The actions and interactions of inhaled platelet-activating factor in man.

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By

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ABSTRACT

The effects and mechanism of action of inhaled platelet activating factor (PAF) were investigated in normal man.

A cumulative dose-response PAF challenge performed on two separate occasions in six normal subjects resulted in a variable degree of bronchoconstriction and transient fall in circulating neutrophil count. There was no significant increase in bronchial responsiveness to methacholine in any subject at any time when measured on five occasions in the two weeks following each PAF challenge.

The potential involvement of platelets in mediating the effects of PAF in man was investigated by measuring changes in the plasma levels of BTG and PF4 following PAF inhalation. There were no increases in BTG or PF4 levels shortly after PAF inhalation to suggest platelet activation.

The effects of pre-treatment with the cysteinyl-leukotriene receptor antagonist SKF 104353-Z_2 and the 5-lipoxygenase inhibitor BW A4C in modifying the acute responses to inhaled PAF were examined in two placebo-controlled double-blind crossover studies.

SKF 104353-Z_2 caused a significant reduction in PAF-induced bronchoconstriction, but with no effects on the PAF-induced fall in circulating neutrophil count.

BW A4C caused a small, statistically insignificant, reduction in PAF-induced bronchoconstriction and a significant reduction in the transient fall in circulating neutrophils. Measurement of ionophore-stimulated whole blood LTB_4 generation *ex vivo* revealed incomplete 5-lipoxygenase inhibition at the time of PAF challenge. There were positive correlations between the degree of 5-lipoxygenase inhibition and the reduction in PAF-induced falls in sGaw, $\dot{V}max_{30}$ and circulating neutrophil count obtained with BW A4C.

The reproducibility of acute changes in pulmonary function and circulating neutrophils following the cumulative dose-response challenges was very poor due to tachyphylaxis. In subsequent studies tachyphylaxis was avoided, and the reproducibility improved, by the use of a single high dose PAF challenge.

DEDICATION

To my Wife, Vivien.

With love.

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PUBLICATIONS

The work described in this thesis has, in part, been published and presented by the author at several scientific meetings as follows:

1. Lack of platelet activation following platelet-activating factor challenge in man. Spencer DA, Evans JM, Piper PJ, Martin JF, Garland LG, Costello JF. Am Rev Respir Dis 1989;139:95A.
2. Reproducibility of acute effects and subsequent changes in bronchial responsiveness following platelet-activating challenge in normal man. Spencer DA, Green SE, Evans JM, Piper PJ, Costello JF. Thorax 1990;45:330-331P.
3. Platelet-activating factor does not cause a reproducible increase in bronchial responsiveness in normal man. Spencer DA, Green SE, Evans JM, Piper PJ, Costello JF. Clinical and Experimental Allergy 1990;20:525-532.
4. Bronchospasm induced by inhaled platelet-activating factor is reduced by a selective cysteinyl-leukotriene antagonist in normal man. Spencer DA, Evans JM, Green SE, Piper PJ, Costello JF. Am Rev Respir Dis. 1990;141:A219.
5. Involvement of the cysteinyl-leukotrienes in the acute bronchoconstrictor response to inhaled platelet-activating factor in man. Spencer DA, Evans JM, Green SE, Piper PJ, Costello JF. Thorax. *In press*.

6. Effects of the 5-lipoxygenase inhibitor, BW A4C, on the acute response to inhaled platelet-activating factor in man. Spencer DA, Sampson AT, Evans JM, Piper PJ, Costello JF. Presented at the joint meeting of the Royal Society of Medicine and the New York Academy of Sciences. Royal College of Surgeons, London, October 1990. Abstract in Proc Natl Acad Sci USA. *In press*.

APPENDICES

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Each abbreviated term is stated in full and followed by the abbreviation in parentheses when first mentioned in the text.

ADP: adenosine diphospate

AGEPC: acetyl glyceryl ether phosphorylcholine

ANOVA: analysis of variance

APRL: antihypertensive polar renomedullary lipid

BAL: bronchoalveolar lavage

BHR: bronchial hyperresponsiveness

ß-TG: ß-thromboglobulin

CI: confidence interval

DMSO: dimethylsulphoxide

ECG: electrocardiogram

ECP: eosinophil cationic protein

FEV_1: forced expiratory volume in one second

HETEs: hydroxyeicosatetraenoic acids

HSA: human serum albumin

LT: leukotriene

MBP: major basic protein

MCh: methacholine

NCA: neutrophil chemotactic activity

NDGA: nordihydroguaiaretic acid

PAF: platelet-activating factor

Palv: alveolar pressure

Pb: box pressure

PF4: platelet factor four

PG: prostaglandin

PKC: protein kinase C

Pm: mouth pressure

p.s.i.: pounds per square inch

s.e.m.: standard error of the mean

sGaw: specific airways resistance

SRS-A: slow reacting substance of anaphylaxis

TGV: thoracic gas volume

Tx: thromboxane

$\dot{V}m$: airflow at the mouth

$\dot{V}max_{30}$: expiratory flow at thirty per cent of vital capacity above residual volume

5-LO: 5-lipoxygenase

CHAPTER ONE

INTRODUCTION

1.1. THE NATURE AND DEFINITION OF ASTHMA

Asthma is a chronic disorder affecting approximately five per cent of the population in most industrialised societies. Surveys in the United Kingdom suggest that the number of respondents with active disease at the time of the survey (the current prevalence rate) is up to 5.4% of the adult population and 6.8% of children, whilst the number of people who have wheezed at some time (the cumulative prevalence rate), irrespective of a firm diagnosis of asthma having been made, is between 11.1-24.7% in children and 12.2-30% in adults (Gregg. 1983). It is therefore one of the commonest chronic diseases in Western society, and the most common chronic disorder in childhood (Martin, McLennan, Landau, et al. 1980).

Asthma is a seriously underdiagnosed and undertreated condition in children (Speight, Lee and Hay. 1983), and this is also a problem in the adult population in whom under-diagnosis is an important factor in "avoidable" deaths from asthma (British Thoracic Society. 1982; Johnson, Nunn, Somner, et al. 1984).

Asthma is the certified cause of death in approximately 2,000 adults and 50 children per annum in the United Kingdom (British Thoracic Society. 1982), and the mortality rate may be increasing (Sears. 1988). There is also evidence that both the incidence and the severity of this condition are increasing (Ayres, 1986; Burney, Chinn and Rona. 1990;

Haahtela, Lindholm, Bjorksten, et al. 1990) leading to increased morbidity in the population despite the availability, and increasing prescriptions of, seemingly effective anti-asthma medications (Hay and Higenbottam. 1987). There is therefore a compelling need to recognise and treat asthma appropriately, and for a greater understanding of the aetiology, epidemiology, genetics, pathophysiology, immunology, pharmacology and therapeutics of this condition.

Asthma has been recognised since ancient times. The Chinese describe a condition which was probably asthma in the Neu Ching from the third millenium B.C. The Ebers papyrus from Egypt (B.C. 1550) refers to a similar condition treated by clysters (enemas) together with the administration of animal excreta including camel and crocodile dung (reviewed by Sakula. 1988).

Asthma is characterised clinically by recurrent and persistent cough, wheeze, increased mucus production and dyspnoea. These symptoms may occur spontaneously, or as a result of exposure to various stimuli. However, in spite of being a familiar, common and ancient disorder, a precise and universally acceptable definition of this condition has proved elusive (Scadding. 1987), and it is probably more appropriate to think of asthma as a syndrome with complex and diverse aetiologies rather than as a single disease entity.

Asthma has been defined as "The condition of subjects with widespread narrowing of the bronchial airways, which changes its severity over short periods of time either spontaneously or under treatment, and is not due to cardiovascular disease" (CIBA Guest Symposium. 1959) and also as "A disease characterised by an increased responsiveness of the trachea and bronchi to various stimuli and manifested by a widespread narrowing of the airways that changes in severity spontaneously or as a result of therapy." (American Thoracic Society. 1962). These two definitions encompass the essential physiological abnormalities in asthma; namely rapid changes in airway calibre and bronchial hyperresponsiveness (BHR).

1.2. REVERSIBILITY OF AIRWAY OBSTRUCTION

Reversible airways obstruction, usually defined as an increase of at least 15% in the peak expiratory flow rate following the administration of inhaled β-agonist, is often used as a diagnostic criterion for asthma. This response can be very useful, especially in the investigation of patients with atypical symptoms, but there are numerous reasons why this reaction can not be used to define asthma.

Reversibility gives no insight into the pathophysiology of the condition other than to imply that there is constriction of bronchial smooth muscle. The degree of airways obstruction is characteristically variable over short periods of time, so that it may not be possible to

demonstrate a significant degree of reversibility when the patient is clinically well. Measurement of peak expiratory flow rate is effort-dependent, and administration of an inhaler may result in a significant placebo effect. There are also inherent problems in demonstrating this response in those unable to perform the manoeuvre reliably, especially the young and the very old. Finally, infants with asthma may demonstrate a paradoxical response to bronchodilators (Prendiville, Green and Silverman. 1987) and irreversible airways obstruction can develop in patients with long-standing asthma (Brown, Neville and Finucane. 1984).

1.3. BRONCHIAL RESPONSIVENESS

BHR has been defined as "the exaggerated bronchoconstrictor response of the airways to many physical, chemical and pharmacological stimuli" (Bleeker. 1985). The terms "bronchial hyperreactivity" and "bronchial hyperresponsiveness" are often used synonymously, although the latter term is preferable as bronchial reactivity refers pharmacologically to steepening of the agonist dose-response curve which is not a feature of BHR in man (Snashall and Pauwels. 1987)

BHR can be divided into specific and non-specific categories. The most common specific agents increasing bronchial responsiveness in susceptible individuals are inhaled allergens such as house dust mite, pollen and animal dander (Cockcroft, Ruffin, Dolovich, et al. 1977). The great

majority of patients with atopic asthma exhibit specific BHR to a wide variety of such allergens. Specific BHR can also be demonstrated to chemical sensitisers in which no immunological basis can be demonstrated, such as Toluene di-isocyanate present in industrial paints (Pepys, Pickering, Breslin, et al. 1972).

When unqualified the term BHR implies the non-specific form. This refers to a state of increased sensitivity of the airways, resulting in bronchoconstriction after exposure to a whole range of apparently dissimilar agents to which the subject is not specifically sensitised. These stimuli include citric acid, distilled water (Foresi, Mattoli, Corbo, et al. 1986), cholinergic agents such as methacholine [MCh],(Juniper, Frith, Dunnett, et al. 1978) and carbachol, and mediators including histamine (Cockcroft, Killian, Mellon, et al. 1977), leukotrienes [LTs](Bisgaard, Groth and Madsen. 1985) and Prostaglandin E_2 [PGE_2]. This phenomenon can also be demonstrated using various physical stimuli including exercise (Silverman and Anderson. 1972), hyperventilation, and inhalation of cold air (fog) (O'Byrne, Ryan, Morris, et al. 1982) and smoke. Bronchial challenge procedures have been thoroughly reviewed by Eiser (1987).

BHR can be demonstrated in most, if not all, patients with untreated asthma, and is now regarded by many authorities as a characteristic feature of this condition (Boushey, Holtzman, Sheller, et al. 1980; Hargreave, Ryan, Thomson, et

al., 1981). In asthmatic patients, there is a strong correlation between asthmatic symptoms, clinical indices of disease severity and the degree of BHR (Makino. 1966). In addition, exposure to stimuli known to increase asthmatic symptoms such as viral upper respiratory tract infections (Lemanske, Dick, Swenson, et al. 1989) and allergen (Cockcroft, Ruffin, Dolovich, et al. 1977) is associated with increases in BHR. Finally, treatment with prophylactic anti-asthma medications, especially steroids and sodium cromoglycate, as well as causing a clinical improvement in symptoms and pulmonary function, is associated with a concomitant decrease in bronchial responsiveness in both children (Kerrebijn, Van Essen-Zandvliet and Neijens. 1987) and adults (Woolcock, Yan and Salome. 1988).

It is apparent that there is a very intimate relationship between asthma and BHR, but BHR is not synonymous with asthma (Britton. 1988). Early studies suggested that bronchial responsiveness is bimodally distributed within the population, making it easy to distinguish between asthma and normality. However, subsequent studies have demonstrated that the distribution within the general population is log-normal and unimodal (Cockcroft, Bersheid and Murdock.1983A). This suggests that there is a considerable overlap in bronchial responsiveness between the "normal" and asthmatic groups. BHR is also a frequent, although not invariable, finding in other respiratory conditions including cystic fibrosis (Mellis and Levison. 1978) and chronic bronchitis

(Ramsdell, Nachtwey and Moser. 1982). Levels of responsiveness to histamine comparable to those found in asthmatic patients have also been reported in asymptomatic atopics (Cockcroft, Bersheid and Murdock.1983), subjects with rhinitis (Cockcroft, Killian, Mellon, et al. 1977) and in young asymptomatic smokers (Cockcroft, Bersheid and Murdock. 1983B).

1.4. THE AETIOLOGY OF ASTHMA

The factors governing the manifestation of asthma in any individual are incompletely understood. Family studies suggest that BHR has a large genetic component, inherited independently from the gene or genes responsible for the expression of atopy (Sibbald, Horn, Brain, et al. 1980). The expression of asthma may then depend on the expression of both sets of genes.

A genetic linkage between IgE responses in asthmatic patients and a DNA polymerism on chromosome 11 has recently been described (Cookson, Sharp, Faux, et al. 1989). This finding could possibly be linked to the discovery of a range of intercellular adhesion molecules controlling the adhesion of leukocytes to microvascular endothelium. One of these, intercellular adhesion molecule-1 (ICAM-1), has been shown to be important in regulating eosinophil migration and adhesiveness. A monoclonal antibody to ICAM-1 has been produced which attenuates the airway eosinophilia and BHR induced by allergen in a baboon model of asthma (Wegner,

Gundel, Reilly, et al. 1989), and this novel approach has interesting therapeutic potential. It is possible that the gene product of the locus on chromosome 11 regulates the expression of intercellular adhesion molecules, or alternatively that it may influence the function of T lymphocytes. Identification of the structure and biological activity of this locus could greatly increase our understanding of the fundamental abnormalities in asthma.

An alternative hypothesis is that genetic factors and multiple environmental agents, especially those operative in childhood, interact to influence the clinical outcome. Relevant environmental factors probably include respiratory infections due to viruses (Horn, Reed and Taylor. 1979; Reviewed by Busse. 1988) and atypical organisms (Mok, Waugh and Simpson. 1979), exposure to pollution especially cigarette smoke and nitrogen dioxide from gas cookers (Ware, Dockery, Spiro, et al. 1984), and living in homes in which there is damp and growth of mould (Strachan. 1988). Bronchiolitis due to the respiratory syncytial virus may be particularly important, as respiratory symptoms and abnormal pulmonary function may persist for up to ten years after this infection in infancy (Pullan and Hey. 1982).

1.5. THE PATHOLOGICAL FEATURES OF ASTHMA

The pathological features of asthma have been documented from post-mortem studies on patients who have died from status asthmaticus as well as from other causes. Information

has also been gathered in living patients from biopsy material obtained at thoracotomy and at bronchoscopy.

At necropsy, the lungs of patients who have died from status asthmaticus are distended with air and remain so after removal from the chest. Areas of atelectasis due to plugging with viscid tenacious mucus are present in about half of the cases (Cardell. 1956; Dunnill. 1960), and may extend from the trachea to the respiratory bronchioles.

The histological features in patients who have died of status asthmaticus are of an inflammatory condition affecting mucous membranes. There is vascular dilatation and increased vascular permeability, a proteinacious exudate containing inflammatory cells, mucous hypersecretion and shedding of the epithelial lining into the lumen (Dunnill. 1960).

The mucus plugs consist of an inflammatory exudate of mucus, fluid, protein and cells (Sanerkin and Evans. 1965). Strands of epithelium may be attached to the plugs, which are often firmly attached to the bronchial wall. The sputum may form a cast of the bronchus, the "Curschmann spiral", and often contains clusters of columnar cells, "Creola Bodies" (Naylor. 1962). There is extensive desquamation of epithelium (Houston, De Navaquez and Trounce. 1953; Cardell. 1956. Dunnill. 1960) with hyperplasia of the basal layer (Takizawa and Thurlbeck. 1971; Heard and Hossain. 1973),

goblet and squamous cell metaplasia and enlargement of tracheobronchial mucous glands (Dunnill. 1960; Dunnill, Massarella and Anderson. 1969).

The "basement membrane" consisting of a true basal lamina and associated collagen, is thickened in asthma because of an increase in collagen and deposition of IgG, IgA, albumin and fibrinogen (McCarter and Vazquez. 1960). There is an increase of up to three-fold in bronchial smooth muscle (Dunnill, Massarella and Anderson. 1969; Takizawa and Thurlbeck. 1971) principally in the medium sized airways, due mainly to hyperplasia rather than to hypertrophy (Heard and Hossain. 1973). The bronchial and bronchiolar walls are thickened to a greater extent than can be accounted for by the muscle hyperplasia, and the difference is probably due to oedema (Hogg. 1988).

The presence of large numbers of eosinophils, particularly in the subepithelial layer, is a frequently reported histological finding in biopsy material, accounting for up to 44% of the cellular infiltrate (Leopold. 1964). However, this finding may be quantitative rather than qualitative, as a variable number of eosinophils may be present in other respiratory conditions including chronic bronchitis, aspergillosis and chronic pulmonary venous congestion (Glynn and Michaels. 1960; Sanerkin and Evans. 1965).

The histological findings quoted are classical. However,

exceptions have been reported. In an earlier study, Earle (1953) reviewed the literature and found atypical findings including pulmonary oedema and alveolar haemorrhages in eight out of a total of 90 cases and described one atypical case in his own series of 15 asthma deaths. Four of these cases were infants, and it may be that these atypical findings partially reflect previous difficulty in obtaining an accurate clinical diagnosis during life. More recently, Lopez-Vidriero and Reid (1983) have stated that on rare occasions they have found no macroscopic or microscopic abnormalities whatsoever in patients who have died suddenly in a severe attack of airways obstruction.

Post-mortem histological findings in patients who have died in status asthmaticus are likely to be severe, and may not be representative of the typical case. Although asthma is an extremely common disease, death from asthma is relatively uncommon, particularly in the younger age group who are more likely to have uncomplicated disease. However, there is ample evidence to show that the pathological features are essentially similar in living patients.

Necropsy studies on patients with asthma who have died from other causes have usually described similar changes to those reported in the fatal case, although mucus plugging is not invariably present (Sobonya. 1984). However, Cardell (1956) reported five cases dying from causes other than asthma, and in some of these the degree of eosinophil infiltration was

very minor. The author suggested that this feature was inversely related to the time that had elapsed since the last asthma attack.

Open lung biopsy has been occasionally performed in cases in which there has been some doubt as to the clinical diagnosis. Cutz, Levison and Cooper (1978) studied the ultrastructure of open lung biopsies taken from two asymptomatic girls with a history of previous asthma and persistently abnormal chest X-rays. The findings were compared to those in two children of similar age dying in status asthmaticus. The over-all histopathological features were remarkably similar with mucus plugging, smooth muscle hypertrophy, goblet cell hyperplasia, thickening of the "basement membrane" and cilial abnormalities. The only difference between the two groups of specimens was the presence of larger numbers of peribronchial eosinophils and focal epithelial denudation in the patients who died in status asthmaticus.

Bronchial biopsies have been obtained at bronchoscopy in living patients with varying degrees of disease severity. In a seminal study, Glynn and Michaels (1960) reported findings in 18 asthmatic patients. They found no consistent changes in the epithelium, attributing most of the cilial loss to artefact. Eosinophil infiltration was present in the lamina propria in every case, often within the lumina of capillaries, around deep glands, within the epithelium and

occasionally straddling the basement membrane. The presence of eosinophils was always accompanied by a variable, but often heavy, infiltrate of plasma cells. Interestingly, thickening of the "basement membrane" was found in only two patients, suggesting that this feature may be an index of disease severity.

Laitenen, Heino, Laitinen, et al (1985) have performed light and electron microscopy on bronchial biopsies in eight patients with asthma of varying severity. They found that epithelial damage and exposed intra-epithelial nerves were present in all cases, and that changes were present at all levels within the lung. Even in biopsies where the ciliated epithelium was present, the cells often appeared swollen with widening of the intercellular spaces. They found no relationship between the degree of BHR and the severity of epithelial damage, but this may have been partially due to the small number of patients studied and because few biopsies were taken at each bronchoscopy.

Lozewicz, Gomez, Ferguson, et al (1988) have obtained bronchial mucosal biopsies at bronchoscopy in ten adult asthmatics hyperresponsive to MCh but with clinically mild disease, and compared the findings to those in biopsies obtained from nine healthy volunteers. They found that there were significantly more mast cells in the lamina propria from the patients compared to the controls.

There were also more eosinophils, but the increase did not reach statistical significance.

Bronchoalveolar lavage (BAL) is increasingly used to obtain material for cytological studies in patients with asthma. De Monchy, Kauffman, Venge et al (1985) performed BAL following early and late asthmatic reactions induced by allergen inhalation challenge. They found that BAL following the late reaction was associated with a significant bronchoalveolar eosinophilia compared to BAL performed at other times in asthmatics and a control group of normal subjects. In addition, this bronchoalveolar eosinophilia was accompanied by elevated eosinophil cationic protein (ECP)/albumin ratio (*vide infra*, Section 1.6.).

1.6. THE PATHOPHYSIOLOGY OF ASTHMA

Although the precise mechanisms responsible for the expression of BHR in asthma remain elusive, there is now considerable evidence to suggest that this characteristic physiological abnormality is intimately linked to the inflammatory process within the lung (Boushey, Holtzman, Sheller, et al. 1980; Chung. 1986).

It is most unlikely that any single pathological process, individual inflammatory cell type or mediator could account for all of the pathophysiological features observed in asthma. Instead, numerous interrelated pathological and physiological mechanisms including bronchial oedema,

increased mucosal permeability, exposure of epithelial sensory nerve endings and the release of numerous individual mediators from inflammatory cells all may contribute to the clinical expression of this disease.

Damage to the bronchial epithelium is a consistent finding in asthma which may directly increase bronchial responsiveness. Epithelial damage results in increased plasma exudation which itself may contribute to BHR (Persson. 1986) by allowing ingress of a range of plasma macromolecules including kinins, complement and inflammatory mediators, causing further stripping of the epithelium, impairing mucociliary transport and resulting in further accumulation of plasma and mucus. The accumulation of plasma, mucus and cellular debris into the lumen will reduce airway calibre, and thereby increase bronchial responsiveness by a physical mechanism, as airway resistance is inversely related to the fourth power of the airway diameter (Freedman. 1972).

Necrosis and sloughing of the respiratory epithelium is a prominent feature in many viral respiratory tract infections (Hers. 1966), which are recognised as a frequent cause of increased asthmatic symptoms, especially in childhood (Horn, Reed and Taylor. 1979). Viral upper respiratory tract infections are also associated with increases in bronchial responsiveness for up to six weeks in normal subjects (Empey, Laitinen, Jacobs, et al. 1976). These authors have

suggested that disruption of the epithelium may result in disturbances of the neuronal control of the airways by exposing peripheral nerve endings, resulting in increased bronchial responsiveness by a cholinergic mechanism.

Barnes (1986) has suggested that epithelial damage may result in activation of the non-adrenergic non-cholinergic nervous system in the lung, secondary to the exposure of C-fibre afferent nerve endings to stimulation by mediators such as bradykinin. It is postulated that this triggers local axon reflexes, resulting in antidromic conduction down afferent nerves, and causes release of sensory neuropeptides with potent biological effects including substance P, neurokinin A and calcitonin gene-related peptide. It may soon be possible to test this hypothesis, as a selective opiod receptor agonist, BW 443C, is currently being assessed for clinical studies in asthma.

A wide variety of resident and circulating cells have been implicated in the pathophysiology of asthma. As previously discussed (Section 1.7), eosinophil infiltration is a prominent histological feature of this disease. Ellis described the association of blood and tissue eosinophilia with asthma in 1908, whilst Durham and Kay (1985), and others, have shown that the peripheral eosinophil count correlates with the degree of BHR.

It has long been recognised that eosinophilia is a frequent

feature of parasitic infection, in particular with helminthic infestation of the gastro-intestinal tract. Butterworth, Sturrock, Houba, et al (1975) demonstrated that the eosinophil was capable of damaging *Schistosoma mansoni* via a cell-mediated antibody dependent mechanism and subsequently showed that this damage involved eosinophil major basic protein (MBP). Parrillo and Fauci (1978) also reported that the eosinophil was capable of damaging mammalian cells. It was therefore suggested that similar damage may be inflicted on the host in other diseases characterised by eosinophilia, including bronchial asthma.

Eosinophil MBP has been demonstrated to be extremely toxic to the bronchial epithelium. At concentrations of 10-100µg/ml it causes features very reminiscent of the changes seen in asthma, with ciliastasis, epithelial disruption and sloughing down to the lamina propria in both guinea pigs and man (reviewed in Frigas and Gleich. 1986). Eosinophil MBP has been detected in the lungs of patients with asthma (Filley, Holley, Kephart, et al. 1982); it is also present in high concentrations in sputum, and these levels correlate with disease severity (Frigas, Loegering, Solley, et al. 1981).

The secondary crystalline granules of the eosinophil have since been shown to possess a range of other proteins which are potentially cytotoxic including ECP, a neurotoxin, a peroxidase and Charcot-Leyden crystal protein.

The eosinophil has also been shown to be capable of producing a number of inflammatory mediators including LTC_4, platelet activating factor (PAF), PGs and oxygen-derived radicals (Frigas and Gleich. 1986).

Although the eosinophil has attracted a great deal of attention as an important inflammatory cell in asthma, numerous other cell types including mast cells, neutrophils, lymphocytes, monocytes, alveolar macrophages and platelets may also be extremely important in the inflammatory process.

The mast cell is a rich source of histamine, and has long been associated with acute IgE-mediated bronchoconstriction (reviewed by Holgate, Hardy, Robinson, et al. 1986). It is also a source of other mediators including LTs and chemotactic factors such as eosinophil chemotactic factor. In addition, this cell produces neutrophil chemotactic activity (NCA) which has been shown to be released following allergen challenge in asthmatics (Carroll, Durham, Walsh, et al. 1985).

The platelet is increasingly recognised as an important proinflammatory cell (reviewed by Page. 1986). Platelet activation may occur following antigen challenge (Knauer, Lichtenstein, Adkinson, et al. 1981) and other platelet abnormalities including prolonged bleeding time and increased platelet mass have been demonstrated in asthmatics and also in non-asthmatic atopic subjects (Szczeklik,

Milner, Birch, et al. 1986).

The presence of a heavy plasma cells infiltrate in the lungs of patients who have died from status asthmaticus has been mentioned. The lymphocyte may play a central role in regulation of the inflammatory response, perhaps through controlling the release of cytokines (reviewed by Kelley. 1990). Increasing attention is being directed to the investigation of immunological mechanisms controlling the inflammatory response. This has promoted studies on the potential benefit of agents which act by inhibiting lymphocyte function, including methotrexate (Shiner, Nunn, Chung, et al. 1990), and to current studies on the more specific agent cyclosporine (Kahan. 1989).

A group of patients with disease resistant to the effects to inhaled or oral corticosteroids has been identified; monocytes from these patients are not suppressed by corticosteroids and express a higher percentage of monocyte C3b receptors than do cells from corticosteroid sensitive asthmatics (Carmichael, Paterson, Diaz, et al. 1981). These findings shed light on the mechanism of action of steroids in asthma, and suggest that the monocyte may also be an important regulatory cell in this condition (reviewed by Kay. 1987).

1.7. INFLAMMATORY MEDIATORS AND ASTHMA

A wide range of substances have been suggested as potential mediators of the inflammatory response in asthma. These include histamine, PGs, Thromboxane A_2 (TxA_2), LTs, lipoxins, hydroxyeicosatetraenoic acids (HETEs), kinins, neuropeptides, oxygen radicals and PAF (comprehensively reviewed by Barnes, Chung and Page. 1988). It is slowly being realised that no single mediator can reproduce all of the pathophysiological features of asthma, and it is more likely that many mediators are involved in this condition. Furthermore, complex interactions may occur between mediators, circulating and resident cells and the nervous system of the lung. These interactions may be important in amplifying the effects of individual mediators, thereby leading to increased bronchial responsiveness. Alternatively some interactions may attenuate acute responses, decreasing bronchial responsiveness and contributing to homeostasis within the lung.

Whilst a wide variety of pharmacological and physiological stimuli can induce acute bronchospasm, only a few mediators have yet been shown to cause an increase in bronchial responsiveness when given by inhalation either to normal man or asthmatics. $PGF_{2\alpha}$ increases responsiveness to histamine in normal subjects and asthmatic patients (Heaton, Henderson, Dunlop, et al. 1984), whilst PGD_2 potentiates airway responsiveness to both histamine and MCh in asthmatics (Fuller, Dixon, Dollery, et al. 1986). However,

in both these studies the increases in responsiveness were small and the duration of these effects was not investigated. Only LTs (Arm, Spur and Lee. 1988) and PAF (Cuss, Dixon and Barnes. 1986) have been found to cause increases in bronchial responsiveness that are comparable to the changes obtained following antigen challenge in asthmatics.

1.8. LTs AND ASTHMA

For many years slow reacting substance of anaphylaxis (SRS-A) has been implicated as a major mediator of immediate hypersensitivity reactions and allergic bronchoconstriction (Brocklehurst. 1960), but progress was previously limited by ignorance of the chemical structure and the absence of a pure preparation. The description of the chemical structure of SRS-A by Morris, Taylor, Piper, et al (1980) triggered a resurgence of interest in this group of compounds. This led to the discovery that the LTs were a group of 5-lipoxygenase (5-LO) products of arachidonic acid, and to recognition that the cysteinyl-LTs (LTC_4, LTD_4 and LTE_4) accounted for the biological activity originally attributed to SRS-A (reviewed in Samuelsson. 1983).

The LTs can be conveniently divided on the basis of chemical structure and differential biological activity into LTB_4 and the cysteinyl-LTs. These compounds have a wide range of biological actions relevant to asthma, and have been implicated in bronchoconstriction, oedema and mucous

formation, impairment of mucociliary clearance, cellular chemotaxis and activation and increases in bronchial responsiveness (reviewed by Piper. 1984; Barnes and Costello. 1986).

The cysteinyl-LTs are potent bronchoconstrictors, with LTC_4 and LTD_4 being between 1000-6000 times more potent than histamine when given by inhalation to normal man (Weiss, Drazen, McFadden, et al. 1983; Barnes, Piper and Costello, 1984). Allergen challenge of human lung tissue *in vitro* elicits contraction which correlates with the release of large amounts of LTC_4, LTD_4 and LTE_4 (Dahlen, Hansson, Hedqvist, et al. 1983).

In normal subjects, pre-inhalation of LTD_4 increases responsiveness to $PGF_{2\alpha}$, but not histamine (Barnes, Watson, Koulouris, et al. 1983), whereas Kern, Smith, Patterson, et al (1986) described a small increase in responsiveness to MCh immediately after LTD_4 challenge. LTD_4 heightens the level of the maximum response to MCh for up to 72 hours without shifting the dose-response curve to the left (Bel, Van Der Veen, Kramps, et al. 1987), and in a complicated series of experiments, Philips and Holgate (1989) showed that small doses of LTC_4 increase responses to both histamine and PGD_2 for up to nine minutes in asthmatic patients.

LTB_4 has little activity as a bronchoconstrictor in man, but is a potent proinflammatory agent involved in chemotaxis and activation of a wide range of inflammatory cells (discussed in Section 7.2.).

Perhaps the most dramatic claim for involvement of the cysteinyl-LTs in asthma has been made by Arm, Spur and Lee (1989). These workers have shown that responsiveness to histamine increases by up to 3.5 fold in asthmatic subjects seven hours after inhalation of LTE_4, with changes persisting for up to four days. There were no changes in bronchial responsiveness in the normal subjects studied. Unfortunately, only four asthmatic subjects were used in this study, and the mechanism by which these effects were produced is unknown. These exciting findings await verification by other investigators.

1.9. PAF AND ASTHMA

Barbaro and Zweifler reported in 1966 that allergen challenge in the rabbit was associated with histamine release via a complement independent mechanism, the platelet being the major source of histamine in this species (Humphrey and Jaques. 1954). However, it was not known at this time if histamine release occurred as a direct consequence of the antibody-antigen reaction or via an indirect mechanism, nor was it appreciated until the 1980's that the platelet contains both IgE and IgG receptors. Benveniste, Henson and Cochrane demonstrated in 1972 that

this phenomenon was dependent on the release of a product from basophils stimulated with IgE. Benveniste christened this substance platelet-activating factor (PAF) and subsequently showed it to be a phospholipid. The chemical structure was determined by three independent groups in 1979 as 1-o-alkyl-2-acetyl-*sn*-glyceryl-phosphorylcholine. This substance was shown to be identical to an antihypertensive polar renomedullary lipid (APRL) described in the same year (Blank, Snyder, Byers, et al. 1979; reviewed by Hanahan and Kumar. 1987).

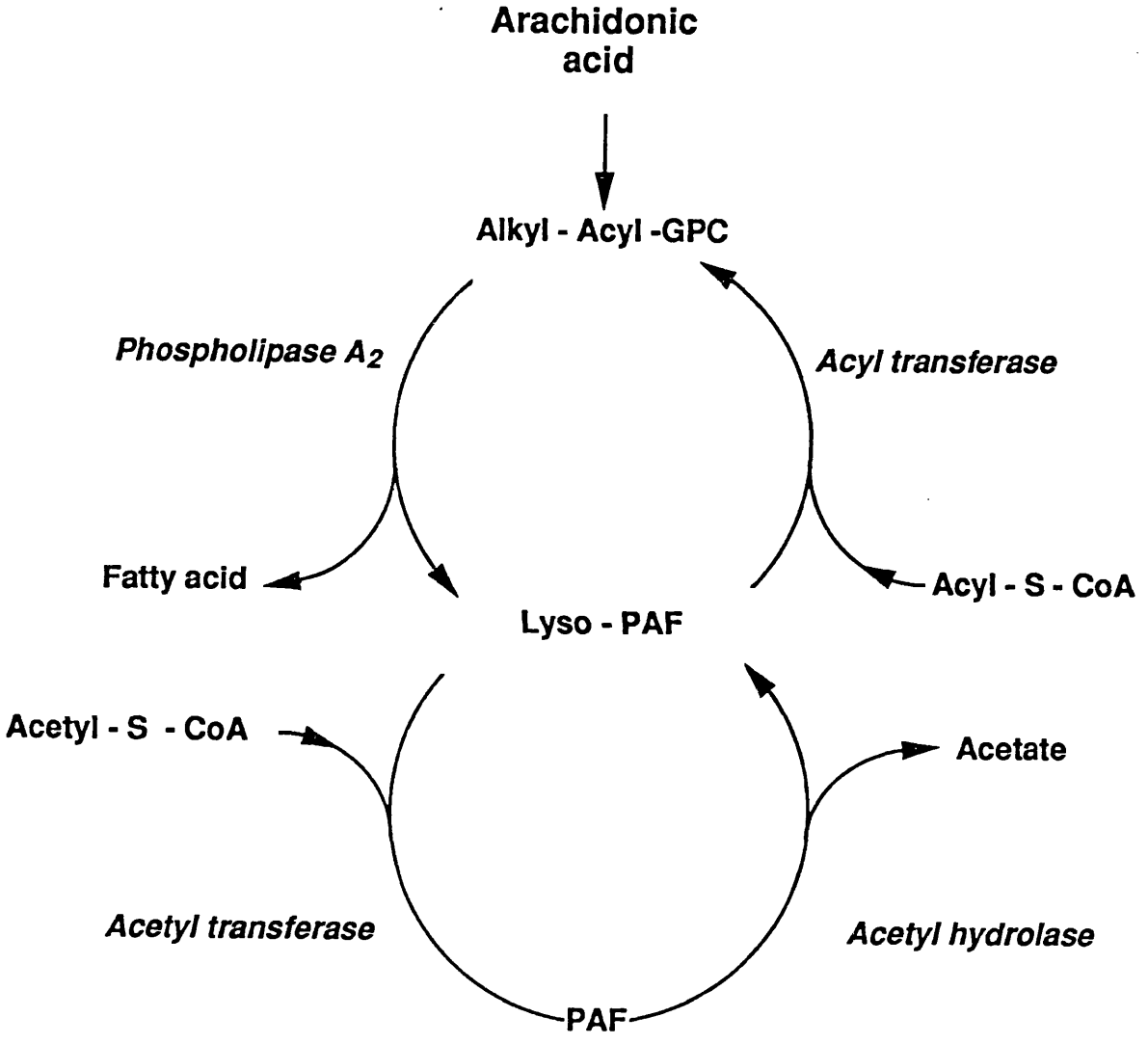
Various groups have used the terms PAF, PAF-acether (ace for acetate and ether for the alkyl bond), AGEPC (acetyl glyceryl ether phosphorylcholine) and APRL to describe this lipid. The familiar term PAF will be used in this thesis, although it is now appreciated that this substance has numerous actions on a wide variety of cells other than the platelet.

PAF was totally synthesised in 1980 (Godfroid, Heymanns, Michel, et al. 1980) and a whole range of structural analogues have since been produced (O'Flaherty, Wykle, Miller, et al. 1981; Snyder. 1985). It is now recognised that there are many naturally occurring compounds with biological activity in this of class of acetylated ether-linked phospholipids, and that there is a degree of cell and species specificity in the biological relevance of individual compounds within this group.

The most important pathway for PAF synthesis in man is probably by the remodelling pathway in which a long chain acyl group is hydrolysed from stored alkylacyl-GPC by the action of a phospholipase A_2 to form lyso-PAF (alkyllyso-GPC). This is then acetylated by an acetyltransferase enzyme to form PAF (Snyder. 1985; Snyder. 1989). A pathway involving de novo synthesis of PAF from alkyllysoglycero-P in four enzymatic has steps also been described.

The main catabolic pathway for PAF is by either a plasma or intracellular acetylhydrolase back to lyso-PAF, which in turn is converted back to alkylacyl-GPC by one of three acyl transferase pathways. Three alternative catabolic pathways involving phospholipase C, lysophospolipase D and phosphohydrolase, and an alkyl monooxygenase have been described, but are not thought to be important in man (reviewed by Lee and Snyder. 1989). It can be seen (Fig 1) that these enzymatic pathways form a cycle by which the major precursor of PAF, lyso-PAF, is also its main breakdown product.

Figure 1. The enzymatic activation and inactivation cycle for PAF.



The biological availability of this potent phospholipid is carefully regulated; lyso-PAF is almost biologically inert and the enzymatic pathways are very efficient, giving PAF a biological half-life of only 90 seconds in human plasma. Furthermore, most PAF remains cell-associated either within the cell or as a component of the cell membrane, and it has been proposed by O' Flaherty and Wykle (1987) that PAF may be transferred from cell to cell by a process of "transmembranous shuttling".

It can be seen that the metabolism of PAF is regulated in a very elegant manner, but this renders it extremely difficult to interpret studies measuring PAF levels in biological fluids and PAF production or release from different cell types. The results of all such studies must therefore be treated with considerable caution (*vide infra*).

PAF has been reported to be produced or released from a wide range of cell types implicated in the pathophysiology of asthma including neutrophils, monocytes, alveolar macrophages, platelets, epithelial cells and eosinophils (reviewed by Bratton and Henson. 1989). The human neutrophil has been extensively studied, and is the most active cell involved in the synthesis of PAF, producing up to 100 pmol/10^6 cells (Jouvin-Marche, Ninio, Beaurain, et al. 1984), of which half is released into the supernatant. Controversy surrounds the ability of the human mast cell to produce PAF; it may be able to synthetise the molecule, but

not release it into the supernatant. The undifferentiated human lymphocyte does not seem to be capable of PAF synthesis, although natural killer cells synthesise and release PAF in response to calcium ionophore or the Fc receptor. The activated human eosinophil releases only small amounts of PAF (approximately $1.2x10^{-15}$ mol/10^6 cells), but the ability of this cell to synthesise PAF is not known, and most synthesised PAF may remain cell-associated (Lee, Lenihan, Malone, et al. 1984).

PAF and lyso-PAF have been detected by bioassay in biological fluids from normal man including blood (Taylor, Sturm, Kendrew, et al. 1989), saliva (Cox, Wardlow, Jorgensen, et al. 1981) and urine (Sanchez-Crespo, Inarrea, Alvarez, et al. 1983). These findings suggest that PAF may have physiological functions, although what these may be remains to be determined.

Antigen stimulates the release of PAF from alveolar macrophages of asthmatics *in vitro* (Arnoux, Joseph, Simoes, et al. 1987), and elevated plasma lyso-PAF levels have been reported in plasma following antigen challenge *in vivo* (Nakamura, Morita, Kuriyama, et al. 1987). PAF and lyso-PAF have also been detected in BAL fluid from asthmatic patients (Court, Goadby, Hendrick, et al. 1987). Elevated levels of PAF in biological fluids has been reported to be associated with several other conditions including bronchopulmonary dysplasia (Stenmark, Eyzaguirre, Westcott, et al. 1987) and

primary acquired cold urticaria (Grandel, Farr, Wanderer et al. 1985). In contrast to this, a deficiency of PAF synthesis has been described in the rare neurological condition, Zellwegers syndrome (Datta, Wilson and Hajra. 1984). However, because of the stated difficulties in interpretation of PAF levels, the true significance of all these findings is unknown.

It was suggested by Morley, Sanjar and Page (1984), and reviewed by Morley (1986) that PAF may be largely responsible for the inflammatory features of asthma. It was initially suggested that these effects were produced as a consequence of the activation of platelets by PAF, although the recognition that PAF has multiple actions on numerous other cell types, especially the eosinophil, has since led other investigators to modify this hypothesis (Barnes, Chung and Page. 1988).

PAF has a very wide variety of relevant biological actions to suggest that it may have a role as a mediator in asthma. It is a potent chemotactic agent for a wide range of inflammatory cells including neutrophils and the eosinophil, for which it is one of the most potent chemotactic agents known (Wardlaw, Moqbel, Cromwell, et al. 1986). Furthermore, it is a potent activator of these cells. For example, PAF causes eosinophils to adhere to endothelial cells (Kimani, Tonnesen and Henson. 1988) and to synthesise LTC_4 and more PAF (Lee, Lenihan, Malone, et al. 1984). In platelets, PAF

causes dose-related aggregation (Chesney, Pifer, Byers, et al. 1982) and also stimulates calcium influx (Lee, Malone, Blank, et al. 1981), whereas in the neutrophil it causes exocytosis, superoxide generation and chemiluminescence (Shaw, Pinckard, Ferrigni, et al. 1981; Poitevin, Roubin and Benveniste. 1984).

PAF has other properties of relevance to the pulmonary inflammatory process. These include stimulation of airway mucus production (Goswami, Ohashi, Panagiotis, et al. 1987), increasing pulmonary microvascular permeability (Evans, Chung, Rogers, et al. 1987), and impairment of mucociliary clearance *in vitro* (Aursudkij, Rogers, Evans, et al. 1987).

PAF induces acute bronchoconstriction in many species including guinea pig (Vargaftig, Lefort, Chignard, et al. 1980), rhesus monkey (Patterson and Harris. 1983) and baboon (Denjean, Arnoux, Masse, et al. 1983). In man, PAF-induced bronchoconstriction is accompanied by facial flushing, tightness of the throat and a dramatic transient fall in circulating neutrophils, but with no change in the circulating platelet count (Cuss, Dixon and Barnes. 1986).

The effect of PAF which has attracted most attention in relation to asthma is its ability to increase bronchial responsiveness. PAF has been shown to cause increases in bronchial responsiveness in several animal species including guinea pig (Mazzoni, Morley, Page, et al. 1986), dog (Chung,

Aizawa, Leikauf, et al. 1986), sheep (Christman, Lefferts and Snapper. 1987), rhesus monkey (Patterson, Bernstein, Harris, et al. 1984) and baboon (Denjean, Arnoux, and Benveniste. 1988).

Cuss, Dixon and Barnes reported that inhalation of PAF caused an increase in bronchial responsiveness to MCh of up to 2.5 fold, maximum at three days and persisting for up to four weeks in five out of six normal subjects. Although other groups have reported similar findings in abstract form, only Rubin, Smith and Patterson (1987) have as yet published any similar findings; reporting an increase in responsiveness to MCh for up to 45 minutes following inhaled PAF in six normal subjects, although the duration of these changes was not apparently investigated.

The mechanism by which PAF produces this multitude of biological effects is not fully understood. It is likely that PAF acts by a variety of direct and indirect mechanisms in different circumstances. High affinity binding sites have been described on the surface of many cells including platelets, neutrophils and in homogenates from human lung membranes (Hwang, Lamb and Shen. 1985 and reviewed by Dent, Ukena and Barnes. 1989). Studies using a radioligand of the specific PAF antagonist WEB 2086 with human neutrophils (Dent, Ukena, Chanez, et al. 1989) and recently in human lung (Goldie, Pedersen, Self, et al. 1990) suggest that these sites represent specific PAF receptors.

Binding of PAF to specific receptors may trigger complex intracellular transduction mechanisms involving activation of G-proteins and protein kinase C, an increase in turnover of inositol phosphates and a rise in intracellular Ca^{2+}. Phospholipase A_2 may then be activated causing release of arachidonate from membrane phospholipids with the subsequent production of a cascade of mediators including LTs, PGs and TxA_2 (O'Flaherty and Wykle. 1987). However, the relative contributions of these different mediators in producing the biological effects attributed to PAF has yet to be determined.

TxA_2 may be an important secondary mediator of PAF in some species, as PAF-induced bronchoconstriction and BHR is inhibited by the Tx synthetase inhibitor OKY-046 in the dog (Chung, Aizawa, Leikauf et al. 1986). This would not seem to be the case in man, in whom the Tx receptor antagonist GR32191B does not reduce PAF-induced bronchoconstriction (Stenton, Ward, Duddridge, et al. 1990).

The synthesis of PAF and LTs are closely linked. The first step of PAF synthesis involves the cleaving of alkylarachidonyl-GPC, effectively liberating lyso-PAF and arachidonic acid. The arachidonic acid may under certain circumstances be channelled down the 5-LO pathway resulting in LT production (reviewed by Bratton and Henson. 1989). This hypothesis is supported by the studies of Sisson, Prescott, McIntytre, et al (1987) who found simultaneous

production of PAF and LTB_4 in activated human neutrophils.

The effects of PAF may also depend on the secondary production of LTs and vice versa. Human eosinophils and neutrophils produce LTB_4 when stimulated by PAF (Chilton, O'Flaherty, Walsh, et al. 1982) whereas human endothelial cells produce PAF when stimulated with LTC_4 or LTD_4 (Mcintyre, Zimmerman and Prescott. 1986).

In some animal models administration of PAF results in LT formation and effects which are reduced by 5-LO inhibitors and LT-receptor antagonists, but other studies have not confirmed these findings. *In vitro* studies suggest that the effects of PAF are dependent on the release of 5-LO products in both the rabbit and the rat (Camussi, Montruchio, Antro, et al. 1983; Voelkel, Worthen, Reeves, et al. 1982).

Many conflicting findings have been reported from studies in the guinea pig. Jancar, Theriault, Lauziere et al (1989) found that PAF induced the release of spasmogens, possibly LTB_4, from lung strips which was blocked by the 5-LO inhibitor nordihydroguaiaretic acid (NDGA) but not by the LT receptor antagonist FPL-55712. However, Bonnet, Thibaudeau and Bessin (1983) found that PAF-induced bronchoconstriction *in vivo* is reduced by NDGA and FPL-55712. In the same species Fitzgerald, Payne, Garland et al (1988) found that PAF-induced bronchoconstriction was not reduced by the 5-LO inhibitor BW A4C *in vivo* and a direct mechanism was

suggested by Stimler and O'Flaherty (1983) as PAF-induced constriction of lung parenchymal strips was not reduced by either NDGA or FPL-55712.

Interpretation of this work in animals is again made difficult because of considerable inter-species variation in the effects of these mediators, and also by the lack of potency and selectivity of earlier LT-antagonists and 5-LO inhibitors. Furthermore, in some animal models the effects of PAF are dependent the route of administration, and on the presence of circulating cells, in particular platelets (Vargaftig, Lefort, Chignard, et al. 1980), whereas in different models a platelet-independent effect can be demonstrated even in the same species (Hamasaki, Mojarad, Saga, et al. 1984).

There have been very few studies on the mechanism of action of PAF in man. Schellenberg, Walker and Snyder (1983) demonstrated a platelet-dependent contraction of human bronchus *in vitro*, whereas Johnson, Armour and Black (1988) found a platelet independent contraction at high concentrations of PAF. PAF-induced mucus production in human airways is reduced by pre-treatment with the cycloxygenase/5-LO inhibitor BW755C *in vitro* (Goswami, Ohashi, Panagiotis, et al. 1987), suggesting that the LTs may be involved in this process.

The only *in vivo* studies on the mechanism of inhaled PAF in man have yielded conflicting results. Smith, Rubin and Patterson (1988) reported that PAF-induced bronchospasm was reduced by pre-treatment with Chlorpheniramine, suggesting that the secondary release of histamine may be important. In contrast to this, Chung, Minette, McCusker, et al (1988) obtained no reduction in PAF-induced bronchospasm after pre-treatment with ketotifen, an orally active antiallergic agent with antihistamine activity.

1.10. ASSESSMENT OF BRONCHIAL RESPONSIVENESS

Bronchial responsiveness is measured by provocation testing. The most widely used and extensively evaluated pharmacological methods employ inhalation of doubling concentrations of aerosols of either histamine or MCh until a predetermined reduction in pulmonary function has been obtained (Cockcroft, Killian, Mellon, et al. 1977; Juniper, Frith, Dunnett, et al. 1978). Challenge procedures have also been devised for other non-specific stimuli such as cold air and exercise, although these are much less frequently used in adults as they are more laborious and tend to be less reproducible than pharmacological techniques. However, exercise testing is a safe technique which is particularly suitable for use in children, in whom it has been comprehensively evaluated (Silverman and Anderson. 1972).

There is a close relationship between the degree of responsiveness obtained following challenge with different

non-specific pharmacological stimuli (Makino. 1966. Cockcroft, Killian, Mellon, et al. 1977; Juniper, Frith, Dunnett, et al. 1978; Juniper, Frith and Hargreave. 1981; Hargreave, Ryan, Thomson, et al. 1981). The degree of responsiveness to MCh also correlates with the response to cold air (O'Byrne, Ryan, Morris, et al. 1982), and with the response to exercise (Kiviloog. 1973). These findings suggest that the various non-specific challenge techniques are all measuring a genuinely non-specific phenomenon, and that it is not of critical significance which type of challenge is performed to assess bronchial responsiveness. Instead, it is probably more important to gain practical expertise in the use of a single technique in order to maximise the reproducibility of each procedure in individual centres.

1.11. THE STUDY OF BRONCHIAL RESPONSIVENESS

The most direct way to investigate the relationships between inflammation and BHR in asthma is to perform morphological and pharmacological studies on pulmonary tissue obtained from asthmatic patients. Ethical and practical considerations limit the frequency with which such studies can practically be performed. Bronchial biopsy and bronchoalveolar lavage are unpleasant procedures for the patient, and can only be performed on a limited number of occasions in each individual.

Pharmacological studies (reviewed by Black and Armour. 1989) have usually failed to demonstrate a correlation between *in*

vivo and *in vitro* responsiveness to any particular inflammatory mediator. Nor have any consistent increases been demonstrated in the responsiveness of isolated bronchial smooth muscle to a variety of factors including physical stimuli, allergen and inflammatory mediators. Exceptions to this rule include potentiation of contraction by $PGF_{2\alpha}$ (Armour, Black and Johnson. 1988) and increased sensitivity to histamine induced by high concentrations of PAF in isolated human bronchus (Johnson, Armour and Black. 1988). The latter finding may be a non-specific effect as it has recently been demonstrated using the ligand [^{3}H]-WEB 2086 that specific binding sites for PAF are scarce on human bronchial smooth muscle (Goldie, Pedersen, Self, et al. 1990).

Given the increasing emphasis on the importance of interactions between mediators, circulating cells and the nervous system of the lung, the relevance of such *in vitro* studies to the study of BHR must be limited.

Physiological and pharmacological studies in man involving inhalation challenge with various physiological and pharmacological stimuli are extremely important in asthma research. However, such studies are particularly difficult to perform on asthmatic patients. In particular, the reproducibility of the challenge procedure is often limited by the variability of baseline pulmonary function, and the dose of agonist must be limited in order to avoid provoking

a severe asthmatic reaction. Furthermore, it may not be feasible or ethically justifiable for patients with significant disease to stop taking their regular medications, potentially modifying the response to the challenge substance.

Studies in animals are generally more easy to perform and allow ready access to tissue. Experiments can be readily performed under reproducible conditions using pure bred animals with precisely defined morphological and genetic characteristics. Experiments can be readily repeated on the same animals or with replacements. The great disadvantage of studies in animals is that asthma is a disease confined to humans, and no satisfactory animal model of exists. In addition, there is a great deal of interspecies variation in immunological and inflammatory mechanisms, and in responses to various inflammatory mediators. Animal models of BHR also differ markedly in many physiological respects from the antigen-induced changes in BHR seen in asthmatic subjects (Schellenberg. 1987). The results of all such studies in animal models must therefore be viewed with extreme caution.

Inhalation challenge studies in normal human volunteers are easier to perform and generally more reproducible than in asthmatic patients, as baseline pulmonary function and bronchial responsiveness are usually stable. Although such studies are vital to increase our understanding of the factors controlling bronchial responsiveness in man, the

nature of the fundamental differences between normals and asthmatics is still poorly understood. Therefore, the results of such studies are not necessarily valid for asthmatic patients.

Various stimuli have been used to study changes in bronchial responsiveness in animals and man. These include physical agents such as non-isotonic solutions (Foresi, Mattoli, Corbo, et al. 1986), exercise (Silverman and Anderson. 1972; Deal, MacFadden, Ingram, et al. 1979), isocapnic hyperventilation, allergens (Cockcroft, Ruffin, Dolovich, et al. 1977), inflammatory mediators (Walters, Parrish, Bevan, et al. 1981; Barnes, Piper and Costello. 1984; Fuller, Dixon, Dollery, et al. 1986), viruses (De Jonste, Degenhart, Niejens, et al. 1984), bacterial toxins, occupational sensitisers such as toluene diisocyanate (Mapp, Polato, Maestrelli, et al. 1985), foods preservatives and drugs (Wilson, Vickers, Taylor, et al. 1982), and pollutants including ozone (O_3), SO_2, NO_2 and cigarette smoke (Cockcroft, Bersheid and Murdoch. 1983B).

Inhalation of ozone is probably the most extensively studied agent capable of causing an increase in bronchial responsiveness (reviewed by Boushey and Holtzman. 1985). Ozone has been shown to cause a transient increase in bronchial responsiveness in dog (Lee, Bleeker and Nadel. 1977), guinea-pig and man.

In the dog, ozone-induced BHR is associated with increase numbers of neutrophils in the airway wall, and with recovery of increased numbers of these cells in BAL fluid. Prior neutrophil depletion with hydroxyurea inhibits the BHR induced by ozone in dogs (O'Byrne, Walters, Gold, et al. 1984) as does pre-treatment with indomethacin (O'Byrne, Walters, Aizawa, et al. 1984). This suggests that the phenomenon is depend on neutrophil-derived cyclooxygenase products. In the guinea-pig, neutrophil infiltration is absent at the time of maximum BHR, but occurs during the recovery phase, emphasing the species specificity of this phenomenon (Murclas and Roum. 1985).

Ozone causes a transient increase in bronchial responsiveness in normal and atopic human subjects, but this has never been demonstrated in asthmatics. This response is blocked by pre-treatment with atropine, suggesting a cholinergic mechanism (Golden, Nadel and Boushey. 1978), and is also associated with increased numbers of neutrophils in bronchial biopsies and BAL fluid, along with increased concentrations of PGE_2, $PGF_{2\alpha}$ and Tx (Seltzer, Bigby, Stulbarg, et al. 1986).

The effect of numerous physical factors known to provoke exacerbation of symptoms in individual asthmatic patients has been extensively investigated. It has been claimed that exercise, inhalation of non-isotonic solutions and isocapnic hyperventilation all increase bronchial responsiveness

through a common mechanism involving changes in the osmolarity of the surface lining of the airways (Smith and Anderson. 1986). However, the effects of these stimuli on bronchial responsiveness are dissimilar.

Exercise caused no consistent increase in BHR in several studies (for example Rosenthal, Laube, Jaeger, et al. 1984), although Suzuki, Chonan, Sasaki, et al (1985) reported a short term increase in sensitivity to MCh in nine asthmatics of whom four did not experience exercise-induced bronchoconstriction. Inhalation of nebulised water causes small, but consistent increases in responsiveness to histamine which persist for up to five days (Foresi, Mattoli, Corbo, et al. 1986) and isocapnic hyperventilation does not induce any changes in subsequent responsiveness to MCh (Rosenthal, Laube, Jaeger, et al. 1984).

The situation regarding airway responses to reduced temperature is also confusing. Malo, Cartier, L'Archeveque et al (1986) have reported minimal increases in responsiveness to MCh, but not to histamine after isocapnic cold air inhalation challenge, whereas Wilson, Dixon and Silverman (1985) have observed increased sensitivity to histamine maximal 90 minutes after the ingestion of ice in six out of seven asthmatic children. The mechanism of this latter response was not investigated, but could be a cholinergically mediated effect as bronchoconstriction associated with gastro-oesophageal reflux may occur through

a vagal mechanism (Mansfield and Stein. 1978).

Other ingested substances reported to increase bronchial responsiveness include common salt (Javaid, Cushley and Bone. 1988) and cola drinks (Wilson, Vickers, Taylor, et al. 1982), although the mechanism by which the changes were induced was not investigated in either of these studies.

Conditions associated with increased intrathoracic blood volume, such as left ventricular failure, are known to increase bronchial responsiveness. Several mechanisms may be important, including congestion of bronchial vessels reducing the calibre of the bronchial lumen (Freedman. 1972) the effects of increased plasma extravasation (Persson. 1986) and reflex increases in vagal tone (Regnard, Baudrillard, Salah, et al. 1990). This phenomenon may be important in wheeze associated with pulmonary oedema ("cardiac asthma"), but the relevance to bronchial asthma has not yet been determined.

Inhalation of allergen is a well recognised precipitant of asthmatic symptoms in many patients. A proportion of patients will develop a dual response after inhaling antigen, with an early response followed by recovery of pulmonary function and then a late response at six to eight hours. The early response is thought to be due to smooth muscle contraction as it is readily reversed by the inhalation of β-agonist. The late response is not completely

abolished by β-agonist, and is presumed to be largely due to an inflammatory process.

There is a strong association between the magnitude of the late response to allergen and the clinical severity of the disease. Cockcroft, Ruffin, Dolovich, et al (1977) have demonstrated that BHR to histamine is increased for up to seven days following antigen challenge in asthmatic patients exhibiting a late response. The magnitude and duration of the subsequent increase in BHR following allergen challenge correlates well with the magnitude of the late response, and is not due to changes in airway calibre (Cartier, Thomson, Frith et al, 1982).

The increase in BHR following inhalation of allergen can be demonstrated before the appearance of the late response (Thorpe, Steinberg, Bernstein, et al. 1987). However, bronchoalveolar eosinophilia with elevated levels of ECP in BAL fluid occurs during, but not before, the late reaction (De Monchy, Kauffman, Venge, et al. 1985); this suggests that eosinophil products may not be not necessary for expression of this phenomenon.

Cockcroft (1983) has postulated that the increase in bronchial responsiveness induced by allergen might in turn enhance the subsequent immediate response to further challenge with allergen, creating a cycle which would in turn lead to further increases in bronchial responsiveness.

This hypothesis is almost impossible to test, mainly because the immediate response to antigen is not very reproducible even under carefully controlled laboratory conditions. However, this concept has important implications for the management of allergen-induced asthma.

CHAPTER TWO

PLAN OF INVESTIGATION

The magnitude and duration of the changes in bronchial responsiveness described by Cuss, Dixon and Barnes (1986) following the inhalation of PAF in normal man are comparable to those observed after antigen challenge in asthmatic patients. This finding in combination with the other known biological properties of PAF suggests that this substance may be of central importance in the pathophysiology of asthma.

There is no satisfactory animal model for asthma and it is difficult to perform inhalation challenge studies in patients with active disease. A PAF inhalation challenge procedure resulting in a reproducible increase in bronchial responsiveness in normal man could therefore be a useful model with which to study the mechanisms of BHR. Furthermore, if PAF proved to be of central importance in the pathophysiology of asthma, such a model could also be useful for the preliminary assessment of potential new anti-asthma drugs in man.

We therefore undertook to develop a PAF inhalation challenge in normal man in order to verify these findings, and to determine the reproducibility of the challenge with respect to both the acute changes and subsequent changes in bronchial responsiveness. It was then our intention to use this PAF challenge procedure to study the mechanisms by which both the acute changes and subsequent changes in bronchial responsiveness were induced in man. In particular,

as experimental evidence suggested that the effects of PAF may depend the secondary release of LTs, we wished to investigate whether selective cysteinyl-LT receptor antagonists and 5-LO inhibitors might modify the effects of inhaled PAF in man.

CHAPTER THREE

SUBJECTS, MATERIALS AND METHODS

3.1. ETHICAL CONSIDERATIONS

Each of the studies described was individually approved by the ethics committee of King's College Hospital. All subjects were volunteers and gave signed informed consent.

As very large doses of PAF are known to have potentially adverse cardiovascular side effects (Gateau, Arnoux, Deriaz, et al. 1984), the safety of the initial PAF inhalation challenge technique, and of each successive modification to the challenge procedure, was initially assessed on myself or on a volunteer member of staff of the Department of Thoracic Medicine.

The safety of both investigational compounds SKF 104353-Z_2 and BW A4C in man has been assessed by other investigators (Evans, Barnes, Piper, et al. 1989; Garland, Jackson and Salmon. 1990)

3.2. SUBJECTS

All studies were performed on healthy, non-smoking adult volunteers. Males and females were used in the initial studies, but no females were allowed to participate in the subsequent studies using SKF 104353-Z_2 and BW A4C because of statutory restrictions confining the administration of investigational pharmaceutical compounds to males.

No subject gave a history of asthma or chronic respiratory disease. All subjects had negative skin prick tests to

inhaled allergens and no history of allergic rhinitis unless specifically stated. Physical examination, chest X-ray, electrocardiogram (ECG) and a routine haematological and biochemical screen were always within normal limits.

All subjects had been previously trained in the performance of the relevant pulmonary function tests and had baseline pulmonary function and bronchial responsiveness within the normal range.

No subject was on any medication during the study period and caffeine-containing beverages were avoided for at least six hours before any challenge (American Thoracic Society. 1980). Any subject with an upper respiratory tract infection was excluded from study for a minimum of six weeks and only recommenced the study after confirmation that bronchial responsiveness had returned to the previously determined baseline level for that subject (Empey, Laitinen, Jacobs, et al. 1976).

3.3. MATERIALS

Doubling dilutions of methacholine hydrochloride [MCh]. (Sigma Chemical Co, St Louis, Mo. USA) from 64 mg/ml to 0.5 mg/ml and of Histamine (Sluka Chemi AG. Switzerland) from 10^{-1}M to 10^{-4}M were prepared in advance by King's College Hospital pharmacy using a diluent of 0.9% saline with 0.5% chlorbutal B.P., and stored at 4°C until required. HSA was supplied by Immuno Ltd, Sevenoaks, Kent.

For the cumulative dose response PAF challenges (Chapter 4), the study on platelet activation (Chapter 5) and the SKF 104353-Z_2 study (Chapter 6), C-18 PAF and lyso-PAF were obtained as a dry powder (100mg) from Ba chem U.K., Saffron Waldron, Essex. This product was used as it induces reproducible local inflammatory changes when injected intradermally into human subjects (Sciberras, Goldenburg, Bolognese, et al. 1987). C-18 PAF or lyso-PAF (100mg) was dissolved in 20ml absolute ethanol and stored in polyropylene tubes as 0.5ml aliquots (2.5mg PAF or lyso-PAF) at -80°C.

For the BW A4C study (Chapter 6), C-18 PAF was supplied by Cascade Biochem Ltd (The Innovation Centre, University of Reading) in vials containing 1.6mg of lyophilised powder and stored at -20°C.

The investigational compounds SKF 104353-Z_2 and BW A4C along with matching placebos were kindly supplied, free of charge, by the manufacturers in the form detailed in the relevant chapters. SKF 104353-Z_2 was supplied by Smith, Kline and French Research Laboratories, Mundells, Herts and BW A4C by Wellcome Research laboratories, Beckenham, Kent.

3.4. PULMONARY FUNCTIONS MEASUREMENTS AND ASSESSMENT OF BRONCHIAL RESPONSIVENESS

3.4.1. Facilities

All pulmonary function measurements and inhalation challenge studies were performed in the bronchial challenge room of the Department of Thoracic Medicine, King's College Hospital. This room is equipped with a full range of rescucitation equipment, and facilities are immediately available for the administration of oxygen and nebulised β-agonists.

Challenges involving the administration of PAF or lyso-PAF were performed by myself with the assistance of either a senior pulmonary function technician (Miss Sally Green) or a nurse. The majority of the histamine and MCh challenges were performed by myself, the remainder were perfomed by Miss Sally Green.

3.4.2. Reproducibility

The usefulness of any measurement for research purposes is largely determined by its reproducibility. This applies both to individual measurements of pulmonary function and to estimates of bronchial responsiveness obtained by inhalation challenge techniques.

If a measurement has poor reproducibility, it implies that variables are operating which are either unpredictable, unrecognised or difficult to quantify and control. This

suggests that the method will have limited useful application. However, the precise degree of reproducibility required will depend on the proposed use for the procedure; for example, a different degree of reproducibility may be required for detailed physiological studies on an individual subject compared to that needed for epidemiological use on a large population.

Factors affecting the reproducibility of a pulmonary function measurement will depend on a large number of variables. These can be divided for convenience into those dependent on the subject, and those dependent on the pulmonary function measurement or challenge technique.

Human influences on reproducibility include the experience and competence of the investigator (Tattersfield and Keeping. 1979), diurnal variation and the degree of training of the subject in the individual manoeuvres. Normal subjects generally give more reproducible results than do asthmatics for reasons discussed in the introduction (Section 1.11.), and only normals were used in all of the studies to be described. Various medications and foodstuffs potentially affect the response to an agonist, including theobromides present in tea, coffee, chocolate and cola drinks, which were therefore avoided for six hours prior to the challenges in these studies (American Thoracic Society. 1980).

The effect of viral upper respiratory tract infections on bronchial responsiveness has been discussed at length. It is not known whether a recent upper respiratory tract infection may alter the acute response to PAF, although responses do not seem to directly correlate with the degree of bronchial responsiveness (Cuss, Dixon and Barnes. 1986; Rubin and Smith. 1987). PAF challenge was not performed within six weeks of an upper respiratory tract infection because of this possibility, and because it would render it impossible to interpret the significance of any subsequent changes in bronchial responsiveness.

Other ill-defined human factors which may affect reproducibility include the motivation of the individual subject and boredom. It is definitely possible to "exhaust" the subject by asking them to perform a large number of manoeuvres (either forced expirations or panting manoeuvres) over a short period of time. This factor may adversely effect the reproducibility of a challenge procedure, but has not to my knowledge been formally investigated. "Exhaustion" is almost certainly dependent on human factors including reduced concentration rather than on respiratory muscle fatigue. Nevertheless, this creates a genuine practical problem in deciding the optimum number of manoeuvres to perform at each time point, as reproducibility may also be improved by taking the mean of a large number of measurements (discussed for sGaw by Tattersfield and Keeping. 1979).

The reproducibility of the different indices of pulmonary function employed will be discussed individually. The reproducibility of the bronchial challenge procedures will depend on the reproducibility of these individual measurements, on the human factors mentioned and also on numerous variables affecting delivery of the challenge substance to the airways. These variables including nebuliser output, aerosol particle size, pattern of breathing and details of the method used for calculating the response (reviewed by Eiser. 1987). The use of a standard technique in each laboratory is necessary to minimise the effects of these multiple variables, and attempts have also been made to introduce international standards (American Thoracic Society. 1980; Eiser. 1987).

3.4.3. Individual measurements of pulmonary function

Three indices of pulmonary function were used in these studies; Forced expiratory volume in one second (FEV_1), specific airways resistance (sGaw) and maximum expiratory flow at 30 per cent above residual volume calculated from a partial expiratory flow-volume manoeuvre ($\dot{V}max_{30}$).

3.4.3.1. Measurement of FEV_1

This measurement was performed (mean of three values) as an index of baseline pulmonary function at the beginning of each bronchial challenge session using a dry wedge bellows spirometer (Vitalograph Ltd, Bucks.). It was repeated following administration of the investigational compounds

SK&F 104353-Z_2 and BW A4C in order to confirm that these compounds had no effect on baseline pulmonary function.

This measurement was employed as it is a reproducible index of expiratory flow for which normal values are readily available (Cotes. 1979; Larsson, Hedenstrom and Malberg. 1987), giving an indication that volunteers had normal baseline pulmonary function. It was also used to confirm that any changes in bronchial responsiveness or responses to agonist challenge observed over the over a period of time were genuine, and not the result of significant changes in baseline airway calibre. Measurement of FEV_1 is not suitable for the assessment of bronchoconstrictor drugs as it is not sensitive to small changes in airway calibre (Pride. 1979). It is also recognised that the initial maximum inspiratory manoeuvre required for this measurement can itself induce bronchodilatation in some subjects (Nadel and Tierney. 1961).

Baseline values for FEV_1 for each subject are quoted in litres and as a percentage of predicted values for white european caucasians from Cotes (1979). All subjects were white europeans except for subject five in the SKF 104353-Z_2 study who was Indian; the low value as a percentage of predicted for this subject reflects the smaller mean lung volumes of this race.

3.4.3.2. Measurement of sGaw

Measurements of airway resistance performed in a body plethysmograph provide a sensitive index of changes in airway calibre, avoiding potential errors introduced by performing a maximum inspiration or dynamic compression due to forced expiration (Tattersfield and Keeping. 1979).

The value obtained for airway resistance is due to the combined resistance in a great number of airways connected in series and in parallel. The airway resistance at any given level in the tracheobronchial tree depends on the total cross-sectional area of the airways at that level. In normal subjects, the total cross-sectional area of the airways is greatly increased towards the periphery, so that during quiet breathing the majority of the resistance occurs in the trachea and large airways.

Airway resistance varies with lung volume which has an approximately linear relationship to the inverse of airway resistance, airway conductance (Gaw). It is therefore convenient to refer to specific airway conductance (sGaw), which is Gaw divided by the thoracic gas volume (TGV).

Airway resistance can be readily measured by three methods: body plethysmography, the oesophageal balloon method and the forced oscillation technique. Body plethysmography is the most frequently used and best validated procedure, and is the method used routinely in our laboratory.

Body plethysmography was first described by DuBois, Botelho and Comroe in 1956. One advantage of this technique is that it gives a simultaneous measurement of TGV. The original method has frequently been modified, notably by Lord and Brooks (1977) who described improved reproducibility by computerising the measurements of slope, thereby reducing observer bias.

An automatic pressure-flow plethysmograph (Gould 2800 autobox, Cardiokinetics Ltd. Salford) was used in these studies. The subject sits in a closed airtight box with a volume of approximately 600 litres and breathes heated air through a pneumotachograph. The box pressure (Pb) is displayed on the X axis of an oscilloscope and the airflow at the mouth ($\dot{V}m$) is displayed on the Y axis. The subject pants at 2 to 3 breaths per second, minimising drift due to differences in temperature, changes in water vapour saturation and RQ effects, and also keeping the glottis open. An "S" shaped curve is produced on the screen, and it's slope ($\dot{V}m/Pb$) is measured by the computer.

The airway is then occluded and the subject continues to make panting efforts with no flow of air at the mouth. Mouth pressure (Pm) is now traced on the Y axis, and the slope of the line Pm/Pb is measured. This slope is also used to calculate TGV and the change in box pressure for the change in alveolar pressure (Palv), as Palv = Pm under conditions of zero flow. Airway resistance is the ratio of alveolar pressure to flow obtained from the ratio of the two slopes:

$$Raw = \frac{Palv}{\dot{V}m} = \frac{\frac{Palv}{Pb}}{\frac{\dot{V}m}{Pb}}$$

The main limitation in using sGaw as an index of airway calibre is that the reproducibility of the measurement is not as good as for conventional spirometry. Coefficients of variation of 10% to 20% are frequently quoted (reviewed in Tattersfield and Keeping. 1979). The reproducibility of the technique can be improved by using well trained subjects, by using the mean value of multiple measurements taken at each time point, and by performing sequential challenges at the same time of day, as airways resistance exhibits considerable diurnal variation.

In these studies results are expressed either as the absolute value ($s^{-1}kPa$) or as a percentage of values obtained after the inhalation of diluent.

3.4.3.3. Measurement of $\dot{V}max_{30}$

The use of maximum expiratory flow-volume curves to study forced expiration was first reported by Hyatt, Schilder and Fry in 1958. These investigators subsequently demonstrated that flow during the final 70% of the forced vital capacity is independent of effort. This phenomenon occurs because of dynamic compression of the central intrathoracic airways resulting in the formation of flow limiting segments. During forced expiration, expiratory muscle effort increases pleural pressure, which as well as tending to increase expiratory flow, also acts on the external surface of the major bronchi resulting in airway compression. As driving pressure increases above a threshold, the diameter of the major airways is reduced leading to increased airway resistance and preventing any further increase in flow (discussed by Pride. 1971).

Mead, Turner, Macklem, et al (1967) demonstrated that in normal subjects maximum expiratory flow becomes increasingly dependent on the calibre of the peripheral airways towards the end of expiration. These findings suggested that measurements of flow derived from partial expiratory flow-volume manoeuvres may be useful for the investigation of changes in small airway calibre (Bouhuys, Hunt, Kim, et al. 1969).

Intrasubject variability is again the main drawback in the use of this measurement, although this can improved by using similar guidelines to those used when measuring sGaw.

The specific measurement of partial expiratory flow used in these studies was expiratory flow at 30% of vital capacity above residual volume ($\dot{V}max_{30}$). At the beginning of each bronchial challenge, maximum expiratory flow-volume loops (mean of five values) were performed to obtain the vital capacity, using a rolling seal spirometer (P.K. Morgan, Gillingham, Kent) attached to a differentiator (P.K. Morgan) and recorded on an X-Y recorder (Rikadenki Mitsui Electronics (U.K.) Ltd. Chessington, Surrey). Five partial expiratory flow volume manoeuvres were then performed to obtain the baseline $\dot{V}max_{30}$, which was derived manually. The timing of subsequent measurements is detailed in the description of measurement of bronchial responsiveness.

Results are expressed either as a the absolute values ($l\ s^{-1}$) or as percentage of the values obtained after the inhalation of diluent.

3.4.4. Measurement of bronchial responsiveness

This was performed using modifications of the protocol described for histamine by Cockcroft, Killian, Mellon, et al (1977). MCh was used as the agonist in the studies investigating the reproducibility of the PAF inhalation challenge and the effects of a cysteinyl-LT receptor

antagonist SK&F 104353-Z_2, whereas histamine was used as the agonist in the study using the 5-lipoxgenase inhibitor, BW A4C.

Bronchial responsiveness was defined as the provocation concentration of agonist causing a 35% fall in sGaw (PC_{35}sGaw) and as the provocation concentration causing a 30% fall in $\dot{V}max_{30}$ ($PC_{30}\dot{V}max_{30}$). Baseline pulmonary function was initially assessed by measurements of FEV_1 (mean of three values), sGaw (mean of 16 values) and $\dot{V}max_{30}$ (mean of five values). The subject subsequently inhaled diluent (0.9% saline with 0.5% chlorbutol B.P.); 2ml of diluent was placed in the reservoir of a Wright nebuliser driven by air at 7 litres per minute and the aerosol was inhaled from a face mask by tidal breathing with the mouth open for two minutes. The output of the nebuliser was assessed gravimetrically as 0.13ml per minute.

Control measurements of sGaw (mean of 16 values) were made at one minute and of $\dot{V}max_{30}$ (mean of 5 values) at three minutes after inhalation of diluent. All subsequent measurements obtained after inhalation of agonist were expressed as a percentage of these control values.

The subject proceeded to inhale the lowest concentration of MCh (0.5 mg/ml) or histamine (10^{-4}M) in the same manner as described for diluent. Measurements of sGaw (mean of 8 values) were made at one minute, and of $\dot{V}max_{30}$ (mean of 2

values) at three minutes after the inhalation when histamine was used as the agonist. When MCh challenge was performed, measurements of sGaw were repeated at six minutes and of $\dot{V}max_{30}$ at eight minutes and the lowest pairs of mean values were retained, as MCh has a delayed onset of action compared to histamine.

If the sGaw remained above 90% of the control value, the next doubling concentration of agonist was inhaled immediately after completing the previous pulmonary function measurements. If the sGaw fell below 90% but was above 65% of the control measurement, the pulmonary function tests were repeated at five minute intervals until sGaw had returned to above 90% of the control value. The challenge continued until either sGaw had fallen to below 65% of the control value or the final concentration of agonist (64mg/ml MCH, 10^{-1}M histamine) had been reached.

The PC_{35}sGaw and $PC_{30}\dot{V}max_{30}$ were derived by linear interpolation from log^{10} dose response curves. If a 35% fall in sGaw was accompanied by a fall of less than 30% in $\dot{V}max_{30}$, $PC_{30}\dot{V}max_{30}$ was obtained by extrapolation of the dose response curves if a definite trend was found. In the absence of a definite fall in the $\dot{V}max_{30}$, the extrapolation was not made and these data were excluded from subsequent analysis.

3.4.5. General description of PAF and lyso-PAF challenges

Baseline measurements of FEV_1 (mean of three values), sGaw (mean of 16 values) and $\dot{V}max_{30}$ (mean of five values), haemoglobin, differential white cell count and platelet count were initially obtained. Blood pressure was measured using an automatic sphygmomanometer (Dynamap) and the subject connected to an ECG monitor.

Ten inhalations of diluent (HSA 2.5mg/ml in 0.9% saline) were taken by tidal breathing with the nose clipped from a "Microneb lll" nebuliser (Lifecare Hospital Supplies Ltd, Market Harborough, Leics.) attached to a "Nebicheck" dosimeter (P.K. Morgan). The dosimeter was driven at a pressure of 30 p.s.i. with a flow of air to the nebuliser of 8 litres per minute; delivery of the aerosol was limited to 1.4 seconds with an obligatory delay between inhalations of 4 seconds. The ECG was monitored continuously during the inhalation of PAF or lyso-PAF, and the blood pressure was recorded on completing the inhalation.

Control measurements of sGaw (mean of 16 values), $\dot{V}max_{30}$ (mean of 5 values) and differential blood count were obtained at one, three and five minutes respectively after the inhalation of diluent. All subsequent measurements were expressed as a percentage of these control values.

There were considerable differences in the subsequent details of the PAF inhalation challenge procedure employed in separate studies. These details will be described in the appropriate chapters.

3.5. STATISTICAL ANALYSIS

The statistical analyses employed will be described in detail in the relevant chapters. Data were analysed using the "Minitab" statistical software package (Minitab Inc., State College, PA. USA) and the confidence interval analysis (CIA) software program (Gardner, Gardner and Winter. 1989) in conjunction with the textbooks "Statistics with confidence" (Gardner and Altman. 1989) and "The Minitab Handbook" (Ryan, Joiner and Ryan. 1976).

CHAPTER FOUR

PRELIMINARY STUDIES AND ASSESSMENT OF THE REPRODUCIBILITY OF THE ACUTE EFFECTS AND ANY SUBSEQUENT CHANGES IN BRONCHIAL RESPONSIVENESS FOLLOWING INHALED PAF IN MAN

4.1. ABSTRACT

The reproducibility of acute effects of inhaled PAF on airway calibre, circulating neutrophil count, and any subsequent increase in bronchial responsiveness was studied in six normal subjects and compared to the effects of inhaled lyso-PAF, the inactive precursor and metabolite of PAF.

Inhalation of PAF resulted in acute bronchoconstriction and a transient fall in neutrophil count on two separate occasions in five out of six subjects (Minimum percentage of baseline values (mean): First PAF challenge; sGaw 69%; $\dot{V}max_{30}$ 72%; neutrophil count 70%. Second PAF challenge; sGaw 61%; $\dot{V}max_{30}$ 74%; neutrophil count 63%). In one subject inhaled PAF caused bronchoconstriction and a transient fall in neutrophil count once, but a second challenge resulted in no detectable changes. The reproducibility of the acute changes induced by PAF on the two occasions was poor (mean difference between the first PAF challenge and the second PAF challenge [coefficient of repeatability]: sGaw; 8.2% [46.9]. $\dot{V}max_{30}$; -1.8% [54.5]. Neutrophil count; 7% [57.2]).

There was no significant increase in bronchial responsiveness to MCh either in the whole group or in any individual subject at any time when measured on five occasions over a two week period following each PAF challenge. Challenge with lyso-PAF did not cause acute effects or any subsequent changes in bronchial

responsiveness.

These findings demonstrate that any effects of inhaled PAF on bronchial responsiveness in normal man are small and probably not of clinical significance. It would be inappropriate to use this human model either to study the mechanisms of bronchial hyperresponsiveness, or for the preliminary assessment of potential new anti-asthma drugs.

4.2. INTRODUCTION

As discussed in the methods section (Section 3.4.2.), in order for any bronchial challenge procedure to be useful for further studies, the induced changes must be reproducible. In relation to PAF challenge this will apply to both the acute changes and to any subsequent increase in bronchial responsiveness. Furthermore, the challenge must be well-tolerated and without severe adverse side-effects. This is particularly important in relation to PAF which may be an important mediator of anaphylaxis, causing sudden death when given intravenously to mice (Terashita, Imura, Shino, et al. 1987) and marked cardiovascular effects when instilled into the trachea of decerebrate man (Gateau, Arnoux, Deriaz, et al. 1984).

We therefore undertook to design a PAF challenge which would induce acute bronchoconstriction and a transient fall in circulating neutrophils without severe adverse effects in normal human subjects. The reproducibility of these acute effects and of any subsequent changes in bronchial responsiveness following this PAF challenge procedure have been assessed, using challenge with lyso-PAF as a negative control.

The reproducibility of MCh and histamine challenges has been shown by Juniper, Frith, Dunnett et al (1978) to be within one doubling concentration using $PC_{20}FEV_1$, and similar findings have been reported using PC_{35}sGaw to histamine

(Cockcroft and Berscheid. 1983). The reproducibility of PC_{35}sGaw MCh and $PC_{30}\dot{V}max_{30}$ MCh has not previously been published. As the ability to detect changes in bronchial responsiveness following inhaled PAF will be critically dependent on the reproducibility of the MCh challenge, it was important to verify the reproducibility of the procedure in our own laboratory. We therefore assessed the reproducibility of PC_{35}sGaw MCh and $PC_{30}\dot{V}max_{30}$ MCh in a separate group of six normal subjects.

The dose of agonist given by nebuliser is conventionally assessed gravimetrically. This is a poor reflection of delivery for any substance, as it does not gauge evaporative loss and it assumes that all of the of the compound remains in solution. Little is known about the delivery of any polar lipid by the nebulised route, although the study by Barnes, Piper and Costello (1984) using LTC_4 and LTD_4 suggests that nebulisation results in a 50% loss of biological activity. PAF adheres strongly to many surfaces, especially glass and is usually prepared in an albumin solution to reduce this problem (Snyder, 1985), although the effect of albumin on delivery of nebulised PAF is not known. Pilot studies were therefore performed to determine the output characteristics of the nebuliser.

4.3. REPRODUCIBILITY OF MCh CHALLENGE

4.3.1. Subjects

Six healthy subjects, four males and two females aged 20-41 years (mean age 27 years) participated in the study.

4.3.2. Method

MCh challenge was performed according to the standard protocol at the same time of day on two occasions separated by one week in each subject.

4.3.3. Statistical analysis

The reproducibility of the MCh challenge was assessed by plotting the difference between the two measurements against their mean. The 95% confidence interval (95% CI) for the mean difference between the two challenges was calculated after logarithmic transformation of the data for both PC_{35}sGaw MCh and $PC_{30}\dot{V}max_{30}$ MCh (Bland and Altman. 1986).

4.3.4. Results

These are shown in Table 1. The mean ratio for PC_{35}sGaw MCh was 1.1 (95% CI= 0.886, 1.38) and all values were within one doubling concentration of MCh. The mean ratio for $PC_{30}\dot{V}max_{30}$ MCh was 0.89 (95% CI 0.48, 1.65). Five of the six subjects reacted within one doubling concentration, but one subject reacted at 64mg/ml once and 28mg/ml on the second occasion.

Table 1. Reproducibility of MCh challenge (mg/ml MCh)

	$PC_{35}sGaw$		$PC_{30}\dot{V}max_{30}$	
SUBJECT	DAY 1	DAY 2	DAY 1	DAY 2
1	34	28	12	19
2	18	20	13	19
3	7	6	5	8
4	62	55	64	28
5	16	19	12	22
6	6	4	12	8

4.4. PILOT STUDY ON PAF DELIVERY

The size of aerosol droplets produced by the nebuliser was assessed using a Malvern laser diffraction sizer 2600HSD (Malvern instruments, Malvern, U.K.). Droplet size, expressed as volume median diameter, was 4-5μm in all of the twelve nebuliser units tested, and did not vary with different concentrations of PAF or albumin.

The output of PAF from the nebuliser was assessed at the concentrations of PAF and albumin used in the challenge studies. The solution was nebulised onto a polytetrafluoroethane filter (Gelman Sciences Inc, Michigan U.S.A.) attached to a suction pump and the dosimeter was activated by a freon spray. The PAF was washed off the filter with absolute alcohol (10ml) and frozen at -80°C. Bioassay was performed by aggregometry of rabbit platelets washed with prostacyclin (Radomski and Monca dℓ. 1983) by the method of Born (1962) on serial dilutions of the samples in phosphate buffered saline. The mean recovery of PAF from the filter was 1.4% (SD 0.22, n=6) of the PAF placed in the nebuliser chamber. The addition of albumin (1.25mg/ml and 2.5mg/ml) did not increase PAF delivery.

The gross nebuliser output was also assessed gravimetrically. The mean output per inhalation was 6.4 x 10^{-3}ml (SD 0.007, n=10).

4.5. PAF AND LYSO-PAF CHALLENGES

4.5.1. SUBJECTS, MATERIALS AND METHODS

4.5.1.1. Subjects

Six healthy subjects, four males and two females aged 20-41 years (mean 25 years) participated in these studies. Demographic details are given in Table 2. One subject had positive skin prick tests to inhaled antigens, but had a normal serum IgE level.

Table 2. Demographic details

Subject	Age	Sex	FEV_1 (l)	FEV_1 (% predicted)	Skin prick tests
1	22	M	5.02	114	+VE
2	20	F	3.47	106	-VE
3	40	F	3.59	117	-VE
4	22	M	4.30	94	-VE
5	22	M	6.45	139	-VE
6	23	M	4.55	103	-VE

4.5.1.2. Materials

Immediately before a challenge, ethanolic C-18 PAF or lyso-PAF was evaporated to dryness under nitrogen and re-suspended in diluent consisting of 0.9% sodium chloride with 2.5mg/ml HSA to produce a concentration of 1600µg/ml ($3.0x10^{-3}M$); concentrations of 800µg/ml ($1.5x10^{-3}M$) and

400μg/ml ($7.3x10^{-4}M$) were obtained by serial dilution.

4.5.1.3. Methods

Baseline bronchial responsiveness to MCh was initially determined in each subject. A PAF inhalation challenge was then performed twice and lyso-PAF inhalation challenge once in random order over a three month period. Bronchial responsiveness to MCh was measured at six hours, one day, three days, one week and two weeks following each PAF or lyso-PAF challenge. The next PAF or lyso-PAF challenge in the series was only performed after bronchial responsiveness to MCh had returned to the previously determined baseline value for each subject.

4.5.1.4. PAF and lyso-PAF challenge

As there was no previous experience of administering inhaled PAF to man in our laboratory, preliminary dosing studies were performed on volunteer members of staff. Challenges initially commenced with ten inhalations of a very low concentration (6.25μg/ml) of PAF because of the potential adverse cardiovascular effects of PAF mentioned above; doubling concentrations were then inhaled up to a final concentration of 800μg/ml or until a 35% fall in sGaw had been obtained. This challenge procedure resulted in only modest falls in sGaw at the lower concentrations of PAF, with tachyphylaxis occurring at the higher concentrations, so that it proved impossible to obtain the required 35% fall in sGaw.

Further pilot studies were therefore performed using a much higher initial concentration of 400µg/ml of PAF, and only two doubling concentrations of PAF up to a maximum of 1600µg/ml were given. This dose range was found to induce the required degree of bronchoconstriction without any serious side effects, and was chosen for use in the subsequent studies.

Baseline and control measurements were performed as detailed under the general description of the PAF challenge (Section 3.4.5.). Ten inhalations of PAF or lyso-PAF (400µg/ml) were then taken. sGaw (mean of eight values) was measured at one and six minute, and $\dot{V}max_{30}$ (mean of two values) was measured at three and eight minutes. Venous blood samples were taken at five minutes for blood count and differential.

The end-point of the challenge was defined as a 35% fall in sGaw below the control value; if the fall in sGaw was less than this, the next concentration of PAF (800µg/ml) was given immediately. Pulmonary function measurements were repeated and if a sufficient reduction in sGaw was still not obtained, the highest concentration of PAF (1600µg/ml) was given.

Following administration of the final concentration of PAF, pulmonary function measurements and venous blood samples were repeated at five minute intervals until recovery of sGaw to above 90% of the control value. One investigator was

aware of which substance was being administered, but the investigator supervising the pulmonary function measurements during the PAF and lyso-PAF challenges was unaware which challenge substance was being used.

4.5.2. Statistical analysis

The maximum mean acute changes in pulmonary function and neutrophil count following PAF and lyso-PAF challenges were calculated as percentages of the control values obtained after challenge with diluent. The reproducibility of these changes was assessed by plotting the difference between the two measurements against their mean, calculating 95% CI for the mean difference and a coefficient of repeatability for each parameter using the formula:

$$2\times\sqrt{\frac{\sum(D1-D2)^2}{n}}$$

Where D1 is the maximum mean reduction obtained following the first challenge, D2 is the maximum mean reduction obtained following the second challenge and n is the number of subjects. This defines 95% of the differences between the two challenges (i.e.two standard deviations), and is the coefficient of repeatability adopted by the British Standards Institution (Bland and Altman. 1986).

The relationship between the maximum PAF-induced changes in sGaw and $\dot{V}max_{30}$ was assessed by calculation of correlation coefficients using the Pearson product moment coefficient

and by linear regression analyses using the least squares method (Ryan, Joiner and Ryan. 1976). The other haematological data were analysed by Wilcoxon signed rank tests. Results are expressed as 95% CI or as the mean [SD].

Changes in bronchial responsiveness were analysed using the geometric mean PC_{35}sGaw MCh and $PC_{30}\dot{V}max_{30}$ MCh before and at each time point after each PAF challenge for the group of subjects; these were compared to the corresponding values after the lyso-PAF challenges. As the number of subjects was small, the use of a parametric analysis of variance (ANOVA) could not be justified and a non-parametric ANOVA, the Kruskal-Wallis test, was therefore employed (Langley. 1979).

4.5.3. RESULTS

4.5.3.1. Baseline Pulmonary Function

There were no significant differences in measurements of baseline pulmonary function before each PAF, lyso-PAF and MCh challenge during the study period. Coefficients of variation were within previously published limits of the reproducibility for each measurement for FEV_1 and sGaw (Larsson, Hedenstrom and Malberg. 1987; Tattersfield and Keeping. 1979), although there are no published data on the reproducibility of $\dot{V}max_{30}$ (coefficients of variation, mean [range]: FEV_1: 3.5% [2.5-5.3]; sGaw: 15% [9.7-26]; $\dot{V}max_{30}$: 9.6% [6.0-11.9]).

4.5.3.2. PAF and lyso-PAF challenges

PAF and lyso-PAF challenge were well-tolerated, and there were no serious adverse side effects. Subjects experienced a variable degree of facial flushing, throat irritation, a desire to cough and mild chest tightness following PAF challenge. These effects were evident within one minute of PAF inhalation, and persisted for up to five minutes. There were no symptoms suggestive of a "late asthmatic" reaction. No symptoms were reported by any subject following lyso-PAF inhalation.

There were no alterations in heart rate or rhythm during the period of continuous ECG monitoring while PAF or lyso-PAF was being inhaled. No changes in blood pressure or pulse rate were observed at any time following the inhalation of PAF or lyso-PAF, although the number of pulmonary function measurements limited the frequency of cardiovascular observations that could practically be performed.

The highest concentration of PAF required to produce the desired fall in sGaw on the two occasions was highly variable and is shown in Table 3. The predicted geometric mean cumulative dose of inhaled PAF for the six subjects using a gravimetric method to assess nebuliser output was 1.3×10^{-7}mol (range 4×10^{-8} to 3×10^{-7}mol).

Table 3. Highest concentrations of PAF administered in the two challenges (μg/ml)

Subject	PAF Challenge 1	PAF Challenge 2
1	1600	800
2	1600	400
3	1600	400
4	1600	400
5	400	800
6	1600	1600

Acute changes in pulmonary function are shown in Table 4 and the reproducibility of changes in sGaw and $\dot{V}max_{30}$ is shown in Figures 2 and 3 respectively. On one occasion, a 35% fall in sGaw was not obtained with Subject 3 even after the highest dose of PAF had been given.

Figure 2. Reproducibility of the acute changes in sGaw following cumulative dose-response PAF challenge

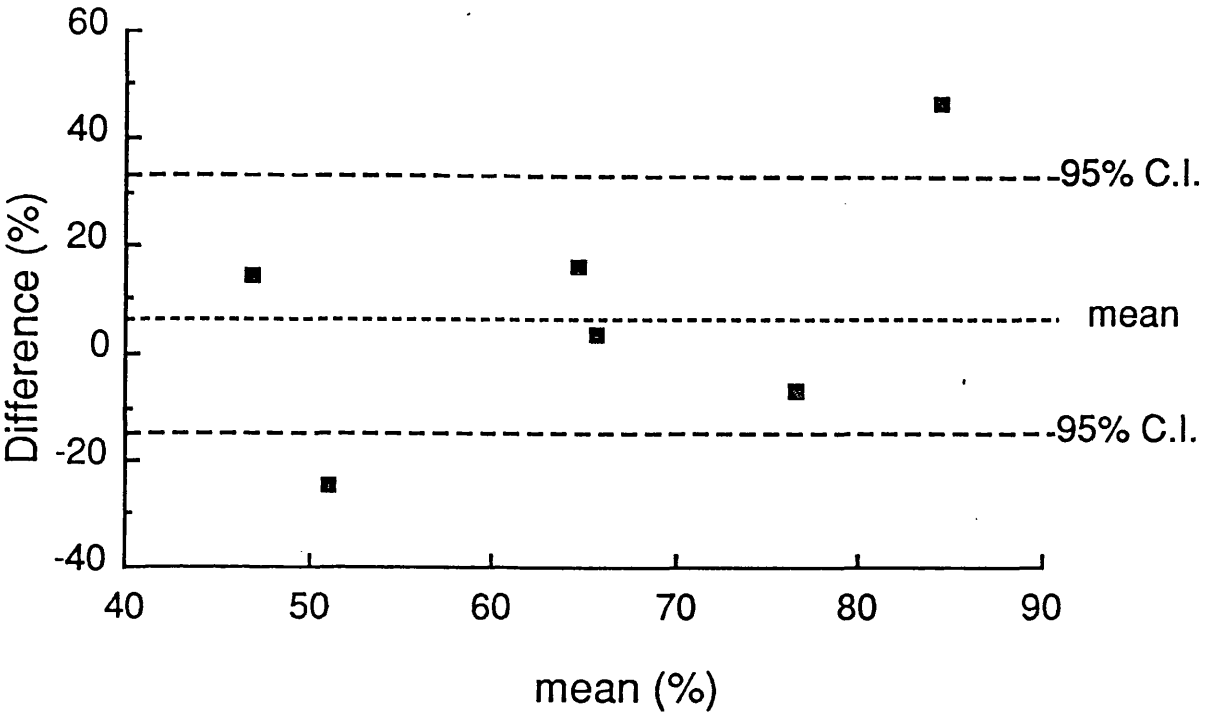
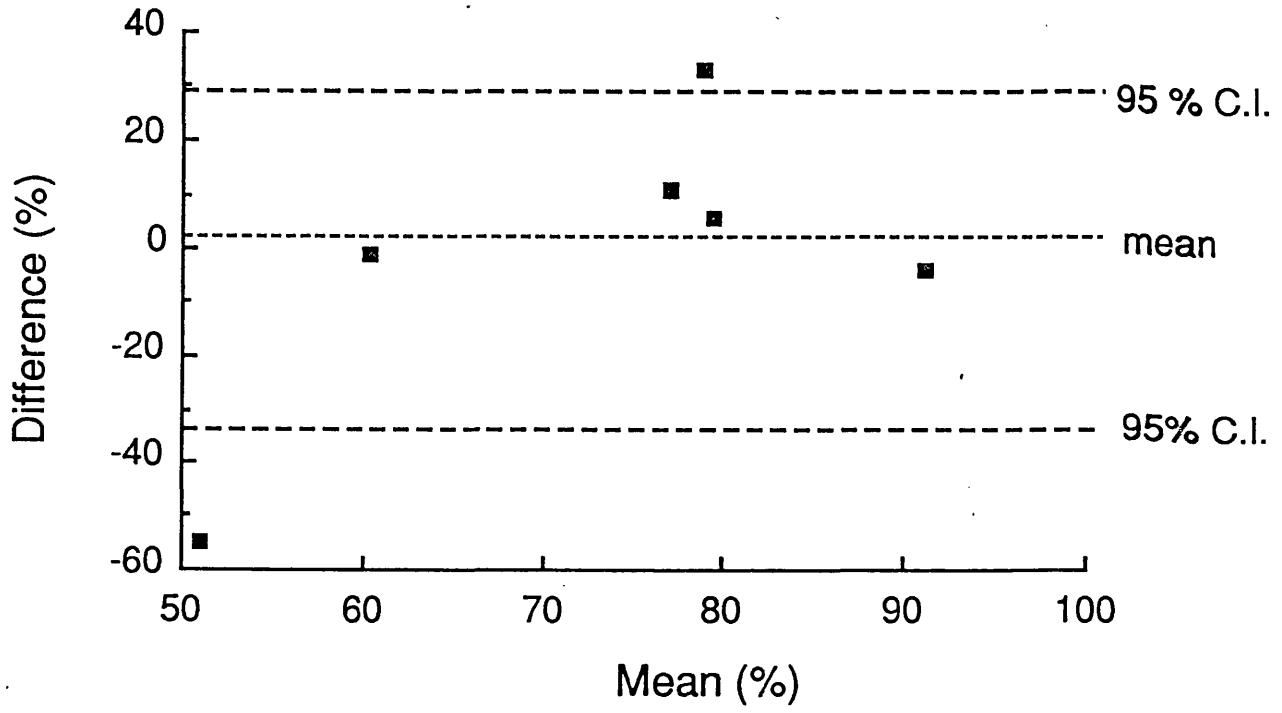


Figure 3. Reproducibility of the acute changes in $\dot{V}max_{30}$ following cumulative dose-response PAF challenge



The mean minimum sGaw after PAF inhalation was 65% of the control value (PAF challenge 1 [SD]: 69% [23], PAF challenge 2: 61% [13]. The mean difference between the two challenges was 8% (95% CI -17, 33) and the coefficient of repeatability was 46.9%. The mean minimum $\dot{V}max_{30}$ was 73% of the control value (PAF challenge 1 [SD]: 72% [27], PAF challenge 2: 74% [12]. The mean difference between the two challenges was 1.8% (95% CI -33, 29.4) and the coefficient of repeatability was 54.5.

The fall in sGaw was maximal at one minute and that in $\dot{V}max_{30}$ at three minutes in all except for two of the challenges when the maximum falls were observed at six and eight minutes respectively. These changes resolved rapidly with sGaw returning to above 90% of control values at between 6-31 minutes after the challenge (mean 15 minutes). The maximum falls in sGaw and $\dot{V}max_{30}$ in the two PAF challenges correlated well ($r=0.809$, $r^2 =65.5\%$, $n=12$, $p=0.001$).

Tachyphylaxis was frequently observed, and further reductions in pulmonary function following inhalation of the higher concentrations of PAF were small compared to the fall induced by the first inhalation. There was no reduction in sGaw or $\dot{V}max_{30}$ following lyso-PAF challenge.

4.5.3.3. Cellular Changes

The falls in circulating neutrophil count are shown in Table 4. Control levels of haemoglobin, differential white cell counts and platelet numbers were within normal limits. A transient fall in circulating neutrophils occurred following PAF challenge in five subjects on both occasions and only once in one subject (subject 3). The fall in neutrophil count was maximal at five minutes after PAF inhalation, and had returned to the baseline value by ten minutes.

The mean minimum neutrophil count following the first PAF challenge was 70% (SD 28) of the control value and was 63% (SD 24) of the control value after the second PAF challenge. The reproducibility of the cellular changes is shown in figure 4. The mean difference between the two challenges was 7% (95% CI -25, 39) and the coefficient of repeatability was 57.2. There were no changes in haemoglobin levels or platelet counts after PAF or lyso-PAF challenges. A small reduction in circulating lymphocytes was observed (mean 10% at 5 minutes) returning to control levels by 10 minutes, but this fall did not reach statistical significance ($p>0.1$).

Figure 4. Reproducibility of the acute changes in circulating neutrophil count following cumulative dose-response PAF challenge

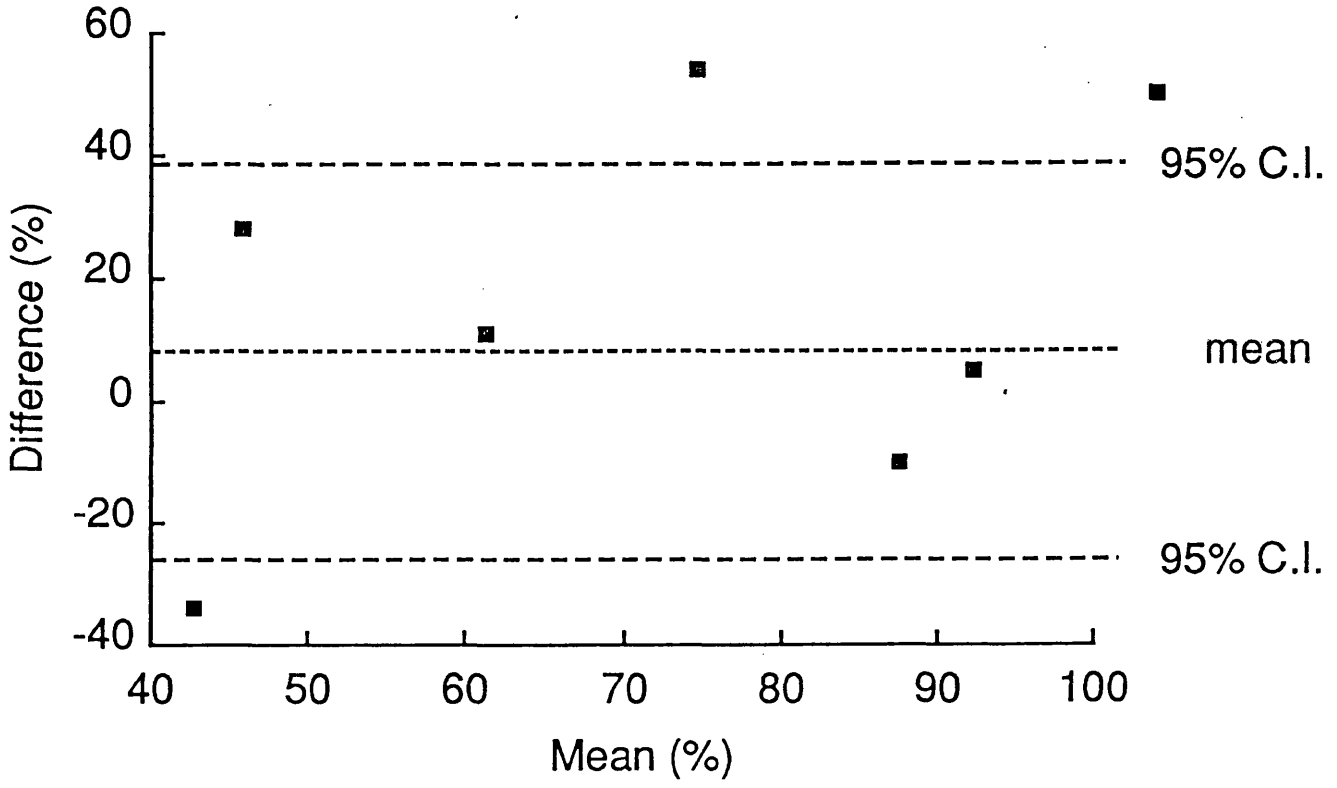


Table 4. Acute changes in pulmonary function and circulating neutrophil count following PAF challenges (% of the control value.

	CHALLENGE 1			CHALLENGE 2		
Subject	sGaw	$\dot{V}max_{30}$	Cell count	sGaw	$\dot{V}max_{30}$	Cell count
1	67	83	65	64	72	56
2	54	60	58	40	61	32
3	108	96	100	61	62	48
4	73	82	81	57	77	93
5	73	89	93	80	93	90
6	39	23	24	63	79	60

4.5.3.4. Changes in bronchial responsiveness following PAF and lyso-PAF challenge

All subjects remained well in the two weeks following PAF and lyso-PAF challenges with no respiratory symptoms suggestive of an increase in bronchial responsiveness. Individual data for PC_{35}sGaw MCh and $PC_{30}\dot{V}max_{30}$ are given in Appendix 1 and the geometric mean data for PC_{35}sGaw MCh and $PC_{30}\dot{V}max_{30}$ MCh for the whole group are shown in Tables 5 and 6. The individual data for PC_{35}sGaw MCh are illustrated in Figure 5, and the geometric mean data for PC_{35}sGaw MCh and $PC_{30}\dot{V}max_{30}$ MCh are illustrated in Figure 6 and 7.

Figure 5. Individual changes in PC_{35}sGaw MCh following the cumulative dose-response PAF and lyso-PAF challenges

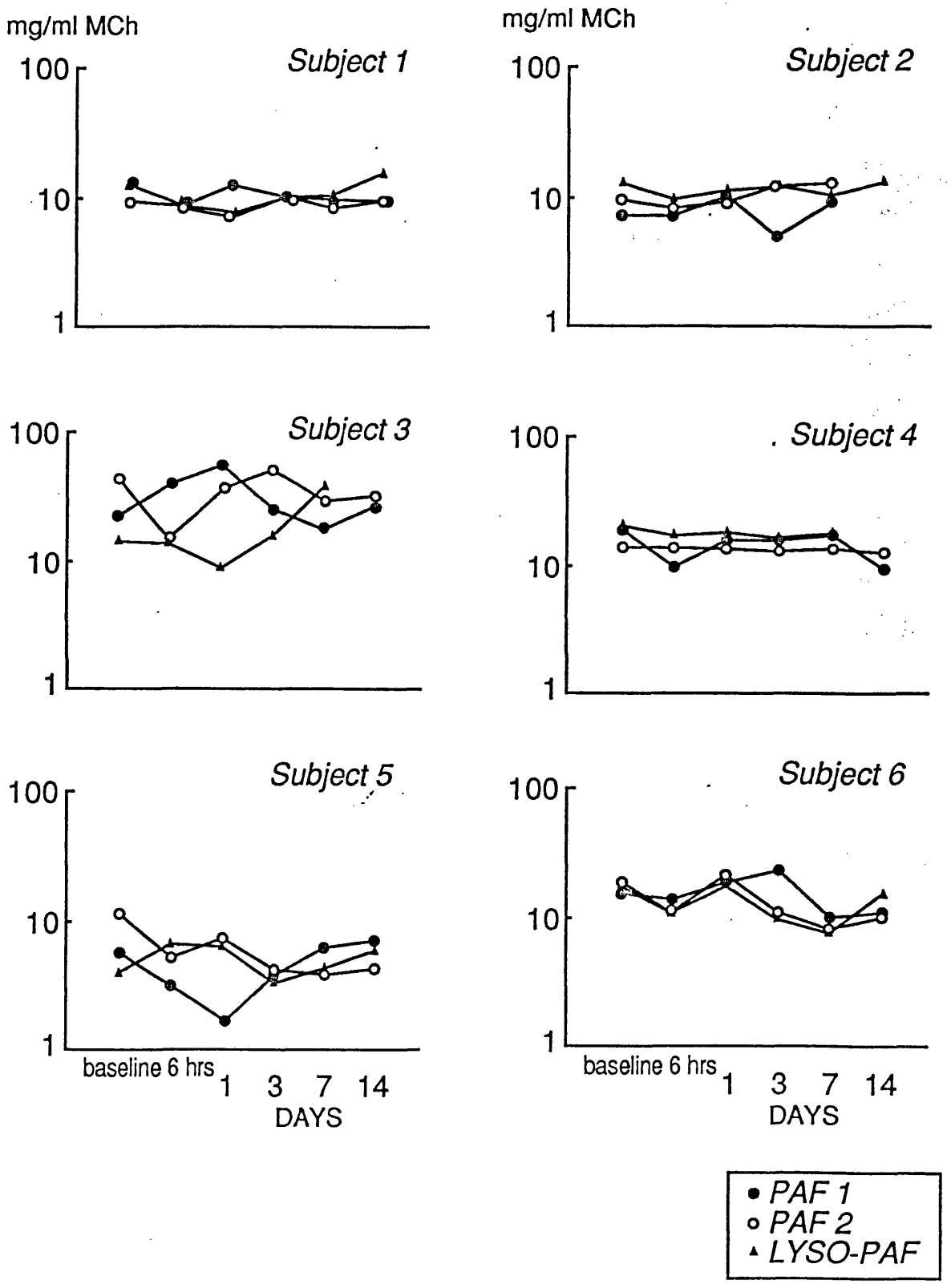


Figure 6. Geometric mean PC_{35}sGaw MCh for the whole group following the cumulative dose-response PAF and lyso-PAF challenges

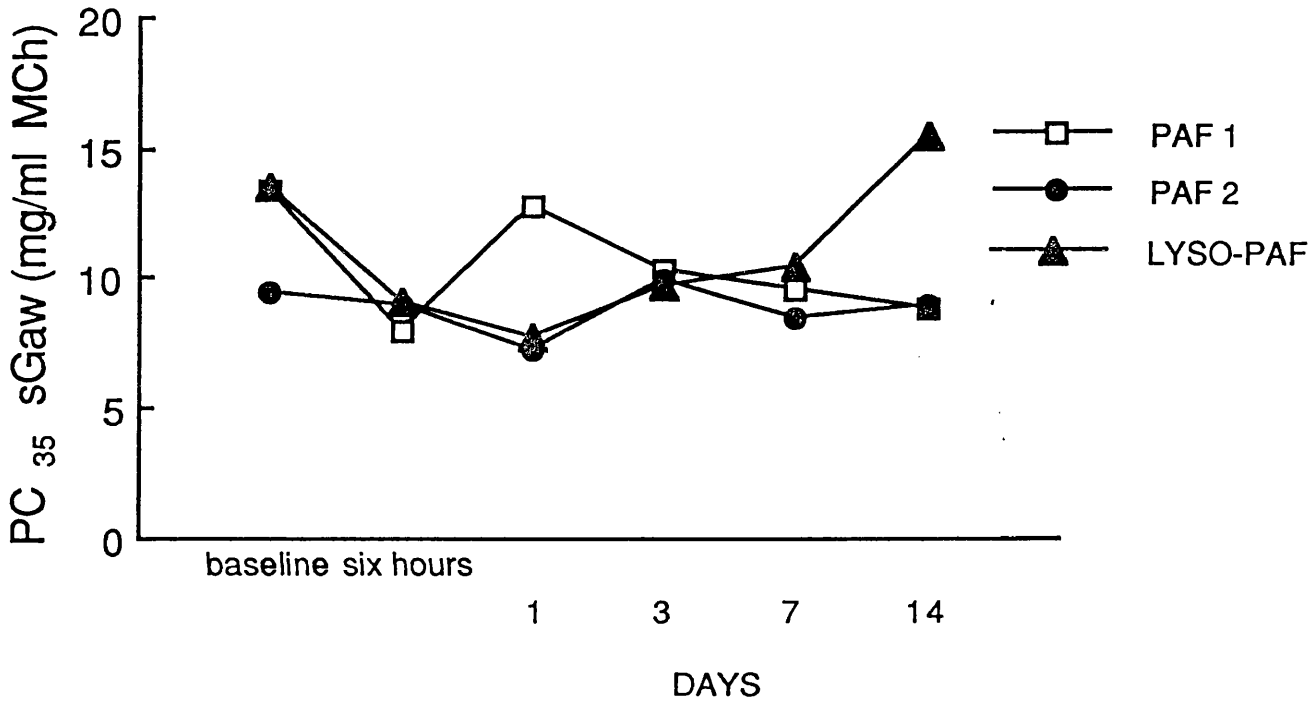
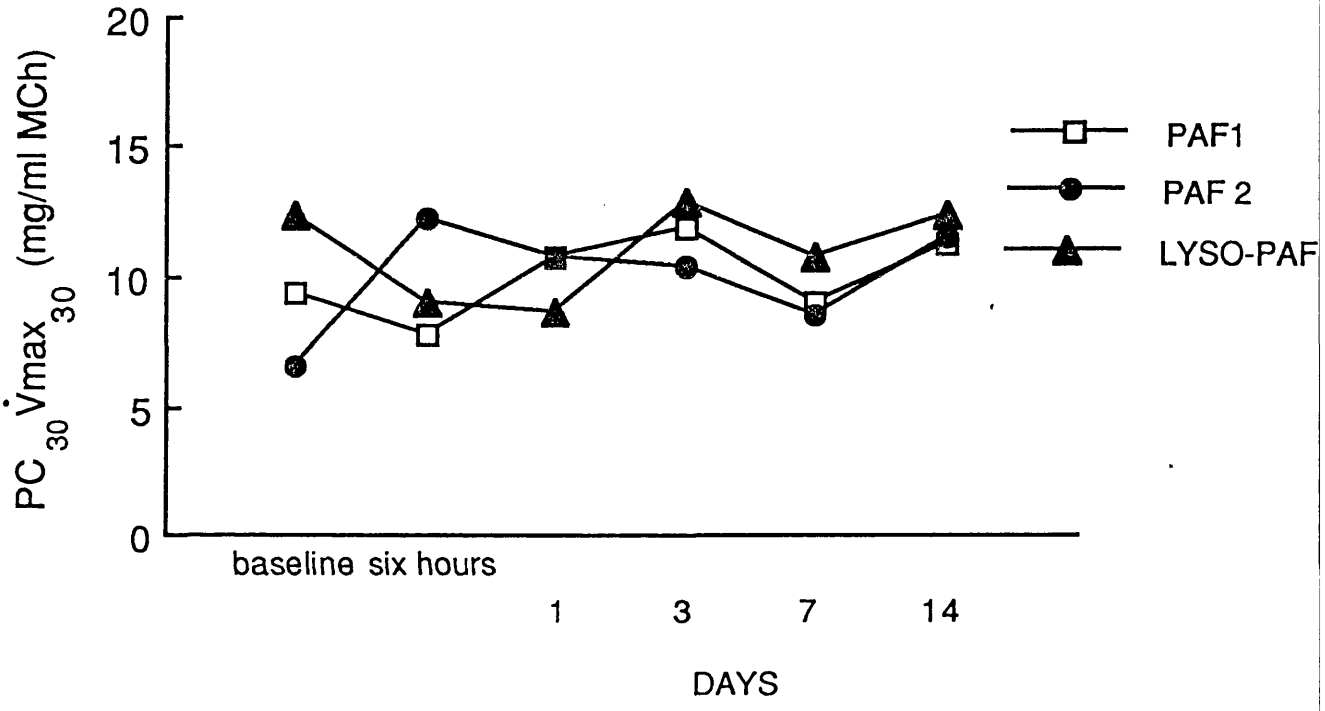


Figure 7. Geometric mean $PC_{30}\dot{V}max_{30}$ MCh for the whole group following the cumulative dose-response PAF and lyso-PAF challenges



There was no clear trend for bronchial responsiveness to increase after either PAF or lyso-PAF challenges and individuals did not have changes in PC_{35}sGaw MCh or $PC_{30}\dot{V}max_{30}$ MCh of more than one doubling concentration of MCh. Changes in the geometric mean data did not reach statistical significance for PC_{35}sGaw MCh ($k=18$, $\chi^2=4.51$, $p>0.1$) or for $PC_{30}\dot{V}max_{30}$ MCh ($k=18$, $\chi^2=14.4$, $p>0.1$) when compared to changes in bronchial responsiveness occurring after lyso-PAF challenge.

Table 5. Geometric mean values for PC_{35}sGaw MCh following PAF and lyso-PAF challenges (mg/ml MCh)

TIME	First PAF challenge	Second PAF challenge	lyso-PAF challenge
Baseline	13	9	14
6 hours	8	9	9
1 day	13	7	8
3 days	10	10	10
1 week	10	9	10
2 weeks	9	9	15

Table 6. Geometric mean values for $PC_{30}\dot{V}max_{30}$ MCh following PAF and lyso-PAF challenges (mg/ml MCh)

TIME	First PAF challenge	Second PAF challenge	lyso-PAF challenge
Baseline	9	7	12
6 hours	8	12	9
1 day	11	10	9
3 days	11	10	13
1 week	9	9	11
2 weeks	11	12	12

4.5.4. Discussion

The doses of inhaled PAF administered in these studies resulted in no significant increase in bronchial responsiveness either in individuals or in the group of subjects as a whole. This finding contrasts with the prolonged increase in bronchial responsiveness following inhalation of PAF described in five of six subjects by Cuss, Dixon and Barnes (1986). There are several possible explanations for these differences, the most obvious being that either the dose of PAF delivered to the airways might have been smaller in our study, or that the two groups of subjects reacted differently to a similar bronchoconstricting dose of PAF.

If dose is assessed gravimetrically, the geometric mean dose of PAF given in our study (1.3×10^{-7}mol) was higher than that of 1.1×10^{-7}mol given in the study of Cuss, Dixon and Barnes. However, the actual delivery of PAF to the airways will depend on many factors including aerosol droplet size, the number of inhalations and the pattern of breathing (Newman and Pavia, 1985). Assessment of delivered dose by a gravimetric method is not satisfactory for the reasons stated in the introduction, and the pilot study on PAF delivery was designed to show whether PAF was nebulised in significant amounts rather than to give an accurate estimate of delivery to the airways.

The amount of PAF recovered as a percentage of that reaching

the target filter was not estimated in this study, nevertheless it is apparent that nebulisation results in a significant loss of biological activity. PAF is conventionally administered in an albumin solution to increase solubility of the lipid, but as the addition of albumin had no affect on PAF delivery, this may not be necessary at the concentrations of PAF used in these studies.

As the challenge techniques employed were not identical, a direct comparison of doses of PAF delivered to the airways in the two studies is extremely difficult, if not impossible. Indirect evidence suggests that similar doses of PAF were given in our study to those used to study the acute effects of inhaled PAF in the Cuss, Dixon and Barnes study. In both studies inhalation of PAF resulted in similar acute physiological effects of bronchoconstriction and a transient fall in circulating neutrophils, and similar symptoms were reported. Unfortunately, Cuss, Dixon and Barnes used different doses of inhaled PAF in the studies of changes in bronchial responsiveness following inhaled PAF (a geometric mean dose 0.11μmol) to those used in the studies of acute effects of inhaled PAF (a cumulative dose of up to 0.77μmol), rendering comparison of dose even more difficult.

An alternative explanation for these dissimilar findings is that the two groups of subjects reacted differently to similar received doses of PAF. This may be because an

increase in bronchial responsiveness following inhaled PAF is a highly variable phenomenon within the normal population, or perhaps because of some other factor affecting this response, such as the presence of atopy.

Patients with asthma are less likely to bronchoconstrict following inhalation of PAF, and unlike normal subjects do not show increased responsiveness to MCh in the first hour following PAF challenge (Rubin, Smith and Patterson. 1987). The effects of PAF on asymptomatic atopic subjects are unknown, but it is possible that such subjects are more likely to demonstrate increased bronchial responsiveness following inhaled PAF than are non-atopic healthy subjects. The subjects in our study gave no history of asthma or allergic disease and had normal circulating eosinophil counts. Skin prick tests to commonly inhaled allergens were negative in all but one subject who had a normal serum IgE level. Although three of the subjects in the Cuss, Dixon and Barnes study were atopic, the atopic status of the subgroup (n=6) in whom changes in bronchial responsiveness following PAF was studied was not quoted.

Finally, the increases in bronchial responsiveness described by Cuss, Dixon and Barnes could conceivably have occurred coincidentally as a result of an unrecognised factor such as seasonal exposure to allergen in atopic subjects, or mild respiratory infection.

Since this study was completed, several other groups have reported that they have also been unable to induce consistent or prolonged increases in bronchial responsiveness in normal man using various doses of inhaled PAF (Gebré-Michael and Leuenberger. 1989; Lai, Jenkins, Polosa, et al. 1990; Stenton, Ward, Duddridge, et al. 1990). There must now be some question that such a prolonged increase in bronchial responsiveness is a reproducible consequence of PAF inhalation in normal non-atopic subjects.

On one occasion in our study, inhalation of PAF did not result in either acute bronchoconstriction or any transient fall in circulating neutrophil count, suggesting that the subject did not receive a significant dose of PAF. We were unable to identify either nebuliser malfunction or variation in the way that the materials were prepared prior to this particular challenge. This result may represent an intrasubject variation in response to PAF.

The reproducibility of the procedure used for the MCh challenge using sGaw was similar to previously published data using $PC_{20}FEV_1$ (Juniper, Frith, Dunnett, et al. 1978), whereas for $PC_{30}\dot{V}max_{30}$ MCh the reproducibility was less good, with only five of the six subjects responding within one doubling concentration. The 95% CIs are wide, reflecting the small number of subjects, and the use of sensitive measurements of pulmonary function. The 95% CIs for reproducibility of the acute changes in pulmonary function

and circulating neutrophil count following PAF are also wide, presumably because of the reasons stated above and also because of tachyphylaxis and the one occasion when PAF had no apparent effect.

The size of the aerosol droplets produced by the nebuliser suggests that deposition would be mostly in medium and small airways (Newman and Pavia. 1985). However, neither the precise site of particle deposition within the airways, nor the distribution of the high affinity PAF binding sites found in homogenised human lung membranes (Hwang, Lamb and Shen. 1985) have yet been determined.

It is likely that PAF is only one of many mediators involved in asthma, and that important interactions occur between the airways, the nervous system of the lung, circulating cells and mediators. Perhaps it is not surprising that a prolonged increase in bronchial responsiveness was not observed in normal subjects on any occasion after a single inhalation challenge with this one mediator.

Although this PAF inhalation challenge procedure could possibly be used to study the acute effects of PAF in man, the reproducibility of these effects was poor, probably due to tachyphylaxis occurring after the initial dose of PAF. This challenge technique will not be a useful model for the study of BHR in normal man.

CHAPTER FIVE

ASSESSMENT OF PLATELET ACTIVATION FOLLOWING PAF CHALLENGE IN MAN

5.1. ABSTRACT

The levels of two alpha-granule secretory products, ß-thromboglobulin (ß-TG) and platelet factor four (PF4) have been measured before and after PAF inhalation challenge in order to investigate the involvement of platelets in PAF-induced bronchoconstriction in normal man. There were no increases in either circulating ß-TG or PF4 levels above baseline values shortly after PAF inhalation. These findings suggest that platelet activation does not occur following PAF inhalation in man.

5.2. INTRODUCTION

Numerous platelet abnormalities including prolonged bleeding time, reduced platelet aggregation, altered PAF sensitivity and increased platelet mass have been described in asthmatics (Szczeklik, Milner, Birch, et al. 1986). Platelet activation has also been observed during antigen-induced bronchoconstriction (Knauer, Lichtenstein, Adkinson, et al. 1981), although other investigators have not always been able to reproduce these findings (reviewed by Gresele, Ribaldi, Grasseli, et al. 1987).

Morley, Sanjar and Page (1984) proposed that exacerbations of asthma result from the inflammatory sequelae of PAF formation within the lung, involving an effect of platelets or platelet products on airway smooth muscle. This hypothesis was strengthened by the discovery that platelets are normally produced by physical fragmentation of circulating megakaryocytes within the pulmonary circulation (Trowbridge, Martin and Slater. 1982), by the presence of large numbers of pulmonary megakaryocytes in lung sections of patients dying from status asthmaticus (Slater, Martin and Trowbridge. 1985) and by the finding that inhaled PAF caused bronchoconstriction and increased bronchial responsiveness in normal man (Cuss, Dixon and Barnes. 1986).

Bronchoconstriction induced by intravenous PAF is platelet dependent in the guinea pig (Vargaftig, Lefort, Chignard, et al. 1980) and intratracheal instillation of PAF results in

accumulation of platelets in the lungs of baboons (Arnoux, Denjean, Page, et al. 1988).

Conflicting results have been reported on the involvement of platelets in PAF-induced human bronchial smooth muscle contraction *in vitro*; Schellenberg, Walker and Snyder (1983) reported a platelet-dependent contraction, whereas Johnson, Armour and Black (1988) obtained contraction in the absence of any cells. There have been no documented changes in platelet count following PAF-inhalation in man, although thrombocytopaenia was observed following intratracheal instillation of very large doses of PAF to decerebrate patients (Gateau, Arnoux, Deriaz et al, 1984). An increase in platelet phosphatidylinositol turnover has been shown to occur after PAF inhalation by PAF in asthmatics (Block, Imhof and Perruchoud, 1988), perhaps indicating platelet activation, although the true significance of this finding is not known.

To assess whether platelet activation occurs following PAF challenge in man, we have measured circulating levels of PF4 and ß-TG before and after PAF inhalation in normal human subjects.

5.3. SUBJECTS, MATERIALS AND METHODS

Nine healthy volunteers (6 male, 3 female) aged 20-50 years participated in the study. No subject gave a history of asthma or hay fever or was studied within six weeks of any

upper respiratory tract infection. The subject took ten inhalations of nebulised C-18 PAF (Bachem. 800µg/ml in 2mls 0.9% NaCl with 2.5mg/HSA) which was prepared and delivered in the same manner as with previous study.

Venous blood samples (5ml) were taken immediately before and five minutes after PAF challenge from an unoccluded antecubital vein using a 19G "Butterfly" needle. Radioimmunoassay for ß-TG (Amersham) and PF4 (Abbott) was performed using the standard procedures recommended by the manufacturers.

5.4. STATISTICAL ANALYSIS

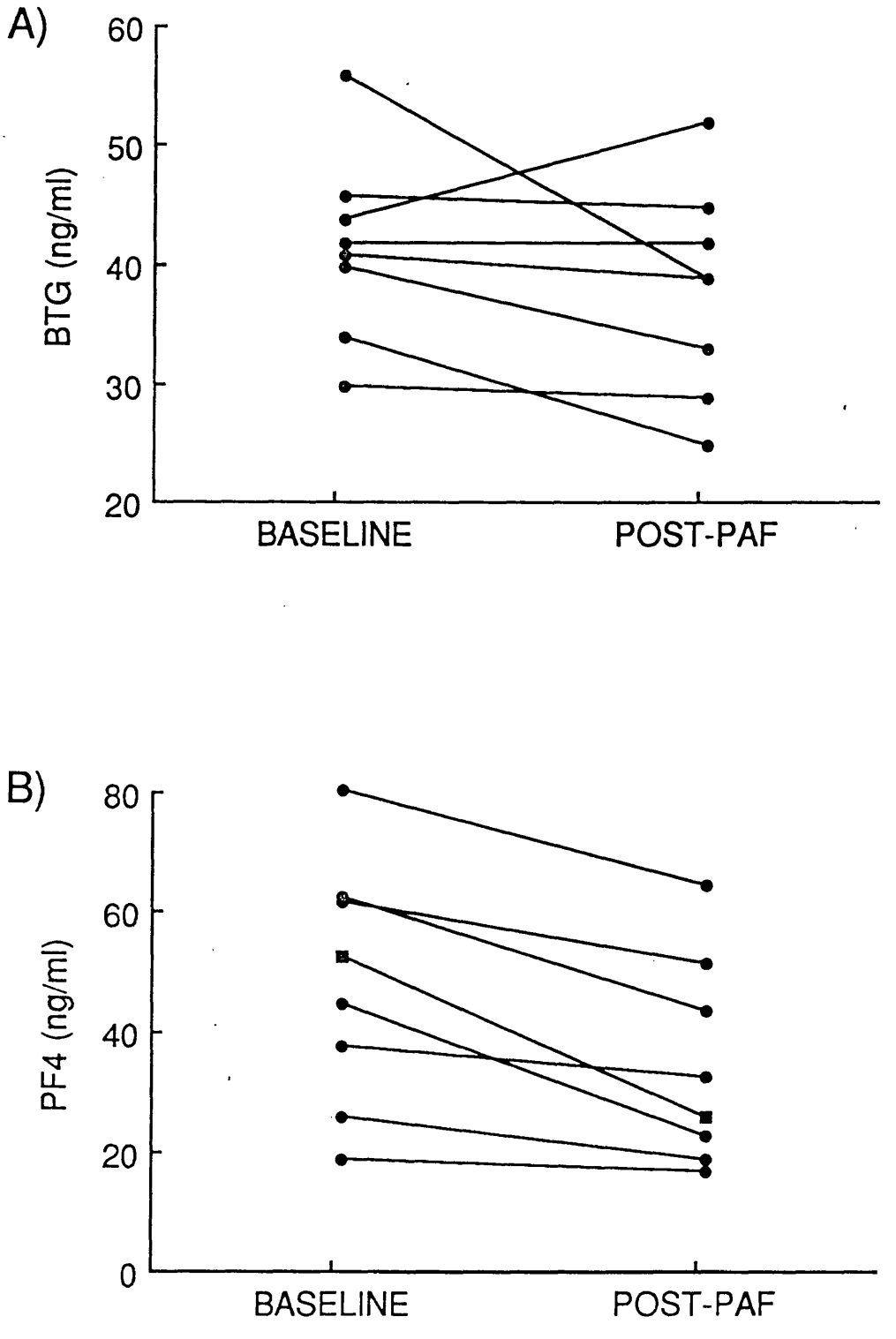
The results are expressed as ng/ml of plasma. The data was analysed by paired Student's t-tests.

5.5. RESULTS

The inhalation of PAF was well tolerated although subjects experienced a variable degree of facial flushing, tightness of the chest and desire to cough. Results are given in appendix 1 and illustrated in Figure 8. Venepuncture was initially difficult in subject 1, which may have resulted in platelet damage and account for the high baseline levels of circulating PF4 and ß-TG found in this subject; these results were therefore excluded from the statistical analysis.

There was no rise in circulating ß-TG or PF4 levels to suggest platelet activation after PAF. Indeed there was a statistically significant fall in the measured values for PF4, with no significant change in PF4 levels (mean [$\pm$ s.e.m.]): ß-TG; 40.6 [2.7] ng/ml baseline vs 37.0 [3.1] ng/ml post-PAF; 95% CI -2.6,9.8; p=0.21. PF4; 46.4 [7.28] ng/ml baseline vs 32.9 [6.09] ng/ml post-PAF; 95% CI 1,19.5; p=0.0037).

Figure 8. A) ß-TG and B) PF4 levels before and after PAF challenge (ng/ml of plasma)



5.6. DISCUSSION

We have found no evidence that platelet activation occurs *in vivo* following PAF inhalation in normal man. These findings are broadly in agreement with those of Kioumis, Lammers, Dent, et al (1988), who found no changes in *in vitro* platelet aggregability to PAF or ADP following inhaled PAF challenge in normal subjects. There was a statistically significant reduction in PF4 levels after PAF although we doubt whether this finding could have any physiological basis, as it was not accompanied by any statistically significant change in ß-TG levels.

The venous blood samples were taken 5 minutes after the PAF was inhaled to coincide with the period of maximum bronchoconstriction and neutropaenia induced by inhaled PAF. It could be argued that any induced changes in PF4 and ß-TG could have resolved by this time, this is unlikely to be the case as Durham, Dawes and Kay (1985) have found the *in vivo* half-life of ß-TG to be 100 minutes and that of PF4 to be immeasurably short. However, Knauer, Lichtenstein, Adkinson, et al (1981) were able to detect increases in PF4 levels for up to ten minutes after antigen challenge of asthmatic subjects.

It would seem that in normal subjects the acute effects of inhaled PAF occur independently of platelet activation. This conclusion is supported by the absence of any change in circulating platelet numbers following PAF challenge, and by

recent confirmation that platelets are not sequestered into the lung following PAF challenge in man (Tam, Clague, Dixon, et al. 1990). Although a single PAF challenge in normal man cannot be accepted as a model of acute asthma, these results do not support the suggestion that the platelet activation observed in acute asthma is due to the actions of PAF.

CHAPTER SIX

THE EFFECTS OF A SELECTIVE CYSTEINYL-LEUKOTRIENE RECEPTOR ANTAGONIST ON THE ACUTE RESPONSES TO INHALED PLATELET-ACTIVATING FACTOR IN NORMAL MAN

6.1. ABSTRACT

The effects of pre-treatment with the selective cysteinyl-LT antagonist SK&F 104353-Z_2 on the airway and cellular responses to inhaled PAF were examined in a randomised, double-blind, placebo controlled, crossover study in eight healthy male subjects.

A single dose of PAF causing a fall of at least 35% in specific airways conductance was initially determined for each subject. PAF challenge was then repeated with this dose of PAF on two further occasions separated by two weeks after pre-treatment with a single nebulised dose of either SK&F 104353-Z_2 or placebo.

SK&F 104353-Z_2 caused a significant reduction in PAF-induced bronchoconstriction (mean maximum fall below control values, SK&F 104353-Z_2 vs placebo, [mean difference; 95% CI for the mean difference]: sGaw; 22% vs 34% [-12.6%; -23.8,-1.4], $p<0.05$. $\dot{V}max_{30}$; 19% vs 31% [-12.5%; -20.8, -4.2], $p<0.01$), but did not reduce the fall in neutrophil count (SK&F 104353-Z_2 vs placebo: 46.1% vs 49.6% [-3.5%; -13.6, 6.6], $p>0.1$). Bronchial responsiveness to MCh did not increase above baseline values in any subject when measured two weeks after each PAF challenge. This study confirms that interactions between PAF and LTs occur in man, and suggests that such interactions may be important in the pathophysiology of asthma.

6.2. INTRODUCTION

The previous study demonstrated that the PAF inhalation challenge used resulted in acute bronchoconstriction and a transient fall in circulating neutrophils, but with no subsequent increase in bronchial responsiveness at any time in any subject studied. These negative findings precluded any further study on the mechanism by which this phenomenon may occur in normal man. However, there is no doubt that PAF has a very wide range of biological activities to suggest that it may be a mediator of great importance in asthma, and it was decided to investigate the mechanism by which the acute effects of inhaled PAF were produced in man.

As has been discussed in the introduction (Section 1.10.), animal and *in vitro* studies suggest that the effects of PAF are produced indirectly, possibly through the secondary production of LTs. However, the evidence from the animal studies is confusing, and no *in vivo* studies have investigated whether this might be the case in man.

SK&F 104353-Z_2 (2(S)-hydoxy-3(R)-[(2-carboxyethyl)thio]-3-[2-(8-phenyloctyl)phenyl]-propanoic acid), a structural analogue of LTD_4, is a potent and selective antagonist of the cysteinyl-LTs active by the inhaled route (Hay, Muccitelli, Tucker, et al, 1987; Evans, Barnes, Zakrzewski, et al. 1988). SK&F 104353-Z_2 inhibits LTD_4-, but not histamine-induced bronchoconstriction in man (Evans, Barnes, Zakrzewski, et al. 1988; Evans, Barnes, Piper, et al. 1989),

and is a potent antagonist of LTC_4- and LTE_4-induced contraction of human bronchus *in vitro*. Unlike previous LT-antagonists (reviewed by Krell. 1989) this compound is extremely selective, and possibly totally specific for the cysteinyl-LT receptor with no effect on PAF- or histamine-induced contraction of guinea pig trachea. Unfortunately, the scarcity of human tissue for pharmacological studies has prevented studies on PAF-induced bronchoconstriction *in vitro* (Dr Douglas Hay, *personal communication*). In contrast to the activity of the earlier purportedly selective cysteinyl-LT antagonists LY 171883 and FPL 55712, SKF 104353-Z_2 does not inhibit cyclic nucleotide phosphodiesterase activity in canine tracheal smooth muscle (Hay, Muccitelli, Tucker, et al., 1987). We have studied the effects of pre-treatment with SKF 104353-Z_2 on the acute responses to inhaled PAF in man.

It was our impression that the poor reproducibility of the acute effects seen with the PAF challenge employed in the first study was because of tachyphylaxis occurring after the initial dose of PAF. A single dose PAF challenge was therefore developed in the hope that this would improve the reproducibility of the challenge procedure.

6.3 SUBJECTS, MATERIALS AND METHODS

6.3.1. Subjects

Eight healthy male subjects aged 22-32 (mean age 25 years) participated in this study. Demographic details are given in

Table 7. Two subjects had positive skin prick tests to inhaled allergens; of whom one gave a history of a history of mild seasonal allergic rhinitis, but was studied outside the pollen season.

Table 7. Demographic details.

Subject	Age	FEV_1 (l)	FEV_1 (% predicted)	Skin prick tests
1	24	3.84	85	-VE
2	24	4.71	112	+VE
3	32	4.24	103	-VE
4	23	4.98	116	-VE
5*	28	3.47	82	-VE
6	23	3.97	96	-VE
7	24	5.45	122	+VE
8	23	4.78	109	-VE

* Subject of Indian race in whom FEV_1 is normally less than for european caucasians, and on which the predicted value is based.

6.3.2. Materials

These are described in Section 3.3.

6.3.3. Methods

6.3.3.1. Protocol

Baseline bronchial responsiveness to MCh was initially assessed and a single dose of inhaled PAF causing a fall of at least 35% in sGaw determined for each subject. On two treatment days, separated by at least two weeks, this dose of PAF was administered after pre-inhalation of either SKF 104353-Z_2 or placebo in a randomised, double-blind crossover study.

As PAF induced no significant changes in bronchial responsiveness in the previous study, it was felt unnecessary to perform repeated measurements of bronchial responsiveness following each PAF challenge in this study. However, in order to confirm that baseline bronchial responsiveness was comparable on each treatment day, MCh challenge was performed two weeks after each PAF challenge, immediately prior to the next treatment day.

6.3.3.2. Dose-finding PAF challenge

Initial measurements and the inhalation of diluent were performed as described in the general description of the PAF challenge (Section 3.4.5.). Ethanolic PAF was dried under nitrogen and re-suspended in diluent to produce a concentration of 800µg/ml immediately before the challenge. This solution (2ml) was placed in the nebuliser reservoir.

Ten inhalations were taken in the same manner as described for diluent; measurements of sGaw (mean of eight values) were made at one and six minutes, of $\dot{V}max_{30}$ (mean of two values) at three and eight minutes, and differential blood count was obtained at five minutes. The predicted dose of PAF delivered from gravimetric assessment of the nebuliser output was 9.6 $x10^{-8}$mol per ten inhalations. If the mean maximum fall in sGaw was less than 35% below the control value, the challenge was repeated two weeks later, after confirmation that bronchial responsiveness remained unaltered, using 20 inhalations of the same concentration of PAF. In view of the potential adverse cardiovascular effects of large doses of PAF, the subject was excluded from further study if the required 35% fall in sGaw did not occur with this larger dose.

The safety and tolerability of this single dose PAF challenge was examined using myself and several volunteer members of the department as experimental subjects prior to being administered to the subjects participating in the SKF 104353-Z_2 study.

6.3.3.3. Treatment Days

Baseline measurements of FEV_1 (mean of three values), sGaw (mean of 16 values) and $\dot{V}max_{30}$ (mean of five values) were initially obtained. Three ml of 400µg/ml SKF 104353-Z_2 or placebo was placed into the reservoir of a "Pulmo-Sonic" ultrasonic nebuliser (DeVilbiss Co. Somerset, PA. USA), and

the subject inhaled the aerosol by tidal breathing with the nose clipped for ten minutes. FEV_1 (mean of three values), sGaw (mean of eight values) and $\dot{V}max_{30}$ (mean of two values) were then repeated. PAF challenge was performed 15 minutes after the inhalation was completed using the previously determined bronchoconstricting dose of PAF for each subject. Drug delivery was estimated volumetrically.

6.4. STATISTICAL ANALYSIS

The reproducibility of baseline values of sGaw, $\dot{V}max_{30}$ and FEV_1 performed prior to each MCh and PAF challenge, and at the beginning of each treatment day, was calculated as a coefficient of variation for each subject. The effects of SKF 104353-Z_2 and placebo on pulmonary function was assessed by paired t-tests on the pre-treatment and immediate post-treatment values of FEV_1, sGaw and $\dot{V}max_{30}$.

The mean of the sGaw and $\dot{V}max_{30}$ measurements at the time of maximum bronchoconstriction following PAF and the neutrophil count at five minutes were expressed as a percentage of control values obtained after inhaling diluent. The values on the two treatment days were compared by paired t-tests and the results expressed as mean difference, 95% CI for the mean difference and p value.

The reproducibility of the PAF challenge was assessed by comparing the data for PAF-induced changes in pulmonary function and circulating neutrophil count on dose-finding

and placebo treatment days. These findings were expressed as the mean difference between the two challenges along with the coefficient of repeatability, as in the previous study.

Changes in bronchial responsiveness two weeks after each PAF challenge were compared to baseline values by paired t-tests and by a one way ANOVA on the log transformed data.

6.5. RESULTS

6.5.1. Baseline Pulmonary function

Coefficients of variation for FEV_1 and sGaw were within previously published values (mean [range]: FEV_1; 3.76% [2.3-5.8%], sGaw; 9.6% [4.3-14.4%] (Larsson, Hedenstrom and Malberg. 1987; Tattersfield and Keeping. 1979) The mean coefficient of variation of $\dot{V}max_{30}$ was 8.9% (range 6.0-20.3%).

6.5.2. PAF Challenge

PAF challenge was well-tolerated with no serious side effects. Four of the subjects required ten inhalations of PAF and four subjects 20 inhalations to produce the required fall in sGaw. The number of inhalations of PAF given along with the maximum falls in pulmonary function and neutrophil count for the dose finding PAF challenge are shown in Table 8.

All subjects experienced transient irritation of the throat, facial flushing and a desire to cough. The greatest mean falls in sGaw and $\dot{V}max_{30}$ were always obtained when first measured at one and three minutes respectively after PAF inhalation, in agreement with previous findings (Rubin, Smith and Patterson. 1987). No changes in heart rate, blood pressure or cardiac rhythm were observed.

Table 8. Dose-finding PAF challenge. Number of inhalations of PAF given with subsequent maximum falls in pulmonary function and circulating neutrophil count (% of control values obtained after the inhalation of diluent)

Subject	Inhalations of PAF	sGaw	$\dot{V}max_{30}$	Neutrophil count
1	10	55	34	18
2	10	57	44	86
3	10	33	16	63
4	20	36	33	90
5	20	58	64	65
6	20	34	28	52
7	20	18	23	51
8	10	34	33	46

6.5.3. Treatment Days

There were no reported symptoms following inhalation of SKF 104353-Z_2 or placebo. The mean volume of drug nebulised was 1.7ml (SD 0.40, range 1.2-2.6ml) equivalent to 680µg of SKF 104353-Z_2 (range 480-1040µg). Pulmonary function measurements performed before and immediately after SKF 104353-Z_2 and placebo are recorded in Table 9. Inhalation of neither SKF 104353-Z_2 or placebo resulted in any significant change in pulmonary function compared to baseline measurements.

Table 9. Mean (SD) values for pulmonary function before and immediately after inhalation of SKF 104353-Z_2 or placebo

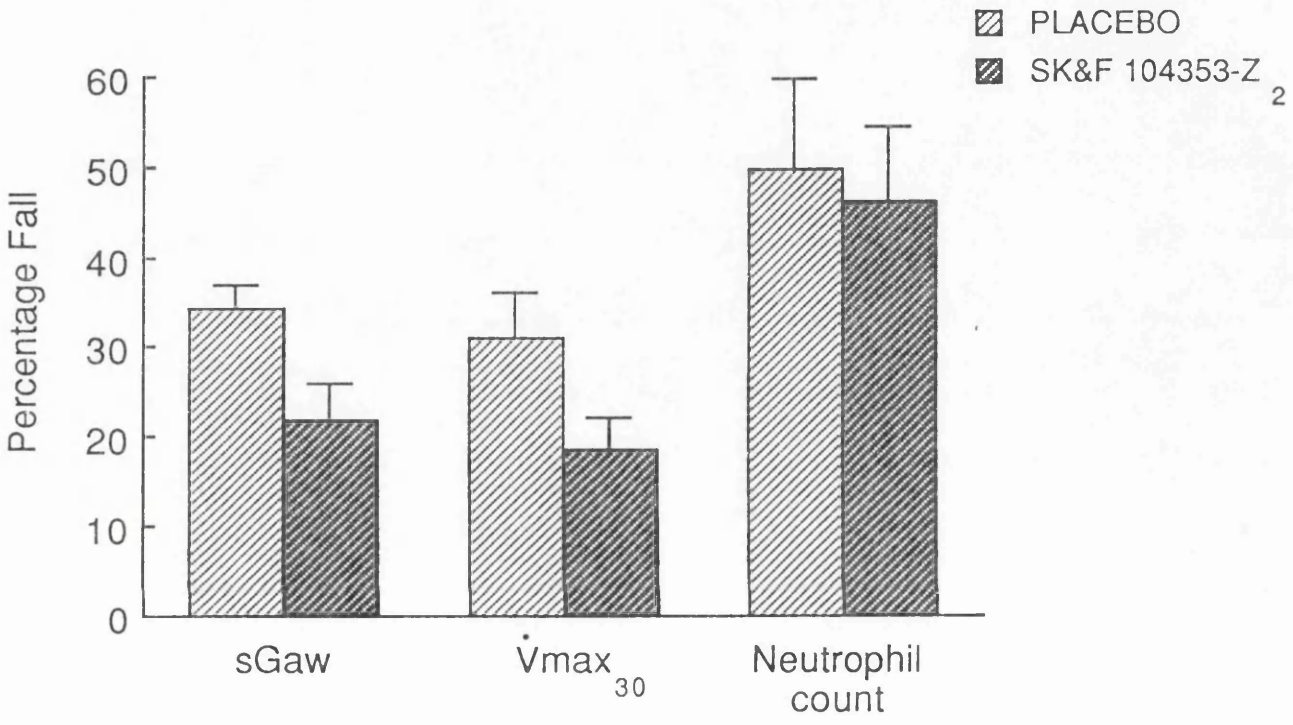
	SKF 104353-Z_2		PLACEBO	
	pre-Rx	post-Rx	pre-Rx	post-Rx
FEV_1	4.54	4.46	4.44	4.42
(l)	(0.75)	(0.71)	(0.69)	(0.70)
$\dot{V}max_{30}$	3.22	3.06	3.30	3.09
(ls^{-1})	(0.85)	(0.92)	(0.84)	(0.91)
sGaw	3.40	3.48	3.26	3.59
($s^{-1}kPa$)	(0.50)	(0.53)	(0.65)	(0.78)

Pulmonary function and circulating neutrophil count following PAF challenge after pre-treatment with SKF 104353-Z_2 or placebo are shown in Table 10 and Fig 9. SKF 104353-Z_2 caused a significant reduction in PAF-induced bronchoconstriction (SKF 104353-Z_2 vs placebo: sGaw; 22% vs 34%. $\dot{V}max_{30}$; 19% vs 31%). The mean difference for sGaw was -12.6% (95% CI for the mean difference -23.8, -1.4), $p<0.05$. and for $\dot{V}max_{30}$ was 12.5% (95% CI for the mean difference -20.8, -4.2), $p<0.01$. The mean difference in the PAF-induced fall in circulating neutrophil count between SKF 104353-Z_2 and placebo was -3.5% (46.1% vs 49.6%. 95% CI for the mean difference -13.6, 6.6), $p>0.10$.

Table 10. The effects of SKF 104353-Z_2 ("SKF") and placebo on the maximum reduction in pulmonary function and circulating neutrophil count induced by inhaled PAF (% of control values)

	sGaw		$\dot{V}max_{30}$		Neutrophil count	
SUBJECT	skf	placebo	skf	placebo	skf	placebo
1	12	31	12	13	8	0
2	41	40	41	54	66	84
3	20	43	19	27	61	68
4	26	37	21	51	80	79
5	19	42	16	25	53	41
6	32	27	8	29	32	42
7	22	22	17	18	29	23
8	2	33	15	32	40	60

Figure 9. Pulmonary function and circulating neutrophil count following PAF challenge after pre-treatment with SKF 104353-Z_2 or placebo (% fall below control values)



6.5.4. Reproducibility of acute changes induced by PAF

PAF challenge resulted in comparable reductions in pulmonary function and circulating neutrophil count on dose-finding and placebo treatment days (mean difference between the two challenge days [coefficient of repeatability]: sGaw; 6% [25]. $\dot{V}max_{30}$; 3% [36]. Neutrophil count; 9% [33]).

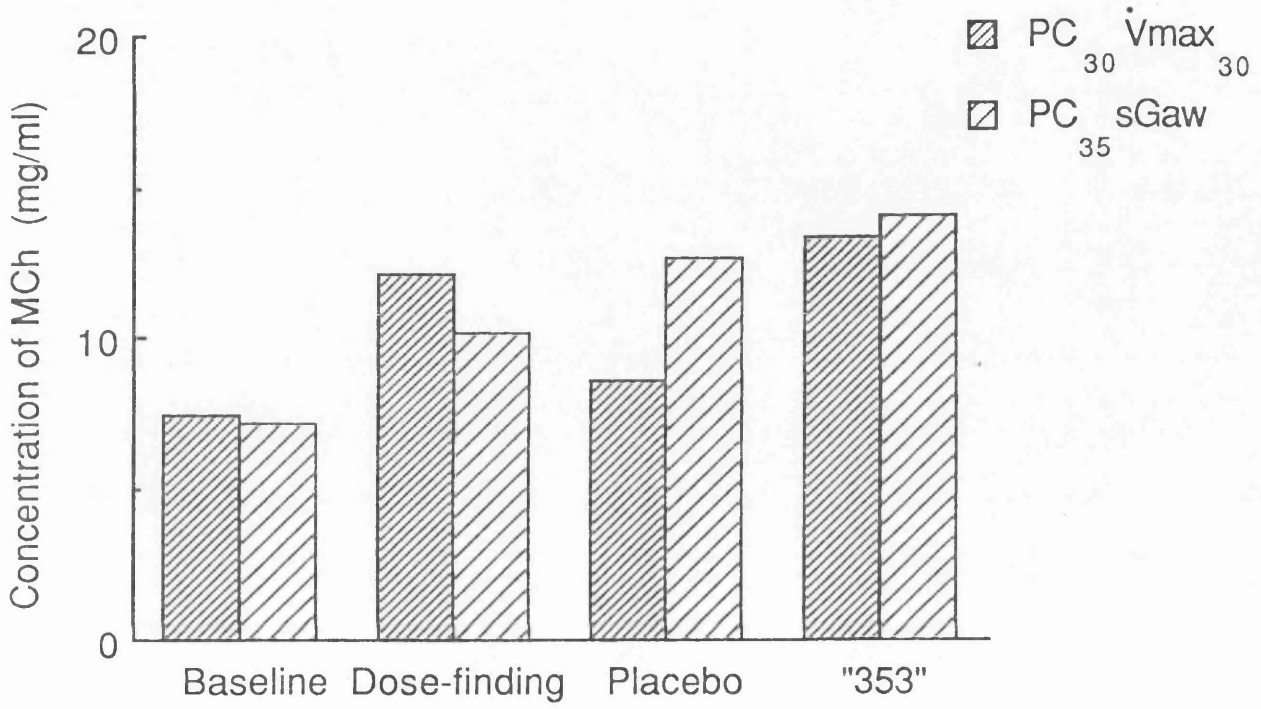
6.5.5. Changes in Bronchial Responsiveness

Changes in bronchial responsiveness are shown in Table 11 and Fig 10. Bronchial responsiveness two weeks after each PAF challenge did not change by more than one doubling concentration of MCh compared to baseline values in any individual. Nor were there any statistically significant changes in the whole group when compared by paired t-tests. There was a trend for bronchial responsiveness two weeks after each PAF challenge to decrease during the course of the study compared to baseline values, but this did not reach statistical significance when analysed by one way ANOVA (PC_{35}sGaw MCh: $F=1.25$, $p=0.31$. $PC_{30}\dot{V}max_{30}$ MCh: $F=0.55$, $p=0.65$).

Table 11. Changes in bronchial responsiveness two weeks after each PAF challenge compared to baseline values, expressed as the geometric mean $PC_{35}sGaw$ and $PC_{30}\dot{V}max_{30}$ (mg/ml MCh)

	$PC_{35}sGaw$	$PC_{30}\dot{V}max_{30}$
Baseline	7.2	7.5
Post dose-finding PAF challenge	10.2	12.2
Post placebo/PAF challenge	12.7	8.6
Post SKF 104353-Z_2 /PAF challenge	14.1	13.4

Figure 10. Bronchial responsiveness two weeks after each of the PAF challenges ("353" = SKF 104353-Z_2)



6.6. DISCUSSION

In this study pre-treatment with a single dose of SKF 104353-Z_2, a structural analogue of LTD_4, reduced the C-18 PAF-induced falls in both sGaw and $\dot{V}max_{30}$ by 30% in normal human subjects. Although *in vitro* studies using SKF 104353-Z_2 against PAF have not been performed on human bronchial smooth muscle, this compound has no activity as a PAF-antagonist in animal studies (Hay, Muccitelli, Tucker, et al. 1987), and is an extremely selective cysteinyl-LT receptor antagonist in animals (Hay, Muccitelli, Tucker, et al. 1987) and in man (Evans, Barnes, Zakrzewski, et al. 1988). These findings therefore suggest that at least a proportion of the bronchoconstriction induced by inhaled PAF in man is dependent on the secondary release of cysteinyl-LTs. The absence of any reduction in the PAF-induced fall in circulating neutrophil suggests that this phenomenon occurs via an independent mechanism.

The single dose PAF challenge was well tolerated, and resulted in more reproducible acute changes than obtained using the cumulative dose-response PAF challenge employed in the initial study (*vide infra*, Chapter 8).

The dose of SKF 104353-Z_2 given in this study (800µg) has been shown to reduce the mean fall in sGaw induced by LTD_4 by 75% when given 15 minutes before LTD_4 challenge in normal man (Evans, Barnes, Piper, et al. 1989). The results of this study suggest that SKF 104353-Z_2 also antagonises the

effects of endogenous cysteinyl-LT production in man. However, as it is not known whether SKF 104353-Z_2 is an equipotent antagonist of endogenous cysteinyl-LTs and exogenous inhaled LTD_4 these findings do not allow us to estimate the actual proportion of PAF-induced bronchoconstriction related to secondary cysteinyl-LT production. In contrast to other species such as the guinea pig, which possess discrete receptors for each of the cysteinyl-LTs, human airways probably contain only a single cysteinyl-LT receptor (Buckner, Krell, Lavaruso, et al. 1986), so that SKF 104353-Z_2 will probably antagonise the effects of all the relevant endogenous cysteinyl-LTs (i.e. LTC_4, LTD_4, and LTE_4).

The transient fall in circulating neutrophils observed after inhaled PAF is due to temporary sequestration of these cells within the pulmonary circulation (Tam, Clague, Dixon, et al. 1990). Cysteinyl-LTs have no known chemotactic properties in man, so it is not surprising that pre-treatment with SKF 104353-Z_2 did not affect this response. However, this cellular effect could be explained by the secondary production of LTB_4, a potent chemotactic agent for the human neutrophil produced in close association with PAF (Sisson, Prescott, McIntytre, et al. 1987). Alternatively, this phenomenon could be due to a direct effect on neutrophils, mediated via specific PAF receptors present on the human neutrophil (Dent, Ukena, Chanez, et al. 1989).

Bronchial responsiveness two weeks after each PAF challenge was unchanged compared to baseline values. These findings are in agreement with those of the previous study. The doses of PAF used in this study (either $9.6x10^{-8}$mol or $1.9x10^{-7}$mol by gravimetric estimation) are comparable to the geometric mean dose of PAF ($1.1x10^{-7}$mol) used in the Cuss, Dixon and Barnes study. Several possible explanations for these different findings have already been discussed (Section 4.5.4.). Recently it has been suggested that PAF-induced increases in bronchial responsiveness could be specifically related to smaller doses of PAF, with "self-tachyphylaxis" occurring at higher doses (Lai, Jenkins, Polosa, et al. 1990). However, it must now be increasingly doubtful that such prolonged increases in bronchial responsiveness as originally described are a reproducible consequence of PAF inhalation in normal man.

The mode of action of PAF in man has not been completely determined. Indirect mechanisms may be important as demonstrated by the findings that PAF-induced contraction of human bronchial smooth muscle is platelet dependent *in vitro* (Schellenberg, Walker, and Snyder. 1983), and that pre-treatment with an antihistamine reduces PAF-induced bronchospasm *in vivo* (Smith, Rubin and Patterson. 1988). PAF may act through binding to specific PAF receptors, triggering complex intracellular transduction mechanisms resulting in activation of phospholipase A_2 with the subsequent release of a cascade of mediators including LTs,

PGs and TxA_2 (as discussed in the Introduction, Section 1.11.). This study supports this hypothesis for the indirect mechanism of action of PAF, although the relative contributions of individual secondary mediators in producing the biological effects attributed to PAF remain to be determined.

The nature of the fundamental difference or differences between asthmatics and normals have not been ascertained, nor is it known if the metabolic pathways mediating interactions between PAF and LTs are comparable in normal man and asthmatics. Any extrapolations from this study of inhaled PAF in normal subjects to the presumed effects of endogenous PAF in asthma must therefore be viewed with great caution. Potent and selective PAF-antagonists, 5-LO inhibitors and LT-receptor antagonists are now becoming available for clinical trials. These studies will help to determine the relative importance of PAF and LTs, and of interactions between them, in the pathophysiology of asthma.

CHAPTER SEVEN

THE EFFECTS OF A SELECTIVE 5-LIPOXYGENASE INHIBITOR ON THE ACUTE RESPONSES TO INHALED PLATELET-ACTIVATING FACTOR IN NORMAL MAN

7.1. ABSTRACT

The effects of pre-treatment with BW A4C, a potent and selective 5-LO inhibitor, on the acute responses to inhaled PAF in normal man were examined in a randomised, placebo controlled double-blind crossover study in eight male subjects.

The dose of inhaled PAF causing a fall of at least 35% in sGaw (10 or 20 inhalations of 800µg/ml C18 PAF) was initially determined for each subject. PAF challenge was then repeated with the dose of PAF on two further occasions separated by two weeks, one hour after pre-treatment with either a single dose of BW A4C (400mg p.o.) or placebo.

BW A4C caused a significant reduction in the PAF-induced fall in neutrophil count 5 minutes after PAF, but only small, statistically insignificant, reductions in the PAF-induced falls in sGaw and $\dot{V}max_{30}$ (mean maximum fall below control values, BW A4C vs placebo [mean difference; 95% CI for the mean difference]: Neutrophil count; 45% vs 58% [-13%;-25,-1], p=0.04. sGaw; 31% vs 41%, [-10%; -34, 10], p >0.1. $\dot{V}max_{30}$; 29% vs 35% [-6%; -18, 6], p>0.1]).

Inhibition of whole blood ionophore-stimulated LTB_4 generation *ex vivo*, as an index of 5-LO activity, demonstrated mean inhibition of 70% at one hour, and 90% inhibition at two hours after ingestion of BW A4C. There were positive correlations between the degree of 5-LO

inhibition and the percentage reduction in PAF-induced falls in sGaw, $\dot{V}max_{30}$ and fall in circulating neutrophil count obtained with BW A4C.

No changes in bronchial responsiveness to MCh were observed two weeks after each PAF challenge.

PAF-induced neutropenia may be dependent on the secondary generation of a 5-LO product, probably LTB_4. Failure to detect a significant reduction in PAF-induced bronchoconstriction could be due to insufficient pulmonary 5-LO inhibition at one hour after ingestion of a single 400mg dose of BW A4C in this group of subjects.

7.2. INTRODUCTION

The previous study confirmed that interactions do occur between PAF and LTs in man. An alternative approach to studying the relationships between these two groups of mediators would be to prevent LT generation by inhibition of 5-LO. The potential advantage of this approach is that the use of a 5-LO inhibitor would allow simultaneous investigation of the potential role of all 5-LO products, including both the cysteinyl-LTs and LTB_4, in mediating the effects of PAF. Furthermore, as an inhaled antagonist is unlikely to be distributed to all pulmonary cysteinyl-LT receptors, effective pulmonary 5-LO inhibition with an orally active agent might prove more effective in preventing secondary mediator release.

The possibility that LTB_4 may account for the chemotactic activity of PAF for human neutrophils, and the close relationship between PAF and LTB_4 have already been mentioned (section 1.10.). LTB_4 and PAF have other similar pro-inflammatory actions (reviewed by Piper. 1984; Samuellson. 1983; Peters, Freeland, Kelly, et al., 1987). LTB_4 is an extremely potent chemotactic agent for the human eosinophil and neutrophil, causes aggregation and activation of polymorphs (Ford-Hutchinson, Bray, Doig, et al. 1980), promotes oedema formation with exudation of plasma and stimulates calcium translocation and phospholipase A_2. LTB_4 is a very weak constrictor of human bronchus, and in common with PAF this phenomenon exhibits marked tachyphylaxis. LTB_4

has been detected in high concentrations in biological fluids from patients with numerous conditions with a prominent inflammatory component, including asthma and cystic fibrosis (Sampson, Spencer, Green, et al. *In Press*). Administration of LTB_4 to dogs leads to increased bronchial responsiveness (O'Byrne, Leikauf, Aizawa, et al. 1985), but this does not occur at the doses which have been given to normal man (Black, Fuller, Taylor, et al. 1989).

BW A4C (N-[(E)-3-(3-phenoxyphenyl)prop-2-enyl] acetohydroxamic acid) is a potent inhibitor of 5-LO which acts by binding to Fe^{3+} at the catalytic site of the enzyme (Jackson, Islip, Kneen, et al. 1988; Salmon, Jackson and Garland. 1989; Darley-Usmar, Hersey and Garland. 1989). This compound is selective, inhibiting the synthesis of LTB_4 by isolated human leukocytes at concentrations 10 to 100 times less than that required to inhibit cyclo-oxygenase, 12-lipoxygenase and 15-lipoxygenase, and inhibiting LTB_4 but not TXB_2 synthesis when administered to rats (Tateson, Randall, Reynolds, et al. 1988).

A single dose of 400mg BW A4C effectively inhibits *ex vivo* ionophore-stimulated LTB_4 generation by whole blood for at least four hours when administered orally one hour previously to healthy human volunteers (Garland, Jackson, Salmon, et al. 1990). We have used this dose of BW A4C to further investigate the involvement of 5-LO products in mediating the acute bronchoconstriction and transient fall

in circulating neutrophils induced by inhaled PAF in normal man.

The single dose PAF challenge used in the previous study was well tolerated and induced reproducible acute changes. The technique was therefore retained for this study, but the preparation of PAF was modified in an attempt to further improve the reproducibility of the procedure.

7.3. SUBJECTS, MATERIALS AND METHODS

7.3.1. Subjects

Eight healthy male subjects (mean age 24 years, range 21-33) participated in this study. Demographic details are given in Table 12. One subject gave a history of mild seasonal allergic rhinitis, but had a normal serum IgE level and was studied outside the pollen season.

Table 12. Demographic details

Subject	Age	FEV_1 (l)	FEV_1 (% predicted)	Skin prick tests
1	23	4.02	98	-VE
2	33	4.46	108	-VE
3	23	4.88	121	-VE
4	21	4.24	96	-VE
5	22	4.61	106	-VE
6	22	4.82	105	-VE
7	24	5.18	125	+VE
8	25	4.30	97	-VE

7.3.2. Materials

Details are given in section 3.3. BW A4C (200mg) and matching placebo were supplied as tablets and stored at 4°c.

7.3.3. Methods

7.3.3.1. Protocol

Baseline responsiveness to histamine was initially measured, and a single dose of inhaled PAF causing a fall of at least 35% in sGaw determined for each subject. On two treatment days, separated by at least two weeks, this dose of PAF was administered one hour after ingestion of either 400mg BW A4C or placebo in a randomised, double-blind crossover manner. Bronchial responsiveness to histamine was reassessed two

weeks after any PAF challenge.

7.3.3.2. Histamine Challenge

Responsiveness to histamine correlates closely with responsiveness to MCh (Juniper, Frith, Dunnett, et al. 1978), but histamine has a more rapid onset of action, making bronchial challenge with this agonist less time consuming. Histamine was therefore used in this study because of increasing demands from other studies for the use of the laboratory facilities.

7.3.3.3. PAF challenge

Lyophilised C-18 PAF 1.6mg was dissolved into 2mls of saline with 2.5mg/ml of HSA and placed in the nebuliser chamber immediately prior to the challenge. PAF challenges were performed on dose-finding and treatment days in the same manner as described for the previous study.

7.3.3.4. Treatment Days

FEV_1 (mean of 3 values), sGaw (mean of 16 values) and $\dot{V}max_{30}$ (mean of 5 values) were initially performed. A venous catheter (Venflon) was inserted, and blood was drawn for baseline blood count and assay of *ex vivo* ionophore-stimulated LTB_4 generation. The subject then ingested the trial medication or placebo. Repeated specimens of blood were obtained for LTB_4 assays at 20, 40 and 60 and 120 minutes. PAF challenge was performed at 60 minutes.

7.3.3.5. Assay of LTB_4 generated ex vivo by ionophore-stimulated whole blood

This was performed using the method described by Sampson, Evans, Garland et al (1990). The incubation of blood samples with ionophore was performed by Dr Tony Sampson. Venous blood samples (2ml) were drawn into heparinised syringes, transferred into test tubes and incubated at 37°C in a water bath. After five minutes, 20µl calcium ionophore A23187 (2mg/ml in dimethylsulphoxide [DMSO]) was added to give a concentration of ionophore in the blood sample of 38µM and 1% v/v DMSO. Samples were mixed thoroughly using a vortex mixer and further incubated at 37°C for 30 minutes. Incubations were terminated by rapid cooling of the tubes on ice. Each specimen was centrifuged at 6000rpm (3000g) for 10 minutes. 1ml of the plasma was removed with a disposable pasteur pipette and stored in screw topped plastic containers at -20°C.

Samples were subsequently transported on dry ice to the Wellcome Research Laboratories, Beckenham, Kent, where radioimmunoassay for LTB_4 (Amersham) was performed by Dr Steven Jeal.

The limit of the sensitivity of the assay was 20ng/ml, the percentage inhibition of LTB_4 was therefore calculated using the formula:

$$\textit{Percentage 5-LO inhibition} = \frac{(\textit{pretreatment } LTB_4 \textit{ level}-20)-(60\text{min } LTB_4 \textit{ level}-20)}{\textit{pretreatment } LTB_4 \textit{ level}-20}$$

7.4. STATISTICAL ANALYSIS

The reproducibility of baseline values of sGaw, $\dot{V}max_{30}$ and FEV_1 performed prior to each histamine and PAF challenge, and at the beginning of each treatment day, was calculated as a coefficient of variation for each subject. The effects of BW A4C and placebo on pulmonary function was assessed by paired t-tests on the pre-treatment and one hour post-treatment values of FEV_1, sGaw and $\dot{V}max_{30}$.

The mean of the sGaw and $\dot{V}max_{30}$ measurements at the time of maximum bronchoconstriction and the neutrophil count at five minutes following PAF were expressed as a percentage of control values obtained after inhaling diluent. The values on the two treatment days were compared by paired t-tests and the results expressed as mean difference, 95% CI for the mean difference and p value.

The reproducibility of the PAF challenge was assessed by comparing the data for pulmonary function and circulating

neutrophil count following PAF challenges on dose-finding and placebo treatment days. These findings were expressed as the mean difference between the two challenges along with the coefficient of repeatability, as in the previous studies.

Correlation between the percentage inhibition of LTB_4 generation in whole blood and any reduction in the PAF induced falls in pulmonary function and circulating neutrophil counts after pre-treatment with BW A4C was assessed using the Pearson product moment coefficient. Linear regression analyses were performed using the least squares method.

Changes in bronchial responsiveness two weeks after each PAF challenge were compared to baseline values, after logarithmic transformation of the data, by paired t-tests and by a one way ANOVA.

7.5. RESULTS

7.5.1. Baseline Pulmonary function

Coefficients of variation for FEV_1 and sGaw for each subject performed prior to each histamine and PAF challenge, and at the beginning of each treatment day, were within previously published values (mean [range]: FEV_1; 2.6% [1.4-4.4%]. sGaw; 9.6% [4.2-13.9%] (Larsson, Hedenstrom and Malberg. 1987; Tattersfield and Keeping. 1979). The mean coefficient of variation for $\dot{V}max_{30}$ was 5.8% (range 2.3-10.0%).

7.5.2. PAF challenge

PAF challenge was generally tolerated well and there were no serious adverse side effects. On one occasion, a subject complained of feeling faint within two minutes of inhaling PAF, and was placed in the supine position for several minutes. No changes in heart rate or blood pressure were observed on this occasion, and the subject was able to continue the study within five minutes.

Four of the subjects required ten inhalations of PAF and four subjects 20 inhalations to produce the required 35% fall in sGaw. The number of inhalations of PAF given along with the maximum falls in pulmonary function and neutrophil count for the dose finding PAF challenge are given in Table 13. All subjects experienced irritation of the throat, facial flushing and a desire to cough; these symptoms occurred within one minute of completing the inhalation and resolved within five minutes. The greatest mean falls in sGaw and $\dot{V}max_{30}$ were always obtained when first measured at one and three minutes respectively after PAF inhalation. No changes in heart rate, blood pressure or cardiac rhythm were observed following inhalation of PAF.

Table 13. Dose-finding PAF challenge. Number of inhalations of PAF given with subsequent maximum falls in pulmonary function and circulating neutrophil count (% of control values obtained after the inhalation of diluent)

Subject	Inhalations of PAF	sGaw	$\dot{V}max_{30}$	Neutrophil count
1	20	35	46	48
2	10	39	44	58
3	20	44	38	88
4	20	34	32	84
5	10	35	18	70
6	20	41	28	54
7	10	46	56	59
8	10	51	28	50

7.5.3. Treatment Days

There were no reported symptoms following ingestion of BW A4C or placebo. Pulmonary function measurements performed before, and one hour after BW A4C and placebo are recorded in Table 14. Ingestion of neither BW A4C or placebo resulted in any significant change in pulmonary function compared to baseline measurements.

Table 14. Mean (SD) values for pulmonary function before and one hour after ingestion of BW A4C or placebo.

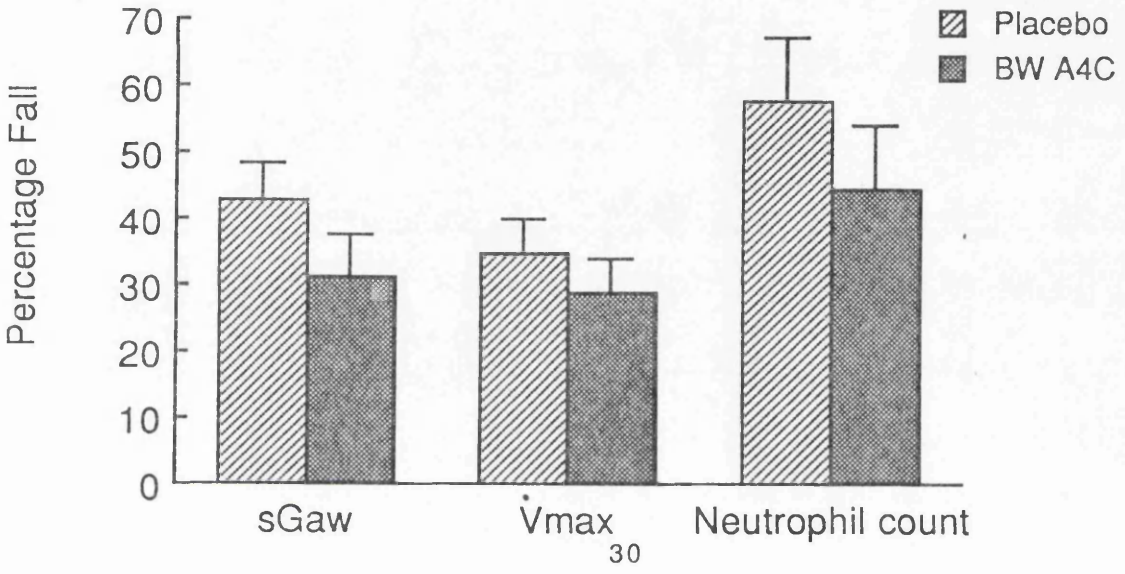
	BW A4C		PLACEBO	
	pre-Rx	post-Rx	pre-Rx	post-Rx
FEV_1	4.51	4.39	4.52	4.43
(l)	(0.36)	(0.35)	(0.37)	(0.37)
$\dot{V}max_{30}$	2.99	2.99	3.06	2.97
(ls^{-1})	(0.65)	(0.80)	(0.86)	(0.83)
sGaw	2.77	2.94	2.82	3.01
($s^{-1}kPa$)	(0.92)	(0.85)	(1.00)	(0.79)

Pulmonary function and circulating neutrophil count following PAF challenge after pre-treatment with BW A4C or placebo are shown in Table 15 and Fig 11. BW A4C caused a statistically significant reduction in the PAF-induced fall in circulating neutrophil count, but with only small, statistically insignificant reductions in the PAF-induced falls in sGaw and $\dot{V}max_{30}$ (BW A4C vs placebo [mean difference; 95% CI for the mean difference]: neutrophil count; 45% vs 58% [-13%; -25, -1), p=0.04; sGaw; 31% vs 41% [-10%; -34, 10], p=>0.1; $\dot{V}max_{30}$; 29% vs 35% [-6%; -18, 6], p>0.1).

Table 15. The effects of BW A4C ("BW") and placebo on the maximum reduction in pulmonary function and circulating neutrophil count induced by inhaled PAF (% of control values)

	sGaw		$\dot{V}max_{30}$		Neutrophil count	
Subject	BW	placebo	BW	placebo	BW	placebo
1	32	30	20	20	39	52
2	17	49	20	37	42	61
3	60	45	58	37	92	95
4	26	27	14	29	33	67
5	41	27	28	25	42	27
6	51	52	37	50	57	78
7	12	73	37	61	56	71
8	10	40	17	18	-5	10

Figure 11. Pulmonary function and circulating neutrophil count following PAF challenge after pre-treatment with BW A4C or placebo (% fall below control values)



7.5.4. *Ex vivo* generation of LTB_4 by ionophore-stimulated whole blood

LTB_4 levels are given in Appendix 5, and the percentage 5-LO inhibition obtained with BW A4C and placebo in Appendix 6. The LTB_4 levels are generally much lower than expected, even in pre-treatment and placebo treatment samples, and in some cases levels were below the detection limit of the assay. These anomalies were probably due to deterioration of the plasma samples, as technical difficulties with the LTB_4 assay system resulted in the samples being stored frozen for five months prior to assay.

These problems render it difficult to interpret the data on the percentage inhibition of LTB_4 generation. The calculated mean percentage inhibition of LTB_4 production following pre-treatment with BW A4C was 70% at 60 minutes and 89% at 120 minutes. The mean percentage inhibition of 54% at 60 minutes and 26% at 120 minutes following pre-treatment with placebo reflect the lack of activity of several of the placebo treated samples.

7.5.5. The relationships between inhibition of LTB_4 generation and reduction in PAF-induced changes by BW A4C or placebo

There was a positive correlation between the percentage reduction in PAF-induced falls in sGaw and $\dot{V}max_{30}$ following pre-treatment with BW A4C at 60 minutes (sGaw; r=0.51, $\dot{V}max_{30}$; r=0.55) but these findings were not significant by linear regression analysis (sGaw: r^2=26%, p=0.2; $\dot{V}max_{30}$; r^2=29.7%, p=0.16), nor were there these analyses significant for the fall in circulating neutrophil count (r=-0.21, r^2=4.5%, p=0.65).

The findings were somewhat more consistent when the reduction in PAF-induced changes were compared to the inhibition of LTB_4 generation at 120 minutes: sGaw; r=0.81, r^2=65.0%, p=0.016; $\dot{V}max_{30}$; r=0.92, r^2=85%, p=0.001; Neutrophil count; r=0.57, r^2=32.3%, p=0.18.

There were no statistically significant correlations at any time between the percentage inhibition of *ex vivo* ionophore-stimulated LTB_4 generation and the PAF-induced changes in pulmonary function and circulating neutrophil count following pre-treatment with placebo.

7.5.6. Reproducibility of acute changes induced by PAF

PAF challenge resulted in comparable reductions in pulmonary function and circulating neutrophil count on dose-finding and placebo treatment days (mean difference [coefficient of repeatability]: sGaw; 2.2% [24.6]; $\dot{V}max_{30}$; 1.6% [26.4]; neutrophil count; 6.3% [47.6]).

7.5.7. Changes in Bronchial Responsiveness

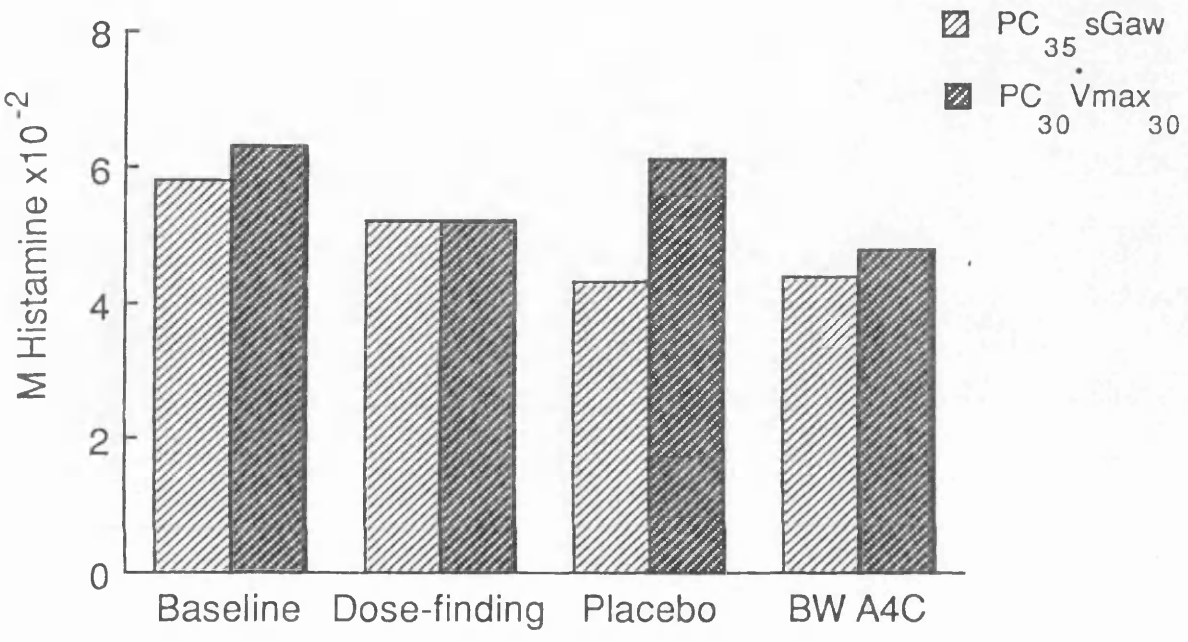
Geometric mean changes in bronchial responsiveness are shown in Table 16 and in Fig 12. Bronchial responsiveness in individual subjects remained within one doubling concentration of the baseline value on all except for two occasions. Bronchial responsiveness increased just over one doubling concentration of histamine following the dose-finding PAF challenge in subject five and from 0.048M to 0.013M of histamine following the placebo treatment/PAF challenge in subject eight. The increase in bronchial responsiveness with the latter subject was associated with symptoms suggestive of a viral upper respiratory tract infection. Neither of these two subjects had any increase in bronchial responsiveness after any other PAF challenge.

There were no statistically significance changes in bronchial responsiveness compared to baseline values for the whole group two weeks after each PAF challenge when analysed by paired t-tests and by one way ANOVA (PC_{35}sGaw Histamine: $F=0.37$, $p=0.77$. $PC_{30}\dot{V}max_{30}$: $F=0.21$, $p=0.89$).

Table 16. Changes in bronchial responsiveness two weeks after each PAF challenge compared to the baseline values, expressed as the geometric mean $PC_{35}sGaw$ and $PC_{30}\dot{V}max_{30}$ (M histamine)

	$PC_{35}sGaw$	$PC_{30}\dot{V}max_{30}$
Baseline	$5.8x10^{-2}$	$6.3x10^{-2}$
Post dose-finding PAF challenge	$5.2x10^{-2}$	$5.2x10^{-2}$
Post placebo/PAF challenge	$4.3x10^{-2}$	$6.1x10^{-2}$
Post BW A4C/PAF challenge	$4.4x10^{-2}$	$4.8x10^{-2}$

Figure 12. Bronchial responsiveness two weeks after each PAF challenge



7.6. DISCUSSION

These results do not confirm the findings of the previous study which suggested that PAF-induced bronchoconstriction is at least partially dependent on the secondary release of cysteinyl-LTs in man. However, they do suggest that the transient fall in circulating neutrophils observed after PAF inhalation could involve 5-LO products, probably LTB_4.

There are several factors which may explain the discrepancies in the findings between these two studies. Eight subjects were used in this study on the basis of power calculations giving an 80% probability of obtaining a similar (30%) reduction in the indices of PAF-induced bronchoconstriction with BW A4C to that obtained in the SKF 104353-Z_2 study. As the mean reductions in the PAF-induced falls in sGaw and $\dot{V}max_{30}$ were less than this, it is possible that if larger numbers of subjects were used, a statistically significant reduction in bronchoconstriction may have been obtained.

The dose of BW A4C (400mg) and the timing of the PAF challenge employed in this study was decided on the basis of findings by Garland, Jackson, Salmon, et al (1990) that administration of this dose of BW A4C to man resulted in greater than 90% inhibition of *ex vivo* ionophore-stimulated LTB_4 generation by whole blood at one hour. Technical problems rendered the results of the LTB_4 assays less than totally satisfactory in the current study, but the mean

percentage inhibition at 60 minutes in our group of subjects was only 70%, rising to 90% at 120 minutes. Although the PAF challenge commenced at 60 min after ingestion of the medication, a series of pulmonary function measurements were initially performed, so that PAF was inhaled approximately 90 minutes after the medication.

Positive correlations were found between the percentage inhibition of LTB_4 generation at 120 minutes and the percentage reductions in PAF-induced falls in sGaw and $\dot{V}max_{30}$ obtained with BW A4C. These findings suggest that if a greater reduction in the degree of 5-LO inhibition could be achieved, either by increasing the time between dosing and PAF challenge, or by using a larger dose of BW A4C, then a statistically significant reduction in PAF-induced bronchoconstriction may have been be obtained.

Additional, although indirect, evidence supporting the hypothesis that PAF acts through secondary mediator release in man has recently been provided by Taylor, Taylor and Fuller (1990). These investigators found that PAF inhalation in asthmatic subjects resulted in a ten-fold increase in urinary LTE_4 secretion. The same group also found smaller increases in urinary TxA_2 metabolites after PAF challenge (Ward, Taylor and Fuller. 1990).

Although *ex vivo* inhibition of ionophore-stimulated LTB_4 generation either from purified preparations of neutrophils or from whole blood is widely used in the assessment of 5-LO inhibitors, Hui, Taylor, Rubin, et al (1990) have now suggested that this may be a relatively poor method for assessing the dose of inhibitor necessary to inhibit the pulmonary effects of LTs *in vivo*. This group have reported the effects of the 5-LO inhibitor A-64077 on *in vivo* urinary LTE_4 secretion and ionophore-stimulated LTB_4 generation in whole blood *ex vivo* following antigen challenge. They found a correlation between the decrease in urinary LTE_4 and the reduction in maximum fall in FEV_1 in the early asthmatic response, but no correlation with the reduction in *ex vivo* ionophore-stimulated LTB_4 generation in whole blood. On the basis of these findings they suggest that measurement of changes in urinary LTE_4 levels may be the more useful index of 5-LO inhibition in man.

As with the previous study, there was no prolonged increase in bronchial responsiveness two weeks after each PAF challenge. It is not possible to make direct dosage comparisons between these studies, as different formulations of PAF were given in the two studies. However, in both studies the same total number of inhalations of C-18 PAF was administered using identical equipment, and similar changes in pulmonary function and circulating neutrophil were induced. Thus the dose of PAF delivered to the airways is likely to have been similar in the two studies. These

findings therefore augment the evidence that a prolonged increase in bronchial responsiveness is not a reproducible consequence of PAF inhalation in normal man.

CHAPTER EIGHT

COMPARISON OF THE REPRODUCIBILITY OF THE SUCCESSIVE PAF CHALLENGE TECHNIQUES

The PAF challenge procedure was modified twice in attempts to improve the reproducibility of the technique without compromising safety or tolerability. Reproducibility was assessed in each of the three studies by calculation of the coefficient of repeatability adopted by the British Standards Institute (Altman and Bland. 1986). Coefficients of repeatability for changes in pulmonary function and circulating neutrophil count in the three relevant studies are shown in Table 17.

Table 17. Comparison of the repeatability of the successive PAF challenges (Coefficient of repeatability)

STUDY	sGaw	$\dot{V}max_{30}$	Neutrophil count
Cumulative dose-response challenge	46.9	54.5	57.2
SKF 104353-Z_2	24.1	36.2	33.7
BW A4C	24.6	26.4	47.6

In the first study, in which a cumulative dose-challenge was employed, the reproducibility of the acute responses was very poor, and it was our impression that this was due to tachyphylaxis. The term tachyphylaxis in this context refers to the phenomenon in which repeated inhalation of the agonist over a short period of time results in a blunting or

abolition of the response. In this instance, if the required degree of bronchoconstriction was not obtained after inhalation of the initial dose of PAF, inhalation of the next (doubling) concentration was accompanied by flattening of the dose-response curve and much less than a doubling of the response. This effect was observed when PAF was first given by inhalation to man (Cuss, Dixon and Barnes. 1986). The mechanism of this phenomenon is unknown, but may be internalisation or down-regulation of receptors, or alternatively to depletion of a stored preformed secondary mediator.

To circumvent the problem of tachyphylaxis, a single dose PAF challenge was devised for the SKF 104353-Z_2 study. This involved inhaling either 10 or 20 inhalations of PAF using double the initial concentration given in the first study. Although symptoms of throat irritation, flushing and a desire to cough were reported more frequently than in the former study, this challenge procedure was generally well-tolerated, and consistently induced the required degree of bronchoconstriction.

Reproducibility of this technique, and the subsequent single dose challenge used in the BW A4C study, was assessed by comparing the maximum falls in sGaw, $Vmax_{30}$ and circulating neutrophil count obtained when the same dose of PAF was given on the dose-finding and placebo treatment days. This is not an absolutely correct method for assessment of

reproducibility, as it is conceivable that the administration of placebo one hour before PAF challenge could somehow influence the response to PAF. Nevertheless, pulmonary function immediately before the two PAF challenges was very similar, and it was impractical to perform another PAF challenge on a separate day solely to assess repeatability.

It can be seen from the table that the coefficient of repeatability for both the pulmonary function measurements and the fall in circulating neutrophil count induced by PAF was greatly reduced in the SKF 104353-Z_2 study compared to the cumulative dose-response PAF challenge. This suggests that tachyphylaxis was indeed a major contributory factor in the poor reproducibility of the cumulative dose-response challenge.

For the final study, using the 5-LO inhibitor BW A4C, particular attention was paid to the preparation of PAF. In the first two studies, 100mg C-18 PAF was divided into aliquots of 2.5mg in ethanol and frozen at -80°c until required. It is not known whether ethanol affects the biological responses to PAF in man, although concentrations above 1% are associated with impairment of PAF-induced aggregation of rabbit platelets *in vitro* (Dr G. Blackwell, *personal communication*). The decision was therefore made to eliminate this potential variable by evaporating off the ethanol under a stream of nitrogen immediately before PAF

challenge. The PAF was then resuspended in 0.9% saline with 2.5mg/ml HSA.

The preparation of PAF in the manner described may itself be a source of errors. It is not possible to obtain exact aliquots of ethanolic PAF, as ethanolic solutions cannot be pippetted with accuracy using standard equipment. PAF adheres to many surfaces, and although polypropylene tubes were used to minimise this problem, the loss of PAF due to surface adhesion during preparation and storage is unknown. Finally, evaporation of the ethanol with a stream of nitrogen could potentially result in evaporation of small quantities of PAF.

In an attempt to minimise these errors from the BW A4C study, a company with special expertise in the synthesis of bioactive lipids (Cascade Biochem Ltd, The Innovation Centre, Univerity of Reading) was approached. They were contracted to supply lyophilised C-18 PAF in individual vials containing 1.6mg of PAF prepared to a high degree of accuracy using industrial techniques. Immediately prior to the challenge the contents of the vial were simply dissolved in to 2mls of saline with 2.5mg/ml of HSA and placed in the nebuliser chamber. If the repeatability of this PAF challenge is compared to that used in the SKF 104353-Z_2 study, it can be seen that this modification was associated with an improved coefficient of repeatability for $\dot{V}max_{30}$, but with no change in that for sGaw and a slight worsening

of repeatability for the neutrophil count measurements.

Any added benefit from the use of a commercial preparation of PAF in the BW A4C study is small compared to the improvement associated with introduction of the single dose challenge. It is difficult to explain the worsening repeatability for the PAF-induced fall in circulating neutrophils observed with the BW A4C study. A single measurement of circulating neutrophil count is not very satisfactory for the assessment of PAF-induced changes, as the reduction in circulating cells occurs and resolves very rapidly. Subtle differences in the timing of the venepuncture could therefore result in considerable changes in repeatability of this measurement. Unfortunately, the performance of multiple pulmonary function measurements immediately after the inhalation of PAF made it impractical to obtain repeated blood samples at this time.

Other factors which may have contributed to changes in repeatability in these three studies need to be considered. Different subjects were employed in each of the three studies, and it is possible that the pulmonary function measurements of the subjects recruited for the latter studies were more reproducible than that of the subjects involved in the initial study. Certainly, pulmonary function was not very reproducible in one subject used in the first study (Subject 3; who also did not bronchoconstrict following one of the PAF challenges), and this subject was

excluded from the latter studies for this reason. Two subjects participated in all three studies, and although it would be interesting to assess sequential changes in reproducibility for these individuals, it would be difficult to separate changes due to differences in the challenge technique from those due to other factors such as a learning effect.

CHAPTER NINE

CONCLUDING REMARKS AND SUGGESTIONS FOR FURTHER STUDIES

Our inability to demonstrate a significant increase in bronchial responsiveness at any time following the inhalation of PAF in any of the subjects studied was disappointing, and effectively precluded the use of this PAF inhalation challenge as a model for the study of BHR in normal man. The original finding of an increase in bronchial responsiveness following inhaled PAF has since been re-iterated in three further papers from the same group (Chung, Minette, McCusker, et al. 1988; Chung, Dent and Barnes. 1989; Wardlaw, Chung, Moqbel, et al. 1990). Several other groups of workers have also published similar positive findings in abstract form only (including Townley, Hopp, Bewtra, et al. 1986; Di Maria, Bellofiore, Ciancio, et al. 1989), whereas Gebré-Michael and Leuenberger (1989) found no such increase. Critical analysis of findings published only in abstract form is impossible as experimental details are inevitably excluded. Although it could be argued that failure to publish these results in full after a period of at least two years has elapsed suggests that the preliminary findings were not confirmed, many other factors influence the publication of results, and I believe that because of the complexity of the subject matter the findings in all of these abstracts (positive *and* negative) should be viewed with great caution.

Two other complete papers have been published reporting increases in bronchial responsiveness after inhaled PAF in man. In the former (Rubin, Smith and Patterson. 1987)

increases in responsiveness lasting for up to 45 minutes were reported. Kaye and Smith (1990) subsequently described an apparent increase of 50% in responsiveness to MCh in six out of eight normal subjects for up to 14 days following inhaled PAF. There are serious reasons to doubt the validity of the claims made in the latter paper; such changes would be at the limit of the detectable changes in responsiveness, as the reproducibility of a MCh challenge is approximately one doubling concentration of the agonist. In addition, the results in this study would seem to be invalid as the data was not apparently log transformed for the analysis. Interestingly, in the same paper similar increases in responsiveness to MCh following LTD_4 challenge in normal subjects were reported, the only time that this has ever been claimed despite numerous previous reports to the contrary.

In contrast to these positive findings, two studies have now been published in full, in addition to our own, in which no increase in bronchial responsiveness has occurred following PAF inhalation in normal subjects (Lai, Jenkins, Polosa et al. 1990; Stenton, Ward, Duddridge, et al. 1990). Several reasons for these divergent findings have already been discussed, but in addition Lai, Jenkins, Polosa, et al (1990) have suggested that it is conceivable that this phenomenon could specifically be related to small doses of PAF. These authors suggest that tachyphylaxis might occur at high doses of PAF, presumably in a similar manner to the

tachyphylaxis observed to the bronchoconstricting properties of PAF.

Morley, Sanjar and Page originally proposed in 1984 that PAF might be responsible for the inflammatory features of asthma as a consequence of platelet activation. Recognition that PAF has multiple actions on many other cell types, particularly the eosinophil, led to modification of this hypothesis (Barnes, Chung and Page. 1988), and to a suggestion that eosinophil infiltration and activation and increased bronchial responsiveness may be induced by PAF in patients with asthma. It has not been explained how the eosinophil could be involved in any PAF-induced increase in bronchial responsiveness in normal man, as normals have very few circulating eosinophils. The dramatic transient fall in circulating neutrophils following PAF inhalation in normal subjects (Cuss, Dixon and Barnes. 1986) suggested that the neutrophil rather than the eosinophil is the more important cell in mediating the response to inhaled PAF in this group. This is supported by recovery of increased numbers of neutrophils, but not eosinophils or platelets in BAL fluid (Wardlaw, Chung, Moqbel, et al. 1990), and by radioisotope studies which have confirmed that neutrophils but neither eosinophils or platelets are sequestered into the lung after PAF inhalation in normals (Tam, Clague, Dixon, et al. 1990).

A supplement to this chapter is contained in the Addendum.

ADDENDUM

The mechanism by which any increases in bronchial responsiveness might occur in normals following PAF is unknown. However, it would certainly seem to be a distinct phenomenon from that occurring in asthmatics as although the neutrophil has been implicated in some forms of industrial asthma, it is not generally regarded as a cell closely associated with the pathophysiology of the more common IgE-mediated form of the disease.

Given the lack of circulating eosinophils in normal subjects, one explanation for the positive findings reported by Cuss, Dixon and Barnes could be the use of atopic subjects (as postulated in Section 4.5.4.); the mechanism of this response may then be that atopics have higher numbers of eosinophils available within the pulmonary circulation for sequestration and activation within the lung than non-atopic subjects. The platelet could be crucial in such a response, as platelet depletion inhibits eosinophil recruitment into the lungs in the guinea pig (Lellouch-Tubiana, Lefort, Simon, et al. 1988), although the nature of the platelet-derived factor (s) mediating this phenomenon have not yet been determined. Investigation of changes in bronchial responsiveness, and of changes in eosinophil and platelet counts in BAL fluid following PAF inhalation challenge using healthy atopic subjects is required to examine this possibility.

The lack of any significant increase in bronchial responsiveness following PAF challenge in asthmatic patients (Rubin, Smith and Patterson. 1987; Chung and Barnes. 1989) is disconcerting, and difficult to reconcile with the suggestion that this mediator is of central importance in the pathophysiology of asthma. These negative findings could be partially related to the use of smaller doses of PAF in asthmatics compared to normal subjects. In addition, Chung and Barnes (1989) have suggested that these results could be due either to self-tachyphylaxis to PAF, or because "it may be more difficult to enhance the inflammatory processes that ... may already be present in the airways of those with mild asthma". These conclusions are difficult to accept; firstly, because it is difficult to conceive how any mediator could exert an important pathophysiological role contemporaneously with the tissue being tachyphylactic to the effect of that mediator. Secondly, clinically important stimuli such as antigen readily increase bronchial responsiveness in patients with asthma (Cockcroft, Ruffin, Dolovich, et al. 1977).

The most convincing proof for or against the involvement of PAF in the pathogenesis of asthma will be provided by studies using potent and specific antagonists in models of asthma and therapeutic studies in patients with active disease. Preliminary results of such studies are now becoming available. The PAF antagonist BN 52063 had no beneficial effects on pulmonary function or isocapnic

hyperventilation of cold, dry air and caused only a very small (although statistically significant) reduction in exercise-induced bronchoconstriction (Wilkens, Wilkens, Uffman, et al. 1990). Dermarkarian, Israel, Rosenberg, et al (1991) found that SCH-37370, a dual PAF and histamine antagonist, had no effect on the bronchoconstriction induced by isocapnic hyperventilation of cold, dry air. The PAF antagonists WEB 2086 BS (Wilkens, Wilkens, Bosse, et al. 1991) and MK-287 (Bel, De Smet, Rossing, et al. 1991) have also been investigated in allergen challenge, and have been shown not to modify early or late asthmatic responses or the subsequent changes in bronchial responsiveness in asthmatic subjects. Therapeutic trials of WEB 2086 are currently in progress.

The mechanisms by which PAF produces its various biological effects are incompletely understood, and it is likely that PAF acts by a variety of direct and indirect mechanisms in different situations. Our demonstration of an indirect component to the acute effects of inhaled PAF in man, mediated by secondary LT production, has been confirmed by a similar study using the selective and potent cysteinyl-LT receptor antagonist ICI 204,219 in normal subjects (Kidney, Ridge, Chung, et al. 1991). In this study a much greater inhibition of PAF-induced bronchoconstriction was obtained than we obtained using SK&F 104353-Z_2, probably reflecting the more potent cysteinyl-LT antagonism of the ICI compound. No reduction in the PAF-induced fall in circulating

neutrophil count was obtained using ICI 204,219, also in agreement with our findings using SK&F 104353-Z_2. Similar studies have yet to be performed in asthmatics, but indirect evidence for the involvement of LTs in the acute response to inhaled PAF is provided by the finding of a nine-fold increase in urinary LTE_4 excretion following PAF compared to that obtained after MCh in patients with mild atopic asthma (Taylor, Ward, Taylor, et al. *In press*). In addition, in a recent study O'Connor, Ridge, Chen-Wordsell, et al (1991) have confirmed that inhaled PAF does result in a large increase in urinary LTE_4 excretion in normals, a response which, not surprisingly, was blocked by pretreatment with the potent oral PAF antagonist UK,74505.

These studies suggest that the LTs may be an important "final common pathway" in the production of biological effects attributed to PAF. However any clinical relevance of these findings and the precise mechanism by which PAF induces secondary mediator generation have not yet been elucidated. It has previously been discussed how binding of PAF to specific receptors may trigger complex intracellular transduction mechanisms involving activation of G-proteins and protein kinase C, resulting in increased turnover of inositol phosphates and a rise in intracellular Ca^{2+}. Phospholipase A_2 may then be activated, releasing arachidonate from membrane phospholipids with generation of a cascade of mediators including LTs, PGs and Tx. It is not known whether specific activation of 5-LO is required in

this sequence of events, but it has recently been demonstrated that expression of a 5-LO activating protein ("FLAP") in addition to expression of the enzyme itself is necessary for LT synthesis to occur (Dixon, Diehl, Opas, et al. 1990).

If an inflammatory stimulus results in both direct and indirect LT production, it could be argued that selective pharmacological inhibition or antagonism of the LTs alone would be more likely to be of therapeutic value than if the metabolism of these two groups of mediators were entirely independent. However, it is not yet known if any of the known actions of PAF other than bronchoconstriction are dependent on secondary mediator production in man. The LTD_4/LTE_4 antagonist LY 171883 has been shown to improve pulmonary function in patients with mild asthma (Cloud, Enas, Kemp, et al. 1989), and clinical studies using significantly more potent cysteinyl-LT receptor antagonists are currently in progress. If these studies do yield positive results it will raise the intriguing question of whether the direct or indirect component of LT generation is the most significant in producing the pathological and physiological abnormalities in asthma.

As has been previously discussed, some workers have found PAF-induced contraction of human bronchus to be platelet dependent *in vitro* (Schellenberg, Walker and Snyder. 1983) and PAF receptors are scarce on human bronchial smooth

muscle (Goldie, Pedersen, Self, et al. 1990). We found no evidence of platelet activation *in vivo* in terms of any increase in the circulating levels of two alpha-granule secretory products following PAF inhalation. However, it has to be accepted that more subtle forms of platelet activation might occur in the absence of classical aggregation and release of granule contents (reviewed by Page. 1989), and that other investigations might be required to detect such differential activation. In particular, IgE-dependent release of free radicals from platelets can occur in the absence of classical platelet aggregation. This release is inhibited by the anti-asthma drugs disodium cromoglycate (Tsicopoulos, Lassale, Joseph, et al. 1988) and nedocromil sodium (Thorel, Joseph, Tsicopoulos, et al. 1988), drugs which have no effect on classical platelet aggregation (Lewis, Dervinis and Chang. 1984). These observations, coupled with our findings on the mechanism of action of PAF-induced bronchoconstriction *in vivo*, suggest that the platelet may be directly or indirectly involved in the production of cysteinyl-LTs when stimulated by PAF. This suggestion could be explored by *in vitro* studies of mediator release from human platelets stimulated by PAF.

The inconclusive findings in the BW A4C study may have been due to incomplete 5-LO inhibition at the time of PAF challenge in the subjects studied. This conclusion is supported by the positive correlations between the degree of 5-LO inhibition and the percentage reduction in the PAF-

induced falls in sGaw, $Vmax_{30}$ and fall in circulating neutrophil count obtained with BW A4C. It would be relevant to repeat this study using either a longer time interval between dosing and PAF challenge, a higher dose of BW A4C or a more potent 5-LO inhibitor. Changes in urinary LTE_4 excretion could be employed to assess the degree of pulmonary 5-LO inhibition, based on the findings of Hui, Taylor, Rubin et al (1990) which suggest that this is a more useful index of pulmonary 5-LO activity than the reduction in *ex vivo* LTB_4 generation from ionophore-stimulated whole blood.

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Appendix 1. Levels of ß-TG and PF4 before and after the inhalation of PAF (ng/ml)

	ß-TG		PF4	
SUBJECT	BASELINE	POST-PAF	BASELINE	POST-PAF
1	>200	21	>100	24
2	39	32	17	15
3	33	24	36	31
4	40	38	60	50
5	29	28	24	17
6	55	38	51	24
7	43	51	43	21
8	41	41	61	42
9	45	44	79	63

Appendix 2. Individual changes in bronchial responsiveness to MCh following cumulative dose response PAF and lyso-PAF challenges

Legend A= $PC_{35}sGaw$ MCh

B= $PC_{30}\dot{V}max_{30}$ MCh

SUBJECT 1	PAF 1		PAF 2		lyso-PAF	
	A	B	A	B	A	B
Baseline	5.8	6.7	3.7	6.8	7.9	
Six hours	3.5	3.4	2.0	6.8	6.8	6.8
One day	5.8	3.6	1.9		4.1	
Three days	3.3	4.2	4.0	4.8	11.0	8.0
One week	4.5	4.7	2.7	2.5	4.0	2.6
Two weeks	3.7	6.8	1.9			

SUBJECT 2	PAF 1		PAF 2		lyso-PAF	
	A	B	A	B	A	B
Baseline	7.7	8.0	10.0		14.0	27.0
Six hours	7.6	9.0	8.6	25.0	11.0	30.0
One day	11.0	18.0	10.0	45.0	11.6	16.5
Three days	5.2	21.0	13.0	70.0	13.5	
One week	10.0		14.0	27.0	11.5	14.0
Two weeks					14.0	15.5

Legend A= PC_{35}sGaw MCh

B=$PC_{30}\dot{V}max_{30}$ MCh

SUBJECT 3	PAF 1		PAF 2		lyso-PAF	
	A	B	A	B	A	B
Baseline	43.0	21.0	22.5		14.5	7.0
Six hours	25.5	11.0	39.0	20.0	14.0	12.0
One day	37.0	26.0	55.0	22.0	9.1	
Three days	52.0	25.0	26.0	26.0	10.0	11.5
One week	30.5	16.5	19.0	15.0	22	19.3
Two weeks	34.5	13.3	28.5	25.5	32	21.0

SUBJECT 4	PAF 1		PAF 2		lyso-PAF	
	A	B	A	B	A	B
Baseline	19.0	23.0	13.5	17.5	20.0	22.0
Six hours	9.6	12.0	13.5	24.0	17.0	13.0
One day	16.0	17.8	13.5	13.5	18.0	16.0
Three days	16.0	13.0	13.0	24.0	17.0	14.5
One week	18.5	16.0	14.0	32.0	19.0	23.0
Two weeks	10.0	11.3	13.5	13.5		

Legend A= PC_{35}sGaw MCh

B=$PC_{30}\dot{V}max_{30}$ MCh

SUBJECT 5	PAF 1		PAF 2		lyso-PAF	
	A	B	A	B	A	B
Baseline	11.4	2.2	4.1	3.7	5.7	5.1
Six hours	5.8	7.8	6.5	5.8	3.1	3.4
One day	7.1	5.0	6.2	6.4	1.6	3.4
Three days	4.1	4.7	3.3	2.2	3.8	2.0
One week	3.4	4.7	4.0	1.9	5.7	2.6
Two weeks	4.1	3.7	5.7	5.1	6.6	5.8

SUBJECT 6	PAF 1		PAF 2		lyso-PAF	
	A	B	A	B	A	B
Baseline	14.0	12.0	16.0	7.6	16.5	6.6
Six hours	12.3	8.8	9.6	7.0	10.0	5.0
One day	16.5	10.0	20.0	9.6	16.5	17.0
Three days	21.0	20.0	16.0	18.5	9.0	30.0
One week	9.0	10.0	9.3	6.0	7.0	4.1
Two weeks	10.0	7.3	16.5	6.6	14.0	12.0

Appendix 3. SKF 104353-Z_2 study: individual changes in PC_{35}sGaw MCh (mg/ml) two weeks after each PAF challenge.

Legend. A) Two weeks after the dose-finding PAF challenge

B) Two weeks after the SKF 104353-Z_2 treatment/PAF challenge

C) Two weeks after the placebo treatment/PAF challenge

Subject	Baseline	A	B	C
1	13.5	14.5	12.5	17.0
2	3.8	2.0	6.4	8.2
3	3.6	7.0	14.0	18.0
4	8.4	18.0	16.0	9.0
5	2.0	6.4	7.9	9.8
6	18.5	26.0	32.0	45.0
7	3.7	10.0	8.2	8.4
8	34.0	20.0	18.0	19.0

Appendix 3. SKF 104353-Z_2 study: individual changes in $PC_{30}\dot{V}max_{30}$ MCh (mg/ml) two weeks after each PAF challenge.

Legend. A) Two weeks after the dose-finding PAF challenge

B) Two weeks after the SKF 104353-Z_2 treatment/PAF challenge

C) Two weeks after the placebo treatment/PAF challenge

Subject	Baseline	A	B	C
1	13.5	13.0	12.5	11.5
2	2.6		3.2	7.2
3	3.2		5.8	
4	9.0		26.0	16.0
5	2.2	7.0	3.0	
6	18.0	20.0	43.0	46.0
7			4.3	7.6
8	32.0			13.0

Appendix 4. BW A4C study: individual changes in PC_{35}sGaw histamine (M) two weeks after each PAF challenge.

Legend. A) Two weeks after the dose-finding PAF challenge

B) Two weeks after the BW A4C treatment/PAF challenge

C) Two weeks after the placebo treatment/PAF challenge

Subject	Baseline	A	B	C
1	0.060	0.065	0.057	0.070
2	0.090	0.105	0.085	0.060
3	0.080	0.074	0.105	0.040
4	0.045	0.060	0.0215	0.033
5	0.0315	0.016	0.027	0.025
6	0.0120	0.068	0.082	0.160
7	0.0340	0.036	0.0245	0.041
8	0.0408	0.042	0.026	0.013

Appendix 4. BW A4C study: individual changes in $PC_{30}\dot{V}max_{30}$ histamine (M) two weeks after each PAF challenge.

Legend. A) Two weeks after the dose-finding PAF challenge

B) Two weeks after the BW A4C treatment/PAF challenge

C) Two weeks after the placebo treatment/PAF challenge

Subject	Baseline	A	B	C
1	0.080	0.090	0.074	0.060
2	0.090		0.100	0.68
3	0.080	0.070	0.0840	0.090
4	0.043	0.032	0.0260	0.038
5		0.026	0.020	0.030
6	0.060	0.044	0.100	0.090
7	0.0380	0.042	0.022	0.048
8	0.072	0.110	0.042	0.011

Appendix 5. *Ex vivo* generation of LTB_4 in ionophore-stimulated whole blood (ng/ml plasma).

Subject		Time (min) 0	20	40	60	120
1	Placebo	<20	<20	27.6	72.1	65.4
	BW A4C	74.0	43.6	75.0	53.1	27.7
2	Placebo	68.6	61.6	39.6	54.2	137.7
	BW A4C	101.6	37.2	<20	<20	<20
3	Placebo	106.5	127.0	123.6	174.2	291.3
	BW A4C	116.3	96.6	126.6	126.9	65.3
4	Placebo	90.8	73.1	65.9	74.3	99.3
	BW A4C	160.3	38.2	31.5	97.8	<20
5	Placebo	200.8	85.0	158.7	131.4	214.0
	BW A4C	37.4	26.4	<20	<20	24.1
6	Placebo	68.9	30.2	29.0	<20	48.4
	BW A4C	58.6	37.8	50.8	28.0	20.2
7	Placebo	45.7	36.9	23.9	<20	<20
	BW A4C	35.3	<20	63.2	<20	<20
8	Placebo	47.8	30.8	39.7	24.6	28.8
	BW A4C	27.7	29.4	20.5	<20	<20

Appendix 6. Percentage inhibition of *ex vivo* ionophore-stimulated LTB_4 generation after pre-treatment with BW A4C or placebo

	Time (min)			
	60	120	60	120
Subject	BW A4C		PLACEBO	
1	39	86	0	0
2	100	100	30	0
3	0	53	0	0
4	45	100	23	0
5	100	76	38	42
6	81	99	100	100
7	100	100	100	68
8	100	100	83	26