	14.6	-	7.0
Iy	7	1.3	1.8	7.6	2.53
AD	8	45.0	3.3	2.1	3.9
DE	9	93.0	-	-	93.0
GA	10	4.5	3.7	3.1	3.5
	m	15.7	12.2	16.1	

FIGURE 4.2

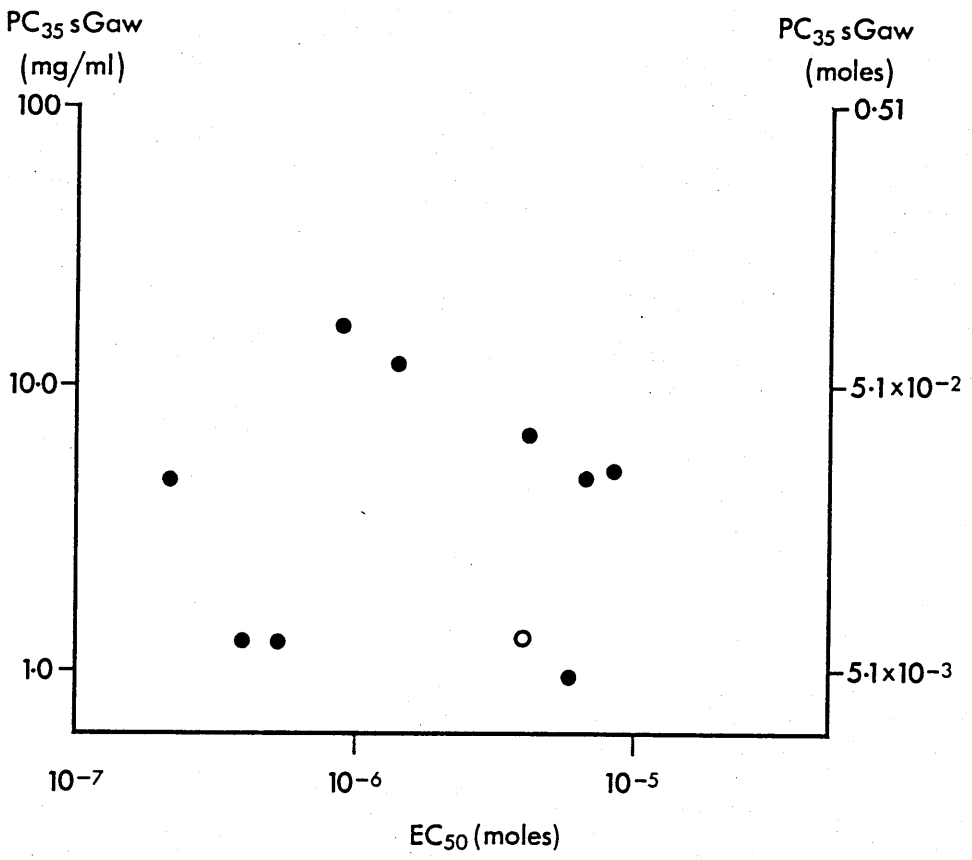
$PC_{20}FEV_1$ against EC_{50} for methacholine



Comparison of *in vivo* airway responsiveness to methacholine expressed as $PC_{20}FEV_1$ and *in vitro* smooth muscle sensitivity expressed as EC_{50} ($r = .-0.35$ $p > 0.05$)

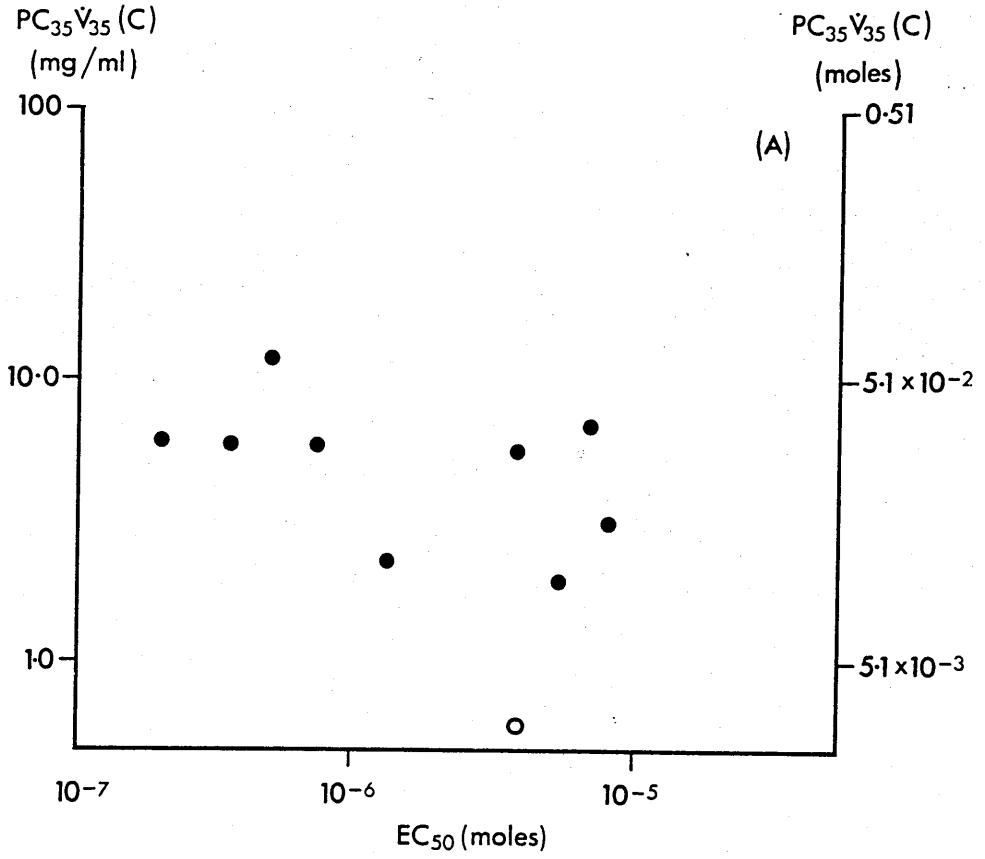
FIGURE 4.3

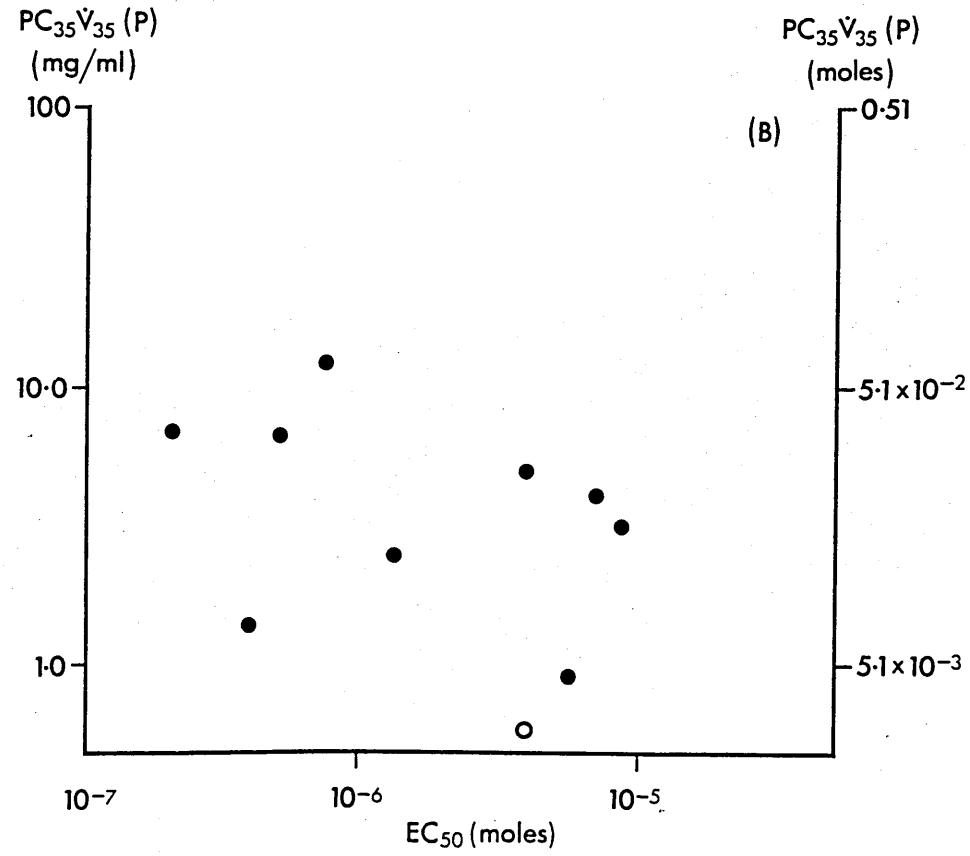
PC_{35}sGaw against EC_{50} for methacholine



Comparison of <u>in vivo</u> airway responsiveness to methacholine expressed as PC_{35}sGaw and <u>in vitro</u> smooth muscle sensitivity, expressed as EC_{50} ($r = -0.03$, $p > 0.05$)

$PC_{35}\dot{V}_{35}(c)$ and $PC_{35}\dot{V}_{35}(p)$ against EC_{50}





Comparison of <u>in vivo</u> responsiveness and <u>in vitro</u> smooth muscle sensitivity to methacholine. $PC_{35}\dot{V}_{35}(c)$ against EC_{50} ($r = -0.33$, $p > 0.05$ $PC_{35}\dot{V}_{35}(p)$ against EC_{50} ($r = -0.30$, $p > 0.05$

4.4 DISCUSSION

This study has demonstrated that no significant relationship exists between measurements of *in vivo* airway responsiveness to methacholine and *in vitro* sensitivity of isolated bronchial smooth muscle obtained from the same patients. Previous studies have examined either *in vivo* human airway responsiveness (Parker et al 1976, Hargreave et al 1981), or *in vitro* human airway smooth muscle sensitivity to this pharmacological stimulus (Hawkins and Schild 1951, Goldie et al 1982) but both *in vivo* and *in vitro* evaluation has not been performed in the same individual.

There are several possible factors which might have influenced both the *in vivo* and *in vitro* measurements. Unlike *in vitro* studies, the dose of methacholine administered to airway smooth muscle *in vivo* cannot be determined accurately. It is dependant on the technique of aerosol generation, particle size and breathing pattern (Brain 1980, Ryan et al 1981). In this study methacholine was administered via the same Wright's nebulizer and by the same method in each individual. Thus, although the dose reaching the lung was unknown it should have been similar in each patient. The site of deposition of the drug within the airways might also influence the response obtained. The patients we studied were current or ex-smokers and had a degree of small airway disease. A Wright nebulizer produces a diffuse pattern of deposition within the lung (Ryan et al 1981), but in individuals with small airway narrowing the aerosol may be deposited to a greater extent in the larger airways, and so apparently increase the response. We examined tissue samples from second to sixth order bronchi. *In vitro* results therefore reflected the sensitivity of larger airways. Comparison of EC_{50} and PC_{35} sGaw, a measurement of large airway responsiveness (Pride 1971), might have been expected to show the closest relationship, had any existed. However, a relationship between *in vivo* airway responsiveness and *in vitro* sensitivity of more peripheral airway smooth muscle cannot be excluded from this study. In addition, the access of a bronchoconstrictor drug to airway smooth muscle, and the response to that drug, may be affected by airway permeability (Hogg 1981). However, although/

although airway permeability has been found to be increased in smokers (Jones et al 1980), no relationship has been found between the level of airway permeability and airway responsiveness in these individuals (Kennedy et al 1983).

It is impossible to produce a full dose response curve to a bronchoconstrictor agent in man, so the position on the curve at which responses are measured is unclear. It may be that all the *in vivo* measurements fall on the lower non-linear region of the full log dose response curve. If so, then *in vivo* meansurements might not be expected to correlate with a response taken from the linear region of the *in vitro* curve (EC_{50}). However, we found no relationship between *in vivo* indices of responsiveness and a measure of *in vitro* sensitivity taken from the lower part of the dose response curve (EC_{20}).

Baseline airway calibre and/or tone may be an important determinant of airway responsiveness (Benson 1975). The same reduction in airway radius will cause different changes in airflow resistance depending on baseline airway calibre, because resistance is inversely proportional to the fourth power of the radius when flow is laminar. We examined the relationship between the four measurements of *in vivo* airway responsiveness and baseline values of FEV_1 and sGaw; only starting FEV_1 significantly correlated with $PC_{35}\dot{V}_{35}(p)$. This result would suggest that the variation in responsiveness to methacholine found between our patients was not due only to differences in baseline airway calibre, although it is possible that small but important differences in baseline airway calibre or tone may not have been detected by the methods used. Furthermore, there may have been differences between subjects in the distribution of resistance within the airways which would also influence *in vivo* measurements of responsiveness.

Variations in upper airway calibre may also affect airway resistance. Higenbottam (1980) demonstrated that aerolised histamine produced a variable reduction in laryngeal calibre with an associated fall in FEV_1. This he proposed was via a vagal reflex. If this hypothesis were correct methacholine would not be expected to produce a similar/

similar effect because it does not act on vagal sensory endings (Vidruk et al 1977).

In normal subjects inhalation to total lung capacity can reduce the effect of induced bronchoconstriction (Fish et al 1981). This would affect FEV_1 and $\dot{V}_{35}(c)$ but not sGaw or $\dot{V}_{35}(p)$ measurements. As we have demonstrated that none of these measurements correlated with *in vitro* sensitivity it is unlikely that this bronchodilation is an important factor in the lack of relationship between *in vivo* and *in vitro* measurements. Finally, other important factors which might influence *in vivo* responsiveness include difference between individuals in the neural and/or humoral control of the airways (Widdicombe 1963, Richardson 1979).

The pre- and peri-operative drugs received by each patient were similar. To minimize the effect of these drugs on the response of smooth muscle to methacholine, all tissues were washed twice on removal, stored for 16 hours in Krebs-Henseleit solution and then washed again both before and after suspension in the organ bath. As precautions were taken to treat all tissues in the same way, and all were washed thoroughly, it seems unlikely that previously administered drugs would influence the results obtained. In particular hyoscine premedication should not have influenced the *in vitro* sensitivity of smooth muscle to methacholine. The effects of atropine, a drug pharmacologically similar to hyoscine can be removed from *in vitro* preparations within minutes by washing (Clark 1926). Furthermore, Brink *et al* (1980) have shown no difference in the sensitivities of fresh and stored human airway smooth muscle preparations.

In addition to finding no relationship between *in vivo* responsiveness to methacholine and *in vitro* sensitivity to this drug as indicated by the EC_{50} value, we found no correlation with the maximum tension generated by bronchial strips from each patient. The maximum tension generated may be related to the quantity of smooth muscle present in each bronchial strip. Ideally, the amount of smooth muscle in each strip should be measured although this is technically difficult. In an attempt to correct for variations in the amount of tissue and hence smooth/

smooth muscle present, the maximum force generated was corrected for the wet weight of tissue in each strip. This was compared with the indices of *in vivo* responsiveness but no relationship was found.

In this chapter methacholine was used as agonist. This drug acts on muscarinic receptors which may be situated in different structures in the airway such as airway smooth muscle (Hawkins and Schild 1951), submucosal glands (Nadel 1981), pre-synaptic receptors on sympathetic nerves (Westfall 1980) and mast cells (Kaliner et al 1972). The bronchoconstrictor response to methacholine may therefore be due to several different mechanisms, and not due solely to a direct effect on airway smooth muscle. The absence of any relationship between *in vivo* and *in vitro* responses to methacholine does not preclude a relationship existing if any agonist which acts more specifically on airway smooth muscle were used. Future chapters will consider other smooth muscle agonists.

Patients selected for this study were due to undergo thoracic surgery. The majority had bronchial carcinoma and were current or ex-smokers. With the exception of one individual, they did not demonstrate *in vivo* hyperresponsiveness, since most symptomatic asthmatics would be expected to have a $PC_{20}FEV_1$ below 8 mg/ml (Hargreave et al 1981). In these patients our findings suggest that smooth muscle sensitivity alone does not determine *in vivo* airway responsiveness to methacholine. It is probable that the measurement of airway responsivness is complex, and that it is influenced by multiple factors.

The cause(s) of airway hyperresponsiveness in asthma is unknown. It could be due to an abnormality in airway smooth muscle and/or to the neural or humoral control of the airways (Thomson 1983). In this study only one patient had some features of asthma. Although we were able to study him only over a short period and were unable to assess the airway response to a "trial of steroids", he had symptoms of wheezy dyspnoea, demonstrated a 14% increase in FEV_1 after bronchodilator, had Curschmann's spirals demonstrated in his sputum and was the most responsive to methacholine. However, the airway smooth muscle strips from/

this individual were not hypersensitive to methacholine. If this finding is confirmed in other patients with definite asthma it would indicate that airway hyperresponsiveness is not attributable to an increased sensitivity to methacholine at the receptor level (as indicated by the EC_{50} value). In support of this finding with methacholine, Dahlen and coworkers (1983) recently reported that the response of bronchial strips to histamine, prostaglandin $F_{2\alpha}$ and leukotriene C_4 were similar in normal and asthmatic subjects. These findings may indicate <u>in vivo</u> hyperresponsiveness is not due to a primary abnormality of airway smooth muscle. However, a post receptor defect in airway smooth muscle, manifest by any increase in maximum tension generated by the bronchial strip, cannot be ruled out as a cause of airway hyperresponsiveness. It is conceivable that an increase in maximum tension generated by the bronchial strips of the patients in this study as compared to the others was not detected because of differences in the quantity of smooth muscle present in the bronchial strips.

C H A P T E R 5

AIRWAY RESPONSIVENESS TO HISTAMINE IN NORMAL AND ASTHMATIC SUBJECTS

AIRWAY RESPONSIVENESS TO HISTAMINE IN NORMAL AND ASTHMATIC SUBJECTS

5.1 INTRODUCTION

The responses of normal and asthmatic subjects to histamine are well established (Cockcroft et al 1977, Laitinen 1974) and, using the protocol devised by Cockcroft et al (1977), there are defined normal and asthmatic ranges for $PC_{20}FEV_1$. In this study a modified protocol was used which entailed the measurement of sGaw, $V_{30}(p)$ and FEV_1 sequentially. Patients tested were due to undergo thoracotomy for bronchial carcinoma. Most were smokers and therefore had varying degrees of chronic bronchitis and chronic airflow obstruction which have been associated with altered airway responsiveness (Taylor et al 1985, Ramsdale et al 1984, Benson et al 1978). It was important to determine an expected range of results for both normal and asthmatic subjects to facilitate interpretation of *in vivo* results. This would put the responsiveness of the surgical patients in perspective when their airway responsiveness was measured by the same protocol.

5.2 METHOD

5.2.1. Subjects

Ten non-smoking normal subjects (7 male and 3 female) with no history of respiratory disease, who had been admitted to hospital for elective surgery were chosen, Table 5.1. None were smokers. Three had positive prick skin tests, but none had increased specific IgE levels.

Ten asthmatic patients (4 male: 6 female) chosen to be in the same age range as surgical patients were recruited from the chest clinic (Table 5.2). They were aged 44 - 75. One was a cigarette smoker and one a pipe smoker. Four had positive prick skin tests and three had raised specific IgE levels. All were taking regular B_2 adrenoceptor agonists, seven were on regular inhaled beclomethasone dipropionate and one patient was taking sodium cromoglycate.

5.2.2. Measurements

Airway/

TABLE 5.1

Patient Characteristics – Normal Subjects

No.	Age	Sex	Height (cm)	FEV_1 L	FEV_1 % Pred	VC % Pred	Atopy	Smoking	Medication	Diagnosis
1	49	M	178	3.88	109	112	–	–	–	Inguinal hernia
2	49	M	183	3.92	105	103	+	–	–	Inguinal hernia
3	61	F	155	2.55	131	117	+	–	navidrex K	–
4	44	F	161	2.83	111	110	–	–	–	haemorrhoids
5	50	M	175	4.59	136	128	–	–	–	–
6	67	M	170	2.50	97	97	–	–	–	villous adenoma of colon
7	55	M	173	3.10	99	102	–	–	–	Inguinal hernia
8	49	M	173	3.04	100	99	+	–	–	haemorrhoids
9	44	M	183	4.57	123	133	–	–	–	Inguinal hernia
10	63	F	163	2.30	108	98	–	–	–	cholecystectomy
m					119					
SD					136					

TABLE 5.2

Patient Characteristics - Asthmatic

Patient	Age	Sex	Height (cm)	FEV L	FEV % Pred	VC % Pred	RV % Pred	TL_{co} Pred	Atopy	Smoker	Medication S B	RAST IgE
1	59	F	159	1.38	65	72	111	88	-	-	S B	0
2	51	F	173	2.72	101	105	95	106	+	_	S	High
3	57	F	163	2.45	108	99	120	115	-	+	S B	0
4	70	M	172	2.79	100	99	94	129	-	-	S, B	0
5	67	M	171	2.15	75	106	121	117	+	-	S, SCG	High
6	75	M	166	1.19	47	86	170	81	-	+ pipe	S, A, B	0
7	44	F	162	1.69	65	84	110	111	+	-	S B	High
8	62	F	151	1.04	57	75	128	90	+	-	S BF	High
9	53	F	157	2.11	94	85	83	115	-	-		0
10	64	M	174	1.04	34	67	194	114	1	-		0
m	60.2				79.1							
SD					22.1							

S = Salbutamol
B = beclomethasone dipropionate (50mg)
BF = beclomethasone dipropionate (250 mg)
SCG = sodium cromoglycate

Airway resistance and thoracic gas volume were measured sequentially in a constant-volume body plethysmograph (Fenyves and Gut) using the methods of Du Bois et al (1956). The results were expressed as specific conductance (sGaw). The mean of eight values recorded was taken as sGaw. Partial and complete expiratory flow-volume (PEFV and CEFV) curves were obtained with a heated pneumotachograph with integration of flow, and recorded on an X-Y recorder. Body plethysmographic measurements always preceded flow volume recordings.

Histamine inhalation tests were carried out using a modification of the method described by Cockcroft et al (1977). Buffered normal saline was inhaled first followed by doubling concentrations of phosphate buffered histamine (2 to 64 mg/ml) for non-asthmatic patients, and 0.06 to 4 mg/ml for the asthmatic patients. Each inhalation was for 2 minutes at 10 minute intervals. Before the first inhalation eight sGaw and five PEFV and CEFV curves were recorded. From 1.5 min after each inhalation responses were measured by sGaw and to PEFV and CEFV curves. Inhalations were continued until the FEV_1 had fallen by 20% or more, or until maximum concentration had been administered.

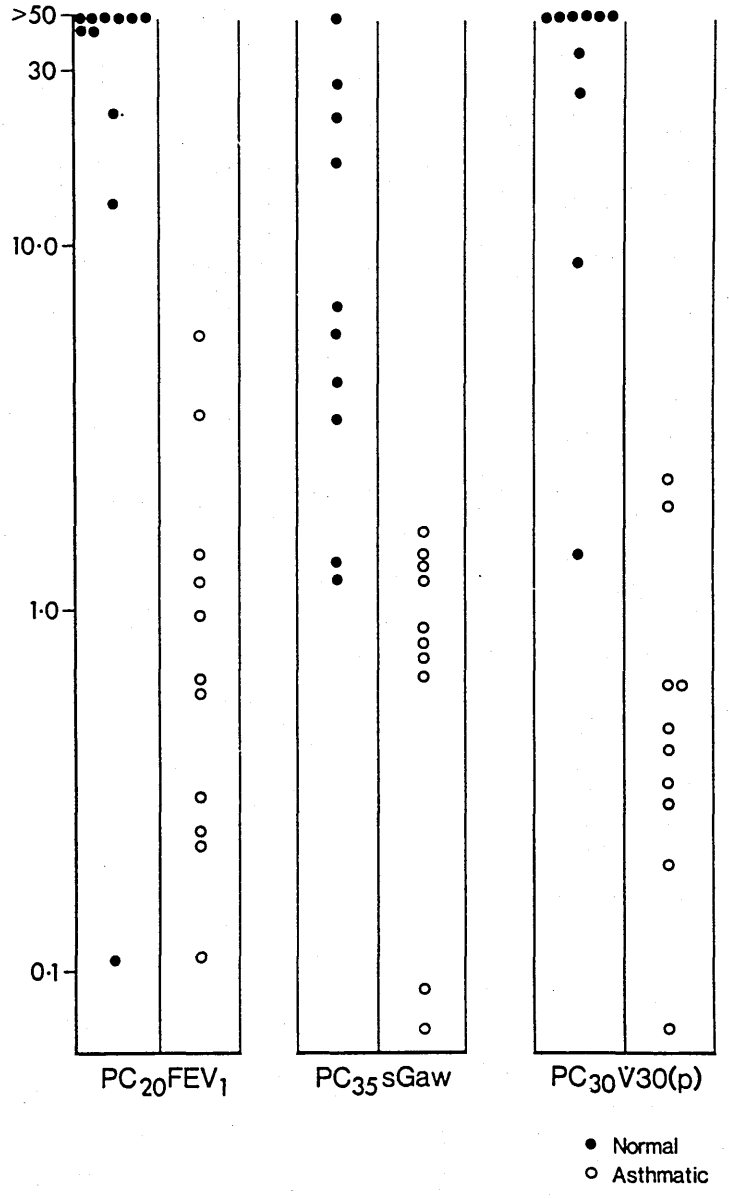
Log concentration-response curves were plotted and results were expressed as the provocation concentration causing a 20% decrease in FEV_1 ($PC_{20}FEV_1$) a 35% fall in sGaw (PC_{35}sGaw), a 35% fall in $\dot{V}_{35}(p)$($PC_{35}\dot{V}_{35}(p)$) and a 35% fall in $\dot{V}_{35}(c)$ ($PC_{35}\dot{V}_{35}(c)$). If the appropriate percentage fall had not occurred by the maximum concentration the provocation concentration is shown as (> 64 mg/ml).

5.3 RESULTS

Airways responsiveness varied within and between the asthmatic patients and the normal subject groups (Figure 5.1). There was no overlap between the groups as assessed by $PC_{20}FEV_1$, slight overlap when assessed by PC_{35}sGaw ($p < 0.01$) and slight overlap between the groups/

FIGURE 5.1

Range of responsiveness in normal and asthmatic subjects



Airway responsiveness shown on a log scale to histamine of 10 subjects due to undergo routine non-thoracic surgery and who had no respiratory symptoms (●) and ten asthmatic patients (0).

Results are expressed as the $PC_{20}FEV_1$ (left) PC_{35}sGaw (centre and the $PC_{30}\dot{V}_{30}(p)$ (right).

groups as assessed by $PC_{35}\dot{V}_{35}(p)$ ($p < 0.001$). Baseline FEV_1 expressed as a percentage of predicted (mean $\pm$ SD) was significantly higher in the normal subjects (119 13.6) than the asthmatic patients 79.1 $\pm$ 22.1 ($p < 0.01$).

In the normal subjects, baseline airway calibre as assessed by FEV_1 expressed as a percentage of the predicted value, was not related to airway responsiveness, the correlation coefficients being $PC_{20}FEV_1$ r= 0.21, PC_{35}sGaw r= -0.33 and $PC_{35}\dot{V}_{35}(p)$ r= -0.16.

Similarly the correlation coefficients for the asthmatic patients between baseline airway calibre and airway responsiveness was $PC_{20}FEV_1$, r= 0.01, PC_{35}sGaw r = 0.01, or $PC_{35}\dot{V}_{35}(p)$ r = -0.13.

5.4 DISCUSSION

In this study there was a 1,000 fold variation in airway responsiveness over a group of twenty human subjects, ten of whom had asthma. A common feature of asthma is an increase in the responsivness of the airways to a variety of different stimuli. This appears to be non-specific for a number of triggers since, in a group of asthmatic patients the airway responses to one stimulus usually correlate with those to another. Individuals highly sensitive to histamine are also generally more sensitive to methacholine (Juniper et al 1978) prostaglandin $F_{2^{\alpha}}$ (Thomson et al 1981) and Leucotriene D_4 (Bisgaard et al 1985).

The protocol used in this project differs from that described by Cockcroft et al (1977) in several respects. They gave inhalations at 5 minute intervals (compared to 10 in this study). This was possible as only FEV_1 was measured. The ten minute period was required to measure sGaw (x8) and $\dot{V}_{30}(p)$ and FEV_1 (x2-3). Less solution was used in the nebuliser (4 ml cf 5 ml) and a flow rate of 8l/min cf 7 l/min by Cockcroft was used. His group nebulised using O_2 as vehicle rather than air. It has been demonstrated that changes in the concentration of O_2 may affect airway calibre (Nadel and Widdicombe 1962). Thus it was important/

important to establish expected values for both normal subjects and asthmatic patients using the protocol used in this thesis.

A highly significant difference was demonstrated between the normal subjects and the asthmatic patients by all three methods of assessment of airway calibre. This confirms the work of numerous other studies (Herxheimer 1951, Townley et al 1965, Cockcroft et al 1977) all of whom measured FEV_1 in normal subjects and asthmatic patients. FEV_1 reflects overall airway function, Raw and hence sGaw reflect large airway function (Pride 1971, Ingram and McFadden 1977) and expiratory flow rates at 25 to 50% of TLC, small airway functions (Tattersall et al 1978).

Differences in response between large and small airways could be due either to differential effect of the site of action of the bronchoconstrictor in use or an effect of distribution in the airway of nebulised solutions. However, by all three methods of measuring airway responsiveness there were highly significant differences between the normal subjects and the asthmatic patients.

Baseline airway calibre has been proposed as a factor in determining human airway responsiveness (Chung et al 1982) althought this has been disputed (Cartier et al 1982). In this study, bronchial responsiveness was unrelated to baseline airway calibre in both the normal subjects and the asthmatic patients.

Thus a normal range and an asthmatic range has been determined for the three measures of airway responsivess used in this thesis.

CHAPTER 6

COMPARISON OF *IN VIVO* AIRWAY RESPONSIVENESS AND *IN VITRO* SMOOTH MUSCLE SENSITIVITY TO HISTAMINE AND THE EFFECT OF *IN VIVO* CHOLINERGIC BLOCKADE ON THIS RELATIONSHIP

COMPARISON OF IN VIVO AIRWAY RESPONSIVENESS AND IN VITRO SMOOTH MUSCLE SENSITIVITY TO HISTAMINE AND THE EFFECT OF IN VIVO CHOLINERGIC BLOCKADE ON THIS RELATIONSHIP

6.1 INTRODUCTION

The bronchoconstrictor response to a stimulus such as histamine involves contraction of the airway smooth muscle (Hawkins and Schild 1951) which is under nervous, humoral and intrinsic muscular control (Widdicombe 1963, Richardson 1963, Davis et al 1982). Patients with asthma and chronic airflow obstruction (Oppenheimer et al 1968, Laitinen et al 1974) exhibit increased airway responsiveness compared with normal subjects. The cause of this increased responsiveness is unknown. Histamine produces bronchoconstriction by acting directly on airway smooth muscle H_1 receptors (Nogrady et al 1978, Thomson and Kerr 1979) and also reflexly via vagal pathways (Simonsson et al 1967). Thus airway hyperresponsiveness to histamine may be due to increased sensitivity of the smooth muscle itself, or may be related to an abnormality of the nervous and/or humoral control of the airways (Thomson 1983). If the primary abnormality resides in the smooth muscle, then increased in vivo airway responsiveness to histamine would be expected to be associated with an enhanced in vitro smooth muscle sensitivity. Alternatively if in vivo airway responsiveness to histamine is modified by an interaction between vagal nerve input and smooth muscle sensitivity then a relationship between in vivo and in vitro responses may only be apparent after cholinergic blockade with atropine.

In this study in vivo responses to histamine, both with and without atropine pretreatment, were compared with in vitro responses of isolated strips on bronchi removed from the same patients in order to assess the contribution of smooth muscle sensitivity to in vivo airway response to inhaled histamine.

6.2 METHODS

6.2.1 Patients

Twenty-one patients due to undergo lobectomy or pneumonectomy were/

were studied (Table 6.1). Twenty had operable bronchial carcinoma and one had an aspergilloma. One had a history of asthma since childhood and had reversible airflow obstruction. Sixteen were current smokers and all others except the asthmatic patient were ex-smokers. Eight fulfilled the MRC criteria for chronic bronchitis (MRC 1965). Seven including the asthmatic patient were atopic as indicated by a $\geq$ 2 mm weal response to prick skin testing with one or more of seven common allergens and three of these, including the asthmatic patient had raised specific IgE levels. Nine patients were on regular drug treatment prior to surgery. The asthmatic patient did not require regular bronchodilator therapy.

6.2.2 In vivo measurements

Histamine inhalation tests were carried out using a modification of the method described by Cockcroft et al (1977). Buffered normal saline was inhaled first followed by doubling concentrations of phosphate buffered histamine (2 to 64 mg/ml) for non-asthmatic patients, and 0.06 to 4 mg/ml for the asthmatic patient. Each inhalation was for 2 min at 10 minute intervals. Before the first inhalation eight sGaw and five PEFV and CEFV curves were recorded. From 1.5 min after each inhalation responses were measured by sGaw and by PEFV and CEFV curves. Inhalations were continued until the FEV_1 had fallen by 20% or more, or until maximum concentration had been administered. In 11 of the patients (No. 11-21) histamine responsiveness was measured after pretreatment with atropine. Following baseline measurements, atropine sulphate (5 mg/ml) was inhaled from the same Wright nebuliser for 5 mins. Thirty min after inhalation eight sGaw and three PEFV and CEFV curves were recorded. The histamine inhalation test was then performed.

Log concentration-response curves were plotted and results were expressed as the provocation concentration causing a 20% decrease in FEV_1 ($PC_{20}FEV_1$) a 35% fall in sGaw (PC_{35}sGaw), a 35% fall in $\dot{V}_{35}(p)$/

Patient characteristics

Patient No.	Age (yr)	Sex	Height (cm)	FEV_1 (L)	FEV_1 (% Pred)	VC (% Pred)	RV (% Pred)	TLCO (% Pred)	Atopic status	Current smokers
1	62	M	169	1.78	61	72	83	90	–	+
2	63	M	181	3.05	99	102	nd	nd	–	+
3	51	M	187	4.30	110	109	134	73	+	+
4	68	M	173	1.65	56	70	85	76	+	–
5	54	M	175	3.19	95	99	120	74	–	+
6	58	M	179	1.78	53	82	111	99	–	+
7	67	M	169	3.37	122	132	98	92	–	–
8	62	F	156	1.31	65	73	103	70	+	+
9	61	M	171	2.81	93	103	135	82	–	+
10	68	M	163	2.18	86	103	144	92	–	+
11*	72	M	173	1.95	70	85	109	77	+	–
12	52	M	171	3.16	94	92	104	48	–	–
13	51	M	176	2.52	72	83	90	97	–	–
14	55	M	171	1.78	57	64	106	119	+	+
15	53	F	165	2.69	109	105	83	105	–	–
16	62	M	173	3.06	98	101	116	69	+	+
17	53	M	185	4.70	126	129	108	73	+	–
18	60	F	158	1.71	87	88	94	117	–	+
19	63	M	163	2.30	82	76	82	89	–	–
20	64	F	165	1.95	94	85	154	84	–	+
21	61	F	160	1.81	88	89	138	115	–	+

*Asthmatic patient

$\dot{V}_{35}(p)$ ($PC_{35}\dot{V}_{35}(p)$) and a 35% fall in $\dot{V}_{35}(c)$ ($PC_{35}\dot{V}_{35}(c)$). If the appropriate percentage fall had not occurred by the maximum concentration the provocation concentration was shown as (> 64 mg/ml). Routine pre-operative pulmonary function tests were performed.

6.2.3 In vitro measurements

Bronchial tissue was obtained immediately following removal at thoracotomy. Samples from second to sixth order bronchi were dissected from macroscopically normal tissue and then maintained overnight at 4°C in well oxygenated Krebs-Hensleit solution. The next day rings of bronchi were dissected and sectioned to produce strips of tissue which were suspended (under a resting tension of 1.5 to 2.0 g) in 20 ml organ baths containing Krebs-Hensleit solution at 37°C bubbled with 5% CO_2 in O_2. At the conclusion of a 60 min equilibration period the tissues were washed three times. Changes in isometric tension were measured using Grass force-displacement transducers (FT03C) and recorded on a Grass (model 7) polygraph.

The normality of the bronchial strips and reproducibility of their contractile changes were assessed by adding histamine (1×10^{-5} moles.l^{-1}) on two separate occasions separated by an interval of 30 min. A cumulative concentration-effect curve was then constructed by adding increasing concentrations of histamine (final concentration 1×10^{-7} to 4×10^{-4} moles.l^{-1}), each addition of the drug being made at the peak of effect produced by the preceding concentration. In each experiment the concentrations of histamine that produced 20% (EC_{20}) and 50% (EC_{50}) of the maximal contraction were calculated. Additionally, the maximum tension generated by each strip was determined and expressed in grammes tension per mg wet weight of tissue (tissue weight was measured at the conclusion of each experiment), and the slope of the dose response curve (%change/log unit) was recorded.

Two/

Two to six bronchial strips from each patient were tested and mean values for EC_{20}, EC_{50} and maximum tension generated were calculated.

6.2.4 Statistical analysis

Comparisons between group values of baseline FEV_1 and sGaw were compared using Student's unpaired t test. Comparison of *in vivo* and *in vitro* results was by calculation of correlation coefficients from linear regression using the method of least squares analysis. PC results were analysed under a natural logarithm transformation. The level of statistical significance was set at $p < 0.05$.

6.3 RESULTS

6.3.1 In vivo

Airway responsivness to histamine varied more than 100 fold within the group of 21 thoractomy patients. Their mean responsiveness was more than the group of non-smoking controls and less than the asthmatic subjects described in Chapter 5 (Figures 6.1, 6.2). Results for the asthmatic patient undergoing thoractomy were within the range determined for the control of asthmatic patients and he was the most responsive of the surgical group. In the group who received atropine there was a significant increase in FEV_1 7.3 $\pm$ 4.5%, sGaw 111.7 $\pm$ 84%, $\dot{V}_{35}(p)$ 55.4 $\pm$ 28.3% and $\dot{V}_{35}(c)$ 31.6 $\pm$ 40.8% after atropine (Figure 6.3).

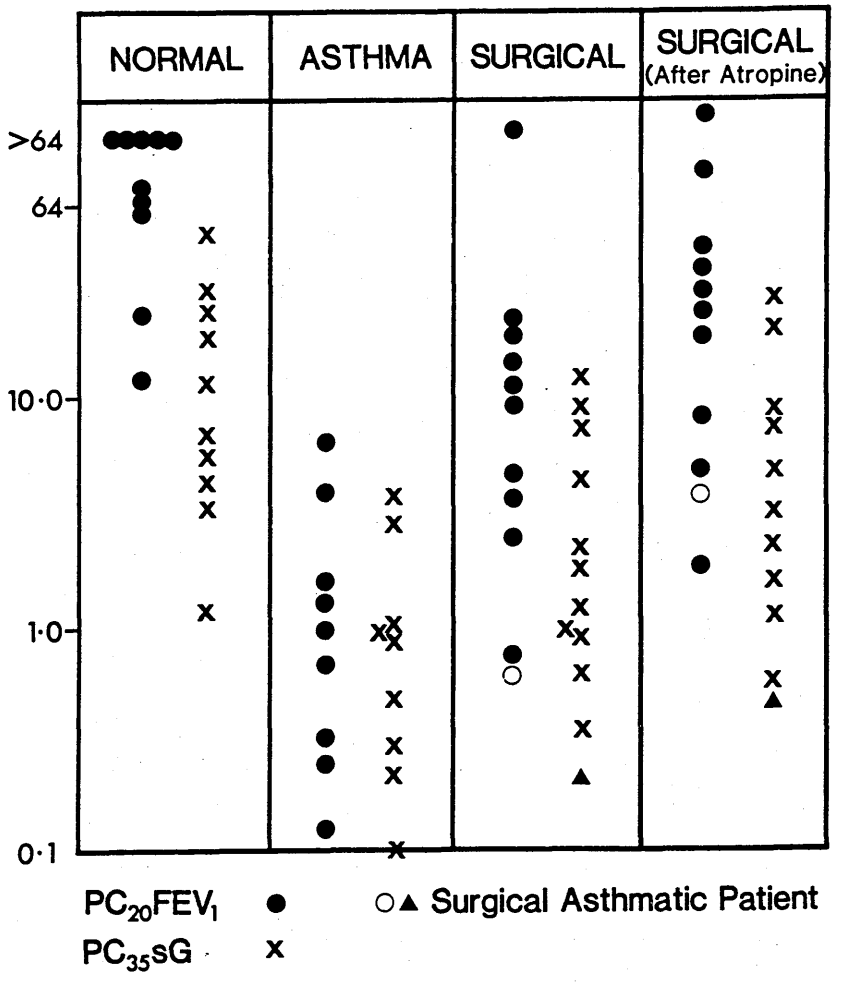
In the surgical group not pre treated with atropine, baseline FEV_1 (% predicted) correlated with PC_{20} FEV_1 $r = 0.66$ (Figure 6.4) but not with $PC_{35}\dot{V}_{35}(p)$ $r = 0.57$ or PC_{35}sGaw $r = 0.08$.

6.3.2 In vitro

The sensitivity to histamine of the bronchial strips from individual patients as assessed by EC_{50} was high/ reproducible ($r = 0.82$). There were small differences in the sensitivity to histamine of bronchial strips prepared from different patients. The/

FIGURE 6.1

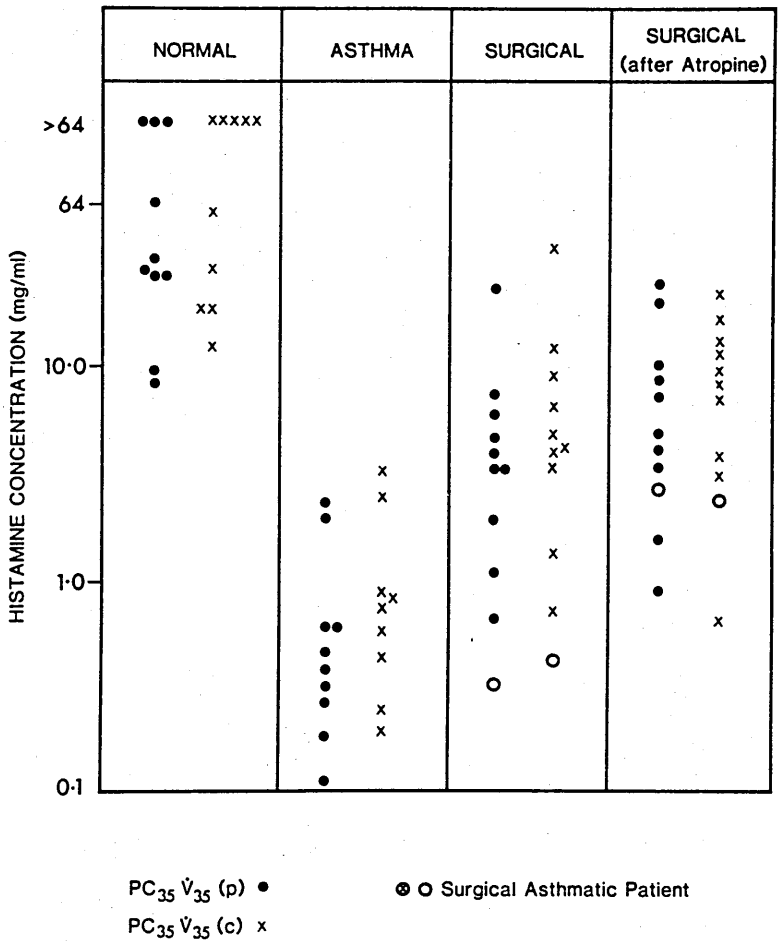
Range of responsiveness to histamine in normal asthmatic and surgical patients.



Airway responsiveness to inhaled histamine in normal (n = 10) and asthmatic subjects (n = 10) described in Chapter 5. In columns 3 and 4, results for the surgical patients (n = 11) and surgical patients after atropine pretreatment are shown. Results are shown for $PC_{20}FEV_1$ (●) and PC_{35}sGaw (X). The results of the asthmatic patient who underwent surgery are also shown (○▲)

FIGURE 6.2

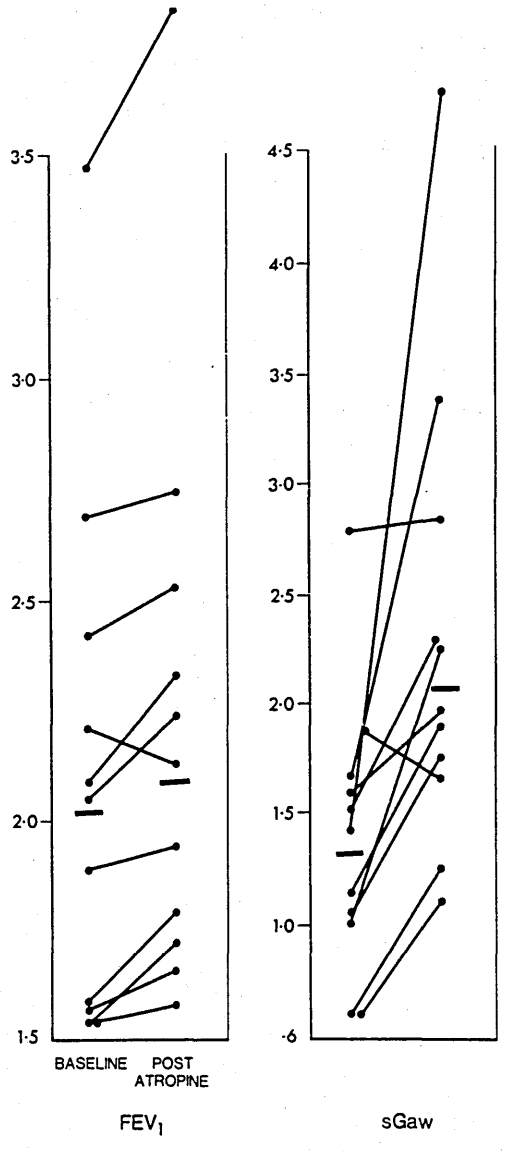
Range of responsiveness to histamine as assessed by $\dot{V}_{30}(p)$ and $\dot{V}_{30}(c)$



Airway responsiveness to histamine in normal (n = 10), and asthmatic subjects (n = 10) as described in Chapter 5. The two surgical patients groups without and after atropine pretreatment are also shown. Results are shown for $PC_{35}\dot{V}_{35}(p)$ and $PC_{35}\dot{V}_{35}(c)$. Results for the asthmatic patient are shown as ⊗ ○

FIGURE 6.3

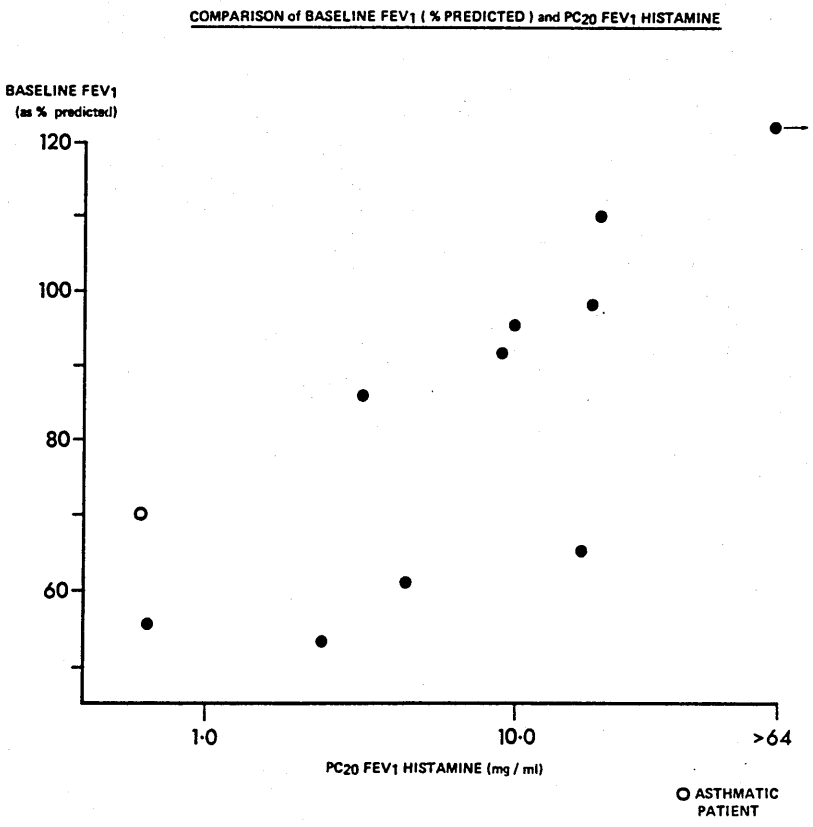
Effect of atropine on baseline FEV_1 and sGaw measurements



Baseline FEV_1 and sGaw in the surgical patients who were given pretreatment with atropine. Figure shows the effect of atropine inhalation on baseline readings.

FIGURE 6.4

Graph of baseline FEV_1 against responsiveness ($PC_{20}FEV_1$) for surgical patients



Graph of baseline FEV_1 (expressed as a % of predicted value) and $PC_{20}FEV_1$ for histamine $n = 11$, $r = 0.83$, $p < 0.05$. The asthmatic patient is shown as an open circle.

The mean EC_{50} value (SEM) from all preparations was $4.0 \pm 0.7 \times 10^{-6}$ moles l^{-1} (range 1.0×10^{-6} moles l^{-1}) to 1.4×10^{-5} moles l^{1} and the mean maximum tension generated was 13.8 ± 1.6 g/mg wet weight (range 4.2 to 30.3 g/mg wet weight). The slope of the dose-response curve varied between 35.6 and 70.3% change/log units (Table 6.2).

6.3.3 Comparison of **in vivo** and **in vitro** responsiveness to histamine.

There was no significant correlation between any of the measurements of in vivo and in vitro responsiveness to histamine. For the patients who did not receive pretreatment with atropine, the lack of relationship is illustrated for $PC_{20}FEV_1$ and $PC_{35}sGaw$ against EC_{50} (Figure 6.5) and for $PC_{35}\dot{V}_{35}(c)$ and $PC_{35}\dot{V}_{35}(p)$ against EC_{50} (Figure 6.6). Using EC_{20} as a measure of in vitro sensitivity did not alter the lack of relationship, correlation co-efficients being $PC_{20}FEV_1$ ($r = 0.32$) $PC_{35}sGaw$ ($r = 0.01$) and $PC_{35}\dot{V}_{35(p)}$ ($r = 0.49$). The slope of the dose response curve did not relate to any of the measures of in vivo responsiveness, the correlation co-efficient ranging from 0.18 to 0.44 for the four measurements. Maximum tension per gram wet weight did not relate to in vivo responsiveness (Figure 6.7).

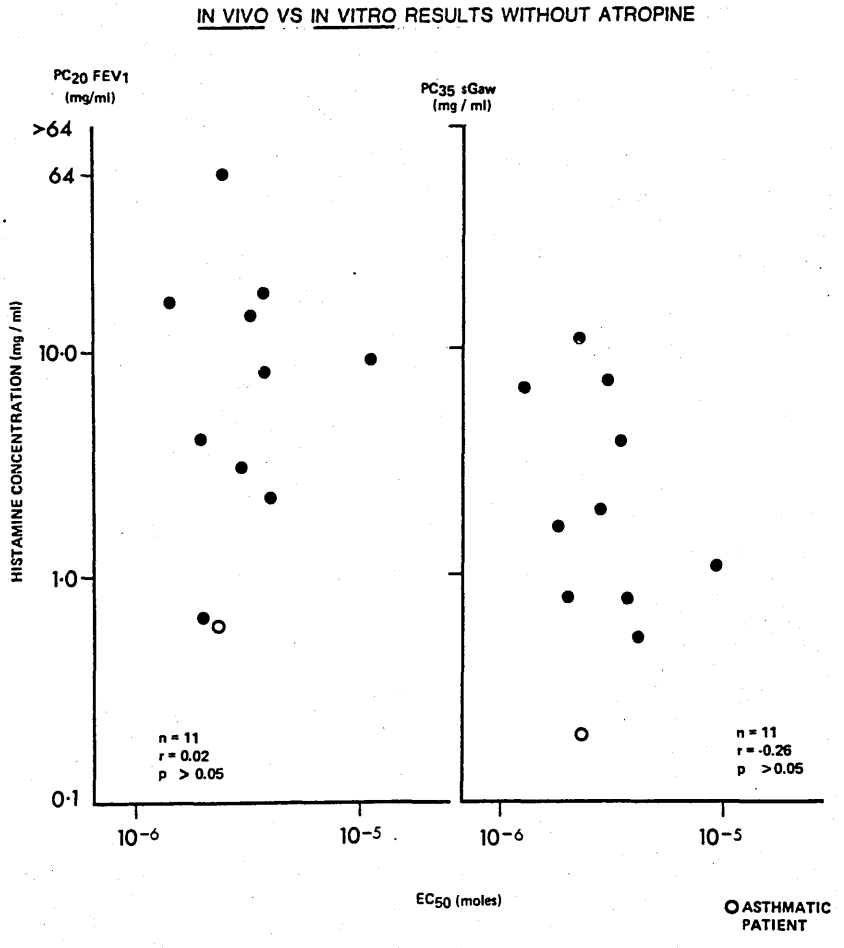
For the patients who received pretreatment with atropine, the lack of relationship is illustrated for $PC_{20}FEV_1$ and $PC_{35}sGaw$ against EC_{50} (Figure 6.8) and for $PC_{35}\dot{V}_{35}(c)$ and $PC_{35}\dot{V}_{35}(p)$ against EC_{50} (Figure 6.9).

Using EC_{20} as the measure of in vitro sensitivity did not change the lack of relationship. Neither the slope of the in vitro dose-response curve nor the tension generated/mg wet weight of tissue were related to the in vivo measurements.

The asthmatic patient who underwent surgery did not exhibit increased in vitro smooth muscle responsiveness to histamine. EC_{50} 2.3×10^{-6} moles l^{-1} EC_{20} 5.7×10^{-7} moles l^{-1} maximum tension generated 18.4 g/mg wet weight and dose-response curve slope = 37.1 % change/log unit.

Patient	EC_{20} ($\times 10^{-6}$ mm/l	EC_{50} ($\times 10^{-6}$ mm/l)	Slope (% change/log unit)	Max Tension Wet Weight Grams/mg.
1	0.46	1.9	42.3	15.9
2	0.31	1.5	39.7	14.2
3	0.38	5.9	49.1	15.8
4	0.41	3.5	35.6	9.1
5	0.62	10.4	44.2	4.8
6	1.02	4.0	43.4	30.3
7	0.5	2.2	45.3	10.4
8	1.35	3.0	51.4	4.2
9	7.9	3.7	45.0	6.5
10	1.09	2.8	51.7	8.6
11	5.7	2.3	43.8	18.4
12	0.39	1.3	50.0	25.8
13	4.6	9.7	70.3	10.4
14	6.1	14.0	70.0	5.3
15	2.3	7.7	47.4	12.0
16	0.86	2.9	49.5	17.7
17	1.09	3.4	49.3	19.6
18	2.14	4.9	62.8	11.0
19	0.42	1.3	61.3	7.6
20	0.2	1.0	39.2	23.9
21	0.43	1.9	37.1	19.0

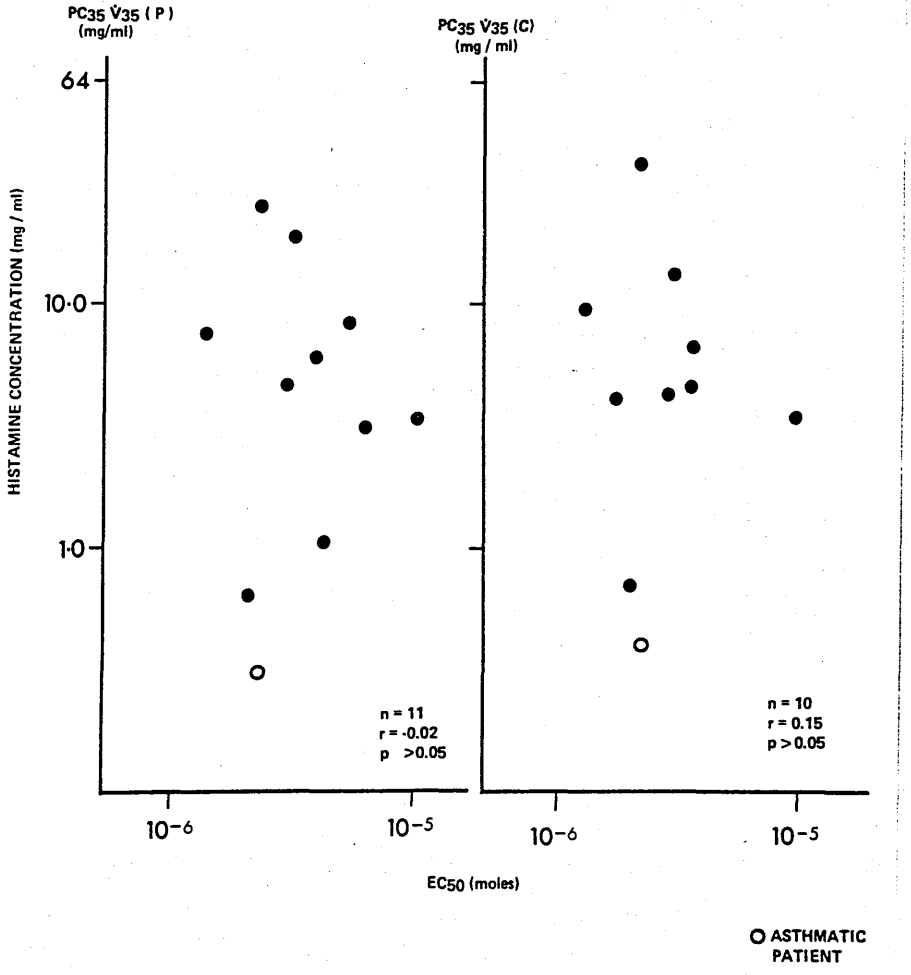
FIGURE 6.5



Graph of in vivo responsiveness to histamine [without atropine pretreatment] expressed as $PC_{20}FEV_1$ and $PC_{35}sGaw$ with in vitro smooth muscle sensitivity to histamine expressed as the concentration of histamine producing a contraction 50% of maximum

(O) result for patient with asthma.

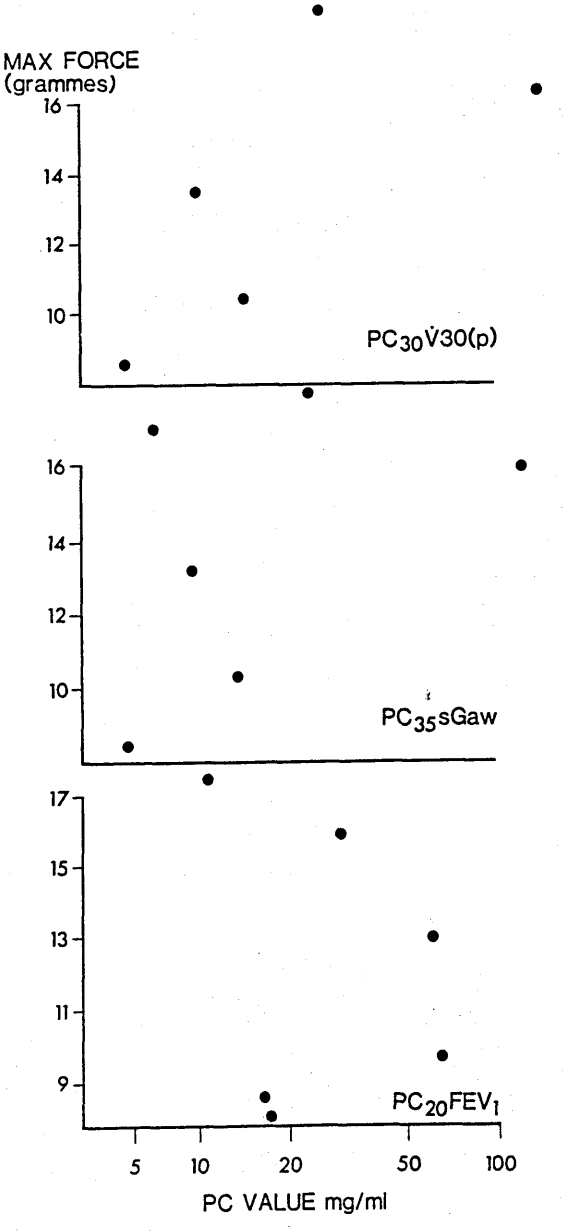
FIGURE 6.6



Graph of <u>in vivo</u> responsiveness to histamine [without atropine pretreatment] expressed as $PC_{35}V_{35}(p)$ and $PC_{35}V_{35}(c)$ and <u>in vitro</u> smooth muscle sensitivity to histamine expressed as EC_{50}

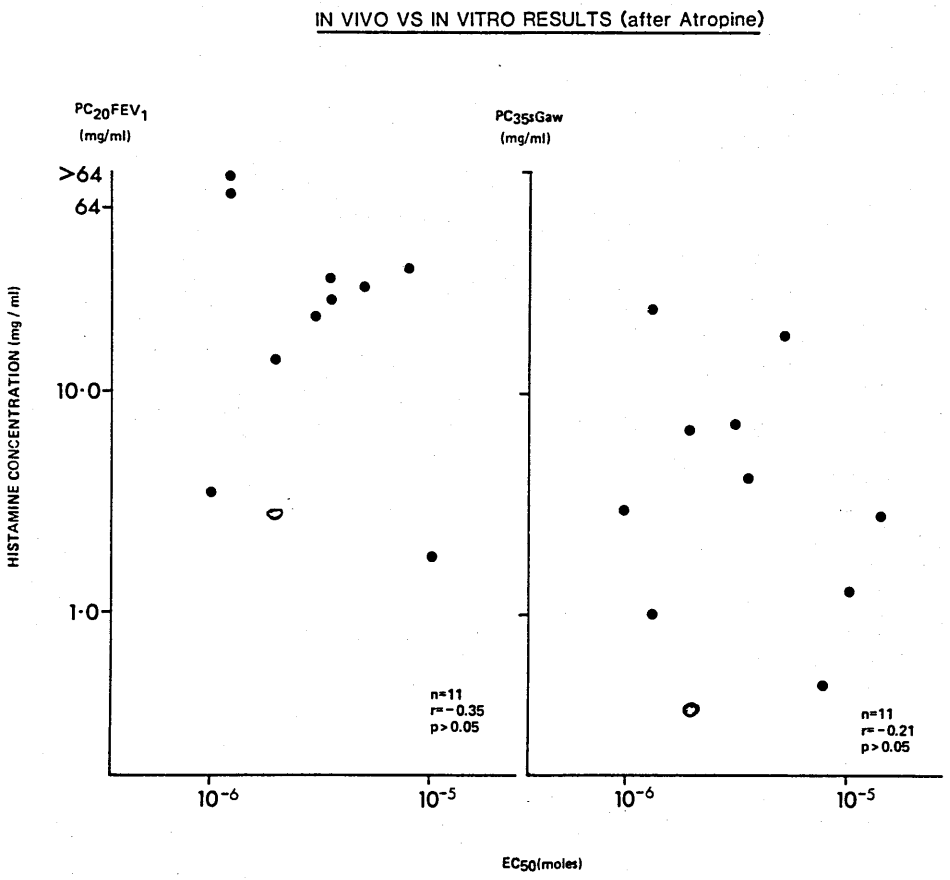
O = Asthmatic patient.

FIGURE 6.7



Graph of average maximum force per mg tissue (wet weight) and PC value for FEV_1, sGaw and $\dot{V}_{30}(p)$ for six surgical subjects (5 for PC_{30}). Results were not statistically analysed because numbers were small, but results are widely scattered with no apparent relationship.

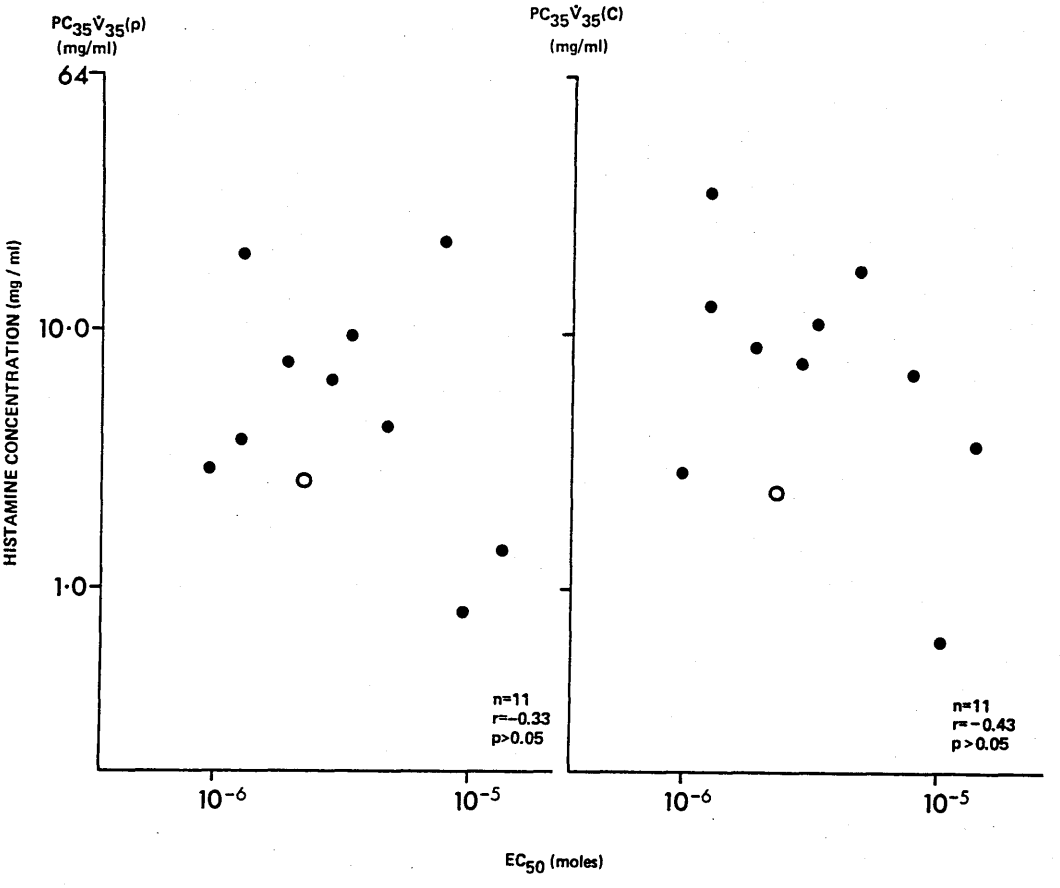
FIGURE 6.8



Graph of in vivo responsiveness to histamine [after atropine] expressed as $PC_{20}FEV_1$ and PC_{35}sGaw and in vitro smooth muscle sensitivity to histamine expressesd as EC_{50}

O = Asthmatic patient.

FIGURE 6.9



Graph of <u>in vivo</u> responsiveness to histamine [after atropine] expressed as $PC_{35}\dot{V}_{35}(p)$ and $PC_{35}\dot{V}_{35}(c)$ and <u>in vitro</u> smooth muscle sensitivity to histamine expressed as EC_{50}

O = Asthmatic patient.

Correlation co-efficients comparing *in vivo* and *in vitro* parameters are tabulated (Table 6.3).

6.4 DISCUSSION

The surgical patients used in this study were more responsive than non-smoking normal subjects of a similar age, but less responsive than a group of asthmatic patients described in Chapter 5. Cigarette smoking, chronic bronchitis and airflow obstruction may have all contributed to the increase in responsiveness in the surgical group. The majority were cigarette smokers, which has been associated with increased non-specific airway responsiveness in asymptomatic subjects without airflow obstruction, (Malo et al 1982) although this finding has not been confirmed by others (Cockcroft *et al* 1983). Eight patients also had chronic bronchitis which has been shown, both with (Oppenheimer *et al* 1968, Laitinen 1974, Ramsdale *et al* 1982, Bahous et al 1984) and without airflow obstruction, (Gerrard *et al* 1980) to be associated with increased airway responsiveness. In the surgical group there was a significant relationship between baseline lung function and $PC_{20}FEV_1$ but this was not true of the normal subjects nor of the asthmatic patients described in Chapter 5. This suggests that baseline airway calibre may be a more important determinant of airway responsiveness in smokers than in patients with asthma.

It is unknown whether the presence of a bronchial neoplasm has any effect on airway responsiveness. However, a comparison with the levels of responsiveness found by Bahous and coworkers (1984) in patients similar to those used in this study but without neoplasms, showed a similar range of airway responsiveness to that reported here.

In this study there was no significant relationship between *in vivo* airway responsiveness to histamine and *in vitro* measurements of smooth muscle responsiveness. *In vitro* measurements were made on tissues which had been washed thoroughly, stored overnight in fresh oxygenated saline at low temperature, and washed thoroughly again before testing. This procedure should have removed all intra-operative medications/

Correlation co-efficients for Histamine + Atropine - in vivo and in vitro measurements

		Log $PC_{20}FEV_1$	Log $PC_{35}sGaw$	Log $PC_{35}V35(p)$	Log $PC_{35}V35(c)$	Log EC_{50}	Log EC_{20}	Slope	Maxforce /wtweight	Initial FEV_1	Initial FEV_1 Pred %	Initial sGaw	
HISTAMINE + ATROPINE	Log $PC_{20}FEV_1$		0.81	0.87	0.92	0.20	0.0	0.44	-0.32	0.56	0.66	0.34	-N = 10
	Log $PC_{35}sGaw$	0.12		0.46	0.56	-0.12	0.21	0.18	0.40	0.07	0.08	-0.02	
	Log $PC_{35}V35(p)$	0.65	0.22		0.98	-0.04	0.16	0.46	-0.5	0.30	0.57	0.55	
	Log $PC_{35}V35(c)$	0.92	0.29	0.61		-0.07	0.08	0.41	-0.35	0.38	0.62	0.56	
	Log EC_{50}	-0.21	-0.27	-0.26.	-0.34		0.44	0.34	-0.17	0.21	0.08	-0.06	
	Log EC_{20}	0.32	0.01	0.49	0.33	0.39		0.63	-0.10	-0.51	-0.35	-0.03	
	SLOPE D%/lg unit	0.45	0.45	0.17	0.35	0.24	0.42		-0.22	0.14	0.27	0.47	
	MAXFORCE/ wet weight	-0.32	-0.52	-0.62	-0.33	-0.15	-0.54	-0.58		-0.09	-0.33	-0.35	
	INITIAL FEV_1	0.07	-0.29	-0.16	-0.05	0.50	0.02	0.06	-0.21		0.90	0.50	
	INITIAL FEV_1/Pred%	0.14	-0.33	0.13	0.06	0.55	0.27	-0.05	0.09	0.81		0.80	
	INITIAL sGaw	0.25	-0.64	0.46	0.07	0.32	0.60	-0.03	-0.11	0.21	0.46		

medications (Clark 1923) which might have otherwise affected the results obtained. In vitro bronchial strips have no functionally active neural input, so the presence of differing degrees of vagal tone in vivo might obviate any relationship between in vivo and in vitro results. Our results demonstrate a variation in the response to atropine suggesting variation in vagal tone between patients. Furthermore, inhaled histamine produces a response, both by direct action on smooth muscle, (Hawkins and Schild 1951, Nogrady and Bevan, 1978, Thomson and Kerr 1980) and via a nervous reflex mediated via vagal pathways (White and Eiser 1983).

Thus, by pretreating patients with atropine, at a dose which has been shown to decrease the response to methacholine by two log units (Thomson et al 1983) any vagal influence which might have confounded a relationship between in vivo and in vitro measurement should have been minimized. Such atropine pretreatment, however, did not enhance the relationship between in vivo and in vitro measurements.

One previous study has compared in vivo and in vitro responses to histamine in human airways (Vincenc et al 1983). These authors used only FEV_1 to measure in vivo responsiveness, and because of the lower dose of inhaled histamine administered, they were unable to record significant changes in airway calibre in all patients. As a result $PC_{20}FEV_1$ values were only obtained for five out of 14 patients, and $PC_{10}FEV_1$ in four more. Pretreatment of patients with atropine was not employed. The conclusion of this limited study was that no relationship existed between the in vivo and in vitro measurements. Our study confirms and extends these findings, and also confirms the lack of relationship found using methacholine as an agonist (Roberts et al) 1984 (Chapter 4).

Thus we have demonstrated that, using histamine as an agonist, no relationship exists between in vivo and in vitro responsiveness of the airways, and that the modifying effect of vagal input to smooth muscle is not the explanation for this lack of relationship. It is likely that the measurements of in vivo airway responsiveness obtained in these patients are the results of a complex interaction between a variety of different technical, physiological and pathological factors.

A common feature of asthma is an increase in the responsiveness of the airways to a variety of different stimuli. This appears to be non-specific for a number of triggers since in a given asthmatic the airway responses to one stimulus usually correlates with that to another. Individuals highly sensitive to histamine are also generally more sensitive to methacholine (Juniper et al 1978), prostaglandin $F_{2\alpha}$ (Thomson et al 1981), cold air (O'Byrne et al 1982) and exercise (Anderson et al 1979). A mechanism which could explain this non-specific hyperresponsiveness is an abnormality in the sensitivity of airway smooth muscle (Thomson 1962). In vitro hyperresponsiveness of airway smooth muscle could be manifest by a lower EC_{50} value and/or by an increase in the maximum tension generated by each smooth muscle strip. However, in this study the surgical patient who had asthma, and showed the expected in vivo hyperresponsiveness to histamine, did not exhibit increased in vitro responsiveness as assessed by EC_{50} or maximum tension generated. The maximum tension generated but not the EC_{50} could be affected by the quantity of smooth muscle present in each strip. It is possible that by chance there was less smooth muscle present in the bronchial strip from the asthmatic patient, which would mask a real increase in maximum tension generated. To offset this problem the maximum tension generated by each piece of tissue was expressed per unit mass of tissue. This failed to alter the conclusion reached. Nevertheless, it would be necessary to accurately determine the size and number of smooth muscle cells present in each bronchial strip to exclude a defect in airway smooth muscle function as a cause of airway hyperresponsiveness.

Another difficulty when comparing in vivo and in vitro responses is that a complete concentration -response curve is performed in vitro, but is not possible in vivo as it would be dangerous to produce further bronchospasm after the patient has become symptomatic. To address this problem the EC_{20} which is on the early region of the in vitro concentration-response curve, was compared with in vivo results.

Another explanation for a lack of relationship between in vivo and in vitro results is that bronchial smooth muscle may be more sensitive over a/

a small range of concentration such as occur *in vivo*. If this were the case the slope of the *in vitro* concentration-response curve would change. Although there was variation in the slope of this curve, it did not relate to *in vivo* results.

CHAPTER 7

ULTRASTRUCTURE OF HUMAN BRONCHIAL SMOOTH MUSCLE

ULTRASTRUCTURE OF HUMAN BRONCHIAL SMOOTH MUSCLE

7.1 INTRODUCTION

The aim of this thesis has been to investigate factors which may alter the responsiveness of human bronchial smooth muscle. The basic approach was to measure the airway responsiveness of patients due to undergo surgery and to compare this with *in vitro* sensitivity of bronchial strips of the same patients. As an adjunct to this study it was proposed to examine bronchial strips with a transmission electron microscope to investigate whether structural differences of the smooth muscle cells or surrounding stroma might be related to *in vivo* responsiveness and thus explain the variation seen in patient responses to bronchconstrictor agents.

There are few ultrastructural studies on human airway smooth muscle (Richardson and Ferguson 1979, Daniel et al 1980), and only the latter study attempted to quantitate features. Airway smooth muscle is under both neural and myogenic control, so airway responsiveness could be affected by differences in one or other of these systems. Possible neural mechanisms affecting responsiveness include an abnormality of vagal pathways (Coburn and Tomita 1973), of sympathetic receptor density (Coburn and Tomita 1973) or of non adrenergic-non cholinergic (NANC) nerves (Coburn and Tomita 1973). The importance of these mechanisms remains controversial.

Possible myogenic mechanisms affecting responsiveness include increased smooth muscle contractility of individual muscle cells or an increase in cell to cell electrical coupling. There is a spectrum of smooth muscle types from those with pure myogenic control modulated by neural signals to those with predominantly neurogenic control (Daniel et al 1986). Systems with largely myogenic control are characterised by spontaneous electrical oscillations that are independent of nerve supply. Action potentials spread through the muscle via low resistance bridges or gap junctions. In contrast, systems with largely neural control do not generate spontaneous electrical activity and most muscle cells are innervated.

In/

In human airway innervation is sparse (Daniel et al 1986). Specimens of human airway taken at post-mortem from accident victims with apparently normal airway show spontaneous contractile activity and numerous gap junctions (Daniel et al 1980).

Increased airway responsiveness could, therefore, be explained by increased cell-to-cell coupling. Subjects with the most responsive airways would be expected to have the largest number of gap junctions analogous to the increased numbers of gap junctions in the rat myometrium at the time of parturition. (Garfield et al 1978).

The hypothesis that airway responsiveness may be related to ultrastructural changes in airway smooth muscle has not been previously tested in man.

The object of this study was to compare in vivo airway responsiveness to various aspects of smooth muscle ultrastructure including the frequency of nerve endings and numbers of gap junctions. Unfortunately, due to difficulties with fixation and preservation of the bronchial specimens this part of the study was delayed. Eventually, collaboration was arranged with Dr. G. Gabella, University College, London. With his help a protocol producing reliable fixation has been developed and good quality electron micrographs are now being obtained. Tissue from the eleven patients who were assessed in vivo with LTD_4 are fixed awaiting processing. Tissue from the asthmatic patient was unfortunately poorly preserved so that no quantitative work will be possible.

A selection of electron micrographs which show particular features of human airway are presented and these features demonstrated.

7.2 METHODS

Initially, bronchial rings from airway obtained at thoracotomy were immediately placed into 2% gluteraldehyde within minutes of removal at surgery. This procedure produced poorly preserved, contracted smooth muscle so the fixation procedure was modified until good results were obtained using the following protocol:

Thin (1 - 2 mm) wide bronchial rings were dissected using a scalpel blade, transversely, from macroscopically normal airway. Isometric tension was maintained with a springed clip. The rings were then suspended in calcium free Krebs-Hensleit physiological saline at 20°C continuously bubbled with 95%O_2- 5%CO_2, to induce smooth muscle relaxation. After ten minutes the bronchial rings still on the springed clips were transferred to freshly made up 4% glutaraldehyde at 22° and left for a minimum of 20mins. Sample were then transferred to Dr. Gabella who prepared sections and stained and examined these under a Philips transmission electron microscope (EM 400) by methods described in Chapter 2.

7.3 RESULTS

Observations here reported are at a preliminary stage. The following electron micrographs demonstrate many of the features of human bronchial ultrastructure.

7.3.1 Arrangement of Structures

The smooth muscle cells lay in the tunica propria of the bronchial mucosa, usually beside cartilage. The muscle tissue commonly lay 100 micrometres (um) or more below the epithelial surface (Figure 7.1).

Sections were orientated to produce, as far as possible, transverse sections of smooth muscle cells. Smooth muscle cells were arranged in bundles or sheets of compact texture (Figure 7.2). Individual muscle cells or loosely arranged groups were only rarely observed. The smooth muscle bundles were separated by spaces containing/

containing an amorphous matrix with collagen bundles and also other cell types such as mast cells and fibroblasts. Figures 7.3 and 7.4 show a low magnification view to demonstrate arrangement of the various structures seen in the bronchial muscle.

7.3.2 Muscle cell size

Smooth muscle cell profiles measured up to 4 micrometres in diameter. About 12% of their profiles, usually with the largest area, displayed a nucleus. The length of nuclei, measured on orthogonal sections of the same blocks, ranged from 16-22 micrometres. The total length of the muscle cells was therefore estimated to be 200-250 micrometres.

Many muscle profiles tended to be round in shape, but had many projections and indentations. In some preparations 4 to 6 muscle cell profiles were flattened and nested together in a way that suggested the presence of small functional units within the muscle (Figure 7.3).

7.3.3 Cell surface

The cell surface was studded with small inpocketings (caveolae). These were usually arranged in rows along the length of the cell. Rows were separated by regions of the cell membrane bearing dense bands in which thin (actin) filaments were inserted. Small cisternae of smooth endoplasmic reticulum were often associated with the caveolae (Figure 7.4).

7.3.4 Intracellular filaments

The preservation of filaments in smooth muscle cells is notoriously difficult and greatly affected by the preparative procedure (see Figure 7.7). This was the case in the material studied in this project. In some preparations only thin (actin) filaments and a few intermediate filaments were found, while in others both thin and thick (myosin) filaments were well represented, together with intermediate filaments (Figure 7.5).

7.3.5 Cytoplasmic organelles

Mitochondria were abundant in all muscle cells. They amounted to more than 5% of the muscle cell volume. Mitochondria were preferentially located immediately beneath the cell membrane or close to the nucleus (Figure 7.6). Rough endoplasmic reticulum was absent from almost all muscle cell profiles although occasionally a cisterna of rough endoplasmic reticulum was found, usually near a pole of a nucleus (Figure 7.6). In this region of the cell a small Golgi apparatus was often present. Smooth endoplasmic reticulum is common and appears as small flat cisternae (Figure 7.6). They are usually found near the caveolae and close to mitochondria.

7.3.6 Mast cells

Mast cells were present in most of the bronchial samples examined. Some were found close to regions of smooth muscle (Figure 7.10). There were projections from the mast cell which were flattened over the cell body. If these were extended, they would reach the surface of the smooth muscle cells.

7.3.7 Nerves

The tunica propria was densely innervated. The nerves ranged from large trunks containing a hundred or more axons, some of which were myelinated, to small nerve bundles consisting of a few unmyelinated axons. Only large nerve trunks were sheathed by an endoneurium (Figure 7.6).

Within the bronchial musculature, only small nerve bundles were found. These contained exclusively unmyelinated axons and were not encapsulated. The axons were associated with processes of Schwann cells. The numbers of intramuscular nerve bundles was small (Figure 7.6, 7.7). Although the density of innervation varied from preparation to preparation, there was not more than one axon per twenty muscle cells. Some intramuscular axons were expanded/

expanded into varicosities and these contained clusters of synaptic vehicles, mainly of the small agranular type (Figure 7.6).

7.3.8 Gap Junctions

Gap junctions were seen in all preparations and were a constant feature of human bronchial musculature (Figures 7.2, 7.4, 7.5, 7.6, 7.7). The junctions were small, rarely exceeding 0.2 micrometres in length, and had the characteristic structure of two closely apposed membranes with an intra cellular gap of around 2 nanometres. This arrangement gives the gap junction a 5 or 7 layered appearance under the electron microscope.

There appeared to be variation in the numbers of gap junctions between preparations although this has not yet been quantified.

7.3.9 Intercellular materials

In the spaces between muscle cells there were collagen fibrils measuring about 50 nanometres in diameter. They were orientated in all directions but predominantly were parallel to the long axis of the cells. In virtually all preparations the muscle cells were surrounded by a thick coat of material similar in texture and electron density to a basal lamina (Figure 7.2, 7.4.). This coat measured from 60-500 nanometres. In some preparations this coat filled the space between adjacent muscle cells, with only some collagen fibrils tunnelling through it.

7.4 DISCUSSION

A reliable method has been developed for the fixation and staining of bronchial samples removed at thoracotomy. Tissue preservation is now such that quantification of various structures is possible, which will allow comparison of numbers of these structures with *in vivo* airway responsiveness. There are several interesting features apparent in this preliminary study.

The/

The caveolae seen in smooth muscle cells were of similar size and shape to those described in smooth muscle cells from other sources (Gabella 1981).

Thick filaments containing myosin and thin filaments containing actin and tropomyosin are important in the contractile response of the smooth muscle cell (Shoenberg and Needham 1976). Intermediate filaments are thought to form part of the Cytoskeleton of the cell and are non-contractile (Cooke 1976). If the relative quantities of these components varied within smooth muscle cells it would cause differences in contractility of the smooth muscle. However, with the inconsistent results in demonstrating filaments by EM, quantification of the different filaments would be impractical.

The role of the endoplasmic reticulum in the regulation of smooth muscle contraction is poorly understood. Rough and some of the smooth ER may be involved in synthetic processes such as that of synthesis of membrane, filaments, glycogen and collagen. Smooth endoplasmic reticulum, by releasing and sequestering calcium, is important in the control of muscle contraction in striated muscle (Ebashi and Endo, 1968). However, the site of Ca^{2+} storage within smooth muscle has not been determined although there is some evidence implicating the smooth ER (Carsten 1969). If this observation is confirmed, then qualitative or quantitative variations in bronchial smooth muscle smooth ER might be important in determining smooth muscle responses to non-specific stimuli.

Mast cells were present in most preparations. They were often found close to groups of smooth muscle cells confirming the observations on airway smooth muscle by Daniel et al (1986). Their proximity to smooth muscle is in keeping with their role in acute bronchoconstriction produced by mediator release (Holgate & Kay 1985). It would be important if there were variations in the quantity and site(s) of mast cells in different preparations as this might indicate why only some atopic individuals develop asthma. Similarly variations in the quantity of cytoplasmic granules where histamine and 5-hydroxytryptamine are stored would also be of interest.

Perhaps the most interesting feature of smooth muscle is the gap junction. Action potentials spread through the muscle via these intercellular connections (Perrechia 1974). Studies on rat uterus have shown that the number of gap junctions between smooth muscle cells of the myometrium increase markedly around parturition, when the uterus is "hyper-responsive" to stimuli, and fall again within two days. (Garfield et al 1977).

Daniel et al (1980) found that samples of bronchi from airway of accident victims with apparently normal airway showed spontaneous contractile activity and numerous gap junctions. More recently the same group have found, using post mortem specimens, that there were greater numbers of gap junctions in tracheal smooth muscle compared to smooth muscle of smaller bronchi. However, they made no attempt to assess whether there were differences in smooth muscle samples obtained from different individuals. Hence, it is important to assess whether variation in gap junction frequency and morphology occurs between individuals, and if differences do exist, whether they relate to <u>in vivo</u> responsiveness.

The thick coat of material surrounding smooth muscle cells in virtually all our preparations has been previously described only once (Daniel et al 1986). Although it is similar in texture and electron density to a basal lamina, the coat around the human bronchial smooth muscle cells measures 60 - 500 nanometres, compared to the 20 to 30 nanometres dimensions of basal lamina in visceral muscle cells from experimental animals (Gabella 1981). This special material, hereinafter referred to as basement membrane, had an amorphous appearance. Its inner aspect was directly adherent to the cell membrane while its outer aspect was associated with collagen fibrils. Bundles or a thin layer of microfibrils of about 10 nanometres diameter, lay close to the outer aspect of the basement membrane. This basement membrane could affect the contraction of the smooth muscle, its nutrient supply, and also the diffusion of bronchconstrictor agents to their site of action on the surface of the smooth muscle cell. Variations in the quantity/

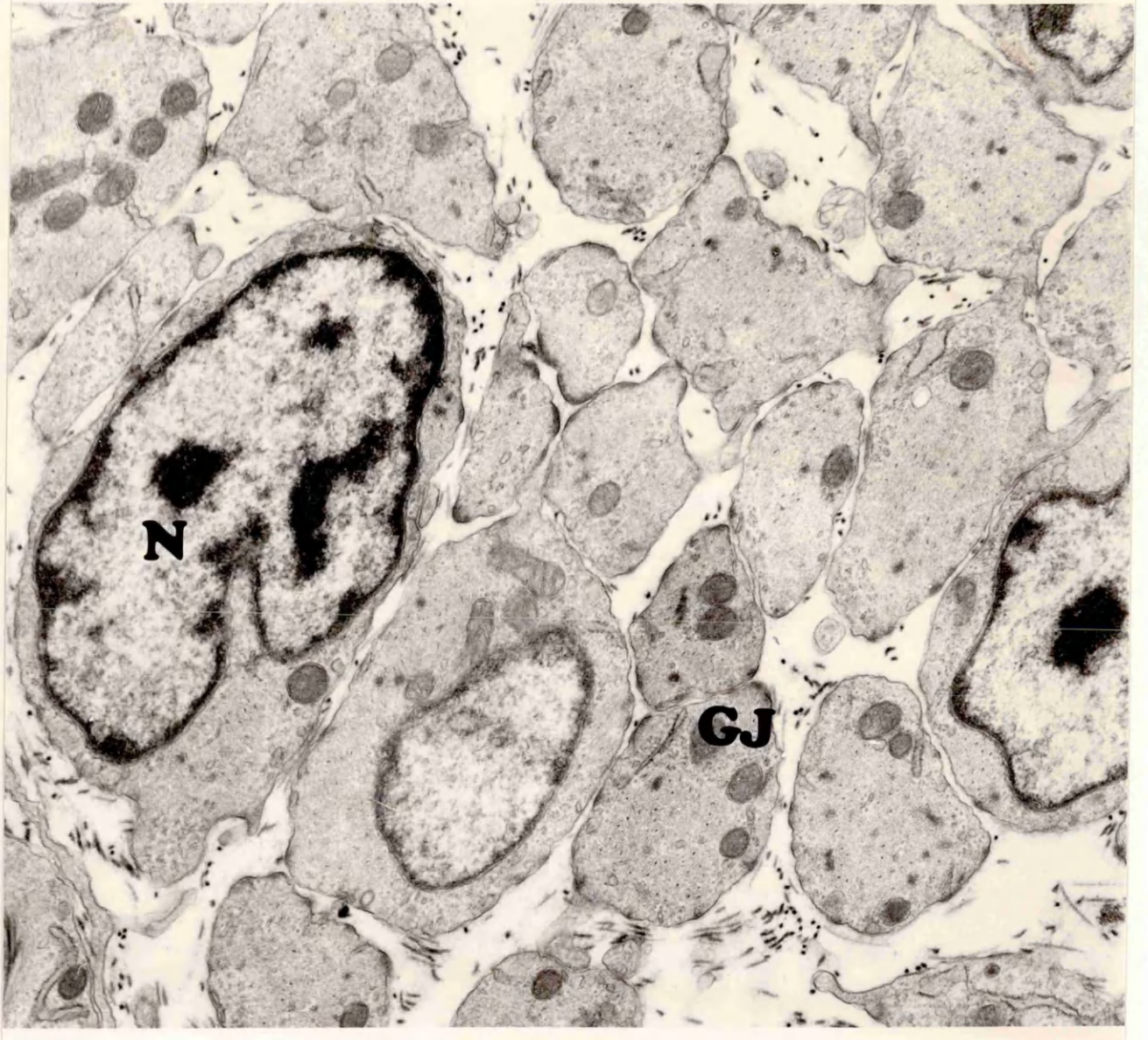
quantity and distribution of this basement membrane could be important in smooth muscle responses.

In summary, reliable electron micrographs of bronchial rings can now be obtained. Several interesting features have been demonstrated which could be important in the contraction of airway smooth muscle. It is hoped that future quantification of these features may yield important insights into the understanding of the control of human airway smooth muscle contraction.

FIGURE 7.1

Electron micrograph of bronchial strip obtained at thoracotomy.
Epithelial cell (A) with associated cilia (B)
Endoplasmic reticulum (er) and mitochondria (m) are seen within the epithelial cell cytoplasm. Bundles of nerves (n) run close to the epithelial surface. Some cilia are seen in transverse section CT showing the 9 rod structure of the cilia. Adjacent to the epithelial cell is a mucous secreting cell with a group of secretory vesicles being discharged into the lumen. [No muscle cells are visible in this micrograph]. (Magnification = 3,700).

FIGURE 7.2

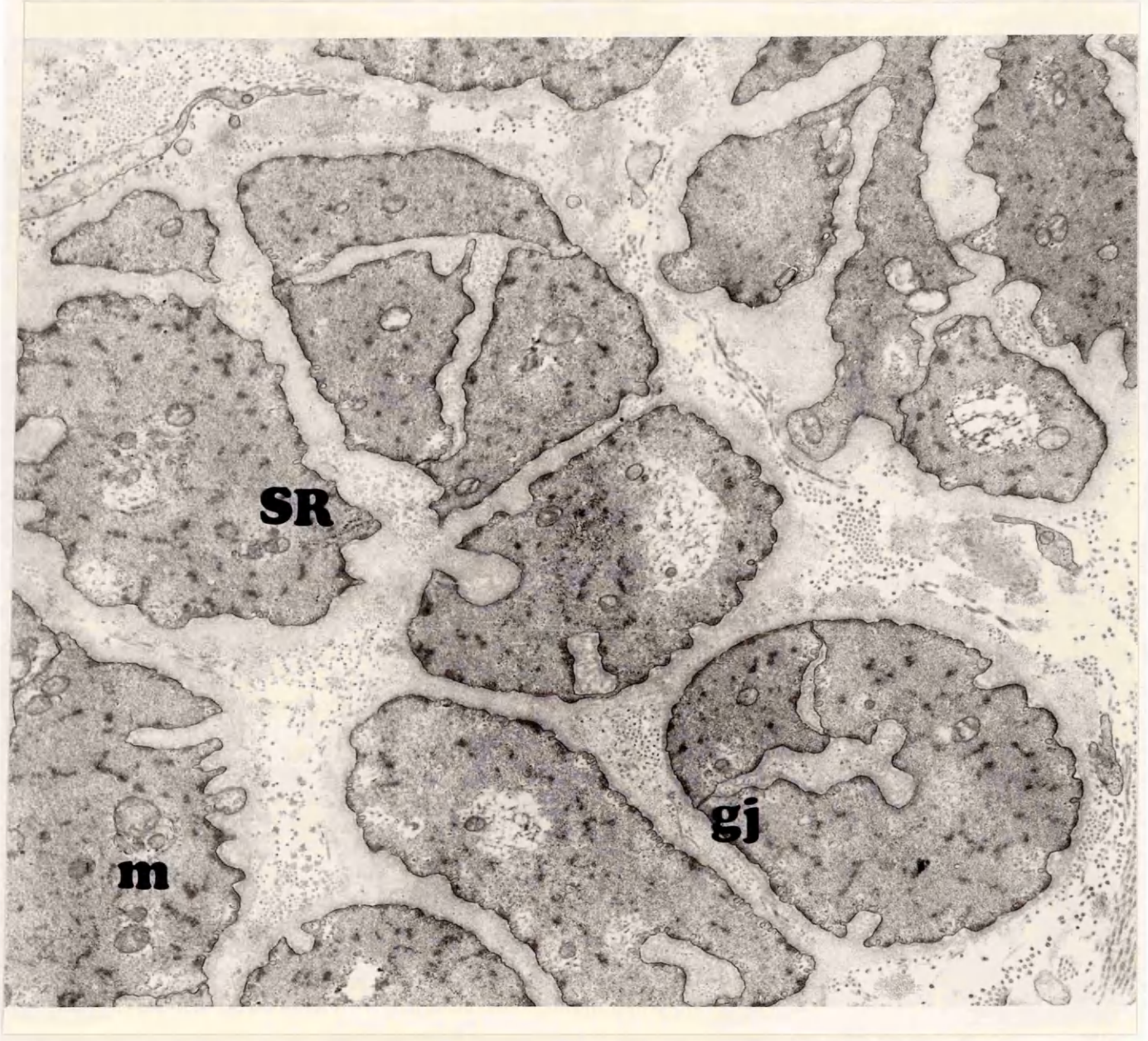


Low magnification electron micrograph view of smooth muscle cells taken from a human bronchus. Nuclei (N) are only seen in some cells because the cells are cut transversely and the nuclei are often missed because of the axial length of the cell.

Even at this magnification cell-to-cell contacts which probably represent gap junctions (GJ) can be recognised.

Magnification = x 8000

FIGURE 7.3

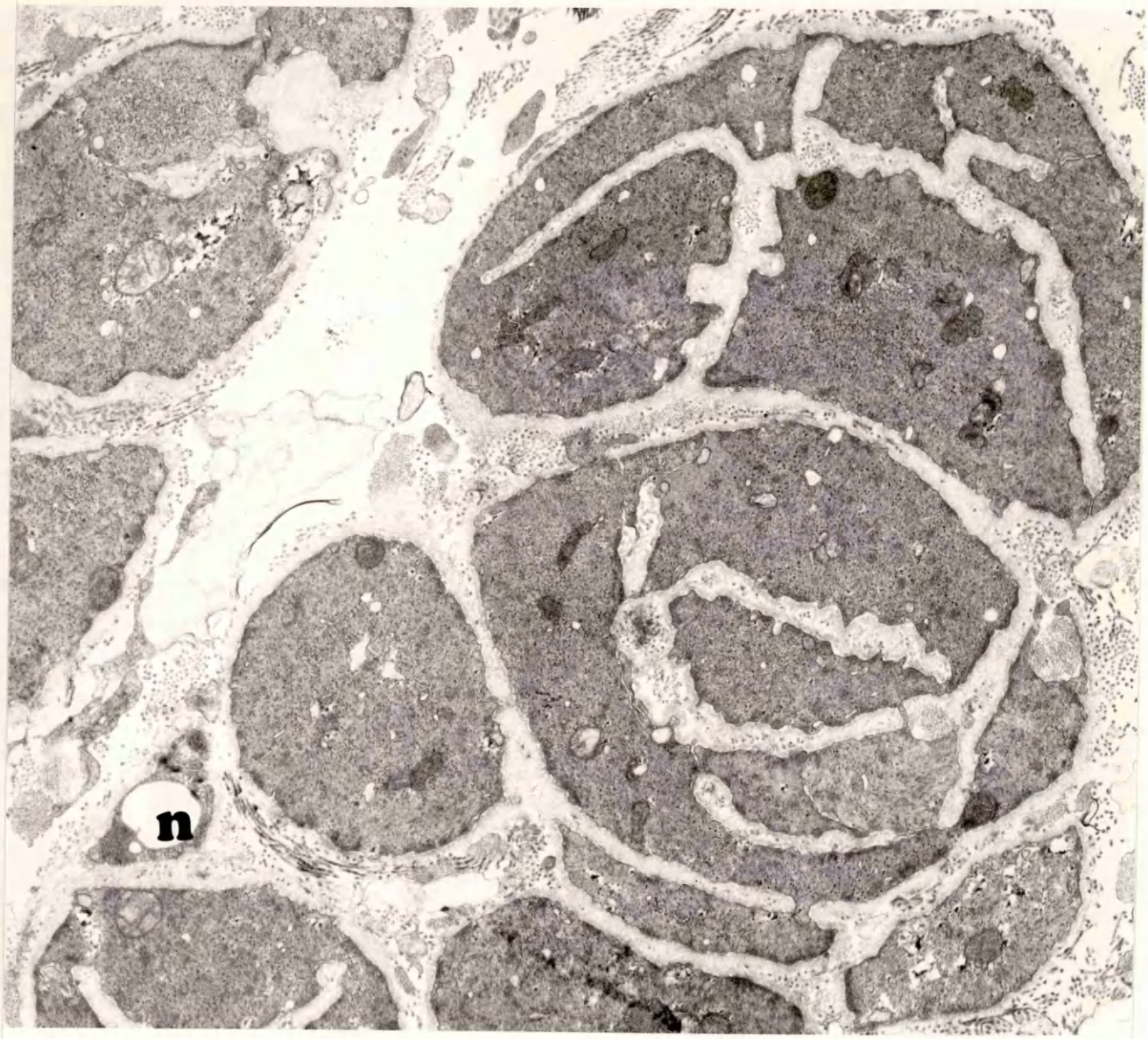


A low power electron micrograph of human bronchial smooth muscle cells which lie in small bundles. These may operate as functional units. Sarcoplasmic reticulum (SR) and mitochondria (M) are present. Several gap junctions (GJ) are also visible. Numerous inpocketings of the cell membrane (caveolae) are present.

The muscle bundles are surrounded by a homogenous material.

Magnification = x6000

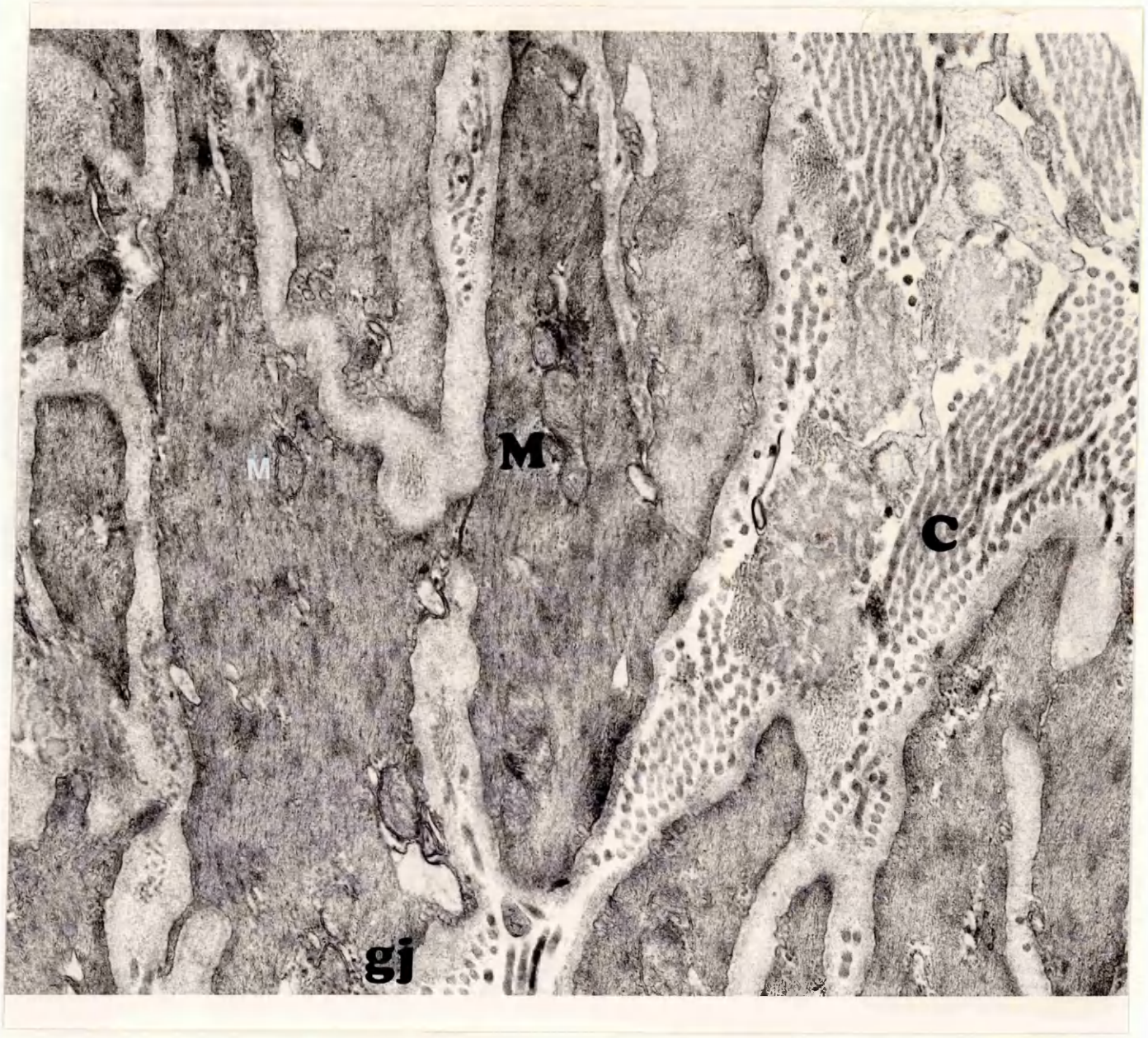
FIGURE 7.4



Bundles of human bronchial smooth muscle cells surrounded by matrix material and collagen fibres seen in longitudinal and transverse section. Intracellular organelles are present and a nerve bundle (n) runs between the muscle cells.

(Magnification = x 8000)

FIGURE 7.5



Low power view of human bronchial smooth muscle cells running longitudinally. Numerous collagen fibres (C) are present surrounding muscle cells.
Mitochondria (M) are present.

(Magnification = x 13,500)

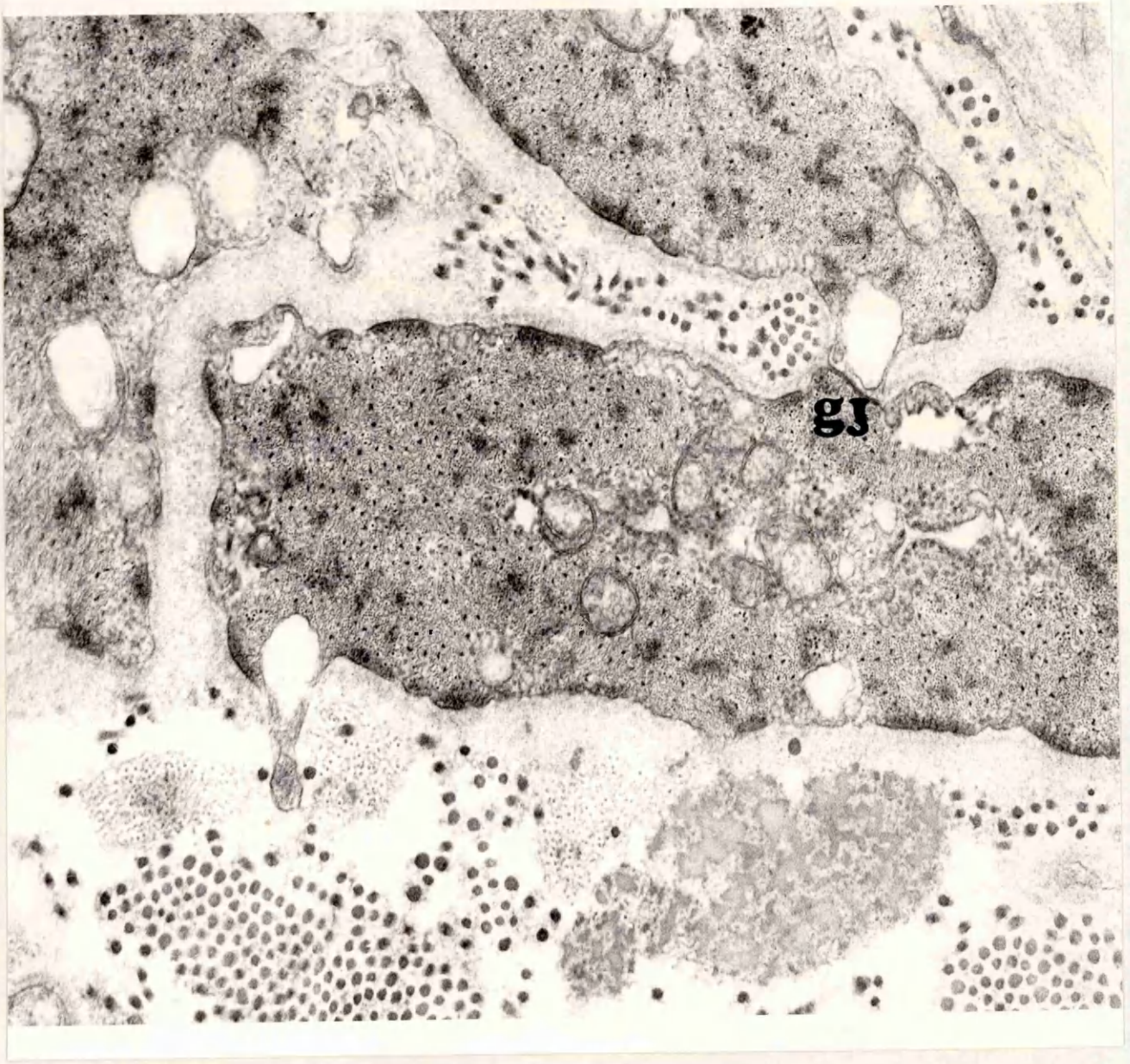
FIGURE 7.6

Numerous caveolae (C) are present at the Electron micrograph of human bronchi smooth muscle cell surface. Sarcoplasmic reticulum and occasional gap junctions are also seen.

Myofilaments are visible within the cell cytoplasm. Homogenous grey material is present in the spaces between the smooth muscle cells. Preservation of structures, particularly mitochondria is poor in this specimen.

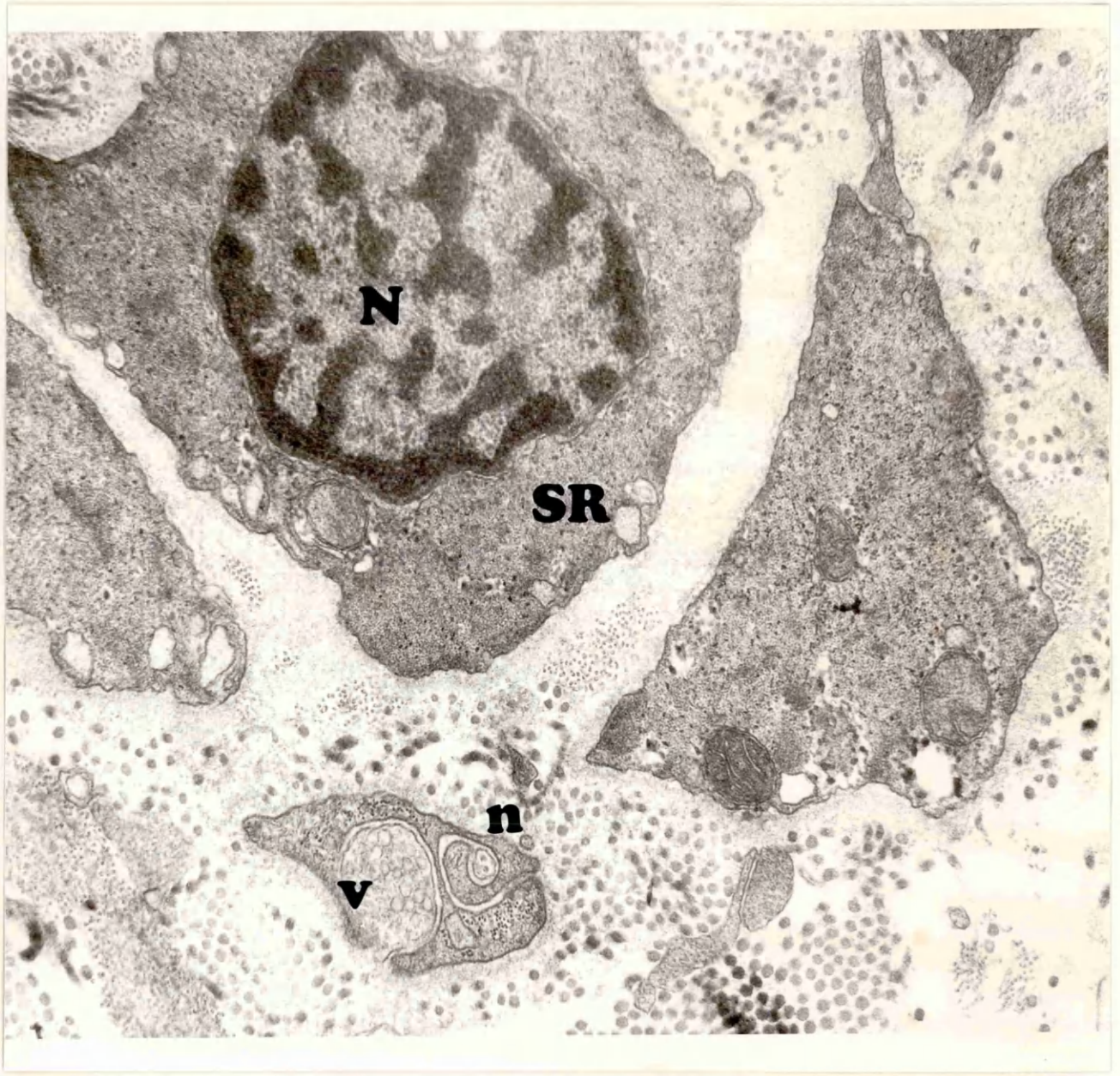
Magnification = x 17,000)

FIGURE 7.7



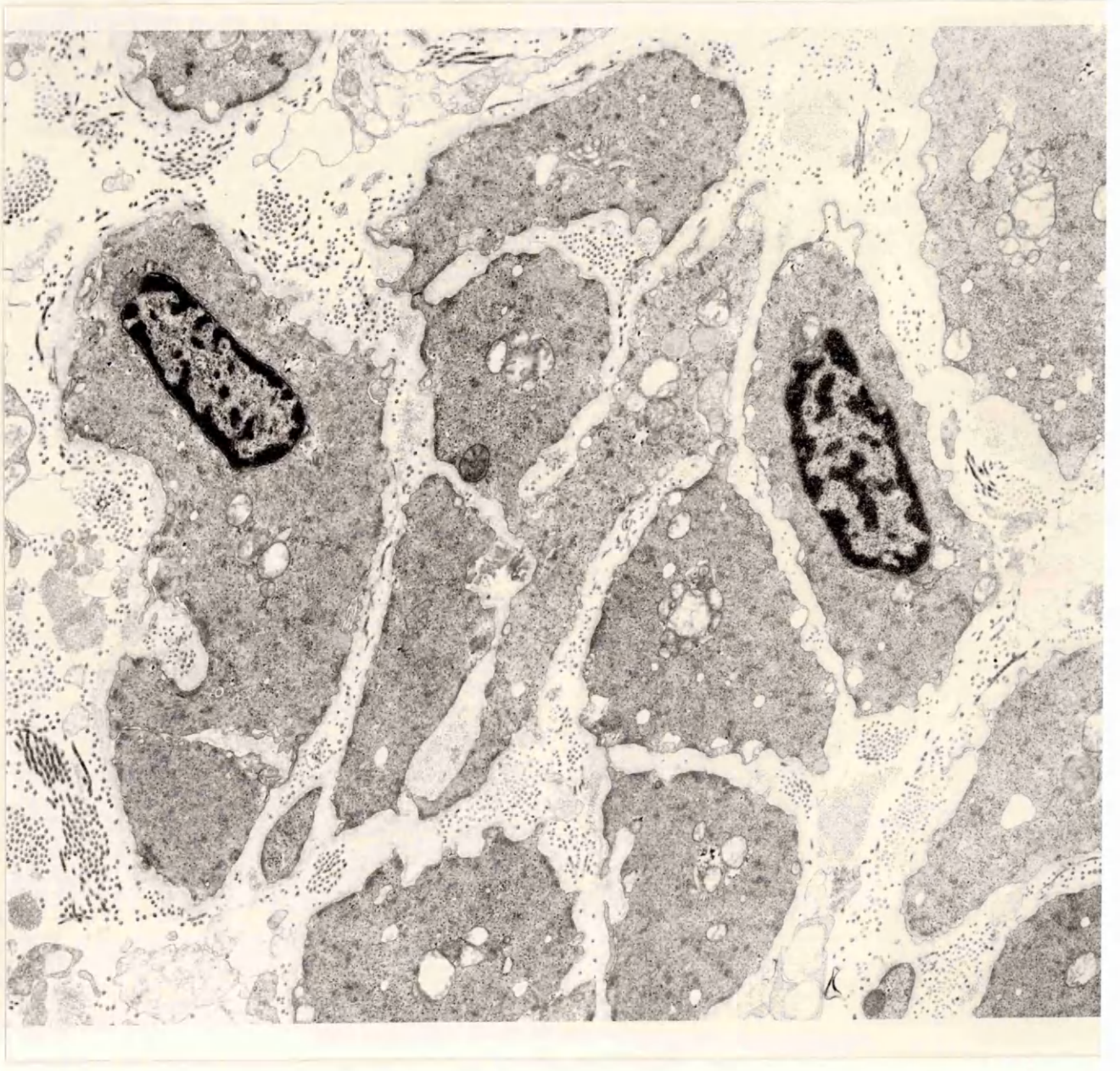
A larger magnification of a human bronchial preparation (17,000) shows details of intracellular structures. Myosin (dark dots) and actin (paler smaller dots) filaments are seen within the cell cytoplasm. Occasional microtubulles are seen. The 5 layer structure of the gap junction is also apparent at this magnification.

FIGURE 7.8



A x 22,000 view of a human bronchial smooth muscle cell showing the nucleus (N) and cell membrane.
Smooth sarcoplasmic reticulum (SR) lies close to the cell membrane and to the nucleus.
Mitochondria are also present.
A small nerve bundle (nb) containing only two axons, one of which is expanded into a varicosity (v) packed with synaptic vesicles

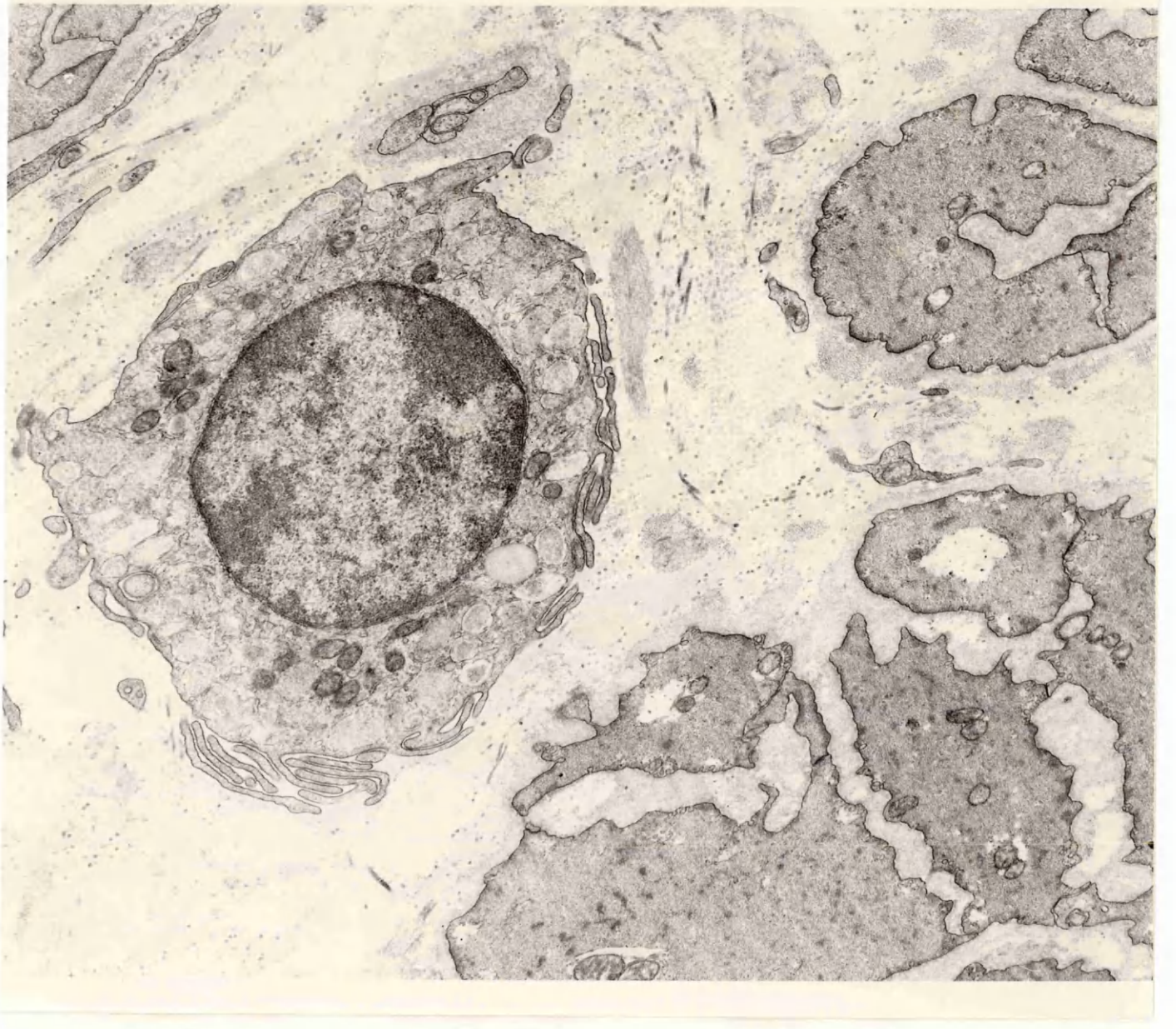
FIGURE 7.9



A low power (x 6000) view of human bronchial smooth muscle bundles. Mitochondria and caveolae are numerous although preservation of mitochondria in this specimen is poor.

There are several gap junctions and a few nerves running within the bundles of muscle cell.

FIGURE 7.10



Electron micrograph of human bronchial strip.
A mast cell lies adjacent to smooth muscle. Secretory granules are present in the cytoplasm. If the cytoplasmic extensions were extended, they would be contiguous with the smooth muscle surface.
Small bundles of smooth muscle show numerous gap junctions.

(Magnification = x 6,000)

C H A P T E R 8

IN VIVO AND IN VITRO EFFECT OF VERAPAMIL ON HUMAN AIRWAY RESPONSIVENESS TO LEUKOTRIENE D_4

IN VIVO AND IN VITRO EFFECT OF VERAPAMIL ON HUMAN AIRWAY RESPONSIVENESS TO LEUKOTRIENE D_4

8.1 INTRODUCTION

In previous chapters of this thesis I have examined the effect of methacholine and histamine on airway smooth muscle tone in vivo and in vitro. Neither of these agonists is likely to have a major role in producing bronchoconstriction in the clinical situation. Methacholine is a pharmacological agonist acting on the smooth muscle receptors normally stimulated via the vagus, and histamine, although present in mast cells and released in allergen and exercise challenge in the asthmatic does not seem to be important in asthma, as potent anti-histamines are ineffective as anti-asthma drugs. Furthermore histamine induced bronchoconstriction is rapid in onset and short in duration, unlike an asthmatic attack.

SRS-A is present in mast cells and released on allergen challenge (Orange and Austin 1969). Recent work has shown that SRS-A consists of a group of related chemicals known as leukotrienes (Simonsson 1980). One of these, Leukotriene D_4 (LTD_4) is a potent bronchoconstrictor in man and may be an important mediator in asthma (Dahlen et al 1980, Barnes et al 1984). The onset of LTD_4 induced bronchial smooth muscle contraction is slow and the duration of contraction prolonged (Barnes et al 1984) akin to bronchoconstriction in asthma.

Specific binding sites (receptors) have been identified in the lungs of different species including man (Pong and De Haven 1983, Lewis et al 1984). Although in vitro contractile responses of airway smooth muscle from these species have been shown to be receptor mediated, the mechanism(s) underlying leukotriene induced bronchoconstriction in man in vivo is uncertain. Since contraction of airway smooth muscle is dependent on calcium ion influx (Triggle 1983, Rodger 1985) this study investigated the effects of the calcium-channel blocking drug verapamil on the airway response to LTD_4 in a group of normal subjects. In parallel, the effects of verapamil on the in vitro LTD_4 concentration-response curve for human bronchial tissue were also examined.

8.2 METHODS

8.2.1 In vitro Study

Bronchial tissue was obtained from patients undergoing lung resection for bronchial carcinoma, immediately following removal at thoracotomy. Samples from second to sixth order bronchi were dissected from areas of macroscopically normal tissue and maintained at $4^{o}C$ overnight in oxygenated Krebs-Hensleit physiological salt solution. On the day following removal the bronchi were dissected free of extraneous connective tissue and sectioned in order to produce transverse strips of tissue which were suspended (under a resting tension of 1.5 to 2.0 g) in a 5 ml organ bath containing Krebs-Hensleit solution at $37^{o}C$ bubbled with 5% CO_2 in O_2. During a 60 minute equilibration period the tissues were washed three times. Changes in isometric tension were measured using force-displacement transducers and recorded on an ink-writing polygraph.

The reproducibility of the contractile changes of the bronchial strips was assessed by adding methacholine (1×10^{-4} moles l^{-1}) on two occasions allowing an interval of 30 minutes for recovery between each drug addition. A cumulative concentration-effect curve to LTD_4 or methacholine was then constructed by adding increasing concentrations of the drug, each addition of the drug being made at the peak of the response produced by the preceding concentration. Following washout of the agonist and full recovery to control resting tension, either verapamil (1×10^{-6} moles l^{-1}) or vehicle was added to the bath. Thirty minutes later a second concentration-effect curve was constructed. The concentration of LTD_4 and methacholine that produced a 50% (EC_{50}) maximal contraction were calculated from the graphically displayed data.

In each experiment four to six bronchial strips from each patient were examined. For each bronchial strip the responses to LTD_4 and methacholine elicited in the presence of verapamil were calculated/

calculated as a percentage of that produced in the absence of the calcium channel blocking drug.

8.2.2 In vivo Study

Six normal subjects were studied (Table 8.1). Five were male. Their ages ranged from 22 to 36 years. All were non-smokers and give no history of respiratory disease. None were taking any drug treatment. All subjects gave informed consent and the experimental protocol was approved by the Western Infirmary Ethical Committee.

Airways resistance (Raw) and Thoracic Gas Volume (TGV) were measured automatically (Chapter 3). The results were expressed as specific airways conductance (sGaw). The mean of 8 values measured was taken as sGaw. The maximum expiratory flow at 70% of expired vital capacity, obtained from a partial flow-volume ($\dot{V}_{30}(p)$) curve, and the forced expiratory volume in one second (FEV_1) were measured automatically. Body plethysmographic measurements always preceded flow volume recordings. Aerosols were generated with a Wrights nebuliser by air at 50 psi (345 kPa) at a flow rate of 8 l/min to an output of 0.15 ml/min.

Each subject received either verapamil (2.5 mg/ml) or normal saline in a randomised single blind manner on four separate days. After baseline measurements of sGaw (mean of 8 readings) and $\dot{V}_{30}(p)$ (mean of 5 readings), the solutions were inhaled for 5 minutes. After 15 minutes lung function tests were repeated. Leukotriene D_4 and methacholine inhalation tests were carried out with a modification of the previously described method. Buffered saline was inhaled first followed by increasing concentrations of LTD_4 (0.4-50 ug/ml) or methacholine (2 - 64 mg/ml). Each concentration was inhaled for 2 minutes. Inhalations were repeated every 15 minutes for LTD_4 and every 10 minutes for methacholine. Two LTD_4 and two methacholine inhalation tests were performed on each subject. Results were expressed as the provocation concentration (PC) producing a 35% fall in sGaw (PC_{35}sGaw) and a 30% fall in $\dot{V}_{30}(p)$)./

TABLE 8.1

SUBJECT CHARACTERISTICS

Subjects	Age (yr)	Sex	Ht (metres)	Baseline FEV_1 Litres	Baseline FEV_1 % Pred
1	32	M	1.74	4.1	102
2	35	M	1.71	3.4	92
3	25	M	1.68	3.7	94
4	22	M	1.75	3.9	93
5	30	F	1.80	3.6	99
6	22	M	1.75	4.4	105

$V_{30}(p)$). Results were compared using analysis of variance and Student's t test.

8.3 RESULTS

8.3.1 In vitro Study

LTD_4 (1×10^{-10} to 2×10^{-7}) and methacholine (1×10^{-9} to 1×10^{-3}) moles l^{-1} elicited concentration-dependent contractions of the bronchial strip preparations (Figure 8.1). There was no significant difference in the sensitivities to LTD_4 or methacholine of bronchial strips prepared from the same patient. EC_{50} values ($\pm$ SE of mean) calculated from all experiments were $2.6 \pm 0.8 \times 10^{-8}$ moles l^{-1} (n = 7) for LTD_4 and $1.7 \pm 0.3 \times 10^{-6}$ moles L^{-1} (n=18) for methacholine.

Contractions to LTD_4 were slow to develop, taking 12 ± 4 minutes to achieve peak effect for each concentration of drug used (Figure 8.2). The mean maximum concentration elicited by LTD_4 at the highest concentration tested (it was not possible to test concentrations in excess of 2×10^{-7} moles l^{-1} because of the limited supply of eicosanoid) was 17 ± 3 m N. This was not significantly different from the maximum contraction produced by methacholine (18 ± 2 m N). LTD_4-induced contractions were slowly reversed, requiring 60 to 80 minutes to return to baseline tension levels with repeated washing of the tissue with fresh physiological salt solution.

Verapamil (1×10^{-6} moles l^{-1}) had no effect on baseline tension during the 30 minute equilibration period. Similarly, verapamil was without significant effect on the concentration-response curve to LTD_4 (Figure 8.1 a). The same concentration of verapamil inhibited contractile responses to methacholine and depressed the maximum contraction by $29 \pm 5\%$ (Figure 8.1 b)

8.3.2 In vivo Study

Inhalation of verapamil did not alter baseline sGaw or $\dot{V}_{30}(p)$ (Table 8.2)/

TABLE 8.2

Effect of verapamil on baseline lung function

	LEUKOTRIENE D_4				METHACHOLINE			
	N = 5				N = 6			
	sGaw		$V_{30(p)}$		sGaw		$V_{30(p)}$	
	Baseline	After Treatment	Baseline	After Treatment	Baseline	After Treatment	Baseline	After Treatment
	($s^{-1}kPa^{-1}$)		$\ell.sec^{-1}$		($s^{-1}kPa^{-1}$)		ℓ sec^{-1}	
Verapamil	1.59 ±0.17	1.78 ±0.28	1.92 ±0.27	1.93 ±0.25	2.00 ±0.25	2.14 ±0.28	1.82 ±0.16	1.82 ±0.16
Control	1.88 ±0.29	2.12 ±0.15	1.82 ±0.23	1.85 ±0.23	2.02 ±0.27	2.11 ±0.40	1.82 ±0.22	1.77 ±0.22
P value		NS		NS		NS		NS

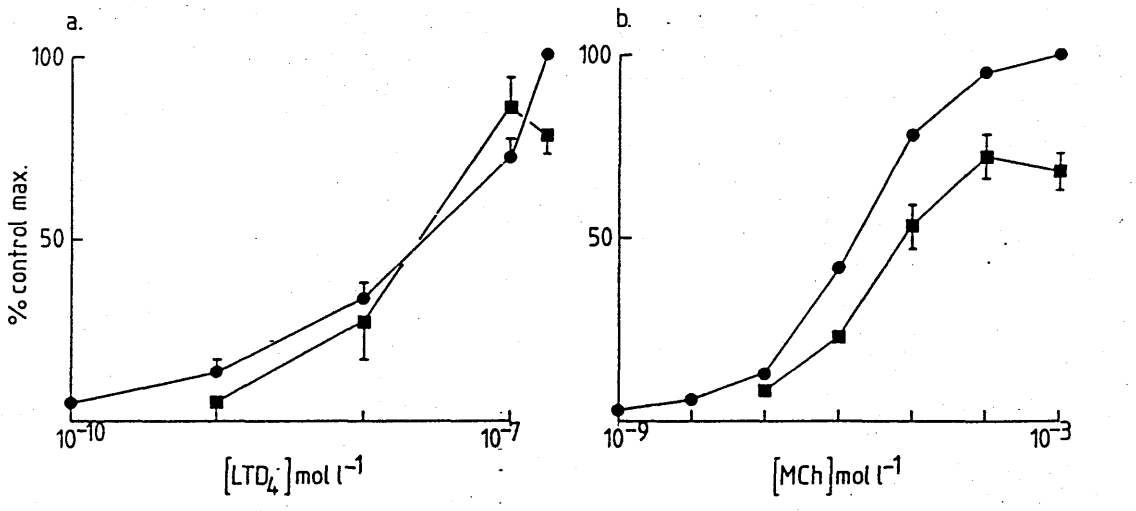
sGaw = Specific airways conductance

$V_{30(p)}$ = partial expiratory flow rate measured at 30% of vital capacity

NS = Not significant

FIGURE 8.1

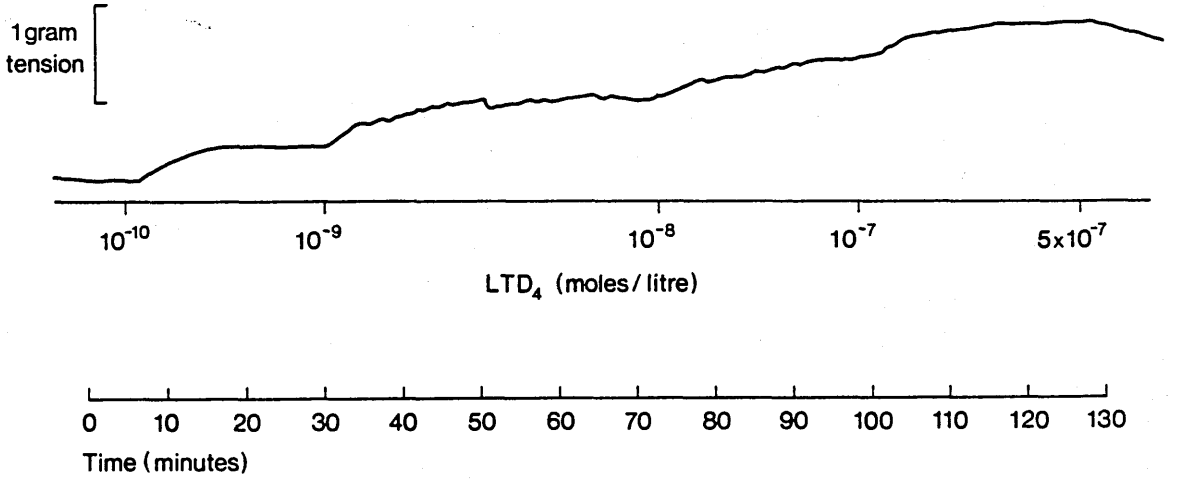
In vitro responses to LTD_4 (a) and methacholine (b)



Graph of response expressed as a percentage of maximum contraction both without (●) and with (■) verapamil 1 x 10^{-6} to in graph a: LTD_4 and graph b: methacholine of in vitro human bronchial preparations.
Each point represents the mean of 7 (graph a) or 18 (graph b) bronchial strips. The standard error of the mean is shown as a vertical bar.

FIGURE 8.2

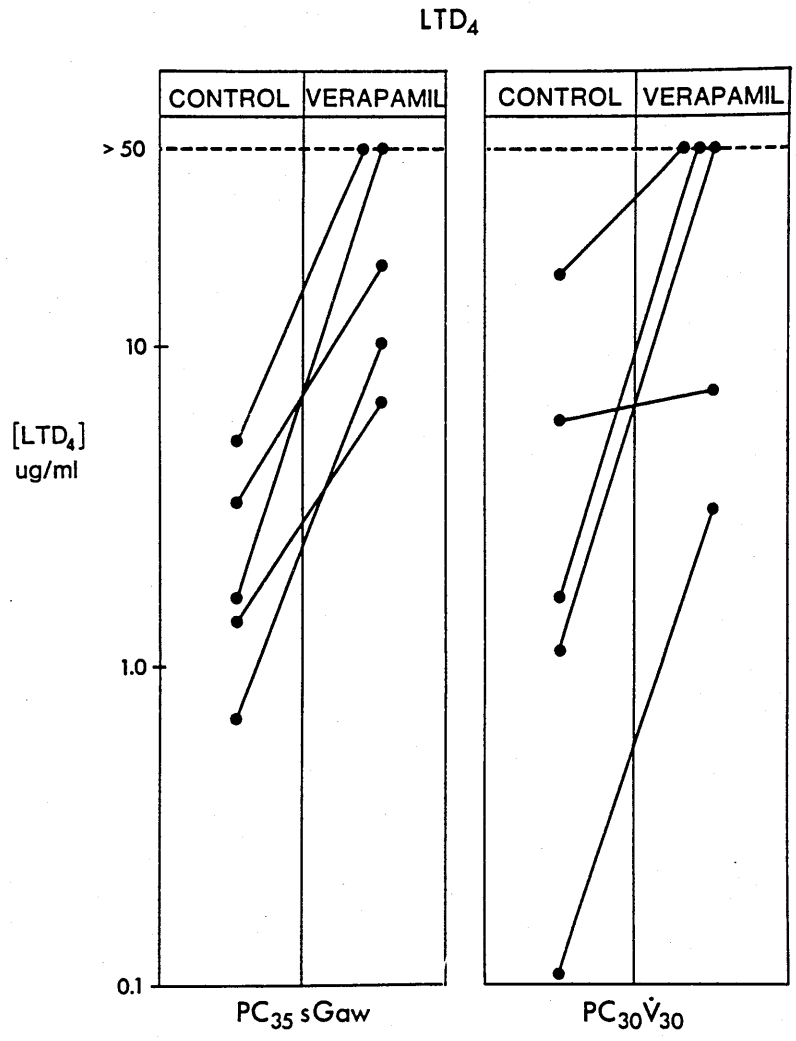
In vitro response to LTD_4



Polygraph tracing of tension generated by smooth muscle strip when LTD_4 in increasing concentrations is added to the organ bath.

FIGURE 8.3

Effect of verapamil on LTD_4 induced bronchoconstriction



Effect of pretreatment with verapamil (2.5 mg/ml) or control (buffered saline) inhalations on airway responsiveness to LTD_4. Results are expressed as the PC_{35}sGaw and the $PC_{30}\dot{V}_{30}$(p).

(Table 8.2). In 5 subjects verapamil significantly inhibited LTD_4-induced bronchoconstriction (Figure 8.3). One subject failed to respond to LTD_4 on either day. The geometric mean PC values underestimate the effect of verapamil on LTD_4-induced bronchoconstriction since 3 subjects did not bronchoconstrict to the highest concentration of LTD_4 administered. These subjects were allocated the highest concentration of LTD_4 (50 ug/ml) as their PC value to allow statistical analysis. Verapamil did not inhibit methacholine-induced bronchoconstriction (Figure 8.4). None of the subjects developed cough after LTD_4.

8.4 DISCUSSION

These results demonstrate that verapamil significantly inhibits LTD_4 but not methacholine-induced bronchoconstriction in man. In contrast verapamil had no inhibitory effect upon the response of human airway smooth muscle strips to LTD_4 and a small though significant inhibitory effect on the response to methacholine.

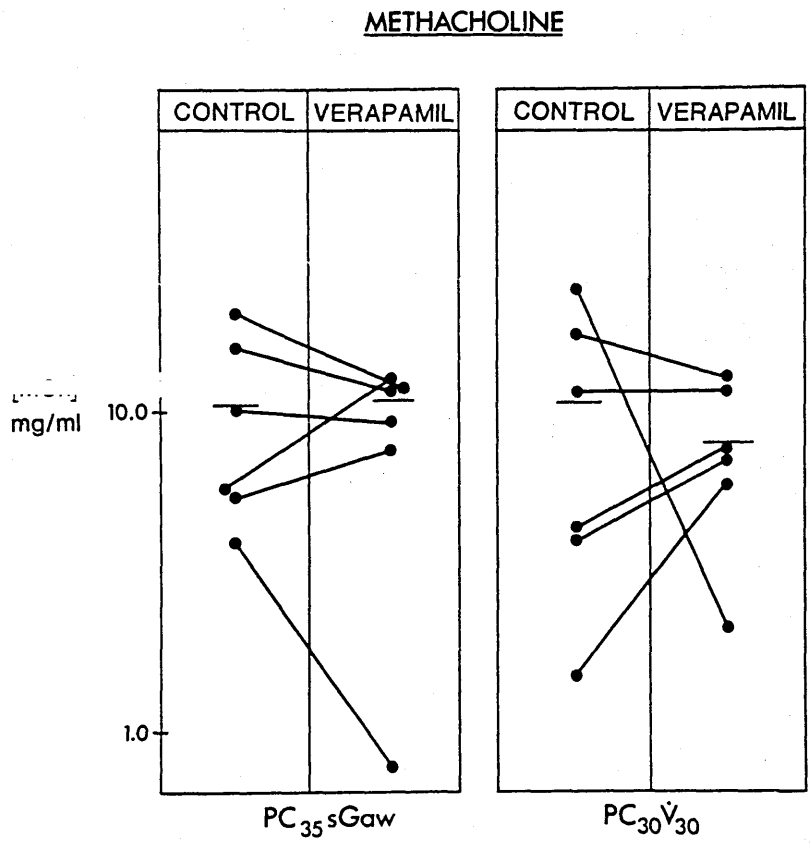
LTD_4 and methacholine elicited concentration-dependent contraction of isolated bronchial smooth muscle. In the case of methacholine these effects were clearly mediated directly via stimulation of muscarinic cholinoreceptors in the smooth muscle since the responses are competitively inhibited by atropine (Raeburn 1984). In the absence of a suitable selective antagonist at LTD_4 receptors a similar experiment was not feasible with LTD_4. However, contractions elicited by LTD_4 were unaffected by mepyramine, atropine or inhibition of the cyclo-oxygenase pathway with flurbiprofen (Raeburn 1984). Similar findings have been reported by another group (Jones et al 1982). This evidence makes the following mechanisms of LTD_4 induced bronchoconstriction unlikely:

1. an indirect effect of LTD_4 mediated via histamine released from mast cells
2. neurotransmitter (acetylcholine) released from vagal nerve terminals in the smooth muscle

3./

FIGURE 8.4

Effect of verapamil on methacholine induced bronchoconstriction



Effect of pretreatment with verapamil or control inhalations on airway responsiveness. Results are expressed as $PC_{35}sGaw$ and $PC_{30}\dot{V}_{30}(p)$.

3. the release of cycle-oxygenase products, e.g: thromboxane A_2 or the constrictor prostaglandins PGF_2 and PGD, derived from arachidonic acid.

It is probable that the observed effects of LTD_4 *in vitro* were directly mediated.

Contraction of airway smooth muscle depends on an increase in the concentration of calcium ions (Ca^{2+}) free within the myoplasm of the cell (Triggle 1982, Rodger 1984). These activator Ca^{2+} molecules may be supplied either from the extracellular fluid surrounding the cell or from storage sites such as the sarcoplasmic reticulum, within the cell (Triggle 1982, Rodger 1984). Contractions of guinea pig airway smooth muscle preparations induced by agonists are highly resistant to inhibition with calcium channel blocking drugs such as verapamil and nifedipine (Raeburn & Rodger 1984, Foster et al 1984). There are few reports of calcium channel blocking drugs on contractions of human airway smooth muscle *in vitro*. It is clear that high concentrations (10^{-6} moles l^{-1}) of nifedipine and gallopamil (D600) are required in order to produce relatively small inhibition of contractions (Jones et al 1982, Drazen et al 1983). The results of our study using verapamil are in agreement with these existing reports. The absence of an inhibitory effect of verapamil on LTD_4-induced contractions is consistent with the hypothesis that this agonist may, in human airway smooth muscle, as occurs in guinea pig airways (Raeburn and Rodger 1984), rely principally upon an intracellular source of activator Ca^{2+} to initiate contraction. In contrast, the significant inhibitory effect of verapamil on methacholine-induced contractions *in vitro* may indicate that in human airway smooth muscle a proportion of the contractile response to this agonist is dependent upon influx of extracellular Ca^{2+} via calcium channels that are sensitive to inhibition by verapamil.

In contrast to our *in vitro* findings, *in vivo* verapamil significantly inhibited LTD_4-induced bronchoconstriction, but not methacholine-induced bronchoconstriction. The marked difference between the effects of verapamil on LTD_4-induced bronchoconstriction *in vivo* and *in*/

in vitro and the selectively of its effect on LTD_4 *in vivo*, suggests that verapamil is not acting solely on airway smooth muscle cells. Thus, the airway response to LTD_4 in man may be mediated both by a direct effect on airway smooth muscle as well as indirectly via a verapamil sensitive pathway.

There are several potential pathways by which LTD_4 might indirectly cause bronchoconstriction. If LTD_4 is capable of stimulating sensory nerve endings in the airway this could result in reflex vagal bronchoconstriction.

In dogs with hyper-reactive airways and in guinea pigs, LTD_4 induced bronchoconstriction is prevented by pretreatment with atropine (Hirschman et al 1983, Advenier et al 1983). However, Holroyde & Jackson (1983) found that LTD_4 had no effect on sensory irritant receptors in cats and dogs, although in cats it potentiated the response to serotonin by a vagal-dependent mechanism. Thus, the role of vagal pathways in the response to LTD_4 in animals is unclear, and in man untested. Furthermore, although neurotransmitter release depends on influx of extracellular Ca^{2+}, this process is insensitive to inhibition by calcium channel blocking drugs (Hausler 1972).

The slow onset on bronchoconstriction with LTD_4 is consistent with the response being due to release of secondary mediators. In guinea pigs, intravenously administered leukotriene produces a reduction in dynamic compliance after pretreatment with cyclo-oxygenase inhibitor suggesting that some of the bronchoconstriction may be due to the generation of thromboxane A_2 (Weichman et al 1982). (Weiss et al 1983) however found that pretreatment with oral aspirin failed to attentuate the response to LTD_4 in man.

LTD_4 may increase airway permeability as well as producing bronchoconstriction. Epithelial transport mechanisms require calcium (Mavin et al 1982) and these could be susceptible to inhibition by verapamil. If verapamil were reducing LTD_4 induced changes in airway permeability and impairing access of LTD_4 to receptors on smooth muscle, the effect of LTD_4 would be reduced *in vivo* but not *in vitro*.

In/

In summary these results suggest that in normal subjects LTD_4-induced bronchoconstriction may occur due to a combination of direct and indirect mechanisms. The pathway mediating the indirect effect of LTD_4 is at present unknown.

C H A P T E R 9

EFFECT OF VERAPAMIL AND SODIUM CROMOGLYCATE ON LEUKOTRIENE D_4 INDUCED BRONCHOCONSTRICTION IN ASTHMATIC PATIENTS

EFFECT OF VERAPAMIL AND DISODIUM CROMOGLYCATE ON LEUKOTRIENE D_4 INDUCED BRONCHOCONSTRICTION IN ASTHMATIC PATIENTS

9.1 INTRODUCTION

The leukotrienes (LT) including LTD_4 may be important mediators in asthma (Orange and Austen 1969, Dahlen et al 1983). LTD_4 is released both *in vitro* and *in vivo* after allergen challenge, and is a potent bronchoconstrictor (Dahlen et al 1980, Holroyde et al 1981). However, the mechanism of LTD_4-induced bronchospasm in asthma has not been established. In guinea pigs (Advenier et al 1983) and normal subjects (Chapter 8) the calcium channel blocker verapamil partially inhibits the bronchoconstrictor response to LTD_4 *in vivo* but not *in vitro* in human bronchi, and only at very high concentrations in guinea pig isolated trachea. These results suggest that in guinea pigs and normal subjects LTD_4-induced bronchconstriction occurs due to both a direct effect on airway smooth muscle which is insensitive to the inhibitory action of verapamil, and to an indirect effect via a verapamil sensitive mechanism. Similarly, in guinea pigs, sodium cromoglycate partially inhibits the contractile response to LTD_4 *in vivo* but not *in vitro* (Advenier et al 1983). Thus, in this species sodium cromoglycate appears capable of inhibiting an indirectly mediated bronchoconstrictor response of LTD_4.

In this study we examined the effect of pretreatment with verapamil and sodium cromoglycate on LTD_4-induced bronchcoconstriction to determine whether, in asthma, LTD_4-induced airway narrowing involves verapamil and/or sodium cromoglycate sensitive mechanisms.

TABLE 9.1

PATIENT CHARACTERISTICS

PATIENT No.	AGE	SEX	FEV_1 (L)	FEV_1 (% PRED)	ATOPIC STATUS	METHACHOLINE $PC_{10}FEV_1$ mg/ml	CURRENT* TREATMENT
1	28	M	2.96	86	+	0.25	S, B
2	49	F	3.43	139	+	0.17	S
3	25	F	2.59	83	+	0.61	S, B
4	42	M	3.00	86	+	0.63	S, B
5	22	F	3.49	103	+	0.16	S, SCG
6	24	M	4.57	113	+	0.64	S
7	24	F	4.21	121	+	0.25	S, SCG

*S = Salbutamol Inhaler

B = Beclomethasone Diproprionate Inhaler

SCG = Sodium Cromoglycate

9.2 METHODS

9.2.1 Patients

Seven asthmatic patients were studied (Table 9.1). Four were female. Their ages ranged from 22 to 49 years. All were atopic and were non smokers. All were taking inhaled B_2 adrenoceptor agonists by pressurised aerosol, three were taking inhaled steroids regularly and two were taking sodium cromoglycate. All B_2 agonists were discontinued 12 hours before testing and sodium cromoglycate 24 hours before testing. Inhaled steroids were continued.

9.2.2 In Vivo Measurements

Airways resistance (Raw) and Thoracic Gas Volume (TGV) were measured in a constant-volume body plethysmograph (Fenyves and Gut) using a computerised data collection and analysis system (Chapter 3). Results were expressed as specific airways conductance (sGaw) (=1/Raw x TGV). The mean of 8 values measured was taken as sGaw. The maximum expiratory flow at 70% of expired vital capacity, obtained from a partial flow-volume ($\dot{V}_{30}(p)$) curve, and the forced expiratory volume in one second (FEV_1) were measured automatically (Collingwood Measurement). The flow-volume curves were performed as previously described.

9.2.3 Dose-Response Curves

The study was performed in two parts. In the first each patient received either verapamil (2.5 mg/ml) or normal saline in a randomised double blind manner on three separate days (two saline). After baseline measurements of sGaw (mean of 8 readings) and $\dot{V}_{30}(p)$ and FEV_1 (mean of 5 readings), the solutions were inhaled for 5 minutes. After 10 minutes lung function tests were repeated and each subject then inhaled increasing concentrations of leukotriene (0.0032 - 10 ug/ml). Each concentration was inhaled for 2 minutes and inhalations were repeated every 15 minutes/

minutes. Results were expressed as the provocation concentration (PC) producing a 35% fall in sGaw (PC_{35}sGaw), a 30% fall in $\dot{V}_{30}(p)$ ($PC_{30}\dot{V}_{30}(p)$) and a 10% fall in FEV_1 ($PC_{10}FEV_1$). The average PC values for the two post saline LTD_4 dose-response curves was used when comparison was made with the post verapamil results.

To confirm that the asthmatic patients had increased non-specific bronchial reactivity, a methacholine challenge was also performed following the method of Cockcroft et al (1977).

In the second half of the study, patients received either sodium cromoglycate (10 mg/ml) or placebo in a randomised double blind manner. Measurements were taken before and 10 minutes after each inhlation. A dose-response curve to LTD_4 was then performed as described above. On a separate day a dose-response curve to methacholine was performed in a single blind manner using the protocol described by Hargreave et al (1981). Results were compared using analysis of variance and Student's t test.

9.3 RESULTS

The $PC_{10}FEV_1$ methacholine (mg/ml) ranged from 0.17 to 0.64 confirming that these asthmatic patients had increased non-specific bronchial reactivity.

9.3.1. Verapamil

Inhalation of verapamil did not alter baseline FEV_1, sGaw, or $\dot{V}_{30}(p)$ (Table (9.2). All patients developed significant bronchoconstriction to LTD_4 as assessed by $PC_{10}FEV_1$, PC_{35}sGaw (Figure 9.1) and $PC_{30}\dot{V}_{30}(p)$. Pretreatment with verapamil did not modify this response (Figure 9.2). Geometric mean $PC_{10}FEV_1$ was 0.35 ug/ml after verapamil compared with 0.47 ug/ml control (NS). PC_{35}sGaw was 0.69 ug/ml after control and 0.37 ug/ml after verapamil (NS) Mean $PC_{30}\dot{V}_{30}(p)$ was 0.41 ug/ml after control and 0.31 ug/ml after verapamil (NS).

TABLE 9.2

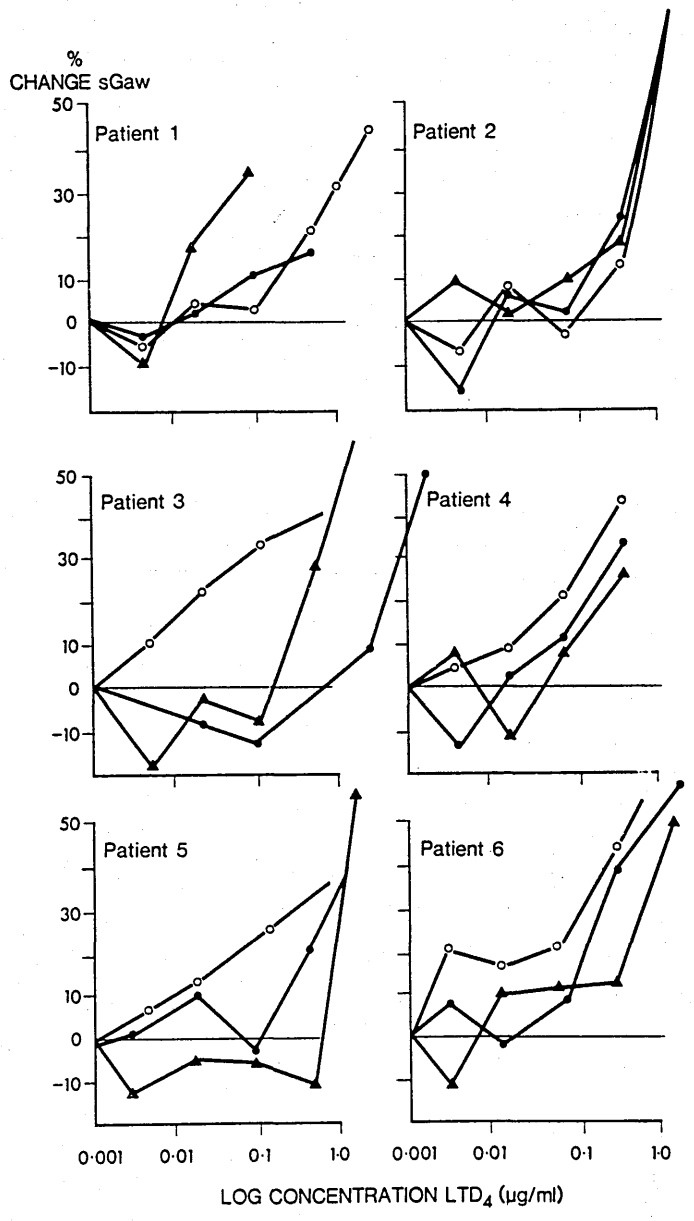
EFFECT OF VERAPAMIL ON BASELINE AIRWAY CALIBRE

	FEV_1(L)		sGaw (s^{-1}ka Pa^{-1})		$\dot{V}_{30(p)}$(LS^{-1})	
	BASELINE	AFTER TREATMENT	BASELINE	AFTER TREATMENT	BASELINE	AFTER TREATMENT
CONTROL ± SE	2.59 (0.36)	2.60 (0.36)	1.13 (0.16)	1.03 (0.13)	1.2 (0.2)	1.36 (0.22)
VERAPAMIL ± SE	2.38 (0.51)	2.37 (0.54)	1.39 (0.49)	1.07 (0.28)	1.05 (0.3)	1.02 (0.63)

NO SIGNIFICANT DIFFERENCES IN BASELINE AND AFTER TREATMENT VALUES OF FEV_1, sGaw and $V_{30(p)}$ BOTH WITHIN AND BETWEEN TREATMENTS

FIGURE 9.1

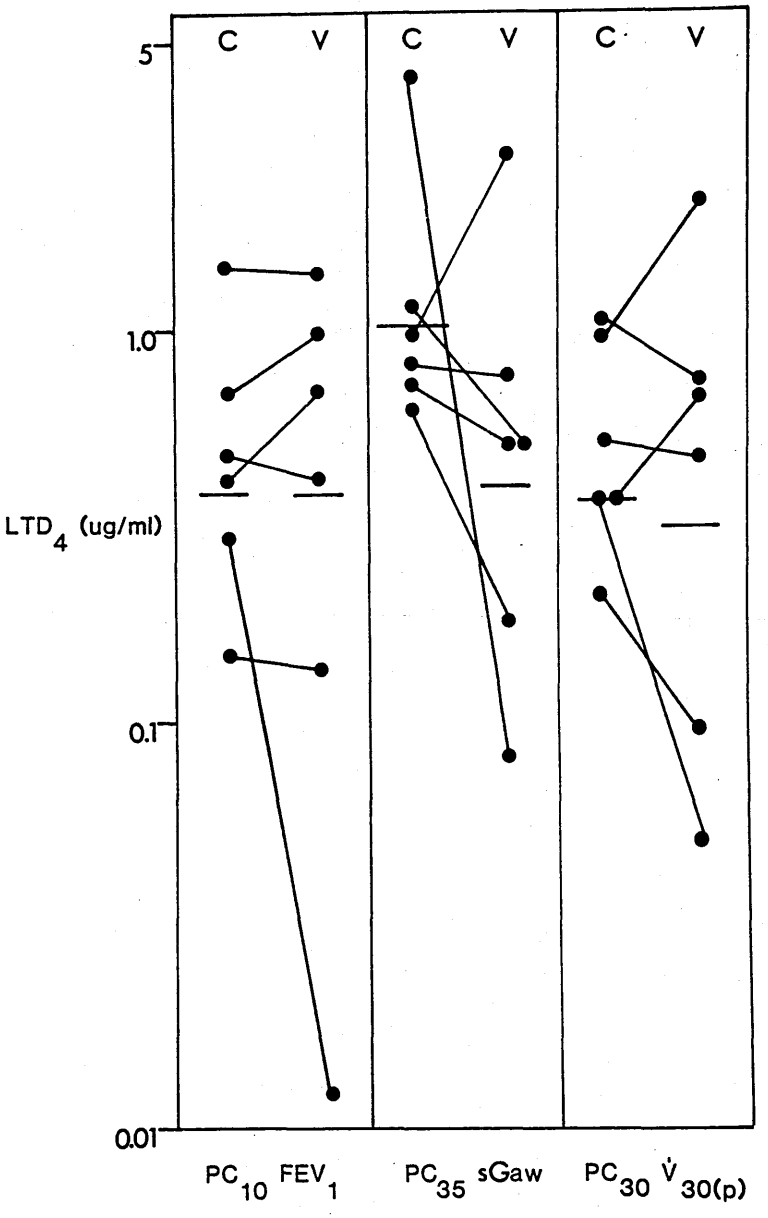
Concentration-response curves for individual patients with and without verapamil pretreatment



Sample concentration-response curves showing percentage change in sGaw against log concentration of inhaled Leukotriene D_4 after placebo 1 (O), placebo 2 (●) and verapamil (▲)

FIGURE 9.2

Effect of verapamil on airway responsiveness



Effect of pretreatment with verapamil (V) (2.5 mg/ml) or control (C) (phosphate buffered saline) on airway responsiveness to LTD_4. Results are expressed as the provocation concentration (PC) producing a decrease in FEV_1 of 10% ($PC_{10}FEV_1$), a 35% decrease in sGaw (PC_{35}sGaw) and a 30% fall in $\dot{V}_{30}(p)$ ($PC_{30}\dot{V}_{30}(p)$). Mean values of $PC_{10}FEV_1$, PC_{35}sGaw and $PC_{30}\dot{V}_{30}(p)$ are shown as horizontal bars. Verapamil did not significantly reduce the response to LTD_4.

9.3.2 Sodium Cromoglycate

Sodium cromoglycate (SCG) did not significantly alter baseline FEV_1 sGaw or $\dot{V}_{30}(p)$ (Table 9.3). All patients developed significant bronchoconstriction as assessed by $PC_{10}FEV_1$, PC_{35}sGaw (Figure 9.3) and $PC_{30}\dot{V}_{30}(p)$ (SCG) did not alter responsiveness to LTD_4 (Figure 9.4). Geometric mean $PC_{10}FEV_1$ was 0.22 ug/ml after control and 0.24 ug/ml after sodium cromoglycate (NS). PC_{35}sGaw was 0.21 ug/ml after control and 0.19 ug/ml after sodium cromoglycate (NS). $PC_{30}\dot{V}_{30(p)}$ was 0.21 ug/ml after control and 0.19 ug/ml after sodium cromoglycate (NS).

Patients inhaling LTD_4 did not develop any side effects such as cough.

9.4 DISCUSSION

These results demonstrate that neither the calcium channel blocker verapamil nor sodium cromoglycate modify LTD_4-induced bronchoconstriction in patients with asthma. This contrasts with results (Chapter 7) in normal subjects where verapamil reduced the constrictor response to LTD_4.

Why should verapamil have a protective effect against LTD_4 induced bronchconstriction in normal subjects but not in asthmatic patients? It has been suggested that there may be heterogeneity of LTD_4 receptors, and that the ability of different drugs to block the response to LTD_4 may vary between the different affinity LTD_4 receptors (Lewis and Austen 1984). In support of this hypothesis, the calcium channel blocker diltiazem inhibits the contraction of guinea pigs lung parenchymal strips to high dose LTD_4 whereas the SRS-A antagonist FPL55712 inhibits only the low dose part of LTD_4 induced contraction (Hirschman et al 1983). Since the normal subjects in this project inhaled higher concentrations of LTD_4 than the asthmatic patients this may have resulted in the calcium channel blocker sensitive low affinity LTD_4 receptors being stimulated only in the former. However, verapamil does/

TABLE 9.3

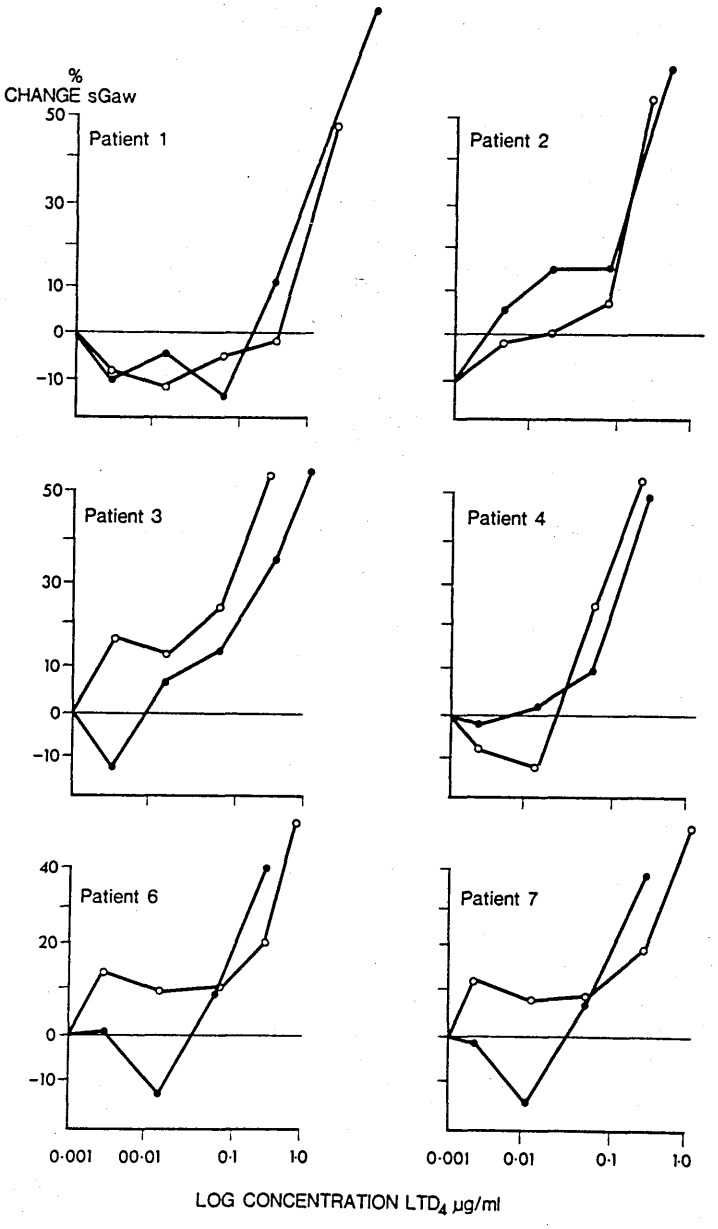
EFFECT OF SODIUM CROMOGLYCATE ON BASELINE AIRWAY FUNCTION

	FEV_1 (L)		sGAW (s^{-1} kPa^{-1})		$\dot{V}_{30(P)}$ (LS^{-1})	
	BEFORE	AFTER TREATMENT	BEFORE	AFTER TREATMENT	BEFORE	AFTER TREATMENT
CONTROL ± SE	2.35 (0.2)	2.46 (0.21)	1.20 (0.9)	1.14 (0.9)	1.00 (0.43)	1.07 (0.2)
SODIUM CROMOGLYCATE ± SE	2.42 (0.18)	2.51 (0.21)	1.2 (0.8)	1.17 (0.9)	0.99 (0.25)	1.09 (0.29)

NO SIGNIFICANT DIFFERENCES IN BASELINE AND AFTER TREATMENT VALUES OF FEV_1, sGaw and $\dot{V}_{30(p)}$ BOTH WITHIN AND BETWEEN TREATMENTS.

FIGURE 9.3

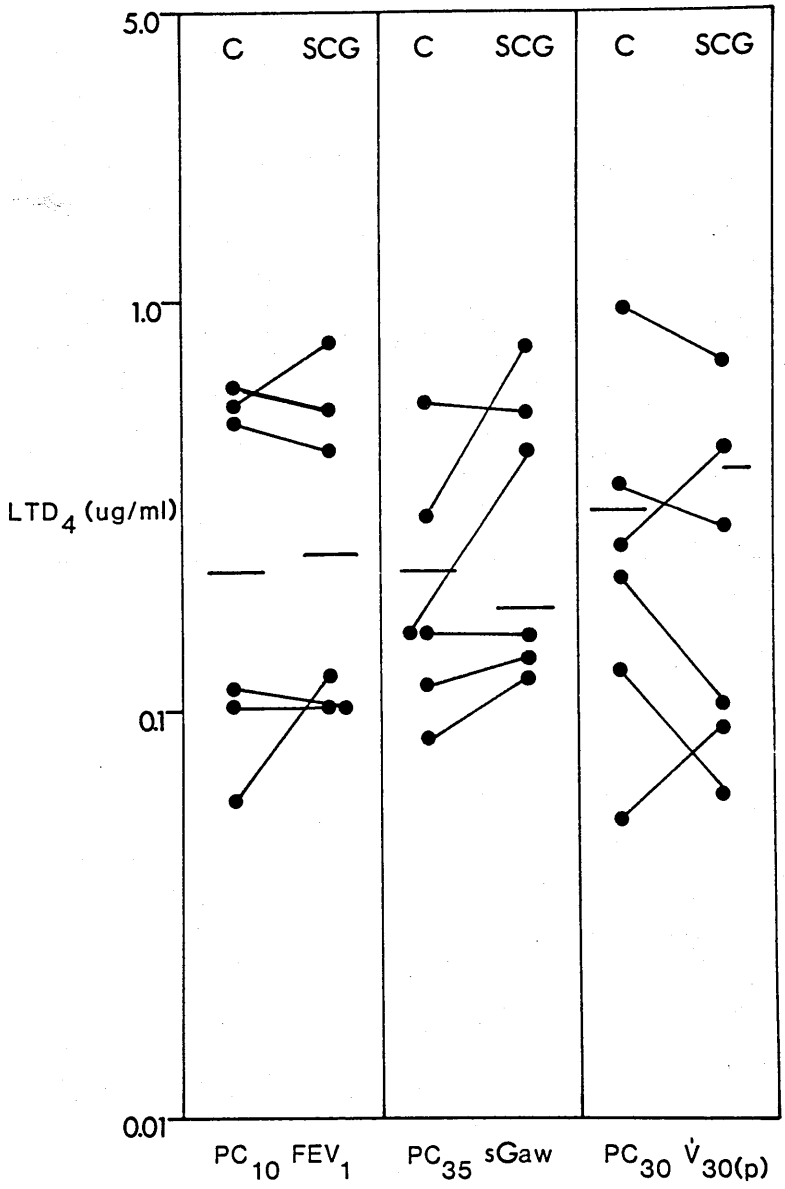
Concentration-response curves for individual patients with and without pretreatment with SCG



Sample graph of percentage change in sGaw against log concentration LTD_4 for 6 asthmatic patients. Results after placebo (0) and sodium cromoglycate (●)

FIGURE 9.4

Effect of SCG on airway responsiveness



Effect of pretreatment with sodium cromoglycate (10 mg/ml) or placebo on airway responsiveness to LTD_4. Responsiveness is expressed as $PC_{10}FEV_1$, $PC_{35}sGaw$, and $PC_{30}\dot{V}_{30}(p)$. Geometric mean values are shown as horizontal bars.

does not significantly inhibit the contractile response of *in vitro* preparations of human bronchi to different doses of LTD_4 although a slight (not statistically significant) reduction of response to the highest concentration of LTD_4 was observed (Chapter 8).

An alternative explanation of these findings is that LTD_4 induced bronchoconstriction in asthmatic patients may be due to a direct effect on airway smooth muscle whereas in normal subjects airway narrowing occurs due to a combination of direct action on bronchial smooth muscle receptors and indirect verapamil sensitive mechanisms. The latter indirect mechanism would have to have a higher threshold to LTD_4 to explain these findings. Possible indirect mechanisms by which LTD_4 might cause bronchoconstriction include reflex vagal bronchoconstriction (Advenier et al 1983, Hirscham et al 1983) or release of secondary mediators (Weichman et al 1982).

A third possibility is that the action of verapamil depends on an intact respiratory epithelium. Recently Raeburn et al (1985) showed that verapamil inhibits LTD_4 induced airway smooth muscle contraction in the rabbit only when the airway mucosa is intact. Even mild asthmatic patients have damaged mucosa (Laitinen et al 1985), so the absence of an inhibitory effect of verapamil on LTD_4-induced bronchconstriction could be explained by a lack of intact mucosa in asthmatics.

If LTD_4 is confirmed as an important mediator in asthma, one would predict from these findings that verapamil would not be an effective drug in asthma. Most studies of calcium channel blocking drugs in asthma support this suggestion. Neither verapamil nor nifedipine significantly alter resting bronchomotor tone and produce little or no protection against bronchoconstriction induced by allergen (Henderson et al 1983, Patel et al 1983). They are moderately effective in inhibiting exercise-induced asthma (Cerrina et al 1981, Patel 1981).

Sodium cromoglycate inhibits mast cell degranulation, but may also have other modes of action (Martelli and Usandivaras 1977). In guinea pigs *in vivo* sodium cromoglycate partially inhibits LTD_4-induced bronchoconstriction (Advenier et al 1983). In patients with aspirin sensitive/

sensitive asthma, the response to aspirin challenge can be blocked by sodium cromoglycate (Martelli and Usandivaras 1977). The pathogenesis of aspirin sensitive asthma is unknown but may involve increased production of lipoxygenase products such as LTD_4 (Seczcklik et al 1975). Sodium cromoglycate may have acted as a leukotriene antagonist. Our results demonstrate that sodium cromoglycate is not a specific inhibitor of LTD_4 in asthmatic patients. This finding confirms and extends the work of Holroyde et al (1981) who showed that in normal subjects sodium cromoglycate had no effect on LTD_4 induced airway narrowing.

In summary, neither verapamil nor sodium cromoglycate inhibits LTD_4-induced bronchospasm in asthmatic patients. This contrasts with the action of verapamil in normal subjects.

CHAPTER 10

THE RELATIONSHIP BETWEEN *IN VIVO* RESPONSIVENESS TO LEUKOTRIENE D_4 AND *IN VITRO* SMOOTH MUSCLE SENSITIVITY, AND THE QUANTITY OF SMOOTH MUSCLE

THE RELATIONSHIP BETWEEN *IN VIVO* RESPONSIVENESS TO LEUKOTRIENE D_4 AND *IN VITRO* SMOOTH MUSCLE SENSITIVITY, AND THE QUANTITY OF SMOOTH MUSCLE

10.1 INTRODUCTION

Airway responses to inhaled methacholine and histamine do not relate to *in vitro* sensitivity of airway smooth muscle obtained at thoracotomy from the same patients. The mechanisms of hyper-responsiveness of the airways remains unexplained, but could be due to enhanced airway smooth muscle sensitivity to certain mediators (Bouchey et al 1980, Thomson 1983). Other investigations designed to examine this hypothesis have not found a relationship between measurements of *in vitro* sensitivity of human smooth muscle to methacholine (Chapter 4, Taylor et al 1985, Armour et al 1984), or histamine (Vincenc et al 1983, Armour et al 1984 Chapter 6) and *in vivo* airway responses to these agents. However, neither of these agonists is an important mediator in clinical asthma. LTD_4 may be important in the pathogenesis of asthma (Lewis & Austen 1984). LTD_4 is a potent bronchconstrictor agent both *in vivo* (Weiss et al 1983, Barnes et al 1984) and *in vitro* (Chapter 8, Dahlen et al 1980) and it is released from sensitized tissue after allergen exposure. (Orange and Austin 1980, Dahlen et al 1981). The slow onset, prolonged contraction produced by LTs have led to the hypothesis that LTs may be important mediators in asthma. This study extends previous *in vivo* and *in vitro* comparisons to leukotriene D_4. In this study *in vivo* airway responses to inhaled LTD_4 in eleven patients undergoing thoracotomy were measured and compared with recordings of *in vitro* sensitivity to LTD_4 of preparations of isolated bronchi taken from the same patients and also with the ability of smooth muscle from these patients to generate tension *in vitro*.

In previous chapters the relative quantity of muscle present was inferred from the wet weight of bronchial strips. In this chapter the amount of smooth muscle in each bronchial strip was assessed by light microscopy and the relationship with *in vivo* responsiveness, maximum tension generated, and sensitivity *in vitro* measured.

10.2 METHODS

10.2.1 *In vivo* Measurements

Eleven patients due to undergo lobectomy or pneumonectomy were studied. All patients gave informed consent. Prick skin testing was undertaken with one or more of seven common allergens.

Airways resistance (Raw) and thoracic gas volume (TGV) were measured in a constant-volume body plethysmograph (Fenyves and Gut) using a computerised data collection and analysis system (Chapter 3) based on the method of Du Bois *et al* (1956).

Leukotriene D_4 inhalation tests were carried out as previously described (Chapter 8). Buffered saline was inhaled first followed by increasing concentrations of LTD_4 (0.06 - 50 ug/ml).

10.2.2 *In vitro* Measurements

Bronchial tissue was obtained from patients undergoing lung resection for bronchial carcinoma, immediately following removal at thoracotomy. Samples from second to sixth order bronchi were dissected from macroscopically normal tissue and maintained at $4^{o}C$ overnight in oxygenated Krebs-Hensleit physiological salt solution. On the day after removal, the bronchi were dissected free of extraneous connective tissue and sectioned to produce transverse strips of tissue which were suspended (under a resting tension of 1.5 to 2.0 g) in a 5 ml organ bath containing Krebs-Hensleit solution at $37^{o}C$ bubbled with 5% CO_2 in O_2. During a 60 minute equilibration period the tissues were washed three times. Changes in isometric tension were measured using force-displacement transducers.

The reproducibility of the contractile changes of the bronchial strips was assessed by adding methacholine (1×10^{-4} moles l^{-1}) on two occasions with an interval of 30 minutes for recovery between each drug addition. A cumulative concentration-effect curve to LTD_4 was then constructed by adding increasing concentrations of the drug, each addition of the drug being made at/

at the peak of response produced by the preceding concentration. Log concentration effects were calculated for each tissue and results expressed as the concentration of LTD_4 producing a contraction 50% of maximum for that tissue. The maximum contraction for each tissue was also recorded. At the completion of the experiment the wet weight of each tissue was recorded.

The amount of smooth muscle present in each strip was then assessed by light microscopy. The pecentage of smooth muscle present, the absolute amount of muscle present in strip (wet weight x% muscle) and the maximum tension per unit mass of smooth muscle was then calculated and maximum tension was compared with the percentage of smooth muscle present for each individual bronchial strip. The average values of these *in vitro* measurements for the four bronchial strips from each patient was compared with *in vivo* measurements.

10.3 RESULTS

10.3.1 *In vivo* Results

Details of the patients who took part in the study are shown in Table 10.1. Their age ranged from 48 to 70 years. Seven patients were male and four were female. Three patients had FEV_1 recordings (expressed as a percentage of the predicted value) below 80%. One patient was atopic. All patients were current or ex-smokers and 4 patients had symptoms of chronic bronchitis. None had a history of asthma. All patients had bronchial carcinoma. One patient was on regular inhaled acebuterol prior to surgery.

Airway responsiveness to LTD_4 varied between individual patients (Table 10.2). $PC_{10}FEV_1$ ranged from 0.15 to > 50 ug/ml, $PC_{35}sGaw$ ranged from 0.18 to >50 ug/ml, and $PC_{30}\dot{V}_{30}(p)$ from 0.04 to > 50 ug/ml. There were significant correlations ($P < 0.05$) between $PC_{10}FEV_1$ and $PC_{30}\dot{V}_{30}(p)$ ($r = 0.68$), and between $PC_{35}sGaw$ and $PC_{35}sGaw$ and $PC_{30}\dot{V}_{30}(p)$ ($r = 0.72$). There was no significant correlation/

TABLE 10.1

PATIENT CHARACTERISTICS

Patient Number	Sex	Age (years)	Height (cm)	FEV_1 Litres	FEV_1 %Pred	VC %Pred	$\frac{FEV_1}{VC}$ %	RV %Pred	TLCO %Pred	Atopy	Current Smoker	Chronic Bronchitis
1	F	62	158	2.1	104	103	76	131	79	–	+	–
2	M	58	177	1.55	48	77	46	216	ND	+	+	+
3	M	63	161	2.35	99	100	70	102	ND	–	–	+
4	M	64	161	2.1	82	106	60	131	109	–	+	–
5	M	66	169	2.32	82	106	67	110	107	–	–	+
6	F	48	159	2.2	91	97	74	91	80	–	+	–
7	M	70	168	1.74	70	87	46	ND	ND	–	–	–
8	M	68	176	4.13	135	125	79	ND	ND	–	+	–
9	M	68	180	3.76	119	173	72	103	80	–	–	–
10	F	57	158	1.55	72	77	71	142	64	–	+	+
11	F	66	159	1.95	101	107	67	105	97	–	+	–

Definition of abbreviations

FEV_1 - forced expiratory volume in 1 second; VC - vital capacity; RV - residual volume;

TLCO - transfer factor for carbon monoxide; ND - not done

TABLE 10.2

IN VIVO AND IN VITRO RESPONSES TO LTD_4

Patient Number	In vivo $PC_{10}FEV_1$ (ug/ml)	In vivo $PC_{35}sGaw$ (ug/ml)	In vivo $PC_{30}\dot{V}_{30(P)}$ (ug/ml)	In vitro EC_{50} (moles x 10^{-8})	In vitro Maximum Tension (mg per gram smooth muscle)
1	1.37	24.3	0.25	1.3	445
2	0.24	0.34	0.26	0.33	191
3	0.56	3.8	0.38	0.34	259
4	0.88	1.21	0.69	0.18	114
5	0.15	0.18	0.04	2.5	255
6	0.19	2.8	0.24	0.54	824
7	>50	2.3	5.5	0.21	104
8	>50	>50	14.7	3.0	93
9	2.1	>50	>50	0.14	196
10	2.1	>50	11.0	0.81	278
11	0.79	3.7	0.35	1.2	242

Definitions of abbreviations $PC_{10}FEV_1$ - Inhaled concentration of LTD_4 producing a 10% fall in FEV_1: $PC_{35}sGaw$ - Inhaled concentration of LTD_4 producing a 35% fall in airways specific conductance: $PC_{30}\dot{V}_{30(P)}$ - Inhaled concentration of LTD_4 producing a 30% fall in $\dot{V}_{30(P)}$: EC_{50} - *in vitro* concentration of LTD_4 producing a 50% of maximum contraction.

correlation between baseline FEV_1 (percentage predicted and $PC_{10}FEV_1$ (r = 0.45), PC_{35}sGaw (r = 0.55) or $PC_{30}\dot{V}_{30}(p)$ (r = 0.45).

10.3.2 **In vitro** Results

There were differences in the responsiveness to LTD_4 of bronchial strips prepared from different patients (Table 10.2). EC_{50} values ranged from 1.8×10^{-9} to 3×10^{-8} umoles/l and the maximum tension (in mg) generated per gram of smooth muscle ranged from 93 to 824.

The tension generated by the bronchial strips (40 samples) ranged from 0.28 to 2.4 G. The percentage of smooth muscle present in the bronchial strips ranged from 1.1 to 16.4%. When the wet weight of tissue was corrected for the percentage of smooth muscle present, the absolute amount of smooth muscle present ranged from 0.54 to 13.8 mg. The absolute amount of smooth muscle present in bronchial strips correlated with the maximum tension generated (r = 0.63; $P<0.01$) but not the EC_{50} (r = -0.08; $P>0.05$). Figure 10.1.

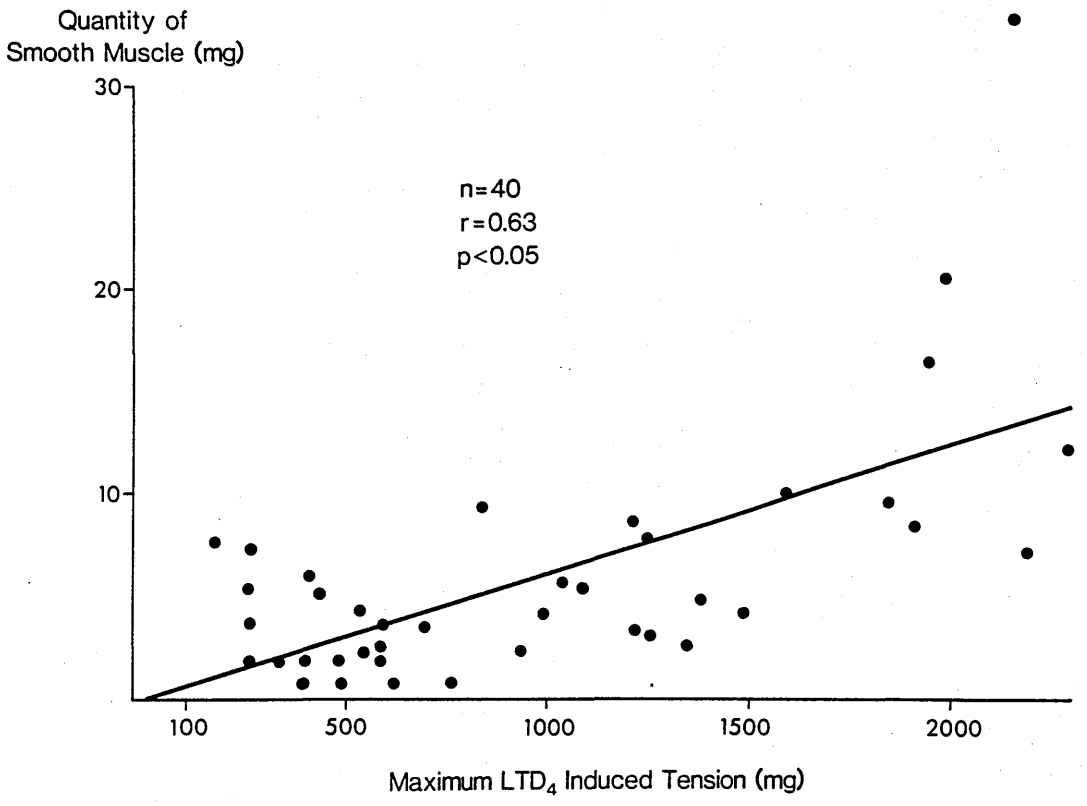
10.3.3 Comparison of **In Vivo** and **In Vitro** Responsiveness to LTD_4

There was no significant correlation between any of the measurements of *in vivo* and *in vitro* responsiveness to LTD_4. The lack of relationship is illustrated for EC_{50} against $PC_{10}FEV_1$ (Figure 10.2) (r = 0.1; $P>0.05$), PC_{35}sGaw (Figure 10.3) (r = 0.26; $P>0.05$) and $PC_{30}\dot{V}_{30}(p)$ (Figure 10.4) (r =-0.12; $P>0.05$). There was no significant relationship between maximum tension generated per mg of smooth muscle and $PC_{10}FEV_1$ (r = 0.44; $P>0.05$), PC_{35}sGaw (r = -0.17; $P>0.05$) and $PC_{30}\dot{V}_{30}(p)$ (r = 0.23; $P>0.05$). The average amount of smooth muscle present in the bronchial tissues did not correlate with any of the *in vivo* measurements of airway responsiveness.

10.4/

FIGURE 10.1

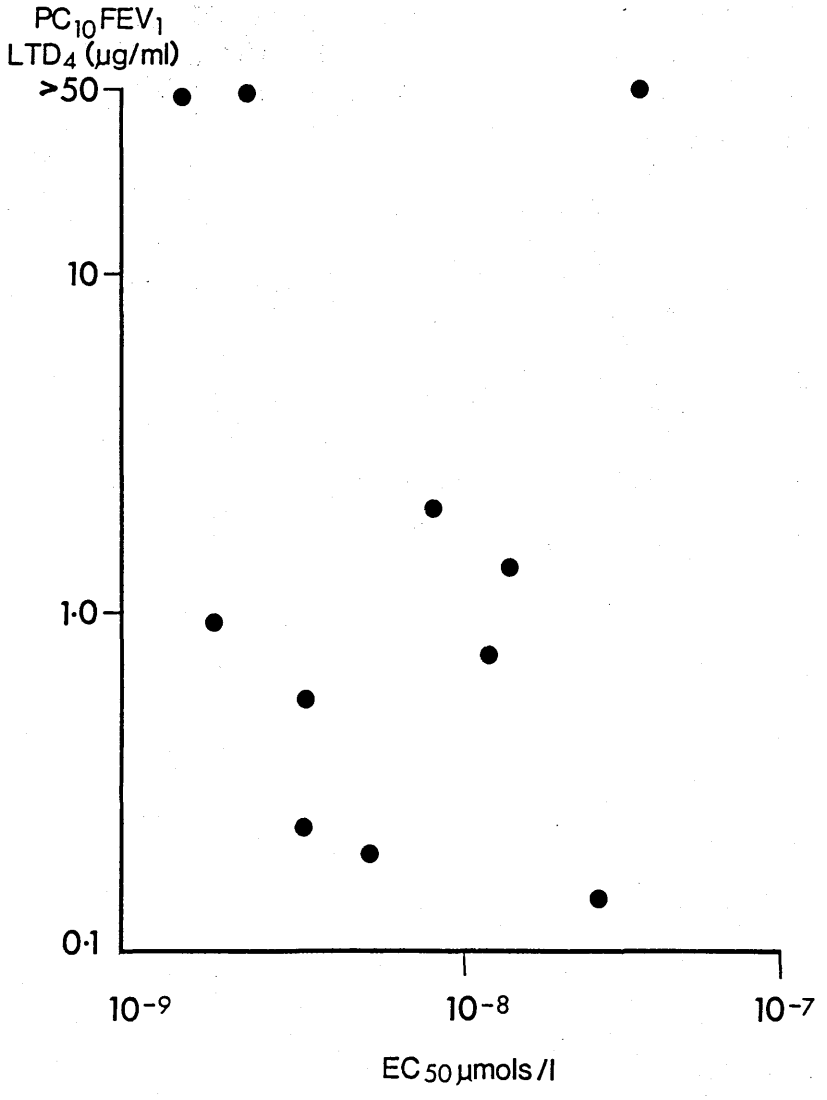
In vitro tension generation versus smooth muscle quantity



Comparison of absolute amount of smooth muscle present (mg) in each bronchial strip (n = 40) assessed by light microscopy and the maximum tension generated by each bronchial strip (in grams).

FIGURE 10.2

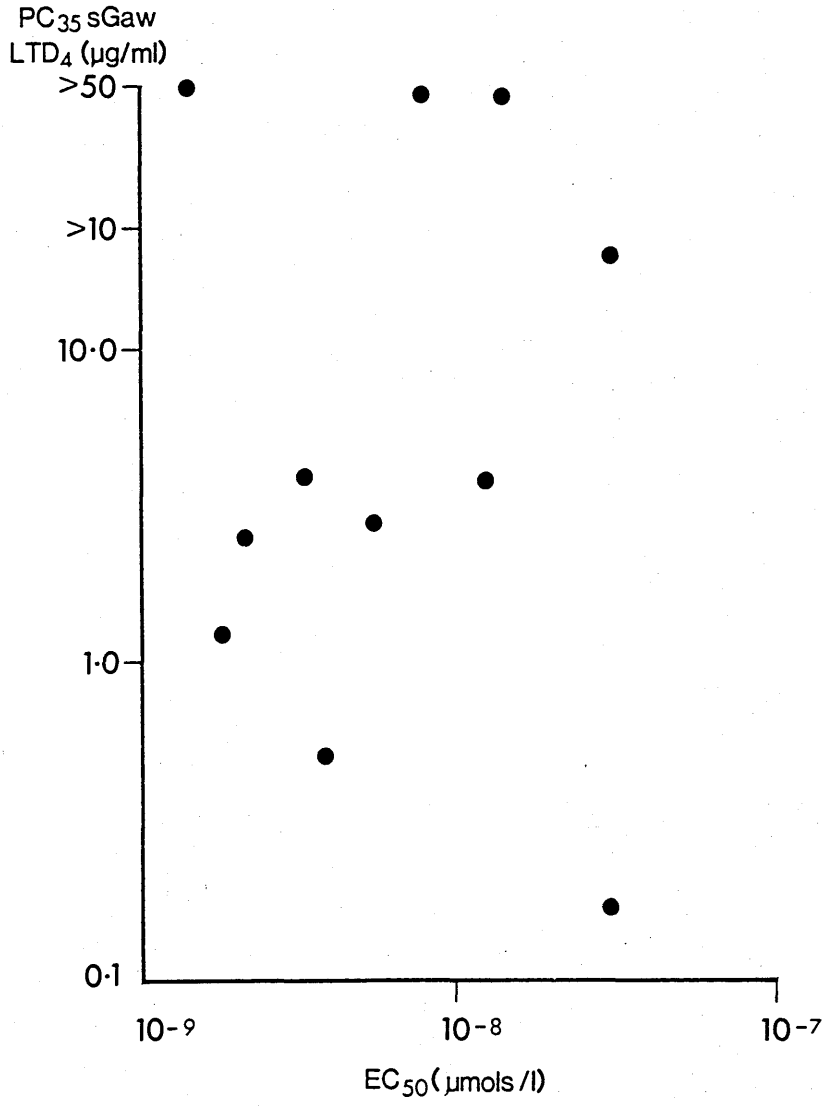
In vivo responsiveness ($PC_{10}FEV_1$) against *in vitro* sensitivity



Comparison of *in vivo* airway responsiveness to LTD_4 expressed as $PC_{10}FEV_1$ with *in vitro* smooth muscle sensitivity to LTD_4 expressed as the concentration that produced a 50% (EC_{50}) maximum contraction $r = 0.1$ $p > 0.05$.

FIGURE 10.3

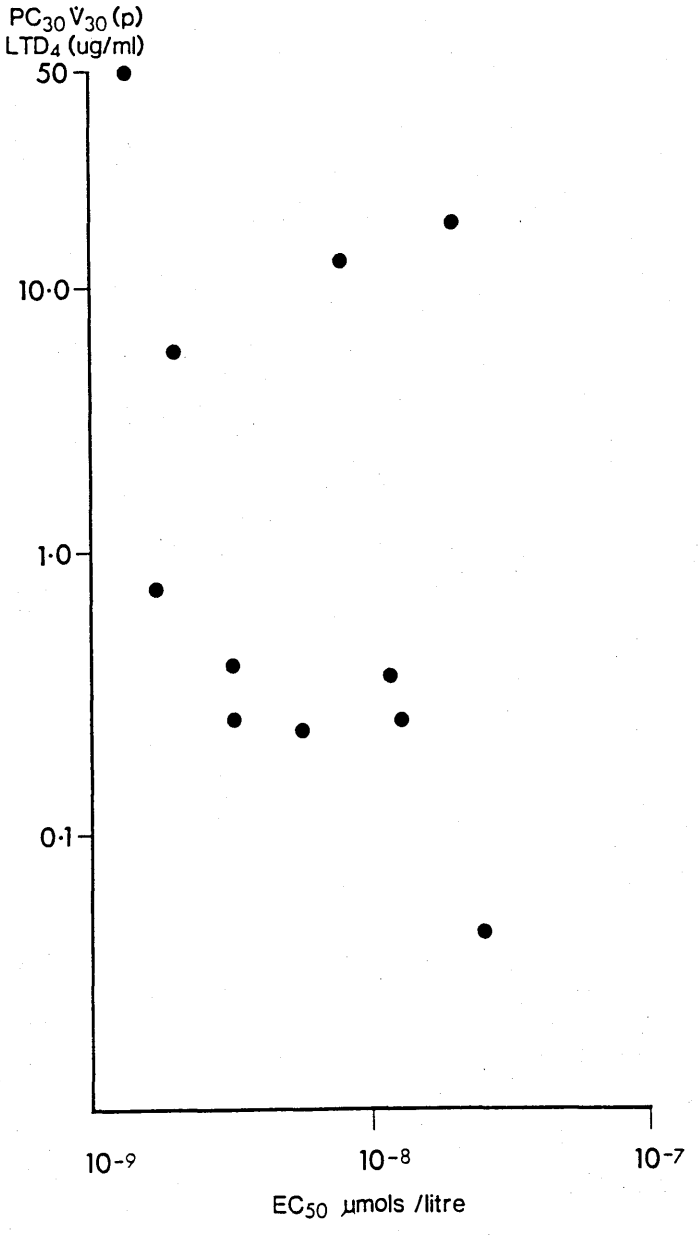
In vivo responsiveness (PC_{35}sGaw) against in vitro sensitivity.



Comparison of in vivo airway responsiveness to LTD_4 expressed as PC_{35}sGaw with in vitro smooth muscle sensitivity to LTD_4 expressed as EC_{50} r =0.26 p 0.05.

FIGURE 10.4

In vivo responsiveness ($PC_{30}\dot{V}_{30}(p)$) against in vitro sensitivity



Comparison of in vivo airway responsiveness to LTD_4 expressed as $PC_{30}V_{30}(p)$ with in vitro smooth muscle sensitivity to LTD_4 expressed as EC_{50} r = -0.12 p 0.05

10.4 DISCUSSION

These results demonstrate that measurements of *in vivo* human airway responsiveness to inhaled LTD_4 are not related to *in vitro* sensitivity to LTD_4 of isolated bronchial smooth muscle obtained from the same patients. Furthermore neither the quantity of bronchial smooth muscle present in the tissue, nor the maximum tension generated by each tissue was related to *in vivo* results. Previous studies have examined *in vivo* (Weiss et al 1983, Griffen et al 1983, Barnes et al 1981) and *in vitro* (Chapter 8, Dahlen et al 1980) responses of human airways to LTD_4 but *in vivo* and *in vitro* responses have not been measured in the same patients.

In human studies it has been reported that LTD_4 has a predominantly peripheral site of action (Weiss et al 1983, Griffen et al 1983), although others have found bronchconstriction induced by LTD_4 to affect both small and large airways (Barnes et al 1984, Smith et al 1985, Bisgaard et al 1985, Chapter 8). In this study LTD_4 produced similar degrees of change in tests thought to reflect small airway function ($V_{30(p)}$) and large airway function (sGaw). *In vitro* results reflected the sensitivity of larger airways. Thus a comparison of measurements of *in vitro* responsinveness with PC_{35}sGaw should have shown the best correlation had any existed.

The patients tested in this study were current or ex-smokers and three had evidence of airflow obstruction. As a group, airway responsiveness to inhaled LTD_4 was similar to a group of normal subjects (Chapter 8). Although the mechanism of LTD_4 induced bronchoconstriction in man is unclear a previous study in normal subjects has suggested that LTD_4 acts by a combination of direct effects on airway smooth muscle and by indirect pathways (Chapter 9). If these patients were responding to inhaled LTD_4 in a similar way to normal subjects, the presence of both direct and indirect pathways would explain the lack of correlation between *in vivo* and *in vitro* results.

An abnormality of the sensitivity of airway smooth muscle, which could produce airway hyper-responsiveness *in vivo*, would be manifest *in*/

in vitro by a lower EC_{50} value for a given agonist or by an increase in maximum agonist-induced tension. Although the quantity of smooth muscle in the bronchial strips might be expected to affect the maximum agonist induced tension, Armour and coworkers (1984) reported a weak correlation between maximum tension change in response to histamine, and the volume of smooth muscle in each bronchial strip. In this study we used the wet weight of bronchial strips together with the measured percentage of smooth muscle in each strip to calculate the absolute amount of smooth muscle present in each strip. The maximum LTD_4 induced tension positively correlated with the absolute amount of smooth muscle. Thus hypertrophy and/or hyperplasia of airway smooth muscle reported in patients with asthma (Takizawa and Thurlbeck 1971, Dunhill et al 1969, Heard and Hossman 1973) and chronic bronchitis (Mullen et al 1986, Takizawa and Thurlbeck 1971) may contribute to in vivo airway hyper-responsiveness to constrictor stimuli. However, it is unlikely that this mechanism is the only explanation for airway hyper-responsiveness. There was no relationship between in vitro measurements of airway sensitivity or the quantity of smooth muscle in the bronchial strips and in vivo measurements of airway responsiveness.

The presence of inflammatory cells and mediators may be important in the development and maintenance of non-specific airway hyper-responsiveness (Holtzmann et al 1983). Mullen and coworkers (1986) demonstrated that airway inflammation was associated with airway responsiveness in patients undergoing thoracotomy for bronchial carcinoma. The processing of bronchial smooth muscle may remove inflammatory cells and mediators which could alter responsiveness in smooth muscle strips and obscure a relationship with in vivo measurements.

None of the patients used in this study had asthma. Results obtained in these non asthmatic patients may not be applicable to patients with asthma. A few previous studies have examined the in vitro responses to bronchoconstrictor stimuli of airway smooth muscle from patients with asthma. The in vitro response to leukotrienes was shown not/

not to be increased in two asthmatics by Dahlen and coworkers (1983) and in one asthmatic by Schellenberg and Foster (1984). In vitro response to cholinergic agonists were similar in asthmatic and non-asthmatic tissue (Chapter 4). A normal in vitro responsiveness to histamine was found (Chapter 5) in a patient who had a history of asthma since childhood.

In summary these results show that in non-asthmatic patients undergoing thoracotomy, the in vivo airway responsiveness to LTD_4 is not related to in vitro airway smooth muscle responsiveness. These findings suggest that factors other than the responsiveness of airway smooth muscle alone must determine in vivo airway responsiveness. Nevertheless there was a positive correlation between the amount of smooth muscle present in bronchial strips and maximal - LTD_4 induced tension suggesting that hypertrophy and/or hyperplasia of airway smooth muscle may contribute to in vivo airway hyper-responsiveness.

C H A P T E R 11

THE EFFECT OF PASSIVE SENSITISATION OF HUMAN BRONCHIAL SMOOTH MUSCLE ON *IN VITRO* SENSITIVITY TO HISTAMINE

THE EFFECT OF PASSIVE SENSITISATION OF HUMAN BRONCHIAL SMOOTH MUSCLE ON *IN VITRO* SENSITIVITY TO HISTAMINE

11.1 INTRODUCTION

Atopy is closely associated with asthma and in sensitised subjects antigen exposure may precipitate acute bronchospasm and increase non-specific bronchial responsiveness. Bronchial hyper-responsiveness is an important feature of asthma. Indeed, some authorities suggest it is a prerequisite.

Increased non-specific responsiveness is related to the late asthmatic response (Cartier et al 1982). If the late response is prevented, the responsiveness of the airway does not change. There are two likely mechanisms for the alteration in airway responsiveness. Mediators released from mast cells may modify the sensitivity of airway smooth muscle or, by chemotactic activity, may attract inflammatory cells which alter the airway, either physically or by the release of secondary mediators. For instance, proteins released from eosinophils can damage epithelial surfaces (Gleich et al 1979) and may increase mediator access to receptors.

In this study, the effect of specific allergen challenge on passively sensitised preparation of human bronchi on their *in vitro* responsiveness to histamine has been investigated.

11.2 METHODS

11.2.1 Sensitising Serum

Sera from atopic asthmatic patients was screened for *in vitro* sensitising activity. A 33 year old male asthmatic patient with positive skin test and elevated specific IgE levels against Dermatophagoides pterronissinus gave serum which was found to be effective. 50 ml of serum was obtained from this subject and was divided into aliquots and maintained at $-40^{o}C$ until use.

11.2.2 Sensitisation procedure

Samples of bronchi were obtained at thoracotomy from six patients undergoing thoracotomy for bronchial carcinoma. From each/

each patient four bronchial rings were dissected from macroscopically normal tissue and washed x 3 in Krebs-Hensleit saline (bubbled with 95% O_2/5% CO_2). From each patient the bronchial rings were treated as follows -

Two rings (Nos 1 and 2) were incubated overnight at room temperature (20°C) in bubbled Krebs-Hensleit solution containing a 10% solution of the above serum. Rings 3 and 4 were also incubated (at 20°C) overnight in oxygenated Krebs-Hensleit saline containing 10% control serum (from a non-atopic, non-asthmatic subject (me)).

TABLE 11.1

PROTOCOL

Tissue	1	2	3	4
Sensitisation	-	-	+	+
Antigen added	-	+	-	+
Histamine dose-response	+	+	+	+
Antigen added	+	+	+	+

11.2.3 Tension measurement

The next day the tissue rings were divided to produce strips of tissue and hung in an organ bath in Krebs-Hensleit solution aerated by 95% O_2/5%CO_2 (as previously described) attached to an isometric force transducer and chart recorder under a resting tension of 1.5 -2.0 grammes. Tissues were allowed to equilibrate for 1 hour. During this period the organ bath fluid was changed three times. After equilibration house dust mite (HDM) allergen extract (Pharmacia) was added to tissues 2 and 4 and control diluent/

diluent solution to tissues 1 and 3. Tissues were rinsed in fresh saline 20, 40 and 60 mins after allergen challenge. Once tension had returned to the resting value, a histamine concentration-response curve was performed. Increasing concentrations of histamine, 10^{-9} - 10^{-4} moles/litre were added to the baths. Each concentration was not added until the response to the previous concentration had plateaued.

At the conclusion of the experiment HDM solution 0.1ml was added to all 4 organ baths and the presence or absence of response noted (Table 11.1).

11.3 RESULTS

All sensitised tissues contracted when exposed to HDM solution and no unsensitised tissue contracted (Figure 11.1) Contraction following antigen took 12 $\pm$ 4 mins to reach maximum and relaxation took 85$\pm$ 40 minutes (m $\pm$ SD). EC_{50} values ranged from 3.2×10^{-7} to 5.3×10^{-6} mM/litre (Table 11.2). The mean EC_{50} for unsensitised tissues was $(2.09 \pm 0.69) \times 10^{-6}$ and for sensitised tissue $(1.88 \pm 0.48) \times 10^{-6}$ (NS). For tissue to which antigen was added before histamine concentration-effect curve the mean values were $(1.92 \pm 0.32) \times 10^{-6}$ and $2.55 \pm 0.47 \times 10^{-6}$ for sensitised tissue (NS).

The maximum tension per gram wet weight tissue m $\pm$ SEM were as follows. For unsensitised tissue not exposed to antigen 13.6 $\pm$ 5.2 and exposed to antigen 18.4 $\pm$ 8.2 and for sensitised tissue 15.0 $\pm$ 2.9 without HDM exposure and 15.8 $\pm$ 6.5 with HDM exposure. None of these differences was significant.

11.4 DISCUSSION

This study has demonstrated that serum from an atopic asthmatic will passively sensitise human bronchial smooth muscle such that exposure of the bronchial smooth muscle to a solution of the specific antigen produces a rapid onset and sustained smooth muscle contraction. Sensitisation of the bronchial muscle does not alter *in vitro* sensitivity/

TABLE 11.2

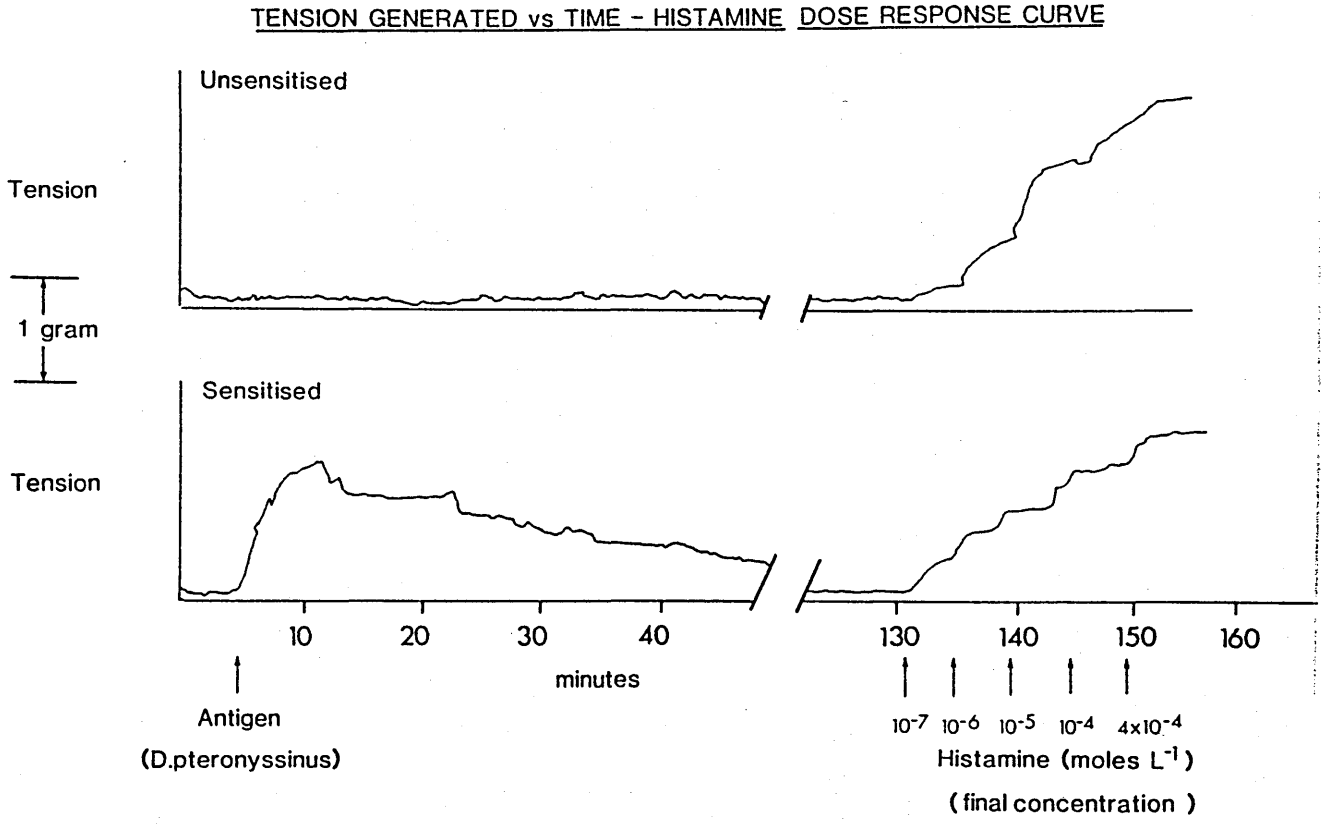
In vitro sensitivity of tissues from six patients

EC_{50} histamine (10^{-6} moles/litre)

	Unsensitised		Sensitised	
	No Ag	+ Ag	No Ag	+ Ag
Patient	1	2	3	4
1	2.3	2.3	4.2	4.4
2	1.1	1.4	1.3	3.2
3	0.46	1.0	0.85	1.0
4	5.3	3.2	1.7	2.6
5	1.6	2.1	1.85	2.1
6	1.8	1.5	1.4	2.0
Mean	2.09	1.92	1.88	2.55
SEM	0.69	0.32	0.48	0.42
	NS		NS	

FIGURE 11.1

Tissue response to allergen followed by concentration-response curve to histamine.



Typical polygraph tracings from unsensitised and sensitised tissue. All sensitised tissue contracted on exposure to antigen, either before (shown above) or after (not shown) histamine concentration-response curve.

sensitivity to methacholine nor the maximum in vitro response of the muscle. Antigen challenge of sensitised tissue produces a sustained contraction but does not alter the sensitivity of the smooth muscle to subsequent histamine exposure.

Other workers have passively sensitised bronchial smooth muscle (Sheard et al 1967, Adams and Lichstensten 1979) and have found that allergen exposure of passively sensitised bronchial tissue produces smooth muscle contraction and causes release of mediators such as histamine (Sheard et al 1967), SRS-A (Sheard et al 1967) and prostaglandins (Murray et al 1986). It has been suggested that this release of mediators may be a factor in the development of bronchial hyper-reactivity (Cockcroft et al 1977, Cartier et al 1982). There are two mechanisms which might cause this increase.

One or several of the mast cell factors could be acting on the airway smooth muscle to increase its sensitivity to agonists. As bronchial hyperresponsiveness is non-specific it would be likely that the alteration would not be on receptor density or sensitivity, but in the contractile mechanism of the smooth muscle cell. These results do not support this hypothesis.

However, the bronchial strips were exposed to mediators for 20 minutes. They were then washed to bring tension back to baseline values. It is possible that mediator induced alteration in smooth muscle could take longer than 20 minutes to occur. Another method by which mediator release could affect smooth muscle responses, is by acting as chemotactic factors (Schemkel et al 1982 O'Driscoll et al 1983) provoking an influx of inflammatory cells to the respiratory tissue. These inflammatory cells could then produce secondary mediators which increase smooth muscle sensitivity. The protocol used in this experiment does not test this hypothesis, but with the system used in this study it would be possible to investigate whether the addition of white blood cells, either as whole blood or as separated cells to antigen exposed passively sensitised smooth muscle, altered in vitro sensitivity.

CHAPTER 12

CONCLUSIONS

IN VIVO VERSUS IN VITRO STUDIES

CONCLUSIONS
IN VIVO VERSUS IN VITRO STUDIES

12.1 INTRODUCTION

This project has compared *in vivo* human airway responsiveness and the sensitivity of smooth muscle obtained from the same patients at thoracotomy. Under the experimental conditions used, no relationship was found. Possible confounding factors were minimised by the design of the trials.

12.2 TRIAL DESIGN

12.2.1 *In vivo*

In vivo results could be affected by factors other than smooth muscle sensitivity. Aerosol deposition varies with disease state of the lung. Patients with small airway disease deposit a greater proportion of nebulised aerosol in the large airways. (Ryan et al 1981). This could lead to an apparent increased responsiveness. Three separate measures of airway responsiveness were used. FEV_1 which reflects overall airway function, sGaw, which reflects mainly large airway calibre (Pride 1971) and $\dot{V}_{30}(p)$ which is measured at 30% of TLC and hence is thought to reflect mainly small airway calibre. Thus if patients tested have small airways disease, sGaw would be the measure of *in vivo* responsiveness to use when comparing responsiveness with *in vitro* sensitivity. However, this will not take account of differences in the pattern of deposition between patients.

Airway permeability could also confound a possible relationship, affecting delivery of the agonist to its site of action. However although alterations of airway mucosal permeability have been demonstrated in smokers (Jones et al 1980), these changes do not relate to variations in airway responsiveness (Kennedy et al 1983).

In vitro a full concentration-response curve is performed and the estimate of muscle sensitivity made from the steep region of the/

the resulting graph (EC_{50}). It is not possible to perform an equivalent complete concentration-response curve *in vivo*. It is therefore possible that *in vivo* measurements are being made on the early part of the curve, where results are less reproducible. This did not appear to be the case in the sGaw measurements performed in this study. Profound falls in sGaw had often occurred before a 20% fall in FEV_1 was reached. In many cases the fall in sGaw appeared to plateau. Another approach taken to counteract this problem was to use the EC_{20} (i.e. a 20% of maximum response *in vitro*) for comparison with *in vitro* results. This did not improve the relationship between results.

12.2.2 *In vitro*

Measurement of *in vitro* responses of smooth muscle may be affected by many variables. These were minimised by the design of *in vitro* protocol.

Intra-operative medications, if present, could modify *in vitro* responses. All tissues were washed thoroughly and maintained overnight in physiological saline. This treatment has been shown to remove intra-operative medications (Clark 1926) but does not alter *in vitro* sensitivity (Thulesius et al 1978). Temperature may also alter responses (Black et al 1984) as may changes in pH (Twort and Cameron 1986). Both parameters were carefully controlled in the organ bath used.

12.2.3 *In vivo - in vitro comparison*

The agonists used in the *in vivo* versus *in vitro* protocol all have several physiological actions besides an action on airway smooth muscle. Methacholine affects muscarinic receptors which are found on submucosal glands (Nadel 1981), mast cells (Kaliner et al 1972) and presynaptic region of sympathetic nerves (Westfall 1980) as well as airway smooth muscle (Hawkins and Schild 1951). Any of these/

these other actions which would effect in vivo but not in vitro results could confound a relationship if present. The second mediator used, histamine, has a direct effect on airway smooth muscle (Nogrady and Bevan 1978, Thomson and Kerr, 1980) but also may induce bronchconstriction via a vagal reflex (White and Eiser 1983). However, the lack of relationship between in vivo and in vitro measures was not improved by pretreating a group of patients with atropine.

The third part of the in vivo versus in vitro study used LTD_4 as a mediator. Recent work has suggested that the leukotrienes may be important mediators in asthma (Barnes et al 1984, Bisgaard 1985) and animal work has shown that LTD_4 increased in vitro airway smooth muscle sensitivity (Creese and Bach 1983).

The mode of action of LTD_4 is unknown. Investigation of LTD_4 responses in this project suggested that, in normal subjects, LTD_4 induced bronchoconstriction was due to a combination of a direct effect on smooth muscle, and an indirect, verapamil sensitive mechanism. There was no relationship between in vivo responses and in vitro sensitivity. Quantification of smooth muscle present in bronchial strip demonstrated wide variations in the proportion of muscle present. The quantity of muscle related to maximum tension generated in vitro, but did not correlate with any of the measures of in vitro responsiveness.

12.3 ASTHMA VERSUS CHORNIC AIRFLOW OBSTRUCTION

Bronchial hyper-responsiveness is closely associated with, and may be a pre-requisite of, asthma (Hargreave et al 1981).

Increased responsiveness also occurs in chronic airflow obstruction but may not be due to the same mechanisms as those found in asthma (Du Toit et al 1986).

Baseline airway calibre (Mullen et al 1986) and cigarette smoking (Du Toit 1986) have both been shown to be factors in determining responsiveness in patients with chronic airflow obatruction/

obstruction but are obviously less important in asthma.

Most of the patients who took part in this study were current or ex smokers, and many had evidence of irreversible airflow obstruction. Thus it is not possible to be categorical in dismissing smooth muscle hypersensitivity in the pathogenesis of asthma. However, two patients included in these studies were asthmatic. This was confirmed by increased responsiveness of *in vivo* measurements. Neither demonstrated increased *in vitro* sensitivity.

12.4 OTHER REPORTS OF IN VITRO ASTHMATIC AIRWAYS

Of the few reports in the literature where bronchial smooth muscle from asthmatic patients has been assessed for *in vitro* sensitivity, only one showed an increase when compared with tissue from non-asthmatic subjects. Dahlen et al (1983) examined bronchial strips from two birch pollen allergic asthmatics and found no increase in smooth muscle sensitivity to histamine or leukotriene D_4.

Patterson et al (1982) studied post mortem tissue from 3 asthmatic patients who died during acute attacks and found no increase in sensitivity to carbachol or histamine.

Schnellenberg and Foster (1984) did demonstrate an increased sensitivity to histamine, but not methacholine or LTD_4 in an asthmatic patient who was operated on for removal of a carcinoid tumour. The presence of a histamine secreting tumour makes this finding difficult to interpret. Thus, the limited data, available from this project, and other published cases does not support the hypothesis that hyper-responsiveness is a primary bronchial smooth muscle abnormality.

12.5 OTHER PUBLISHED IN VIVO AND IN VITRO COMPARISONS

Since this project was commenced several other groups have compared *in vivo* airway responsiveness with *in vitro* smooth muscle sensitivity. Vincenc et al (1983) used histamine as agonist. They measured FEV_1 as an index/

index of *in vivo* response but only obtained $PC_{20}FEV_1$ values in 5 out of 14 patients. No relationship was found between *in vivo* and *in vitro* parameters. Armour et al (1984) confirmed the lack of relationship using histamine as agonist. A similar lack of relationship has also been found using methacholine as agonist (Armour et al 1984, Taylor et al 1985). A recent paper by De Jongste et al (1987) found increased histamine sensitivity in smooth muscle obtained from patients with CAO compared to a group with normal lung function. Patients were divided into the two groups on the basis of *in vivo* spirometry.

In vitro tissue was then assessed by exposure to methacholine, histamine, and LTC_4. The tissue from CAO patients had an increased maximum response (per dry wt of tissue), compared to the non CAO patients. Their results are puzzling in that the maximum tension produced to LTC_4 and methacholine was similar for both groups. The histamine maximum were less than that produced by LTC_4 and methacholine suggesting a reduced response in the normal subjects. This may be due to a decreased number of histamine receptors in the generation of bronchi chosen (10th to 13th) but further investigation is required before true increased sensitivity is demonstrated. The same group have also reported that tissue from an asthmatic subject was more contractile than control specimens. The main difference was in maximum response rather than increased sensitivity. Cerrina *et al* (1986) found no evidence of increased smooth muscle responses *in vitro* in asthmatic patients but did find reduced responses to a B2 agonist. The asthmatic patients in this study were poorly characterised. The result is puzzling as B blockers do not alter *in vivo* NSBR. However it is an interesting possibility that the abnormality in asthma is a failure to respond chemicals to bronchodilator rather than an increased sensitivity to bronchoconstrictor agents. More work is required to further investigate this possibility.

The bulk of evidence to date suggests that *in vitro* tissue from patients with increased NSBR is no different from that from non responsive subjects. The data is fragmentary because it is unusual for patients with asthma to require thoracotomy as almost all are non-smokers/

smokers.

Thus apart from the work of De Jongste and coworkers, there is general agreement in the literature that airway smooth muscle is not the sole determinant of airway responsiveness.

12.6 FUTURE WORK

Although airway smooth muscle is not a major determinant of hyper-responsiveness, it clearly is an important part of the airway response. Future research will look at changes to the *in vitro* environment which might increase *in vitro* sensitivity. Neither sensitisation per se nor subsequent specific allergen challenge altered sensitivity to histamine. However, the current view of airway response to allergen is of a secondary influx of inflammatory cells stimulated by chemotactic factors released by the primary response. Addition of plasma factors and leukocytes to the organ bath might allow an *in vitro* inflammatory response to occur in the bronchial tissue. It would be of great interest to see whether this modified the *in vitro* sensitivity of airway smooth muscle.

12.7 MECHANISM OF ACTION OF LTD_4

Verapamil was used as a probe to assess whether extracellular calcium influx through voltage operated calcium channels was important in the production of LTD_4 induced bronchoconstriction. Verapamil inhibited *in vivo* but not *in vitro* constriction in normal subjects, but did not affect LTD_4 induced bronchconstriction in asthmatic patients. Thus a clear difference in the responses of normal and asthmatic subjects to verapamil was demonstrated. Clear differences between the normal and asthmatic response are of interest as they suggest fundamental differences between the two states. There are several possible explanations for this observed difference. Two groups of LTD_4 receptors have been demonstrated in human airway (Lewis and Austen 1983). One a high affinity, and the other a low affinity receptor. If verapamil were inhibiting/

inhibiting the action of the low affinity receptor its effect might only be seen in normal subjects, who were able to tolerate higher concentrations of LTD_4.

LTD_4 may be acting directly on smooth muscle receptors in asthmatic patients, and by both a direct action and via indirect verapamil sensitive mechanisms, e.g. neural reflexes. A third, and intriguing possibility is that verapamil could be stimulating the release of an epithelial cell relaxant factor from intact mucosa.

Epithelial factors which modulate airway responses have been demonstrated in animal models of asthma (Flavahan et al 1985). The action of verapamil on isolated bronchial preparations is reduced by damage to epithelium (Raeburn et al 1986). As epithelium is damaged even in mild asthma (Laitinen et al 1986), this may explain the observations described above. Further work characterising the nature of epithelial derived factors is necessary.

12.8 ULTRASTRUCTURAL STUDIES

There are few studies of the ultrastructure of human airway (Daniel et al 1980, Daniel et al 1986) and none of these have examined differences between normal and hyper-responsive airways. Classification and quantification of ultrastructural features of airway is now practical. This will allow comparison of normal and hyper-reactive airway. It will also be possible to assess whether *in vitro* changes made to smooth muscle will alter its sensitivity and ultrastructure, e.g. increase the numbers of gap junctions present.

In conclusion, this project has demonstrated that differences in human airway responsiveness cannot be explained simply in terms of different smooth muscle sensitivity. *In vitro* smooth muscle research holds many exciting possibilities for the future in investigating factors which change smooth muscle sensitivity, and in time, in developing an *in vitro* model for human bronchial hyper-responsiveness.

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APPENDIX 1

RESPIRATORY QUESTIONNAIRE

QUESTIONNAIRE ON RESPIRATORY SYMPTOMS

Hospital No. ..... Date of Interview .....

Name ..... Date of Birth .....
(surname)

..... Sex ..... Phone No. .....
(First names)

s m w

Address ..... Civil state

..... Standard Height .....

..... Weight .....

..... Occupation .....

..... Race .....

Name of Interviewer .....

Diagnosis .....

COUGH

1a Do you usually cough first thing in the morning (on getting up*)?

(count a cough with first smoke or on first going out of doors. Exclude clearing throat or a single cough).

Yes/No

1b If yes - Is this worse in the Winter? Yes/No

2a Do you usually cough during the day -or at night (Ignore an occasional cough). Yes/No

2b If yes - are symptoms worse during winter? Yes/No

If No to both questions go to 4

If Yes:

3. Do you cough like this on most days (or nights*) for as much as 3 months each year?

Yes/No

PHLEGM(SPUTUM

4a Do you usually bring up any phlegm from your chest first thing in the morning (on getting up*)?

Yes/No

(Count phlegm with the first smoke or on first going out of doors. Exclude phlegm from the nose. Count swallowed phlegm).

4b If yes - Is it worse in the Winter? Yes/No

5a Do you usually bring up any phlegm from your chest during the day or night?

Yes/No

5b If yes - Is it worse in the Winter? Yes/No

5a Do you bring up phlegm like this on most days (or nights*) for as much as 3

* For subjects who work by night

TOBACCO SMOKING

33. Are you smoking now? Yes/No

if 'Yes' i) Cigarettes/Pipe/Cigars .....

ii) How many (No.) or How much (oz. tobacco) .....

iii) If cigarettes - Low/High tar

- Filter tipped .....

iv) Age of starting smoking? .....

If 'No': Have you smoked previously? Yes/No

i) Cigarettes/Pipe/Cigars .....

ii) How many (No.) or How much (oz.) .....

iii) When stopped .....

OCCUPATION

34. What is your job? .....

How long have you been doing it? .....

Previous occupations (with duration):

Have you ever worked in a dusty atmosphere? Yes/No

Have you ever given up a job for health reasons? Yes/No

FAMILY HISTORY

35.

	FATHER	MOTHER	Siblings 1	2	
Chronic bronchitis					
Emphysema					
Asthma					
Lung Cancer					
Other Chest Conditions					
Alive?					
Age?					
If 'No' - cause of death?					

MEDICATION

36. List all pills amd medicines taken over past month:

36a. List current medication:

37. How is your home heated? Coal/gas/electricity/other.

38. Do you drink alcohol? Yes/No

If 'yes' i) In what form?
ii) How much (per day)?
iii) How often?

LABORATORY TESTS

NAME: .....

HOSPITAL NO.: .....

FBC ☐

Hb ☐
HCT
Wbc
Eosinophil count

Sputum ☐

Eosinophil count ☐

Most recent culture result .....

Date .....

Skin Testing ☐ ☐

Allergen	Wheal Diameter
Control	
House Dust	
Dermatophagoids pteronyssinus	
Cat	
Dog	
Feathers	
Grass pollen	
Aspergillus fumigatus	

IgE ☐

Total ☐

Specific

Carboxyhaemoglobin (No smoking for 12 hrs) ☐ ☐

Routine PFTs ☐ ☐

☐ Tick box when test carried out

6a as 3 months each year? Yes/No

If yes - go to 6b

6b What colour is your phlegm? Grey/White
Green/Yellow

7a In the past 3 years have you had a period of (increased*) cough and phlegm lasting for 3 weeks or more?

Yes/No

If no - go to question 8

if yes:

7b/c Have you had more than one such period. If so how many? Yes .....

* For subjects who usually have phlegm.

8a Have you ever coughed up blood? Yes/No

Details
Fresh/altered
Volume e.g. streaks/teaspoon/egg cup
Frequency .....
Duration .....
When .....

BREATHLESSNESS

9a Are you troubled by shortness of breath when hurrying on level ground or walking up a slight hill?

Yes/No

9b Do you get short of breath walking with other people of your own age on level ground?

Yes/No

If no - go to question 10a
If yes:

9c Do you have to stop for breath when walking at your own pace on level ground?

Yes/No

9d If yes - how far can you walk before having to stop? .....

9e Are you breathless on dressing? .....

WHEEZING

10a Does your chest ever sound wheezing or whistling? Yes/No

If no - go to question 11a
If yes:

10b Do you get this most days -or nights? Yes/No

10c Is wheezing worse at night? Yes/No

10d Does wheezing occur after exercise? Yes/No

10e Are you allergic to aspirin? If yes - what are your symptoms?

11a Have you ever had attacks of shortness of breath with wheezing? Yes/No

If no - go to question 12
If yes:

11b Is/was your breathing absolutely normal between attacks? Yes/No

WEATHER

12. Does the weather affect your chest? Yes/No
(Only record Yes if adverse weather definitely and regularly causes chest symptoms).

If no - go to question 14.
If yes:

13a Does the weather make you short of breath? Yes/No

13b Specify type of weather e.g. fog, damp, cold, heat or other .....

HAY FEVER (ALLERGIC RHINITIS)

14. Do you suffer from hay fever? Yes/No
" " " " summer sneezing? Yes/No

If yes - age at onset .....

15. Do you have non-seasonal nasal symptoms? Yes/No

Describe them:

If yes - age at onset? .....

16a Have you ever had eczema? Yes/No

Age at onset .....

Age at end .....

16b Any other Allergies?

16c. Any pets at home?

CHEST ILLNESSES

17 During the past three years have you had any chest illness which has kept you from your usual activities for as much as a week? Yes/No

If no - go to question 20
If yes:

18 Did you bring up more phlegm than usual in any of these illnesses? Yes/No

If no - go to question 20
If yes:

19 How many illnesses like this have you had in the past 3 years? .....

HAVE YOU EVER HAD:

20 Any injury or operation affecting your chest? Yes/No

21 Heart trouble/Angina? Yes/No

If yes i) confirmed by Doctor? Yes/No

ii) Age at first diagnosis? .....

22. Bronchitis? Yes/No

If 'yes' i) Confirmed by Doctor? Yes/No
ii) Age at first diagnosis? .....

23. Pneumonia? Yes/No

If 'Yes' i) Confirmed by Doctor? Yes/No
ii) When did you have it? .....

24. Pleurisy? Yes/No

If 'Yes' i) Confirmed by Doctor? Yes/No
ii) When did you have it? .....

25. Pulmonary tuberculosis? Yes/No

If 'Yes' i) Confirmed by Doctor? Yes/No
ii) When did you have it? .....

26. Bronchial asthma? Yes/No

If 'Yes' i) Confirmed by doctor? Yes/No
ii) When diagnosed? .....
iii) Date of last attack .....

27. Emphysema? Yes/No

If 'Yes' i) Confirmed by doctor Yes/No
ii) When diagnosed .....

28. Bronchiectasis? Yes/No

If 'Yes' i) Confirmed by Dcotor? Yes/No
ii) When diagnosed .....

29. Other chest trouble? Yes/No

If 'Yes' i) What? .....
ii) Diagnosed by doctor? Yes/No
iii) When Diagnosed? .....

30. Any arthritis? Yes/No

If 'Yes' i) Diagnosis? .....
ii) When diagnosed? .....

31. Any lung problems before the age of 16 Yes/No

If 'Yes' i) What was diagnosis? .....
ii)Confirmed by doctor? Yes/No

32. Any high blood/pressure/hypertension Yes/No

If'Yes' i) When diagnosed? .....

APPENDIX 2

TABLES OF RESULTS

TABLE 4.2

SURGICAL PATIENTS

Change in FEV_1 after methacholine inhalation (mg/ml)

Patient \ Methacholine Concentration	Pre	0	2	4	8	16	32	64	128	$PC_{20}FEV_1$ (mg/ml)
1.	1.35	1.40	2.35	1.55	1.50	1.65				
2.	1.80	1.90	1.20							0.86
3.	1.95	2.0	1.90	1.85	1.70	1.45				10.6
4.	2.3	2.35	2.28	2.28	1.93	1.90	1.83	1.78		29.2
5.	2.25	2.28	2.10	1.85	1.85	1.45				4.1
6.	1.41	1.38	1.40	1.20	1.0	0.95				5.85
7.	1.28	1.27	1.21	1.14	1.08	1.06	1.04			61.3
8.	1.15	0.99	0.92	0.64	0.45	0.32				6.0
9.	2.38	2.37	2.28	2.22	2.02	1.90				16.5
10.	2.43	2.40	2.50	2.38	2.33	2.18	2.13	1.90	2.0	56.1

TABLE 4.3

SURGICAL PATIENTS

Change in sGaw following methacholine inhalation

sGaw $S^{-1}kPa^{-1}$ / No.	Pre	0	2	4	8	16	32	64	128	PC_{35}sGaw (mg/ml)
1.	1.16	1.07	1.23	1.07	0.81	0.80				16.5
2.	0.42	0.41	0.14							1.27
3.	1.61	1.19	1.53	0.95	0.64	0.63				4.7
4.	3.9	5.2	1.2	0.59	0.50	0.45	0.41	0.31		.093
5.	0.48	0.78	0.78	0.42	0.25	0.26				5.0
6.	0.69	0.82	0.71	0.55	0.53	0.45				11.2
7.	0.84	0.65	0.43	0.43	0.38	0.33	0.35			1.2
8.	1.9	1.75	1.75	1.67	1.52	1.37				8.2
9.	0.82	1.05	0.94	0.67	0.41	0.32				4.7
10.	2.46	4.10	1.93	1.40	0.89	0.70	0.62	0.51	0.55	1.2

TABLE 4.4

SURGICAL PATIENTS

Change in $V_{30}(p)$ (litres sec^{-1}) after methacholine inhalation

Patient \ Methacholine Concentration	Pre	0	2	4	8	16	32	64	128	$PC_{30}V_{30}(p)$ (mg/ml)
1.	1.26	0.95	0.90	0.75	0.80	0.50				12.2
2.	0.73	0.75	0.30							0.6
3.	0.99	1.3	1.1	1.2	0.58					6.7
4.	1.74	2.18	0.95	1.10	0.80	0.55	0.35	0.60		0.9
5.	0.97	1.05	0.85	0.50	0.25	0.10				3.1
6.	1.40	1.50	1.20	0.45	0.35	0.35				2.4
7.	1.05	1.02	0.62	0.45	0.35	0.28				1.3
8.	0.94	0.85	0.85	0.70	0.25	0.20				4.8
9.	1.58	1.85	1.75	1.10	0.75	0.60				4.0
10.	1.68	1.70	2.03	1.28	1.02	0.75	0.88	0.48	0.32	6.5

SURGICAL PATIENTS - CHAPTER 4

Summary of **in vivo** methacholine challenge results

Patient	No.	PC methacholine mg/ml			
		$PC_{20}FEV_1$	$PC_{35}sGaw$	$PC_{35}\dot{V}35(p)$	$PC_{35}\dot{V}35(c)$
JA	1	-	16.5	12.2	5.7
DMcK	2	0.86	1.3	0.6	0.6
MW	3	10.6	4.7	6.7	5.9
WT	4	29.2	0.9	0.9	1.9
AF	5	10.4	5.0	3.1	3.0
MK	6	5.9	11.2	2.4	2.4
ly	7	61.3	1.2	1.3	5.9
AD	8	8.2	6.0	4.8	5.5
DE	9	16.5	4.7	4.0	6.8
GA	10	56.1	1.2	6.5	11.9

TABLE 4.6

Tension produced (% max) for *in vitro* bronchial strips for each concentration of methacholine – bronchial strips from surgical patients

Patient	1				2			3		4			5				6				7					8					9			10			
Tissue																																				
Methacholine concentration	1	2	3	4	1	2	3	3	4	1	2	3	1	3	4	5	1	2	3	4	1	2	3	4	5	1	2	3	4	5	1	1	2	3	4	
10-1	0	4	3	2	0	0	0	0	0	0	0	0	0	10.3	0	0	0	0	0	21.1	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
10-8	0	9	10	4	0	0	0	15.8	6.9	0	0	0	12	17.9	5.6	6.6	5.3	0	0	26.3	25	5.3	28.6	0	0	1.3	0	0	0	0	0	0	0	0	0	
10-7	5	26	20	10	5.8	10	6.5	42.1	34.5	2.2	7.1	0	30	28.2	333	33	15.8	11.1	0	42.1	45	15.8	28.6	33.3	40	2.6	1.5	1.8	5.2	7.9	0	0	0	42.3	45.8	32.1	33.3	2
10-6	18	61	63	47	23.5	30	25.8	73.7	72.4	22.2	38.5	17.4	55	46.2	66.7	67	63.2	22.2	28.6	47.4	70	47.4	71.4	66.7	60	17.1	10.3	24.3	31	368	319	36	10.5	51.7	62.5	57.1	62.5	5
10-5	86	100	100	98	64.7	70	61.3	89.5	86.2	44.4	74.4	65.2	87	79.5	91.1	100	89.5	444	64.3	57.9	100	79	100	88.9	100	72.4	61.8	66.7	69	789	70.2	92	44.2	78.8	87.5	82.1	83.3	8
10-4	100	100	100	100	94.1	100	96.8	100	96.6	91.1	100	91.3	100	97.4	100	100	100	83.3	85.7	84.2	100	100	100	100	100	100	91.2	81.8	86.2	94.7	100	100	73.7	92.3	100	96.4	100	1
10-3					100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	1

TABLE 5.A

NORMAL CONTROLS

Changes in FEV_1 (litres) after histamine inhalation

Concentration mg/ml / Patient	Pre	0	2	4	8	16	32	64	$PC_{20}FEV_1$
AD	3.5	3.45	3.40	3.35	3.15	2.90	2.65		23.6
PB	3.47	3.49	3.33	3.05	2.83	2.78	2.63	2.45	13.8
JN	2.25	2.27	2.28	2.18	2.08	2.05	1.98		16.0
CS	2.50	2.52	2.53	2.50	2.48	2.48	2.48	2.42	> 64
JT	4.12	4.08	4.10	4.35	4.10	4.00	3.93	3.83	> 64
RC	2.19	2.22	2.25	2.25	2.23	2.08	1.75		60.5
AB	2.74	2.76	2.60	2.83	2.80	2.90	2.83	2.83	> 64
RW	2.68	2.70	2.70	2.75	2.75	2.68	2.58	2.53	> 64
JP	4.05	4.07	4.18	3.78	3.88	3.60	3.25		63.5
JM	2.05	2.05	2.05	2.03	2.00	1.75	1.78	1.65	67.6

TABLE 5.B

Normal controls

Changes in sGaw ($S^{-1}kPa^{-1}$) after histamine inhalation - (normal controls)

Concentration mg/ml / Patient	Pre	0	2	4	8	16	32	64	PC_{35}sGaw
AD	1.47	2.43	1.74	1.58	1.17	0.96	0.70		6.8
PB	1.96	1.34	0.98	1.01	0.54	0.52	0.49	0.48	1.3
JN	2.28	1.95	1.96	1.87	1.30	0.91	0.85		5.6
CS	3.61	2.30	2.46	2.42	2.08	1.94	1.43	1.31	16.5
JT	3.53	3.25	2.69	3.18	2.77	2.56	1.78	1.68	50.4
RC	1.32	1.24	1.64	1.50	1.15	1.51	0.92	0.78	49.4
AB	3.12	3.56	2.42	2.68	2.53	1.98	1.39	0.93	12.6
RW	2.13	2.37	3.01	2.44	2.18	1.66	1.41	1.03	27.7
JP	2.78	1.96	2.12	1.89	1.52	1.03	0.70	0.54	4.2
JM	2.02	2.09	1.98	1.07	0.95	0.67	0.62	0.52	3.3

TABLE 5.C

Normal controls

Changes in $\dot{V}_{30}(p)$ (litre/sec) after histamine inhalation (normal controls)

Concentration mg/ml / Patient	Pre	0	2	4	8	16	32	64	$PC_{30}V_{30}(p)$
AD	1.83	1.78	2.1	1.95	1.65	1.48	1.40		32.9
PB	2.0	1.8	1.6	1.42	1.35	1.05	0.9	0.95	8.8
JN	2.2	2.16	1.9	1.7	1.86	2.1	1.05		26.2
CS	1.52	1.68	1.52	1.52	1.52	1.62	1.38	1.25	> 64
JT	3.03	2.95	2.90	2.92	3.08	2.95	2.40	2.18	> 64
RC	1.50	1.65	1.90	1.75	1.85	1.75	1.55	1.15	> 64 80 by extrapolation
AB	1.6	1.7	1.78	1.4	1.65	1.8	0.95	0.55	29.7
RW	1.4	1.62	1.62	1.76	1.62	1.50	1.30	1.10	> 64
JP	2.1	2.1	2.00	2.02	2.12	1.6	1.30	1.24	27.4
JM	1.55	1.58	1.60	1.60	1.12	0.5	0.72	0.5	9.9

TABLE 5.D

Asthmatic Patients

Changes in FEV_1 (litres) after histamine inhalation

histamine concentration mg/ml / Patient	0	0.06	0.125	0.25	0.5	1	2	4	8	$PC_{20}FEV_1$
MS	1.06	1.10	0.9	0.85	0.8					0.3
MR	2.12	2.05	2.08	2.03	1.88	1.83	1.53			1.4
AM	1.8	1.73	1.70	1.70	1.48	1.33				0.6
FK	2.44	2.38	2.45	2.33	2.18	1.95	1.68			0.99
JI	1.80	1.93	1.95	1.98	1.93	1.83	1.83	1.55	1.33	5.7
WB	1.20	1.18	1.13	1.05	1.05	1.05	0.93			3.36
IK	1.54	1.53	1.45	1.45	1.50	1.30	1.20			1.23
MJ	1.15	1.1	0.9	0.75						0.11
MF	2.00	1.98	2.00	1.55						0.23
MD	1.15	1.10	1.10	1.00	1.1	0.78	0.60			0.62

TABLE 5.E

Asthmatic patients

Changes in sGaw($S^{-1}kPa^{-1}$) after histamine inhalation

histamine concentration mg/ml / Patient	0	0.06	0.125	0.25	0.5	1	2	4	8	PC_{35}sGaw
MS	0.41	0.43	0.48	0.43	0.35	0.23				0.72
MR	0.39	0.37	0.35	0.31	0.28	0.27	0.22			1.25
AM	0.40	0.38	0.53	0.49	0.40	0.31				1.39
FK	0.98	0.94	0.88	0.79	0.72	0.62	0.37			0.89
JI	0.51	0.46	0.46	0.41	0.35	0.29	0.31	0.24	0.20	0.64
WB	0.36	0.34	0.31	0.28	0.31	0.26	0.22	0.20		1.56
IK	0.74	0.68	0.66	0.64	0.55	0.43	0.41			0.74
MJ	0.43	0.29	0.22	0.16						0.066
MF	2.00	1.6	1.1	0.95						0.09
MD	0.95	1.20	0.87	1.25	0.8	0.65				1.2

TABLE 5.F

Asthmatic patients

Changes in $\dot{V}_{30}(p)$ (litres sec^{-1}) after histamine inhalation

histamine concentration mg/ml / Patient	0	0.06	0.125	0.25	0.5	1	2	4	8	$PC_{30}V_{30}(p)$ (mg/ml)
MS	0.89	0.6	0.75	0.8	0.5	0.5				0.42
MR	0.9	0.92	0.66	0.7	0.6	0.45	0.38			0.60
AM	0.98	1.35	0.95	1.05	0.6	0.65				0.46
FK	1.1	1.05	1.0	0.9	0.48	0.38	0.08			0.33
JI	1.1	1.55	1.45	1.40	1.50	0.9	0.85	0.2	0.6	2.35
WB	0.6	0.55	0.4	0.65	0.45	0.4	0.4	0.3		2.14
IK	1.25	1.05	0.95	0.90	0.50	0.45	0.35			0.29
MJ	0.93	0.70	0.35	0.25						0.07
MF	1.48	1.37	1.40	0.8						0.20
MD	0.7	0.5	0.5	0.55	0.55	0.2				0.60

TABLE 5.3

Summary of responsiveness: normal and asthmatic subjects

Results (mg/ml)

	Normal Subjects				Asthmatic Patients		
Patient	$PC_{20}FEV_1$	$PC_{35}sGAW$	$PC_{35}\dot{V}_{35(p)}$		$PC_{20}FEV_1$	$PC_{35}sGaw$	$PC_{35}\dot{V}_{35(p)}$
1	23.6	6.8	32.9		0.3	0.8	0.4
2	13.8	1.3	8.8		1.4	1.3	0.6
3	> 64	5.6	26.2		0.6	1.4	0.46
4	> 64	16.5	> 64		0.99	0.89	0.33
5	> 64	22	> 64		5.7	0.64	2.3
6	60.5	49.4	80.0		3.4	1.6	2.1
7	> 64	12.6	29.7		1.2	0.74	0.29
8	> 64	27.7	76.4		0.11	0.07	0.07
9	63.5	4.2	27.4		0.23	0.09	0.2
10	67.6	3.3	9.9		0.62	1.2	0.6
mean		14.9				0.87	
S: D		14.9				0.52	

TABLE 6.A

Surgical patients

FEV_1 (L) during histamine challenge

	Pre	0	2	4	8	16	32	64	$PC_{20}FEV_1$
JD	1.43	1.65	1.45	1.15	0.95				4.08
DMcN	2.9	3.1	2.9	2.95	2.8	2.45	2.35		17.04
AS	3.14	3.05	2.85	2.95	2.80	2.60	2.15		18.3
JD	1.18	1.25	0.8						0.64
EH	2.2	2.2	2.15	2.05	1.82	1.58			9.5
DMcC	1.26	1.25	1.02						2.3
PG	2.65	2.60	2.60	2.60	2.58	2.55	2.45	2.35	> 64
MMcG	3.15	3.25	3.27	3.25	3.20	2.58	1.93		15.7
JL	2.12	2.10	2.0	1.75	1.73	1.30			8.6
RT	1.76	1.75	1.55	1.32	1.26				3.1
histamine concentration	Pre	0	0.25	0.5	1				
*WW	2.18	2.10	1.95	1.85	1.50				0.61

*Asthmatic patient

Surgical patients

sGaw ($S^{-1}kPa^{-1}$) during histamine challenge

histamine concentration / Patient	Pre	0	2	4	8	16	32	64	PC_{35}sGaw
JD	0.50	0.51	0.32	0.29	0.19				1.66
DMcN	1.21	0.91	1.06	1.08	0.59	0.40	0.35		6.91
AS	1.08	1.16	0.77	0.73	0.57	0.46	0.39		4.03
JD	0.45	0.41	0.21						0.78
EH	0.77	0.97	0.49	0.38	0.35	0.24			1.1
DMcC	0.4	0.51	0.17						0.53
PG	1.75	1.99	1.95	1.84	1.33	1.11	0.71	0.64	11.3
MMcG	1.16	0.88	0.92	1.02	0.65	0.52	0.46		7.8
JL	1.39	1.16	0.64	0.72	0.53	0.35			0.8
RT	1.27	1.31	0.82	0.49	0.45				1.9
histamine concentration			0.25	0.5	1				
*WW	1.47	0.97	0.73	0.56	0.27				0.2

*Asthmatic patient

TABLE 6.C

Surgical patients

$PC_{30}\dot{V}_{35}(p)$ (1 sec^{-1}) during histamine challenge

histamine concentration / Patient	Pre	0	2	4	8	16	32	$PC_{35}V_{35}(p)$
JD	0.62	0.50	0.50	0.55	0.30			6.12
DMcN	1.91	1.60	1.45	1.55	1.6	1.2	1.1	14.56
AS	1.56	1.40	1.25	1.05	1.2	1.7	0.8	1.00
JD	2.0	2.5	1.5					18.1
EH	1.73	1.65	1.6	1.55	1.2	1.0		11.4
DMcC	1.83	1.75	1.7					
PG	1.78	1.85	1.82	1.65	1.75	1.8	1.7	> 64 (79.7)
MMcG	1.75	2.3	2.5	2.45	2.65	2.00	1.30	26.4
JL	1.2	1.15	1.1	1.25	1.0	0.85		16.9
	1.18	1.3	0.28	0.6				4.5
histamine concentration	Pre	0	0.25	0.5	1			
*WW	1.24	1.1	0.9	0.6	0.3			

*Asthmatic patient

TABLE 6.D

Histamine only **in vivo** patients

In vitro results - histamine (% change)

Concentration moles l^{-1} / Patient	10^{-7}	4 x 10^{-7}	10^{-6}	4 x 10^{-6}	10^{-5}	4 x 10^{-5}	10^{-4}	EC_{50} (x 10^{-6})
1	6.9	21.4	37.4	69.0	85.4	98.5	100	1.8
2	6.8	27.0	45.6	74.5	85.3	99.3	100	1.3
3	4.1	11.2	27.1	53.3	76.1	90.5	100	3.4
4	10.6		26.9		64.9		100	2.0
5	3.1	4.7	11.6	32.6	56.1	76.8	98.5	9.4
6	0	8.0	20.6	50	74.2	93.5	100	4.0
7	0	14.1	35.9	49.8	71.6	83.3	94.4	2.2
8	2.2	7.1	26.7	55.2	84.8	99.0	100	3.0
9	0	14.5	28.8	53.0	73.8	88.3	100	3.7
10	0	2.9	24.5	52.0	73.1	89.0	100	2.8
*11	0	3.5	19.7	40.1	64.5	83.8	100	2.3

*Asthmatic patient

TABLE 6.E

Surgical patients after atropine

Changes in FEV_1 (litres) after histmine inhalation

histamine concentration mg/ml Patient	Pre Atropine	Post Atropine	0	2	4	8	16	32	64	$PC_{20}FEV_1$ (mg/ml)
JJ	2.68	2.76	2.74	2.78	2.78	2.72	2.58	2.48	2.38	> 64
WP	2.05	2.28	2.26	1.8	1.83	1.63				1.8
JF	1.61	1.62	1.70	1.65	1.45	1.20				6.7
EH	2.2	2.12	2.15	2.03	1.75	1.85	1.90	1.73		36.8
WB	2.42	2.52	2.50	2.50	2.75	2.38	2.18	1.80		21.6
RD	3.46	3.83	3.95	3.8	3.6	3.58	3.45	2.85		26.7
AS	1.53	1.75	1.72	1.62	1.63	1.67	1.62	1.36	1.26	30
HM	1.89	1.92	1.94	1.90	1.85	1.83	1.80	1.75	1.60	> 64
*WW	2.08	2.16	2.31	2.1	1.73					3.2
RG	1.78	1.78	1.78	1.78	1.38	1.18				3.7
IL	1.60	1.65	1.60	1.65	1.63	1.48	1.30			14.8

*Asthmatic patient

TABLE 6.F

Surgical patients (after atropine)

Changes in sGaw ($S^{-1}kPa^{-1}$) after histamine inhalation

histamine concentration mg/ml Patient	Pre Atropine	Post Atropine	0	2	4	8	16	32	64	PC_{35}sGaw
JJ	1.67	3.73	2.80	1.81	1.60	1.31	0.87	0.68	0.59	1.00
WP	1.8	2.1	1.51	1.33	1.02	0.67				1.3
JF	1.02	1.60	1.91	1.31	1.01	0.45				2.9
EH	2.78	4.75	4.89	3.20	4.24	2.73	3.01	1.09		0.5
WB	0.38	1.03	1.54	1.28	1.28	0.77	0.62	0.29		7.3
RD	2.44	4.30	5.26	6.02	3.16	2.27	1.29	0.67		4.2
AS	1.13	1.85	1.95	1.40	1.46	1.64	1.27	0.99	0.72	17.4
HM	2.00	1.43	1.96	1.62	1.55	1.85	1.23	0.99	0.72	23.2
*WW	1.53	2.2	2.31	1.05	0.4					0.7
RG	0.7	1.07	1.14	1.01	0.43	0.24				3.00
IL	0.88	1.02	0.94	0.95	0.84	0.54	0.40			0.8

*Asthmatic patient

TABLE 6.G

Surgical patients - after atropine

Changes in $\dot{V}_{30}(p)$ (1 sec^{-1}) after histamine inhalation

histamine concentration mg/ml Patient	Pre Atropine	Post Atropine	0	2	4	8	16	32	64	$PC_{20}FEV_1$ (mg/ml)
JJ	1.5	2.16	2.10	1.78	1.58	1.60	1.25	1.35	1.18	11.9
WP	1.96	1.04	0.98	0.8	0.72	0.6				6.5
JF	1.36	1.80	1.86	2.1	1.22	1.2				7.00
EH	2.4	2.9	2.56	2.55	2.50	1.60	1.95	1.95		7.6
WB	0.8	2.2	2.6	3.5	2.9	2.85	1.85	1.60		25.8
RD	1.4	2.7	2.68	2.25	2.75	2.25	1.73	1.22		16.4
AS	1.87	2.97	3.00	2.95	2.65	2.8	1.75	21.1	1.4	14.1
HM	0.98	1.26	1.38	1.40	1.35	1.15	1.35	1.35	0.75	27.9
*WW	1.16	2.05	2.00	1.73	0.55					2.6
RG	1.3	1.8	1.4	1.35	1.00	0.75				2.8
IL	1.55	2.5	2.2	1.9	1.6	0.85				7.6

*Asthmatic patient

TABLE 7.A

PC values for patients with and without pretreatment with verapamil

inhalation		LTD_4 (mg/ml)				methacholine (mg/ml)	
Subject	Test	C	C	V		C	V
JAR	$PC_{10}FEV_1$	7.5	20.7	> 10		> 50	> 50
	$PC_{35}sGaw$	4.7	1.7	18.9		9.3	10.3
	$PC_{30}\dot{V}_{30}(p)$	5.6	1.2	> 10		17.00	12.7
NCT	$PC_{10}FEV_1$	> 50	38.6	> 50		32.4	21.7
	$PC_{35}sGaw$	7.4	2.7	> 50		16.1	11.9
	$PC_{30}\dot{V}_{30}(p)$	3.8	18.7	> 50		11.2	11.6
MM	$PC_{10}FEV_1$	2.00	2.7	19.9		9.7	11.3
	$PC_{35}sGaw$	0.3	1.1	10.4		5.5	7.7
	$PC_{30}\dot{V}_{30}(p)$	0.7	0.1	3.5		4.2	7.7
SSR	$PC_{10}FEV_1$	3.3	1.1	9.4		10.8	16.5
	$PC_{35}sGaw$	-1.1	12.1	8.2		20.9	11.5
	$PC_{30}\dot{V}_{30}(p)$	-10.00	1.7	> 50		24.1	2.00
KB	$PC_{10}FEV_1$	-2.6	> 50	> 50		11.2	> 50
	$PC_{35}sGaw$	-0.9	22.6	20.1		3.9	0.8
	$PC_{30}\dot{V}_{30}(p)$	-1.4	> 50	> 50		1.5	5.9
MG	$PC_{10}FEV_1$	-1.5	5.9	12.3		9.0	26.1
	$PC_{35}sGaw$	-8.1	5.6	> 50		5.7	12.7
	$PC_{30}\dot{V}_{30}(p)$	-1.4	1.8	> 50		4.0	7.2

C = after control (buffered saline) (2 control days for LTD_4)

V = after verapamil 2.5 mg/ml inhalation

TABLE 9.A

PC values to LTD_4 in asthmatic patients

		VERAPAMIL				SCG	
Patient	Test	C_1	C_2	V		C	SCG
SB	$PC_{10}FEV_1$	0.78	0.04	0.77		0.57	0.79
	$PC_{35}sGaw$	0.96	1.12	2.6		1.12	1.3
	$PC_{30}\dot{V}_{30}(p)$	1.0	0.02	0.69		0.25	0.43
AT	$PC_{10}FEV_1$	0.16	0.15	0.14		0.11	0.10
	$PC_{35}sGaw$	0.86	0.01	0.42		0.15	0.15
	$PC_{30}\dot{V}_{30}(p)$	0.85	0.49	0.96		0.12	0.06
KMcN	$PC_{10}FEV_1$	0.86	0.01	0.42		0.62	0.53
	$PC_{35}sGaw$	0.51	5.6	0.08		0.15	0.43
	$PC_{30}\dot{V}_{30}(p)$	0.87	0.14	0.45		0.20	0.10
CB	$PC_{10}FEV_1$	0.85	0.49	0.96		0.06	0.12
	$PC_{35}sGaw$	0.96	0.46	0.22		0.08	0.12
	$PC_{30}\dot{V}_{30}(p)$	0.35	0.41	0.36		0.05	0.09
PMcN	$PC_{10}FEV_1$	0.09	0.47	0.03		0.51	0.42
	$PC_{35}sGaw$	1.00	1.2	0.49		0.56	0.56
	$PC_{30}\dot{V}_{30}(p)$	0.66	0.09	0.05		0.35	0.29
GG	$PC_{10}FEV_1$	0.62	1.78	1.42		0.10	0.10
	$PC_{35}sGaw$	0.83	0.37	0.17		0.29	0.79
	$PC_{30}\dot{V}_{30}(p)$	0.02	0.20	1.92		0.90	0.70

TABLE 10.A

Surgical patients

Changes in FEV_1 (litres) after LTD_4 inhalation

LTD_4 Concentration (ug/ml) / Patient	Pre	0	0.016	0.08	0.4	2	10	50	$PC_{10}FEV_1$ (ug/ml)
EK	1.51	1.58	1.70	1.66	1.22	1.52			1.37
RN	1.31	1.36	1.36	1.34	1.13				0.24
DS	2.11	2.20	2.01	2.00	2.01	1.78	1.65		0.56
AT	1.75	1.73	1.70	1.70	1.42				0.88
AM	1.70	1.68	1.66	1.68	1.26				0.15
HB	1.52	1.62	1.62	1.64	1.38	1.29			0.19
GA	1.35	1.46	1.49	1.65	1.55	1.52	1.49		> 50
JB	3.75	3.52	-	3.63	3.63	3.38	3.47	3.31	> 50
EB	2.78	3.01	-	2.96	2.82	2.76	2.85	2.69	> 50
CH	1.44	1.41	-	1.48	1.40	1.29	1.23	1.07	2.1
MC	1.61	1.64	-	1.61	1.58	1.41			0.79

TABLE 10.B

Surgical patients

Changes in sGaw ($S^{-1}kPa^{-1}$) after LTD_4 inhalation

LTD_4 Concentration (ug/ml) / Patient	Pre	0	0.016	0.08	0.4	2	10	50	PC_{35}sGaw
EK	1.84	1.30	1.15	1.21	1.15	1.30	1.12		24.3
RN	0.45	0.31	0.37	0.34	0.24				0.24
DS	1.61	1.50	1.90	1.63	1.57	1.26	0.63		3.8
AT	0.88	1.53	1.19	1.47	0.79				1.21
AM	1.27	1.16	0.93	1.01	0.56				0.177
HB	0.79	0.88	0.87	0.76	0.76	0.58			2.8
GA	1.23	1.66	1.35	1.31	1.28	0.96	0.69		2.33
JB	1.16	1.04	-	1.03	0.96	1.2	0.87	0.96	> 50
EB	1.71	1.82	-	1.67	1.83	1.46	1.91	1.62	> 50
CH	0.55	0.68	-	0.54	0.58	0.49	0.45	0.53	> 50
MC	0.83	0.88	-	0.80	0.79	0.42			3.5

TABLE 10.C

Surgical patients

Changes in $V_{30}(p)$ (1 sec^{-1}) after LTD_4 inhalation

LTD_4 Concentration (ug/ml) / Patient	Pre	0	0.016	0.08	0.4	2	10	50	$PC_{30}V_{30}(p)$
EK	0.75	0.70	0.91	0.92	0.57				0.25
RN	0.40	0.42	0.47	0.42	0.26				0.26
DS	1.19	1.11	1.02	0.98	0.75	0.63	0.44		0.38
AT	0.36	0.35	0.32	0.33	0.18				0.69
AM	0.65	0.59	0.67	0.42	0.30				0.04
HB	0.36	0.46	0.60	0.70	0.26	0.16			0.28
GA	0.69	0.83	0.71	0.61	0.61	0.58	0.43		5.5
JB	1.79	1.73	-	1.74	1.63	1.30	1.42	1.19	14.7
EB	1.02	0.98	-	1.02	0.92	0.93	0.72		> 50
CH	0.46	0.50	-	0.70	0.63	0.42	0.36	0.23	11.00
MC	1.12	1.06	-	1.16	1.08	0.73			3.7