THE ROLE OF PLATELET ACTIVATING FACTOR IN ALLERGIC DISEASE.

A thesis submitted for the degree of

Doctor of Medicine

in the

University of London

by

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DEDICATION

To Catrin and Gwyn, in the hope that they will one day appreciate that M.D. does not simply mean "Mummy disappearing".
ACKNOWLEDGEMENTS

I wish to thank all those, too numerous to mention by name, who contributed to this thesis, but in particular must acknowledge the influence of my "top ten mentors". There is no doubt that it would not have occurred to me to pursue clinical research were it not for the stimulation and encouragement of Prof. Fabio Magrini. Clearly, none of the work carried out in these studies would have been possible had Prof. Peter Barnes not taken me under his wing, providing not only the appropriate academic environment and financial security, but also tirelessly setting up links with industry which made the early trials of new drugs possible. I wonder if P.A.F., known to King's College students as Page activating factor, would have activated my interest were it not for the enthusiasm with which my husband, Clive, embraced this mediator before I ever entered the field! He also takes credit for introducing me to the arena of experimental pharmacology, not only teaching me the essentials of experimental design but also providing the equipment to pursue my studies within his laboratories. For the latter, I am grateful not only to Clive, but to the entire Pharmacology Department at King's College, London who not only welcomed me to their midst, but provided excellent technical assistance at all times. In particular, I wish to thank Prof. Mel Schachter and Dr. Jack Botting whose interest in my work and suggestions for further experiments were invaluable. I must also acknowledge the Australian influence of Dr. Roy Goldie. Not only did he teach me all the practical tips I know during his brief sabbatical in London, but he also blessed me with his post-doctoral fellow, Dr. Dom Spina. Unfortunate enough to have to share a laboratory with me, Dom soon became a pillar of strength in the seemingly endless months when experiments didn't seem to go my way, and his willingness to share his knowledge of statistics and computers was greatly appreciated.

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Book Chapters

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Relationship between bronchoconstriction, bronchial hyperresponsiveness and microvascular leakage induced by inhaled PAF and antigen.

Presented at the American Thoracic Society, 1989

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<th>Description</th>
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>APRIL</td>
<td>antihypertensive polar renomedullary lipid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>A23187</td>
<td>Calcium ionophore</td>
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<tr>
<td>B.C.</td>
<td>Before Christ</td>
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<tr>
<td>BHR</td>
<td>bronchial hyperresponsiveness</td>
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<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BP</td>
<td>blood pressure</td>
</tr>
<tr>
<td>BU</td>
<td>biological units</td>
</tr>
<tr>
<td>B</td>
<td>Bursa (derived lymphocytes)</td>
</tr>
<tr>
<td>beta-TG</td>
<td>beta thromboglobulin</td>
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<tr>
<td>C</td>
<td>complement</td>
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<tr>
<td>CD</td>
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<tr>
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<td>CLC</td>
<td>Charcot Leyden Crystals</td>
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<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>copy ribonucleic acid</td>
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<td>Da</td>
<td>Daltons</td>
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<tr>
<td>DAG</td>
<td>diacylglycerol</td>
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<tr>
<td>DSGG</td>
<td>disodium cromoglycate</td>
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EAR  early airways response
EB   Evans blue
ECF-A Eosinophil chemotactic factor of anaphylaxis
ECF  eosinophil cationic protein
ECR  early cutaneous response
EDN  eosinophil derived neurotoxin
EDTA ethylenediamine tetra acetic acid
eos. eosinophils
EpDRF epithelium derived relaxant factor
EPO  eosinophil peroxidase

f femto
Fab  variable region of immunoglobulin
Fc   Constant region of immunoglobulin
FEV₁ Forced expiratory volume in 1 sec
FMLP Formyl methionyl leucine phenylalanine

g gram
G   gauge
GM-CSF granulocyte-colony stimulating factor
GMP-140 granule membrane protein 140
GPC  glyceryl phosphocholine
GSEM geometric standard error of the mean
GTP  guanyl triphosphate

H   histamine
HCl hydrochloride
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>HETE</td>
<td>hydroxy eicosatetraenoic acid</td>
</tr>
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<td>HSA</td>
<td>human serum albumin</td>
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<tr>
<td>HLA</td>
<td>human leucocyte antigen</td>
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<tr>
<td>HPETE</td>
<td>hydroperoxy eicosatetraenoic acid</td>
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<tr>
<td>hr.</td>
<td>hour(s)</td>
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<tr>
<td>HSA</td>
<td>human serum albumin</td>
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<td>H₂O₂</td>
<td>hydrogen peroxide</td>
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<td>Hz</td>
<td>Hertz</td>
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<td>ICAM-1</td>
<td>intercellular adhesion molecule 1</td>
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<td>IFN</td>
<td>interferon</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>interleukin</td>
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<td>IL-2R</td>
<td>interleukin 2 receptor</td>
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<tr>
<td>Ir</td>
<td>immune response</td>
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<tr>
<td>IU</td>
<td>international units</td>
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<tr>
<td>i.d.</td>
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<td>i.m.</td>
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<td>intraperitoneal</td>
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<td>intratracheal</td>
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<td>i.v.</td>
<td>intravenous</td>
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<tr>
<td>Ka</td>
<td>Affinity coefficient</td>
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<tr>
<td>kD</td>
<td>kiloDalton</td>
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<tr>
<td>kg</td>
<td>kilogram</td>
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IAC local antigen challenge
LAR late airways response
LCR late cutaneous response
LFA-1 lymphocyte function associated antigen 1
log logarithm
LPS lipopolysaccharide
LT Leukotriene
lyso-PAF lyso platelet activating factor
L-NAME L-nitroagrinine methylester
Mab monoclonal antibody
MBP major basic protein
MCP major cationic protein
min. minute(s)
mRNA messenger RNA
μ micro
M Molar
mg milligram
ml millilitre

NANC non-adrenergic non-cholinergic
NCF-A neutrophil chemotactic factor of anaphylaxis
n number of experiments
ng nanograms
nmol nanomol
NO nitric oxide
NSAID non steroidal anti inflammatory drug
OD  outside diameter

P   pico

p value  probability value

PAF  Platelet activating factor

PASL  platelet activation suppression lymphokine

PCIF  platelet cytotoxicity inducing factor

PDHRF  platelet derived histamine releasing factor

PEFR  peak expiratory flow rate

PF4  platelet factor 4

PG  Prostaglandin

PIP  pulmonary inflation pressure

PLC  phospholipase C

PMA  phorbol myristate acetate

PPE  plasma protein extravasation

PC40  Provocative concentration 40%, i.e. the concentration inducing a 40% change in lung function

PD40  provocative dose 40% i.e. the dose inducing a 40% change in lung function

PT100  provocative time 100% i.e. duration of exposure required to induce a 100% change in lung function.

%  percent

RL  resistance

RSV  respiratory syncytial virus
s.c.  subcutaneous
sec.  second(s)
s.e.m.  standard error of the mean
SGaw  specific conductance
Subst. P  substance P

T  thymus (derived lymphocytes)
TDI  toluene diisocyanate
Tds  three times a day
TH1  T helper type 1 lymphocytes
TH2  T helper type 2 lymphocytes
TNF  tumour necrosis factor
TX  thromboxane

URTI  upper respiratory tract infection

VC  vital capacity
VCAM-1  vascular cell adhesion molecule 1
VLA-40  very late antigen 40
VP30  Expiratory flow rate at 30% vital capacity
Vmax30  Expiratory flow rate at 30% residual volume from partial expiratory flow volume curves
vWF  von Willebrand factor

w/v  weight/volume
Asthma is a chronic inflammatory disease. In genetically predisposed individuals airborne allergens can trigger symptoms. The sensitivity of the lungs to allergens can be paralleled by skin sensitivity, and the similarity in clinical, pathological and pharmacological profiles of these two reactions has led to the hypothesis that similar mechanisms may underlie cutaneous and airway allergic reactions.

Numerous inflammatory mediators have been implicated in the pathogenesis of allergic disease including platelet activating factor (PAF) which mimics certain aspects of the allergic response in the skin and airways.

The aims of this thesis were to evaluate available antagonists of PAF in man, and to use these drugs to evaluate the potential role of PAF in allergic reactions in the skin and lungs in double-blind cross-over studies.

Intradermal PAF induced dose-related weal and flare reactions in normal subjects. Triazolam failed to inhibit these responses at clinically effective sedative doses. Intradermal allergen induced dose related acute and late responses. BN 52063, which had previously been shown to inhibit the skin responses to PAF, inhibited the late, but not the early response to antigen.

Inhaled PAF induced bronchoconstriction and neutropenia but not bronchial hyperresponsiveness (BHR) in normal subjects. BN52063 partially inhibited the effects of inhaled PAF. Changes in PAF challenge methodology failed to induce clinically significant changes
in BHR. A pilot study suggested that atopic status may be an important determinant of the response.

In view of these results a guinea pig model which closely mimicked accepted clinical allergen challenge procedures was developed. Using this technique, inhaled PAF failed to induce BHR, but inhaled allergen induced significant BHR, which was inhibited by WEB 2086. PAF induced oedema has been implicated in the genesis of BHR, but neither PAF nor allergen induced significant oedema at times of maximal bronchoconstriction or BHR.

These results suggest that PAF may play a role in allergen-induced cutaneous responses and airway hyperresponsiveness but not through inducing bronchial oedema.
SECTION 1

INTRODUCTION
CHAPTER 1

ASTHMA

1.1. Definition of asthma

Asthma has been recognised since ancient times. The Chinese describe a condition which was probably asthma in the Neu Ching from the third millennium B.C. The word 'asthma' has been used in the past to refer to almost any sort of difficulty in breathing, especially if it was paroxysmal or episodic. Although it is now generally agreed that the term should only be used to refer to a disorder of the respiratory system characterised by episodes of difficulty in breathing, usually with wheezing, agreement has not been reached on a formal definition of asthma. In 1959, a Ciba Foundation Guest Symposium suggested a functional definition:- "Asthma refers to the condition of subjects with widespread narrowing of the bronchial airways, which changes in severity over short periods of time either spontaneously or under treatment, and is not due to cardiovascular disease. The clinical characteristics are abnormal breathlessness, which may be paroxysmal or persistent, wheezing, and in most cases relief by bronchodilator drugs".

The American Thoracic Society in 1962 put a greater emphasis on bronchial hyperresponsiveness (BHR), suggesting that "Asthma is a disease characterized by an increased responsiveness of the trachea and bronchi to a variety of stimuli and manifested by widespread
narrowing of the airways that changes in severity either spontaneously or as a result of therapy”.

Various bronchial challenge tests have been developed to measure the sensitivity of the airways to inhaled irritants (Curry, 1947; Orehek & Gayrard, 1976) and have been standardized to a reasonable degree (Hargreave et al, 1985). These tests have been used to study the relationship between BHR and clinical asthma.

BHR is certainly present in virtually all subjects with current symptoms of asthma (Tiffeneau, 1955; Cockcroft et al, 1977a). Asymptomatic asthmatics may (Parker et al, 1965; Townley et al, 1975) or may not (Cockcroft et al, 1977a) have continued BHR. Evidence suggests that the degree of increase is related to the severity of symptoms (Makino, 1966), the number of previous hospital admissions (Townley et al, 1975) and the ease with which asthma is induced by allergens (Cockcroft et al, 1979a), exercise (Kiviloog, 1973), cold air (O'Byrne et al, 1982; Ramsdale et al, 1985) and emotion (Horton et al, 1978). Furthermore there is a correlation between BHR and the minimum treatment needed to control symptoms (Herxheimer, 1951; Cockcroft et al, 1977a). The degree of airway obstruction and the diurnal variation of peak flow rates (Ryan et al, 1982; Ramsdale et al, 1985) as well as the response to bronchodilator drugs (Ryan et al, 1982) are also related to the degree of BHR. Indeed, BHR correlates more closely with variable airflow obstruction than an increase in the diurnal variation of peak flow rates or a fall in FEV₁ after the natural stimulus of hyperventilation of cold dry air (Ramsdale et al, 1985) or exercise (Anderton et al, 1979). A correlation also exists with IgE levels and positive allergen prick
Despite the suggestion that BHR should be the ultimate standard for the diagnosis of asthma (Gregg, 1983) the definition of asthma put forward in 1962 has not been universally accepted. It was rejected by a Ciba Foundation Study Group in 1971 on the grounds that it did not represent what they would accept as a 'disease', and more recently it has been proposed that the term asthma should be abandoned in favour of a concise description of the underlying pathology "chronic desquamating eosinophilic bronchitis" (Reed, 1986).
1.2. Epidemiology of asthma

The disagreement over the definition of asthma has hampered epidemiological studies of asthma, and the incorporation into the 1962 definition of physiological testing to determine the degree of bronchial responsiveness to non-specific agents has been difficult to assess in population studies.

Dodge and Burrows (1980) reported the findings of a longitudinal study of the prevalence, incidence and distribution of asthma in a general population sample of 3860 adults and children in Tuscon, Arizona. Defining asthma as a "yes" response to "Have you ever had asthma?" and "Have you ever seen a doctor about your asthma?", and considering the asthma to be active if there had been at least 1 attack or a need for medication or treatment during the last year, they found a point prevalence (the prevalence of active disease) for asthma of 6.6% with a peak in childhood, and a high prevalence in older subjects, in most of whom "chronic bronchitis and/or emphysema" had been concomitantly diagnosed.

New asthma developed in 1.4% of the subjects who were followed over a period of approximately 4 years. New attacks of shortness of breath with wheeze occurred in 10.3% of the subjects at risk over the same time period. The incidence of asthma was greater in young children, was least in late adolescence, and increased again in early adult life. The incidence was 1.5 times greater in young boys than in young girls but was much greater in women over 40 years of age, which the authors suggest may reflect the diagnostic biases of physicians.

In subjects younger than 40 years of age, onset of the disease was
strongly associated with previously demonstrated allergy skin test reactivity and eosinophilia. New disease in this age group occurred de novo, primarily within the first few years of life and during early adult life. Subjects in whom asthma developed after 40 years of age usually had prior symptoms of chronic bronchial irritation and often had obvious spirometric abnormalities. The disease in these subjects was not associated with positive allergy skin test reactions.

The distribution of allergy skin test reactivity and serum IgE levels in the Tuscon epidemiological study population have been documented by Cline & Burrows (1989), and the changes in IgE levels in infants enrolled at birth in the Children's Respiratory Study in Tuscon were reported by Halonen et al (1991). Positive allergen skin tests were found in 36.2% of 3136 subjects aged 3 or more at the time of enrolment. There was no relationship between positive tests and gender but both the frequency and the size of the positive reactions was strongly related to age, with increasing reactivity during childhood, a peak between 20-45 and a fall in reactivity in later adult life.

In subjects re-tested 8 years later a high risk of conversion from negative to positive responses was noted, but no reversion from positive to negative was recorded in subjects aged less than 15 years on entry. In contrast, there were high reversion rates and relatively low conversion rates in subjects aged 55 or more.

Serum IgE levels showed an almost perfect Gaussian distribution when expressed in logarithmic form, except in the newborn. The median IgE level at birth was below 0.1 international unit (IU). About one third
of newborn infants had no detectable IgE in their cord blood and less than 5% had more than 1 IU. By nine months of age the geometric mean IgE level had increased to 3.8 IU. Serum IgE levels peaked between 8-12 years, after which geometric mean levels decline rapidly over the next few decades and then more gradually throughout adult life. In subjects studied again after 8 years, a substantial decline in IgE levels in individuals first studied when they were less than 15 years was observed, a lesser but still significant decline in subjects aged 15-34 but no significant change over the 8 years in subjects over the age of 35 at enrolment. Women had slightly lower IgE levels than men at all ages. IgE levels at enrolment correlated with those at 8 years follow up and the presence of any measurable IgE in cord blood at birth predicts a relatively high IgE at 9 months.

A very close overall correlation between serum IgE levels and skin test reactivity to allergens was observed. For instance only 8.1% of subjects with a serum IgE level of less than 4 IU had positive skin reactions whereas 75.2% of subjects with IgE level of 250 IU or more had positive skin reactions. Furthermore, the size of the reactions correlated with IgE levels. However, the low rate of atopy in children cannot be explained by their IgE levels, which tend to peak during childhood, and the fall in IgE in the elderly only partially accounts for the reduction in reactivity. A further study of the value of cord IgE levels in the prediction of atopic disease concluded that neonatal IgE determinations, used in conjunction with the family history was useful in identifying high risk subjects whilst IgE levels alone had poor sensitivity (Croner & Kjellman, 1991).
More recently, data regarding the prevalence of BHR in the population have become available. Whilst BHR provides an objective measure of airway responsiveness, a number of epidemiological investigations have suggested that bronchial responsiveness follows a unimodal log normal distribution (Cockcroft and Bersheid, 1983; Weiss et al, 1984; Salome et al, 1987). There is therefore no clear cut-off point which can be used to distinguish normal responsiveness from hyperresponsiveness, so that the difficulties arising from different definitions of asthma used in epidemiological studies based on questionnaires cannot be overcome by measuring BHR unless a particular level of BHR is consistently regarded as indicating the presence of asthma. Attempts to establish such a cut-off point based on diagnosed asthma or symptoms of asthma have repeatedly shown that whilst there is an association between asthma and its severity and BHR, this also gives rise to considerable false positive and false negative results (Cockcroft and Bersheid, 1983; Sears et al, 1986; Salome et al, 1987).

It is possible that the false negative results i.e. the failure to detect BHR in those with a history of asthma may result from treatment which has reduced the severity of BHR to a range not detectable by the histamine challenge tests used. It is also possible, that they represent children who have outgrown their asthma. Preliminary studies of children with asymptomatic BHR (false positives) have shown that the majority have a transitory abnormality (Salome et al, 1986) and Sears et al (1984) found that of a group of children with asymptomatic BHR 50% were no longer hyperresponsive, 25% were less responsive and less than 20% had developed asthma after...
2 years follow up.

Nonetheless, there does appear to be a genetic influence on the presence of BHR (Longo et al, 1987; Clifford et al, 1987) as well as some interaction with atopy in its causation in both cross sectional (Clifford et al, 1987) and longitudinal (Van Asperen et al, 1990) studies in children.

BHR is not specific for asthma, and also occurs in chronic bronchitis (Bahous et al, 1984), cystic fibrosis (Mellis & Levison, 1978), farmer’s lung disease (Moenkaere et al, 1981), cigarette smokers (Gerrard et al, 1980) and after upper respiratory tract infections (Empey et al, 1976).

Despite these shortcomings, Burney et al (1987) have recently described the distribution of BHR to histamine in a randomly selected sample of adults aged 18-64 living in two districts of England. BHR to < 8 umol histamine was present in 14%. Positive skin tests to common allergens were the most important associated feature in the young, whilst smoking became a more important determinant of BHR in older subjects (>45 years). No independent effect on responsiveness of sex, social class or area of residence was detected and no significant effect from recent upper respiratory tract infections was observed. BHR was least prevalent in the 35-44 year age group.

In conclusion, epidemiological studies have shown that there is a significant, but not complete association, between BHR and both diagnosed asthma and respiratory symptoms. The prevalence of BHR in
Community studies is generally higher than the prevalence of
diagnosed asthma but lower than the prevalence of wheeze or
respiratory symptoms in general.
1.3. **Aetiology of asthma**

The aetiology of asthma remains unknown. Genetic factors are undoubtedly important, but numerous environmental factors have also been implicated.

1.3.a. Genetic factors

A genetic predisposition to asthma undoubtedly exists. Family studies have shown an increased prevalence of asthma in relatives and offspring of both atopic, and to a lesser extent non-atopic, asthmatics (Charpin & Arnaud, 1971; Sibbald & Turner-Warwick, 1979; Sibbald et al, 1980). Although this familial aggregation may arise partly from shared family environments, twin studies have shown that shared genetic factors must also play an important role. In a large study of 7000 twin pairs undertaken in Sweden, the concordance of asthma in monozygotic twins was 19% (Edfors-Iubs, 1971).

Whilst asthma is frequently associated with atopy (Coca & Cooke, 1923), the converse is not necessarily true (Sibbald et al, 1980). It has therefore been suggested that atopy and asthma are inherited independently and that atopy may increase the susceptibility to asthma of patients who inherit a predisposition to both asthma and atopy (Sibbald & Turner-Warwick, 1979).

Detailed research into the familial aggregation and probable genetic basis of atopic allergy dates primarily from the study of Cooke and Van der Veer in 1916 who concluded that allergy was
inherited as an autosomal dominant trait. Familial aggregation of atopy is now well established, and Cookson & Hopkins (1988) confirmed, using a definition of atopy based on specific IgE responses to common antigens or on an abnormally raised total serum IgE, that atopy was inherited as an autosomal dominant character. Furthermore, by molecular genetic linkage analysis it has been possible to demonstrate a link between the inheritance of atopy and a DNA marker on chromosome 11q (Cookson et al, 1989). Chromosome 11q is known to carry a number of genes for cell surface antigens some of which occur on T lymphocytes. Since B lymphocyte IgE production is controlled by interleukin 4, a cytokine produced by T helper lymphocytes (Snapper et al, 1988) and in view of the observation that the tendency to IgE hyperresponsiveness and to asthma can be transmitted in bone marrow transplants (Agosti et al, 1988), Higgenbottam and Varma (1989) have postulated that the primary abnormality in asthma may be an autosomally dominantly inherited abnormality of a T cell subset which can lead to IgE hyperresponsiveness.

However the transmission of atopy in the families studied by Cookson, and by others, is associated with variable patterns of response to antigen and disease expression so that other factors - either genetic or environmental - must influence the clinical expression of atopy. Histocompatibility variability has been implicated. Significant association between specific immune responses to most inhaled antigens and the two most common histocompatibility haplotypes (A1B8Dw3 and A3B7Dw2) have been reported, the association being most striking for low molecular weight antigens (Marsh et al,
Furthermore, there is evidence of an interaction between HLA-linked genes and the non-HLA linked IgE regulating gene (Marsh et al, 1979; Hsu et al, 1981).

Since the early observations of Ishizaka (1971) and Johansson et al (1972) that "high" serum levels of total IgE show a marked correlation with allergy, studies of total IgE have been used to provide an insight into the genetic factors underlying allergic disease. The predictive value of serum total IgE in umbilical cord blood in subjects with a family history of atopy in determining the risk of atopic disease suggests that IgE responsiveness is not determined by allergen exposure or other insults to the respiratory tree in early postnatal life (Croner & Kjellman, 1991). Furthermore, IgE levels show a greater concordance in monozygotic than in dizygotic twins (Bazaral et al, 1974) and recessive inheritance of elevated levels of IgE has been postulated (Marsh et al, 1974; Gerrard et al, 1978). It has not been possible to prove the existence of a major IgE regulating locus (Meyers et al, 1982).

The evidence that BHR is inherited is indirect. Niejens et al (1983) showed that BHR may persist in asthmatic patients who are symptom free for years and suggested that a genetically determined predisposition to develop BHR exists that is induced or enhanced by environmental factors. The observation of Peat et al (1987a) that parental asthma is a risk factor in the development of BHR in childhood would support this concept. Evidence from twin studies also points to a genetic influence. Hopp et al (1984) found a greater
concordance in monozygotic twins than dizygotic twins in respect to BHR, total serum IgE levels and total skin test scores. Several studies have reported a positive association between atopy and BHR (Cockcroft et al, 1984; Peat et al, 1987a) whereas others have failed to confirm a relationship (Bryant and Burns, 1976, Woolcock et al, 1987).

1.3.b. Environmental factors

Despite the convincing evidence of a genetic component in asthma, and the strong genetic predisposition to atopy and BHR, it is clear that clinical expression of asthma is significantly influenced by environmental factors. In addition to allergen exposure, infections, sodium intake, and exposure to sensitising chemicals or drugs have been implicated.

Allergen exposure

In subjects with a genetic predisposition, allergen exposure e.g. the seasonal exposure to grass pollen, induces increased symptoms and BHR (Altounyan 1970; Weiss et al, 1984; Platts-Mills & de Weck, 1989). Conversely, removal from environmental allergens improves symptoms and reduces BHR (Platts Mills et al, 1982; Weiss et al, 1984; Warner & Boner, 1988). On a world wide basis, mites (Dermatophagoides sp.) are among the most ubiquitous allergens and
there is now strong epidemiologic evidence that sensitization and exposure to mite allergens is a highly significant risk factor for the development of asthma (Sporik et al, 1990).

The association between atopy and asthma was first suggested in 1923 by Coca & Cooke, and has been confirmed by subsequent studies (McNichol & Williams, 1973; Kreukniet & Pijper, 1973).

A relationship between atopy and BHR has also been described by a number of groups (Townley et al, 1975; Bryant & Burns, 1976; Cockcroft et al, 1979a; Weiss et al, 1984; Cockcroft et al, 1984; Britton et al, 1986; Witt et al, 1986). More recently, Peat et al (1987a) were able to show a quantitative relationship between atopic status, as assessed by a positive skin reaction to one or more prick tests to antigens, and BHR. The risk of BHR was further increased in children atopic to both pollens and house dust mites, and in children with a high index of atopy (derived from the number and size of the skin reactions to four allergen groups). In addition to the degree of atopy and the quantity of aeroallergens present in the environment (Britton et al, 1986) the type of allergen to which subjects are exposed (Peat et al, 1987b) are important in determining BHR. Thus, in two areas of Australia where the prevalence of atopy in the population was similar, the prevalence of BHR was higher in an area where grass pollen predominates than in an area where house dust mites are the predominant allergen. Taken together, these data suggest that atopy and BHR are governed by common mechanisms but that there is no causal relationship between atopy and BHR.
Respiratory infections

It has been suggested that respiratory infections, particularly croup and bronchiolitis may predispose to atopy. However, population based, prospective studies have not confirmed the early findings of hospital-based retrospective cohort studies, and it is now thought that bronchiolitis is more severe in atopic children but that a causal relationship between atopy and the acute respiratory illness does not exist (Frick et al, 1979; Sims et al, 1981; Weiss et al, 1985).

In contrast, an association between upper respiratory tract infections and the development of BHR in children has been demonstrated in both hospital and population based studies (Gurwitz et al, 1980; Gurwitz et al, 1981; Weiss et al, 1985; Peat et al, 1987b).

The induction of BHR by an upper respiratory tract infection (URTI) was noted in atopic subjects by Parker et al (1965) who also noted BHR in 4 otherwise normal subjects with URTIs. Empey et al (1976) confirmed the association between URTIs and BHR in normal subjects, and were able to show that the heightened responsiveness resolves within 7 weeks. Similar results were reported by Little et al (1978) who studied college students with known influenza A infections, and Hall et al (1978) who found a link between adult respiratory syncytial virus infection and BHR. Aquilina et al (1980) extended these observations to show that URTIs induced increased sensitivity to cold air and exercise as well as histamine for at least 3 weeks after viral RTIs. However, Jenkins & Breslin (1984) in

In addition to their potential role in the development of BHR and asthma, there is convincing evidence that viral infections precipitate attacks of asthma in known asthmatics, particularly in children (McIntosh et al, 1973), but also in adults (Beasley et al, 1988). In general, data to implicate bacterial infections other than sinusitis (Slavin et al, 1980) as causes of wheezing are scant.

The mechanism underlying viral induced asthma and BHR remain obscure. However, viral induced damage of the epithelium resulting in increased sensitivity of rapidly adapting sensory fibres of the vagus nerve to stimuli such as histamine have been implicated (Empey et al, 1976). Furthermore, Welliver et al (1980, 1981) were able to demonstrate the production of virus-specific IgE in nasal secretions from patients with respiratory syncytial virus-associated asthma or bronchiolitis, but not from those with pneumonia or upper respiratory tract infections due to respiratory syncytial virus.

Sodium intake

A strong association has been noted between regional morbidity
from asthma and purchases of table salt per person in England and Wales, for adult men and children of both sexes but not for adult women (Burney, 1987). Furthermore the airway response to inhaled histamine is correlated with the 24 hour urinary excretion of sodium (Burney et al, 1986) and a study designed to increase sodium excretion resulted in an increase in the bronchial response to histamine in men but not women (Burney et al, 1989) suggesting that there is a direct causal association between sodium intake and BHR.

Sensitizing chemicals

Asthma following exposure to a variety of organic and inorganic substances is being increasingly recognised. Pepys & Hutchcroft (1975) described reactions to chemical dusts, such as the complex salts of platinum, antimicrobial drugs, piperazine dihydrochloride, wood dusts, fumes from soldering fluxes and from natural and synthetic resins and toluene diisocyanate (TDI). Butcher (1979) concluded that asthma developed in 5% of people occupationally exposed to TDI, and subsequently demonstrated that TDI exposure induces BHR which gradually resolves on avoidance of exposure (Butcher et al, 1982). Vallieres et al (1977) reported BHR induced by dimethylethanomaline in paint.

Cockcroft et al (1979b) reported a case of a patient sensitive to red cedar who developed a late asthmatic response and transient BHR following exposure to red cedar in the laboratory. This observation was confirmed by Lam et al (1979) who also observed that patients with symptomatic occupational asthma (to grain dust, TDI, California...
redwood or western red cedar) had marked increases in BHR comparable to non-occupational asthmatics following natural exposure to the sensitizing chemicals. Furthermore, the degree of BHR decreased after removal from exposure and increased following re-exposure to the offending agents.

Air pollution

There is some controversy regarding the effect of air pollutants such as ozone and nitrogen dioxide on BHR. Thus, whilst Holtzman et al (1979) failed to demonstrate a difference in the inducibility or time course of bronchial hyperresponsiveness between non-atopic and atopic adult subjects following exposure to ozone, ozone has been reported to increase BHR in normal (Golden et al, 1978) and asthmatic subjects (Kreit et al, 1989). Furthermore, Molfino et al (1991) reported that low concentrations of ozone, similar to those commonly occurring in urban areas, can increase the bronchial responsiveness to allergen in atopic asthmatic subjects.

Hazucha et al (1983) found no change in BHR after exposure of normal and asthmatic adults to nitrogen dioxide, whilst a number of investigators have demonstrated an increase in BHR in asthmatic subjects after exposure to nitrogen dioxide (Orehek et al, 1976; Kleinman et al, 1983; Bylin et al, 1988).

There is now considerable evidence that passive smoking, particularly maternal smoking, increases the risk of development of allergy and asthma in infants and young children which may be in part
explained by the induction of IgE antibody production in utero (Kjellman, 1981; Holt & Turner, 1984; Magnusson, 1986; Kershaw, 1987).

Drugs

Aspirin is the most common drug implicated in the exacerbation of asthma, precipitating symptoms in some 10% of adult asthmatics (Samster & Beers, 1968). The mechanism of this reaction is unknown. Three major hypotheses have been proposed. Firstly, it has been suggested that inhibition of pulmonary cyclo-oxygenase results in shunting of arachidonic acid to the 5-lipoxygenase pathway, leading to overproduction of bronchoconstrictor leukotrienes (Undem et al, 1987).

Secondly, Szczeklik put forward the hypothesis that aspirin induced asthma results from chronic viral infection. He suggested that in response to a virus, specific cytotoxic lymphocytes are produced whose activity is suppressed by prostaglandin E2 (PGE2) produced by pulmonary alveolar macrophages. Cyclo-oxygenase inhibitors block PGE2 production and allow cytotoxic lymphocytes to attack and kill their target cells - the virus-infected cells of the respiratory tract. During this reaction toxic oxygen intermediates, lysosomal enzymes and mediators are released which precipitate attacks of asthma (Szczeklik, 1988).

Thirdly, Ameisen et al (1985) have produced strong evidence that a specific abnormality of platelet reactivity occurs in aspirin induced asthma. This involves aspirin/non-steroidal anti-inflammatory drug
induced release of oxygen radicals as well as the hydrogen peroxide
(H$_2$O$_2$)-dependent generation of other mediators.

At a biochemical level, low whole blood peroxidase activity has been
observed in patients with aspirin intolerance and more recently
lowered levels of intracellular glutathione peroxidase activity have
been demonstrated (Malmgren et al, 1986). The mechanism by which such
an enzyme defect induces asthma remains to be elucidated. Since
aspirin affects both enzymatic and non-enzymatic free radical
processes, it is possible that aspirin either has a direct effect on
eicosanoid metabolism (via downregulation of lipoxygenase and
cyclo-oxygenase or via metabolism of hydroperoxide mediators or
intermediates) or affects the removal of platelet or leucocyte
produced H$_2$O$_2$ which could be involved in the inflammatory process
directly or through the generation of hydroxyl groups (Pearson et al,

The beta-adrenoceptor antagonist, propanolol, has been reported
to induce BHR when administered intravenously (Zaid & Beall, 1966) or
by inhalation (Townley et al, 1976). It is also of interest that
beta-adrenoceptor antagonists result in an elevation of the blood
eosinophil count and eosinophil cationic protein (ECP) levels
(Koch-Weser, 1968; Dahl & Venge 1978).

A variety of other factors may trigger clinical asthma, such as
exercise, emotion, cold air and hyperventilation. However, these are
associated with bronchoconstriction but not BHR, and are therefore
thought to indicate the severity of the disease and reflect the
degree of BHR, rather than represent causative factors in the genesis
of clinical disease (Cockcroft, 1988).

In conclusion, it is clear that there is a complex interaction
between inherited and acquired factors in the development of asthma.
Levels of IgE, atopy and BHR are partly genetically determined.
Exposure to allergens, infections and chemicals can induce the
expression of asthma. In turn, children who inherit a predisposition
to BHR have increased susceptibility to the environmental factors
such as infection and allergens which are associated with BHR, and a
combination of atopy and asthma susceptibility can confound each
other increasing the risk of disease expression.
1.4 Pathology of asthma

Chronic inflammation is a well established feature of asthma (Dunnill, 1960; Hogg, 1985), and has been recognised since the first autopsy study of asthmatic lungs (Huber & Köessler, 1922). However, knowledge of the pathology of living asthmatics was confined to the examination of the sputum of asthmatics since pathological specimens could only be obtained from subjects who died of their asthma, or rarely from asthmatic patients who died of unrelated causes. Thus, it was not until the advent of bronchoscopic techniques enabling sampling of bronchoalveolar lavage fluid, and providing biopsy specimens from living asthmatics, that the changes in mild asthmatic subjects were appreciated.

1.4.a. Sputum changes

One of the first pathological abnormalities of asthma was described by Curschmann in 1883. He found respiratory epithelial cells in the sputum, some of which are typical ciliated columnar cells, but abnormal squames may also be found (Cohen & Prentice, 1959) which occasionally show mitotic activity. Creola bodies, compact clusters of columnar epithelial cells, were described by Naylor in 1962. Curschmann’s spirals, which are long twisting casts of small airways may be seen in gross specimens of sputum and have been described as non-mucoid elements organising into strips on paraffin sections (Sanerkin & Evans, 1965).

In addition to the epithelial element, asthmatic sputa contain
Eosinophils, metachromatic cells with the features of mast cells and non-cellular elements composed of mucus and exuded plasma proteins. Eosinophils may account for 46-92% of all cells in the sputum of asthmatics undergoing an exacerbation of their disease, compared to 0.5% in chronic bronchitic patients (Gibson et al, 1989). The degree of sputum eosinophilia correlates with symptoms (Brown, 1958). The presence of eosinophil granule products, particularly major basic protein (MBP) (Frigas et al, 1981) have been reported in asthmatic sputum. In addition, Charcot Leyden crystals (CLC) have recently been characterised and have been shown to be a protein constituent of eosinophil cell membranes (Ackerman et al, 1980). Interestingly, the CLC protein displays lysophospholipase activity. Thus the protein may be active in neutralization of toxic lysophospholipids which are formed as a consequence of phospholipase A₂ activity in stimulated inflammatory cells. However, the CLC is not specific to the eosinophil, and may also be released by basophils (Ackerman et al, 1982), nor to asthma, and has also been reported in the sputum of patients with bronchopulmonary infections (Dor et al, 1984).

Metachromatic cells make up 1.5% of the cell population, compared to 0.14% in chronic bronchitis patients (Gibson et al, 1989).

1.4.b. Necropsy findings

1.4.b.i. Macroscopic appearance

Asthmatic lungs fail to collapse when the pleural cavities are opened and appear over-inflated, often covering the pericardial sac
anteriorly. Focal areas of collapse may also be seen. The cut surface of the lung reveals plugs of exudate in the air passages, occluding small as well as large bronchi (Dunnill, 1978). The lungs of asthmatics who did not die of their asthma show similar but less marked exudation and hyper-inflation (Tschopp & Turner-Warwick, 1984).

1.4.b.ii Microscopic appearance

The airway lumen contains exudate which has cellular and non-cellular components. Desquamation of the respiratory columnar epithelial cells which may appear normal, degenerate or metaplastic, and may be arranged either in strips or singly has been described (Dunnill, 1960; James et al, 1989). However, Reid et al (1989) found that neither epithelial desquamation nor goblet cell metaplasia were found more often in 39 autopsied asthmatics when compared with control subjects. Eosinophils have also been observed (Houston et al, 1953) and more recently, major basic protein has also been detected in bronchial exudates (Filley et al, 1982). The non-cellular component consists of mucus from the glands in the bronchial wall and serous transudate resulting from increased permeability of dilated post capillary venules.

The bronchial mucous membrane is abnormal. There is marked oedema with separation of the mucosal cells and in many areas they are completely detached leaving behind a layer of basal or reserve cells. Much of the epithelium is of the simple stratified non-ciliated variety and exhibits fairly frequent mitoses. Marked folding of the
Epithelium has been noted in some airways with a prominent circular layer of muscle (James et al, 1989). There is an increased incidence of goblet cells (Messer et al, 1960; James et al, 1989). Enlargement of the mucous glands, which is largely due to hypertrophy, has been observed in asthma, in contrast to chronic bronchitis where there is considerable hyperplasia.

Eosinophils are often seen between mucosal cells. Mast cells are found in both normal and asthmatic subjects (Salvato, 1968; Lamb & Lumsden, 1982).

A very typical feature of the asthmatic bronchus is pronounced thickening of the basement membrane (Unger, 1945; Bullen, 1952; Messer et al, 1960; James et al, 1989). Ultrastructurally the basement membrane consists of oedematous collagen with the fibrils exhibiting a plexiform arrangement (Cutz et al, 1978). Immunofluorescence studies have failed to distinguish asthmatic tissues from normal tissues on the basis of IgE bearing cells, although IgG, IgM or IgA was found in the thickened basement membrane of 11 of 19 asthmatics (Callerame et al, 1971). Similar studies have demonstrated eosinophil granule MBP on damaged epithelial surfaces and in necrotic tissue below the basement membrane (Filley et al, 1982).

There is also thickening of the airway wall. In 1922, Huber & Koessler showed that asthmatics had increased airway wall thickness when airways were matched using the external diameter. Dunnill (1969) reported an increase in smooth muscle in the major bronchi of
asthmatic subjects, and Heard & Hossain (1973) showed that this was due to hyperplasia rather than to hypertrophy of the muscle. James et al (1989) demonstrated an increase in smooth muscle volume as well as an increase in the volume of the non-muscular wall components.

The submucosa is characterised by capillary dilatation and swelling of the endothelial cells, oedema and cellular infiltration, mainly of eosinophils and lymphocytes (Bullen, 1952; Dunnill, 1960; James et al, 1989). Mast cells may be detected, particularly adjacent to small blood vessels and bronchial smooth muscle fibres.

The lung parenchyma shows areas of collapse, and in a few cases there is evidence of eosinophilic pneumonitis. Small quantities of exudate appear in alveolar spaces and are surrounded by a cellular reaction characterised by eosinophils and histiocytes (Scadding, 1971). Peribronchiolar fibrosis has been observed very occasionally in long standing asthma (Sobonya, 1984) but destructive emphysema is not observed.

Slater et al (1985) reported large numbers of pulmonary megakaryocytes in the lung parenchyma in 3 subjects who died from status asthmaticus. Trowbridge et al (1982) and Martin et al (1983) have produced evidence that all platelets are produced by the physical fragmentation of circulating megakaryocytes in the pulmonary circulation. Slater et al (1985) therefore speculated that these changes may result from increased thrombopoiesis in status asthmaticus following localised pulmonary activation and consumption of platelets.
I.4.c. Biopsy findings

The advent of bronchoscopy has led to a number of investigations of the nature and extent of inflammation of the airways in living asthmatics. Scant but viscous mucus was observed in the lumen by Salvato (1976). Cutz et al (1978) described the ultrastructural features of the airways of two asthmatic children undergoing open lung biopsy during clinical remission and compared them with lung tissue from two children who had died from status asthmaticus. Mucus plugging was comparable in the two groups.

In contrast to Glynn & Michaels (1960) who reported that epithelial cell loss was not a consistent feature of asthma, Cutz et al (1978) reported epithelial denudation in all subjects, with more extensive changes in fatal asthma. Laitinen et al (1985) found evidence of epithelial cell destruction at all levels in the bronchial tree even in patients with asymptomatic asthma. Ciliated cells were most severely affected and mast cells and intraepithelial nerves were exposed. Regenerative changes were seen and varying stages of ciliogenesis and non-ciliated metaplastic surface epithelium was noted. Lundgren et al (1988) confirmed these findings. Biopsies taken at bronchoscopy from 6 subjects with severe intrinsic asthma showed reduced coverage by cilia, with large areas of non-ciliated cells in 3 subjects and areas where the normal ciliated epithelium was mixed with areas showing both ciliated and non-ciliated cells in the remainder. In all patients small areas with abnormal, usually short, cilia were found. In contrast, Lozewicz et
al (1988) failed to detect a difference in the epithelium with regard to loss or swelling of epithelial cells or widening of intercellular spaces by light microscopy or with regard to loss of cilia, ultrastructure of cilia or vacuolization in biopsies taken from mild asthmatics.

Lundgren et al (1988) failed to demonstrate an increase in the thickness of the epithelium or in the basement membrane compared to the normal controls, although basement membrane thickening was observed by Salvato (1976) and noted by Cutz et al (1978). A detailed study of biopsy material obtained from 8 asthmatic subjects by fibreoptic bronchoscopy was recently reported by Roche et al (1989). The nature and extent of subepithelial thickening was examined by electron microscopy and the nature of the contributing proteins determined by immunohistochemistry. The authors noted extensive collagen deposition beneath the bronchial epithelium. Basement membrane components (collagen IV and laminin) were absent, rather there was an abundance of interstitial structural proteins, collagens III and V, and fibronectin. This led the authors to conclude that the thickening is attributable to fibroblast activation rather than epithelial damage, particularly since no correlation was observed between the subepithelial fibrosis and disease duration, severity or epithelial damage.

Cellular infiltration with eosinophils was a feature of asymptomatic asthmatics (Cutz et al, 1978), mild asthmatics requiring
only intermittent beta-agonist treatment (Beasley et al, 1989) and more severe asthmatics (Lundgren et al, 1988). Furthermore, the eosinophils exhibited morphologic evidence of activation (Beasley et al, 1989) and a significant increase in the number of ‘activated’ eosinophils was demonstrated using the monoclonal antibody (Mab) EG2+ (Kay, 1991). Mast cell numbers are also increased in the lamina propria of mild asthmatics (Lozewicz et al, 1988; Beasley et al, 1989).

The infiltration of lymphocytes has been noted by several investigators (Gerber et al, 1985; Lundgren et al, 1988; Bousquet et al, 1990; Poulter et al, 1990). Jeffery et al (1989) reported an increased number of irregularly shaped lymphocytes in biopsies from asthmatics. An increase of T lymphocytes (CD3+) as well as the subsets CD4+ and CD8+ has been shown in biopsies from allergic asthmatics, emphasizing the importance of cell activation (Azzawi et al, 1992). IL-2R+ cells were also significantly increased in asthmatic subjects (Kay, 1991). Chronic T-cell mediated immune responses in the bronchial tissue of asymptomatic asthmatics has recently been reported (Poulter et al, 1990).

In situ hybridization techniques using an IL-5 cRNA probe have recently been employed to investigate the expression of IL-5 mRNA and the pattern of distribution of IL-5 producing cells in bronchial tissue biopsied from asthmatic and normal individuals. Specific hybridization signals for IL-5 mRNA were not demonstrated in control subjects, but were demonstrated within the bronchial mucosa in 6 out of 10 asthmatic subjects who had clinically more severe disease. Furthermore there was a correlation between IL-5 mRNA expression and
the number of activated (CD 25+) lymphocytes and eosinophils (BG2+ and total) (Hamid et al, 1991).

Goblet cell hyperplasia (Cutz et al, 1978) and increased collagen deposition (Cutz et al, 1978; Beasley et al, 1989) has also been reported in living asthmatics, but smooth muscle hypertrophy was not a consistent finding in the study of Glynn & Michaels (1960).

Endothelial cell abnormalities have also been noted. Transmission electron microscopy of biopsied specimens from asthmatics showed that the postcapillary venules have gaps up to 1 um between endothelial cells, which are not evident in non-asthmatics (Laitinen et al, 1985). Eosinophil, monocyte and platelet adherence to venular endothelial cells was observed by Beasley et al (1989) and platelet aggregates were noted in the vessels within the bronchial mucosa.

Taken together, these studies provide evidence that the changes described in autopsy studies prevail in subjects with asthma even if this is clinically mild, stressing the presence of inflammation in the airways of asthmatic subjects.

1.4.d. Bronchoalveolar lavage findings

Bronchoalveolar lavage (BAL) of volunteers with mild asthma has provided supportive evidence of epithelial damage and eosinophil infiltration.
Beasley et al. (1989) noted increased numbers of epithelial cells in BAL fluid, which correlated with BHR. The percentage epithelial cell count correlated with BHR in the study of Wardlaw et al. (1988). In contrast, Kirby et al. (1987) found no increase in epithelial cell numbers in stable asthmatics with no symptoms of cough, or sputum.

Increased numbers of eosinophils have been found in BAL by a number of investigators (Godard et al., 1981; Tomioka et al., 1984; Agius et al., 1985; Flint et al., 1985; Casale et al., 1987; Kirby et al., 1987; Wardlaw et al., 1988; Kelly et al., 1989; Beasley et al., 1989; Adelroth et al., 1990). Although Casale et al. (1987) and Kelly et al. (1989) failed to find a correlation between BHR and eosinophil count, when expressed as a percentage of the total cell count, a correlation with eosinophil numbers was found by Wardlaw et al. (1988). Kirby et al. (1987) measured the levels of the eosinophil granule product, MBP, in BAL fluid and peripheral blood. No difference in the levels of MBP could be demonstrated between asthmatics and normal subjects. In contrast, Wardlaw et al. (1988) found that levels of MBP provided the best correlation with BHR. Furthermore, in non-asthmatics who did have eosinophils in their BAL, Wardlaw et al. (1988) were unable to demonstrate raised levels of the eosinophil granule derived protein, MBP, suggesting that in these subjects the eosinophils, although present, were not degranulating to the same extent as in the asthmatics. Moreover, only one non-asthmatic subject had levels of MBP in the range found in the symptomatic asthmatics, whereas 5 control subjects had an eosinophil count in the asthmatic range. It is possible that the lack of
symptoms in the patients studied by Kirby et al (1987) accounts for the different findings, although the authors point out that since their subjects had a wide range of BHR it is unlikely that MBP is primarily involved in the pathogenesis of BHR and that shedding of airway epithelium is not involved in persistent BHR.

A recent study of the density of the eosinophils found in BAL fluid has shown that 60% are hypodense and that the BAL fluid eosinophils are less dense than the peripheral blood eosinophils. Furthermore the eosinophils show partial or complete lucency of the granule matrix suggesting that the cells are degranulated (Fukuda & Gleich, 1989).

Increased levels of mast cells in the BAL fluid obtained from asthmatics have been reported by a number of investigators (Tomioka et al, 1984; Flint et al, 1985; Casale et al, 1987; Wardlaw et al, 1988; Laitinen & Laitinen, 1988; Lozewicz et al, 1988; Adelroth et al, 1990). A correlation has been noted between cell numbers (Tomioka et al, 1984; Kirby et al, 1987), total cell histamine (Flint et al, 1985) and BAL fluid histamine levels (Casale et al, 1987). Furthermore spontaneous histamine release from BAL mast cells was observed, and when BAL mast cells from asthmatics were incubated with anti-human IgE in vitro they released a significantly increased proportion of total cellular histamine than cells from control subjects (Flint et al, 1985).

Lymphocyte counts have been variable. Increased numbers of lymphocytes in BAL fluid obtained from asthmatics (Godard et al,
1981) have been reported to correlate with BHR (Kelly et al, 1989). However, the other investigators have shown similar numbers of lymphocytes in asthmatics and control subjects (Tomioka et al, 1984; Kirby et al, 1987; Wardlaw et al, 1988) whilst Beasley et al (1989) reported fewer lymphocytes in the BAL fluid of mild atopic asthmatics compared to normal controls. Robinson et al (1992) recently demonstrated a preponderance of TH$_2$-lymphocytes in BAL from atopic asthmatics.

Although measurements of inflammatory mediators in BAL fluid obtained from asthmatic subjects after various challenges are well documented, there is scant data on the release of inflammatory mediators in asthmatic patients compared with non-asthmatic subjects. However, Wenzel et al (1991) documented significant levels of LTC$_4$ in atopic subjects with asthma compared to atopic subjects without asthma.

In vitro studies of the function of inflammatory cells collected at BAL have revealed increased production of superoxide radicals by alveolar macrophages and neutrophils (Kelly et al, 1989) and increased release of leukotriene B$_4$ and 5-HETE from asthmatic alveolar macrophages compared to normal subjects (Damon et al, 1989).
1.5 **Pathophysiology of allergic asthma**

1.5.a. **Allergen inhalation as a model of asthma**

It is clear from the pathological changes described above that inflammatory changes are a characteristic feature of asthmatic lungs even if the clinical syndrome is mild. The question therefore is whether a link can be established between the known triggers of asthma and the observed pathological changes.

Since allergen is thought to be one of the major causative factors in the induction of clinical disease associated with increases in BHR, the consequences of allergen inhalation will be considered.

1.5.a.i. **Lung function**

In 1873, Blackley reported symptoms for a number of days in himself as a result of a brief accidental inhalant exposure to grass pollen allergen to which he was allergic. Since then, techniques have been developed which allow the safe exposure of sensitised individuals to low doses of allergen by inhalation under controlled conditions and it is well documented that this induces bronchoconstriction. The reaction may have several phases. There may be an isolated early reaction or an early reaction followed by a late reaction (dual asthmatic response). The early airway reaction (EAR) occurs within minutes, is maximal at approximately 10-30 min. and resolves within 3 hours (Herxheimer, 1951). This is followed in some
47-73% of subjects by a late airway reaction (LAR) which usually begins between 3 and 4 hours after allergen exposure and is maximal between 8 and 12 hours. The LAR usually resolves within 24 hours but may persist for days or weeks (Herxheimer 1952).

BHR increases progressively for several hours after allergen challenge, and may persist for several days. It has been suggested that the increase in BHR is associated in some way with the onset of the LAR (Cockcroft et al, 1977b; Cartier et al, 1982) although recent evidence suggests it may well precede it (Thorpe et al, 1987; Durham et al, 1988).

The dose of antigen required to induce such a reaction is related to the level of IgE (Bryant et al, 1975), skin sensitivity (Makino, 1964; Bruce et al, 1975; Bryant et al, 1975) and the degree of BHR (Makino, 1964; Kreukniet & Pijper, 1973; Killian et al, 1976). Zuidema (1966) suggested that using skin sensitivity and BHR together it should be possible to predict the amount of antigen needed to induce a reaction. Cockcroft et al (1979a) were able to demonstrate that this is the case, and developed a formula for calculating the challenge dose of antigen (Cockcroft et al, 1987).

1.5.a.ii. Pathology

Recently, the pathological changes induced in the lung by laboratory allergen challenge have been investigated.

Beasley et al (1989) found biopsy findings identical to those seen in unchallenged atopic asthmatics, namely extensive epithelial
fragility, sub-basement membrane collagen deposition and mucosal infiltration with activated eosinophils, degranulated mast cells, lymphocytes and macrophages.

De Monchy et al (1985) bronchoscoped allergic asthmatics following allergen challenge with house dust mite. Patients who suffered isolated early responses did not have increased eosinophil counts in BAL fluid collected 6 hours after challenge. In contrast patients who suffered dual reactions did not have increased eosinophil counts in BAL fluid after the EAR but had significantly elevated cell counts and levels of the eosinophil derived protein eosinophil cationic protein (ECP) following the EAR. No increase in the number of neutrophils was observed. Similar findings were reported by Metzger et al (1987a). These authors described a technique of local allergen challenge (LAC) and local BAL via a bronchoscope. They were able to compare BAL findings in the challenged lung with those in the unchallenged lung of the same patient. They noted that immediately after LAC there was mucosal pallor, followed by reactive hyperaemia, oedema and bronchial narrowing. Cellular inflammation characterised by a significant increase in helper T-lymphocytes, neutrophils and eosinophils was most prominent in BAL fluid recovered 48 hours after challenge, but lymphocyte and eosinophil counts remained elevated 4 days later. Macrophages were also observed at all three time points. Electron microscopy revealed degranulation of mast cells and eosinophils both immediately and 2 to 4 days later. Degranulation of eosinophils resulted in the loss of core material and the formation of vesicles that carried core material from the interstices of the cells to their
peripheral membranes, presumably with the release of the granule contents. Macrophages showed activation and had phagocytosed partially intact granules from both mast cells and eosinophils. Alveolar macrophages collected after local provocation with allergen were recently found to be depleted of lysosomal enzymes when compared with alveolar macrophages from the unchallenged, contralateral lung (Tonnel et al, 1983) suggesting in vivo enzyme release.

Platelets were also observed in the BAL fluid following allergen challenge suggesting extravascular migration of platelets. Platelets were most numerous during the immediate response but were also detected up to 48 and 96 hours after challenge. Sometimes, platelets were seen in close apposition to macrophages and eosinophils (Metzger et al, 1986, 1987b). Furthermore, biochemical analysis of BAL fluid after local allergen challenge revealed platelet granule contents (Metzger et al, 1983).

Increased lymphocyte numbers in BAL fluid after allergen challenge have been noted (Diaz et al, 1989). Segmental bronchial allergen challenge (Metzger et al, 1987a) resulted in a selective increase in CD4+ cells in BAL fluid observed 48 hours after challenge in subjects who had previously been shown to develop a late-phase asthmatic reaction. Gonzalez et al (1987) have analysed lymphocyte subsets in BAL fluid and peripheral blood after allergen challenge. Where a single early asthmatic reaction occurs, there is a fall in the ratio of helper to suppressor cells and a decrease in the proportion of suppressor to helper cells in the peripheral blood. These changes in T-cell subset ratios are not observed where a late reaction occurs, and this has been interpreted as showing that a
preferential recruitment of suppressor T cells into the airways following allergen challenge protects against the subsequent development of a late asthmatic reaction.

A number of cell products have been assayed in BAL fluid following allergen challenge. Levels of mast cell derived histamine (De Monchy et al, 1985; Casale et al, 1987; Wenzel et al, 1988), PGD$_2$ (Murray et al, 1986; Wenzel et al, 1989), thromboxane (Wenzel et al, 1989), and neutrophil chemotactic factor (Metzger et al, 1986) are raised. Increased beta-glucuronidase (Tonnel et al, 1983) and leukotrienes (Wenzel et al, 1991) have been reported. Eosinophil chemotactic activity (Rak et al, 1988; De Monchy et al, 1988) and eosinophil derived ECP (De Monchy et al, 1985) is elevated, and a rise in platelet derived PF$_4$ and beta-thromboglobulin has been documented (Metzger et al, 1983).

Wenzel et al, 1991 investigated the relationship between the amount of mediators released during the EAR and the amount of antigen administered, the subsequent development of IAR, and the degree of BHR. Isolated EAR was associated with significantly higher levels of histamine, thromboxane, LTC$_4$ and PGD$_2$ than dual reactions or non-responders, and no correlation was found with BHR.

1.5.a.iii. Pharmacology

The effect of drugs on the EAR and IAR responses to allergen challenge and on the subsequent increase in responsiveness has been
extensively studied. The findings are compared with the effects of the drugs on the clinical course of asthma and BHR in Table 1. Overall, the effect of drugs on the course of laboratory-induced asthma correlates with their clinical efficacy, suggesting that this is a worthwhile model of asthma.

1.5.a.iv. Mechanisms

The pathology and pharmacology of the EAR and LAR phases of allergen challenge have led to the belief that the early asthmatic reaction is primarily caused by airway smooth muscle constriction, in response to released or newly synthesised mediators, principally derived from mast cells and possibly alveolar macrophages, and may be augmented by vagal cholinergic reflexes (Holgate et al, 1986).

The mechanism of the late response is less well understood but is considered to be the result of the cellular phase of inflammation and besides muscle constriction involves structural changes in the airways such as sputum production and mucus plugging.

The EAR is a Type I hypersensitivity reaction (Coombs & Gell, 1968) as evidenced by passive transfer experiments (Prausnitz and Kuestner (1921). Passive transfer has been confirmed repeatedly with serum from atopic subjects and has been shown to be antigen-specific. Evidence that reagin sticks to various tissues and not just to skin has been provided by the demonstration that fresh bronchial tissue removed from an atopic subject undergoes contraction of the smooth muscle when exposed to the appropriate antigen, and it has been shown
Table 1

**Pharmacology of airway allergen challenge.**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Antigen challenge</th>
<th>Asthma</th>
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<tbody>
<tr>
<td></td>
<td>EAR</td>
<td>IAR</td>
</tr>
<tr>
<td>beta-adrenoceptor agonists</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>long-acting</td>
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<tr>
<td>Corticosteroids</td>
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<tr>
<td>DSCG</td>
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<tr>
<td>Nedocromil sodium</td>
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<td>*</td>
</tr>
<tr>
<td>Atropine</td>
<td>*/-</td>
<td>-</td>
</tr>
<tr>
<td>Histamine H1 antagonists</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>Histamine H2 antagonists</td>
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<td>long term, low dose</td>
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<tr>
<td>Ketotifen</td>
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<td>Cetirizine</td>
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<td>Xanthines</td>
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<tr>
<td>NSAIDs</td>
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<tr>
<td>Thromboxane synthetase inhibitor (OKY-046)</td>
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<tr>
<td>Lipooxygenase inhibitors</td>
<td>*/-</td>
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<td>Leukotriene Antagonist</td>
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<tr>
<td>Nifedipine</td>
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References

Dorsch, 1990
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**KEY**

* inhibition
- no effect
that normal tissues of various types can be sensitised passively by the serum of atopic individuals. Ishizaka et al (1966) demonstrated that this serum factor was an antibody, since termed IgE. It has now been shown that the intradermal injection of anti-IgE antiserum induces a weal and flare response (Ishizaka & Ishizaka, 1968; Dolovich et al, 1973), and inhalation of anti-IgE antiserum induces both acute and late onset reactions in allergic asthmatics (Kirby et al, 1986).

IgE is produced by all individuals in response to large quantities of certain antigens, such as helminthic gut infestation, and is thought to play a role in defence against such infection. By contrast, an individual's ability to produce IgE antibody against very small amounts of one or more common antigens, such as inhaled house dust or grass pollen appears to be an idiosyncratic response (Ishizaka, 1971).

The existence of receptors for the Fc portion of IgE on mast cells and basophils is well established. More recently, IgE receptors have also been found on blood monocytes (Melewicz & Spiegelberg, 1980), macrophages (Joseph et al, 1983), eosinophils (Capron et al, 1981), lymphocytes (Melewicz et al, 1982) and platelets (Joseph et al, 1986).

The majority of lymphocytes bearing these receptors are B cells. However, in a study of children with atopic asthma Matsumoto et al (1990) noted not only an increase in receptor-positive B cells, but also a small proportion (1.6%) of receptor positive T cells. Furthermore, the level of IgE binding factors, the soluble form of the receptor, was also elevated in children with atopic asthma. In
normal subjects some 20% of platelets are IgE positive, whereas in allergic subjects the mean is increased to 35% (Joseph et al, 1986).

IgE receptors on inflammatory cells fall into two classes. Those on mast cells and basophils are of high affinity ($K_a = 10^{-10}$M$^{-1}$) so that binding is rapid and dissociation slow, whereas those on the macrophage (Melewicz et al, 1982), eosinophil (Capron et al, 1985), lymphocyte (Melewicz et al, 1982) and platelet (Joseph et al, 1986) are of low affinity and it is thought that these receptors are triggered by IgE immune complexes rather than monomeric IgE. The platelet receptor has been shown to consist of two subunits of 43kD and 30kD, the latter differing from the smaller subunit in eosinophils and monocytes generally identified as a 23-25kD molecule.

Adherence of IgE to mast cells and basophils by its Fc component leaves the Fab end of the antibody free to react with the corresponding antigen, and when this occurs, cross-linking of surface-bound antibody molecules by bound antigen results in degranulation. The discharged granules in turn release their stored histamine, heparin, tryptase, neutrophil chemotactic factor (NCF-A), eosinophil chemotactic factor (ECF-A) and membrane derived mediators such as leukotrienes and PGD$_2$ (Holgate et al, 1986). IgE dependent kininogenase release from human lung mast cells has recently been described (Proud et al, 1985).

The IgE mediated reactions to inhalation tests may possibly be occurring with sensitized basophils lying on the surface of the bronchial mucosa since there is doubt as to whether, or how far, allergens penetrate into the mucosa to reach the tissue mast cells.
(Richardson et al, 1973).

It is also possible that the major IgE bearing inflammatory cell to which inhaled antigens bind is the alveolar macrophage, since this is the major inflammatory cell type in the lung. In macrophages, IgE dimers induce the discharge of lysosomal enzymes, production of superoxide and other oxygen products (Harada et al, 1982) and secretion of interleukin 1 (IL-1) and plasminogen activator as well as products of arachidonic acid metabolism (Rankin et al, 1982; Martin et al, 1984) and PAF (Arnoux et al, 1987). IgE-dependent cytotoxicity is also expressed by activated macrophages (Melewicz et al, 1981).

The production of cytokines by alveolar macrophages may play an important role in the migration of blood derived inflammatory cells. IL-1, tumour necrosis factor (TNF) alpha, TNF beta and lipopolysaccharide strongly upregulate the expression of the adhesion molecule ICAM-1 on endothelial cells (Dustin et al, 1986; Pober et al, 1986). Whilst ICAM-1 expression has not been demonstrated on human bronchial tissue, bronchial endothelial and epithelial cells of monkeys and mice have been reported to express ICAM-1. In addition, ICAM-1 was shown to stain intensely on the basolateral portion of the epithelium and endothelium of monkey tracheal sections taken 20 minutes after the third of alternate day allergen inhalations which was accompanied by an 8 fold increase in BHR to methacholine. This increase in BHR was preceded by an intense eosinophil infiltrate and both were inhibited by intravenous Mab to ICAM-1 (Mab RR1/1).
Furthermore, Mab RRI/1 inhibits eosinophil adhesion to stimulated human umbilical vein endothelial cells in vitro. Another Mab to ICAM-1 (Mab R6.5) also inhibited eosinophil adhesion to monkey airway epithelial cells (Wegner et al., 1990).

Induction of ICAM-1 expression is also likely to be important in the activation of T lymphocytes since ICAM-1 interacts with lymphocyte function-associated antigen (LFA-1), since LFA-1 is expressed by memory T cells and its expression is 'switched on' when lymphocytes encounter antigen. In a study using a murine ICAM-1 gene, Siu et al. (1989) have shown that LFA-1 - ICAM-1 interaction leads to an enhanced antigen-specific T-cell activation with accompanying intracellular signalling by T-cell second messengers.

IL-1 and TNF alpha also induce the expression of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells (Osborn et al., 1989). VCAM-1 has been shown to bind lymphocytes and its receptor on these cells is the lymphocyte-homing receptor and integrin VLA-40 (Elices et al., 1990).

The activation of mast cells, basophils and macrophages results in the release of a number of inflammatory mediators. Histamine (Curry, 1946), leukotrienes (Barnes et al., 1984; Adelroth et al., 1986) and PAF (Cuss et al., 1986) can all induce bronchoconstriction and increase vascular permeability (Lewis & Grant, 1924; Williams & Piper, 1980; Pinckard et al., 1980).

Furthermore, these mediators may play a role in cell infiltration since they have been shown to induce chemotaxis in vitro (Clark et al., 1975; Nagy et al., 1982; Camussi et al., 1983). Additionally
PGD\textsubscript{2} which is a bronchoconstrictor (Hardy et al, 1985) and other factors which are chemotactic for neutrophils and eosinophils, such as NCF-A (Paterson et al, 1976) and ECF-A (Kay & Austen, 1971) are released from mast cells. Furthermore, histamine influences the release of prostaglandins from pulmonary cells (Platshon & Kaliner, 1978) and has been postulated to have an immunoregulatory role (Beer & Rocklin, 1984).

Recently, it has become evident that these mediators, including histamine and LTC\textsubscript{4}, can mobilize the selectin, granule-membrane protein 140 (GMP-140) to the cell surface of platelets and endothelial cells. Its function is to promote the rapid onset of adhesion between the endothelium and platelets and leucocytes (Geng et al, 1990).

Mediator release from mast cells, basophils and macrophages may therefore not only result in the manifestations of acute inflammation, but also play a central role in the expression of adhesion molecules on the surface of the endothelium which is thought to represent the first step in the migration of leucocytes and platelets from the blood. Infiltration of inflammatory cells into the lung is certainly a characteristic feature of allergen challenge and asthma. Recent evidence has suggested that lymphocytes and platelets, in addition to providing a further source of inflammatory mediators, may play a pivotal role in the recruitment of eosinophils. In turn, the eosinophil will release mediators which will attract further inflammatory cells, but the eosinophil is also thought to be the main
culprit in inducing the tissue damage characteristic of asthma.

Evidence to date suggests that allergic responses are mediated by the subgroup of CD4+ lymphocytes known as TH2 (Robinson et al, 1992). These, in addition to secreting granulocyte/macrophage colony stimulating (GM-CSF), TNF and IL-3 (like TH1 cells) are responsible for the secretion of IL-4, IL-5, IL-6 and IL-10 (Street & Mosmann, 1991).

GM-CSF and interleukin-5 (IL-5) both have pronounced effects on eosinophils including prolongation of survival, enhanced adherence and activation to the hypodense phenotype (Owen et al, 1987; Lopez et al, 1988; Yamaguchi et al, 1988). Many of the effects of IL-5 are selective for eosinophils and are not observed with neutrophils, such as the hyperadherence of eosinophils to vascular endothelium (Walsh et al, 1990). Enhanced eosinophil survival may be one mechanism whereby these cells accumulate in the mucosa. A new lymphokine, called HILDA, has recently been characterised which has potent effects on eosinophil chemotaxis and chemiluminescence (Moreau et al, 1987).

Moreover, T-lymphocyte clones from subjects with hypereosinophilic syndrome have been shown to elaborate eosinophil differentiation factors, providing evidence that the number and function of eosinophils are directly regulated by T lymphocyte products (Raghavacher et al, 1987) and Wierenga et al (1990) have demonstrated cytokine release by allergen specific T cell clones (Wierenga et al, 1990).

IL-5 and IL-6 also induce B cell growth and differentiation. The
lymphokine IL-4 stimulates antibody production in general and particularly the IgE isotype of antibody and induces the expression of the Fc receptor for IgE and the cell surface expression of proteins such as MHC class II, whilst IFN-gamma, a TH\textsubscript{1} product inhibits the effects of IL-4 on B cells (Coffman & Carty, 1986; Finkelman et al, 1986).

Furthermore, IL-3, IL-4 and IL-10 promote mast cell growth (Street & Mosmann, 1991).

It has been postulated that platelets behave like inflammatory cells, and may play a pivotal role in the recruitment of eosinophils into the airways (Page, 1989). Platelets adhere to the endothelium (mediated by GMP-140), and have been shown to undergo chemotaxis in vitro (Lowenhaupt et al, 1973) and diapedesis in vivo in experimental animals (Lellouch Tubiana et al, 1985). Platelets have been noted in asthmatic lungs both at autopsy and in biopsy specimens and have been reported in BAL fluid after allergen challenge (see section 1.5). Platelets may be activated via their IgE receptors, by neuropeptides, and by inflammatory mediators such as PAF (Benveniste et al, 1975; Joseph et al, 1986; Dammonville et al, 1990). This results in the release of substances which have been shown to be chemotactic for eosinophils in vitro, namely PF\textsubscript{4} (Chihara et al, 1988) and 12-HETE, a product of 12-lipoxygenase metabolism that is specifically released from platelets (Marcus et al, 1984) and is chemotactic for eosinophils and neutrophils (Goetzl et al, 1977). Thus, the release of PF\textsubscript{4} and 12-HETE may contribute to the influx of eosinophils into the lungs. Support for this hypothesis has been derived from
histological and BAL studies which have shown that eosinophil accumulation in sensitised experimental animals exposed to allergen is diminished in animals depleted of platelets by lytic antisera, or subjected to infusion of prostacyclin (Lellouch Tubiana et al, 1988). Similar results were reported when intravenous PAF was used as the stimulus for eosinophil recruitment (Lellouch Tubiana et al, 1988). In sensitised rabbits, selective depletion of platelets also resulted in decreased numbers of eosinophils in BAL fluid during an allergic reaction and in addition resulted in an inhibition of the late onset airway response and subsequent BHR (Coyle et al, 1990a).

In addition to a possible role in cell infiltration, a novel function of platelets, namely IgE mediated cytotoxicity, resulting in the production of free radicals, has recently been delineated. The IgE dependence of the cytotoxic reaction of platelets isolated from patients with allergic asthma has been demonstrated by isotype-specific adsorption of antibodies from the serum of allergic donors, by the lack of effect of anti-IgG antibody and by the inhibitory role of polyclonal and monoclonal antibodies against the Fc receptor on the passive sensitisation process (Joseph, 1988).

It has recently been demonstrated that the neuropeptide, substance P, will activate platelet cytotoxicity. It is possible that this effect is related to the existence of limited but significant conformational homology between the primary structure of substance P and IgE since the reaction is competitively inhibited by IgE (Damonneville et al, 1990). It is possible that cytotoxicity of platelets is regulated or induced by T-lymphocyte factors. Thus, gamma interferon will activate normal
platelets in vitro (Pancre et al, 1987) and enhances the expression of the IgE receptor on the platelet membrane (Pancre et al, 1988), and tumour necrosis factor will induce cytotoxicity in normal platelets against S. mansoni larvae (Damonneville et al, 1988) showing an additive effect with interferon in this model. Furthermore, a factor termed platelet cytotoxicity inducing factor (PCIF) which has not yet been characterised will induce cytotoxicity in platelets (Joseph, 1988). On the other hand, T cells stimulated with antigen release a factor able to inhibit IgE dependent cytotoxicity of human platelets (Pancre et al, 1986) which has been called platelet activity suppressor lymphokine (PASL). A general feature of platelet stimulating factors such as IgE, gamma interferon and PCIF is that cytotoxic functions are independent of aggregation, suggesting that an activation pathway distinct from that which accounts for the classical aggregatory response to thrombin, ADP and PAF may exist.

In addition to their role in recruiting eosinophils and to their cytotoxic capacity, activated platelets release potent mediators of inflammation. Thromboxanes, cyclic endoperoxides, leukotrienes, PAF, 5-HT and histamine will induce bronchoconstriction (Page, 1989). In addition, PF₄ will evoke a concentration-dependent release of histamine from basophils (Brindley et al, 1983) and more recently another soluble factor stimulating histamine release from human basophils (PDHRF) has been described (Orchard et al, 1986). Platelet derived growth factor is chemotactic for neutrophils (Deuel et al, 1982), and may play a part in the smooth muscle hyperplasia (Ross et
which characterises asthma.

Eosinophils are thought to play a central role in the pathogenesis of asthma. Adhesion to the endothelium, promoted by ICAM-1 expression and the cytokines IL-5 and GM-CSF, and subsequent migration in response to chemotactic stimuli such as leukotrienes, PAF, ECF-A, PF₄ and 12-HETE are thought to result in the infiltration of eosinophils into the airway tissues.

The activation of eosinophils by inflammatory mediators or via their IgE receptors results in cytotoxicity, granule product release and the release of newly formed mediators. The latter include PAF (Lee et al, 1984), leukotrienes (Owen et al, 1987; Kauffman et al, 1987) and prostaglandins (Smith et al, 1987) as well as oxygen free radicals (Agosti et al, 1987).

The IgE-dependent cytotoxic capacity appears restricted to hypodense eosinophils and is likely to be linked to an increase in the number of Fc receptors. Indeed the expression of these receptors appears to be increased on hypodense eosinophils and the proportion of cells bearing cytophilic IgE is increased in hypodense pulmonary alveolar eosinophils (Capron et al, 1988).

The release of eosinophil granule products is believed to be particularly relevant to the pathology of asthma. Major basic protein induces ciliastasis and is cytotoxic to epithelial cells (Frigas & Gleich, 1986). Furthermore, MBP causes a gradual release of about 60% of mast cell histamine content over 30 minutes (O'Donnell et al, 1983) and has recently been reported to inhibit the release or action of epithelial-derived relaxant factor (EpDRF), a substance released

Current hypotheses implicate the granule product-induced damage of the epithelium in the pathogenesis of BHR. Eosinophil derived MBP- or ECP-induced damage of the epithelium results in greater access of inhaled substances to airway smooth muscle (Ranga et al, 1983). Furthermore the epithelium is believed to be an important source of inflammatory mediators such as leukotriene B_4 and 15-HETE, which are chemotactic for inflammatory cells (Hunter et al, 1985) and produces a smooth muscle relaxant, epithelial derived relaxant factor, EpDRF (Flavahan et al, 1985). The mucociliary escalator will be disrupted, leading to an inability to clear bronchial secretions (Frigas & Gleich, 1986).

Finally, damage to airway epithelium may expose unmyelinated C-fibre afferent nerves (Laitinen et al, 1985), which may then be triggered by inflammatory mediators such as bradykinin and prostaglandins. This might release sensory neuropeptides [substance P, neurokinin A & B, calcitonin gene-related peptide] from collaterals of the excitatory NANC nerves, with resultant bronchoconstriction, vasodilatation, microvascular leakage and mucus secretion (Barnes, 1986). Substance P may also degranulate mast cells and have chemotactic activity (Payan
et al, 1984). In addition, neuropeptides facilitate neurotransmission at postganglionic nerves thus enhancing the release of acetylcholine (Hall et al, 1989).

Axon reflexes may thus spread the inflammatory changes from areas of localised epithelial damage up and down the airway and may therefore contribute to BHR (Barnes, 1986). To this end, it is interesting to note that substance P containing fibres are more abundant in the airways of asthmatics when compared to normals (Ollerenshaw et al, 1989).

It has been suggested that the threshold to stimulation of sensory nerves may also be decreased due to local inflammation in the lung, similar to hyperalgesia in other inflamed tissues (Garland, 1984). Mediators such as 15-HPETE, PAF and LTB4 are thought to be particularly relevant in this regard, particularly since 15-lipoxygenase is the predominant route of metabolism for arachidonic acid in lung epithelial cells (Sigal and Nadel, 1988).

In conclusion, it is becoming increasingly clear that allergic asthma is not simply due to mast cell histamine release, but rather is the result of complex interactions between resident pulmonary cells, infiltrating inflammatory cells and their released mediators and the nervous system.
1.6. Cutaneous allergen challenge

1.6.a. Historical background

In 1921, Prausnitz and Kuestner noted that a residual local swelling persisted at the site of local injection of boiled fish antigens the morning after the reaction was induced. Cooke (1922) confirmed this observation, reporting experiments performed in himself involving the intracutaneous injection of antigen to which he was allergic. He noted that local skin swelling and erythema were often present many hours after the administration of antigen.

It is now well established that the injection of a variety of allergen extracts into the skin of allergic individuals may produce a biphasic response. The immediate weal and flare reaction (early cutaneous reaction, ECR) is generally maximal in 15-20 min. and then subsides within a few hours. The late cutaneous reaction (LCR) appears after an interval of 2 or more hours, usually reaches maximal intensity by 6 to 8 hours and subsides within 24 to 48 hours (Walker, 1919; Dolovich et al, 1973). The LCR is characterised by diffuse oedema, erythema, pruritus, heat and tenderness.

1.6.b. Pathology

Histologically, the LCR is characterised by oedema (Dolovich et al, 1973; Solley et al, 1976), mast cell degranulation of varying intensities (Dolovich et al, 1973) and perivascular cellular
infiltration. Four to six hours after challenge eosinophils predominate with only scant neutrophil and no basophil infiltration (Kline et al, 1932; Atkins et al, 1973). Mononuclear cells including lymphocytes and monocytes, but not plasma cells, have been noted (De Shazo et al, 1979). Recent immunocytochemical analyses of cells infiltrating cutaneous LCRs using Mabs have demonstrated increased numbers of T lymphocytes of the CD 4+ (helper) phenotype in perivascular locations (Frew & Kay, 1988), which are predominantly memory T cells (Frew & Kay, 1991). Dolovich et al (1973) commented on the absence of thrombi, necrosis of blood vessels and fibrin deposition. However, De Shazo et al (1979) found similar cellular changes in biopsies taken from subjects undergoing only ECR and those undergoing dual responses. Fibrin deposition was observed only in subjects with a visible LCR, forming a fibrillar meshwork interspersed between collagen bundles of the reticular dermis, the subcutis and to a lesser extent the papillary dermis. Individual fibrin strands formed a loosely knit, three-dimensional gelatinous network but were not found within vessel lumina nor vessel walls and spared a narrow rim around the vessels cuffed by inflammatory cells. This led to the suggestion that activation of the coagulation system may be important in the production of the LCR.

18 hours after challenge eosinophils and basophils were reported by Felcara & Lowell (1971) although Richerson et al (1979) failed to confirm the presence of basophils at 24 hours. Solley et al (1976) observed mainly lymphocytes although eosinophils, neutrophils and basophils were also present. Immunohistochemical studies have confirmed the presence of activated eosinophils (Frew & Kay, 1988).
1.6.c. Mechanisms

The investigations which established that the ECR is an IgE mediated type I hypersensitivity reaction has been outlined in section 1.5. The role of IgE in the LCR was demonstrated by Dolovich et al (1973) who induced a LCR by the intradermal injection of a monospecific antiserum to IgE. The studies of Solley et al (1976) provided further evidence. They showed that the LCR could be elicited by passive transfer of allergic serum and that it could be abolished by heating the allergic serum to denature the Fc portion of IgE antibodies, by removing IgE by specific immunoadsorption and by competitively inhibiting the reaction of specific IgE antibodies with the cell receptor sites by IgE myeloma protein. Furthermore a LCR could be elicited by an affinity-chromatography purified IgE protein containing specific IgE antibodies to ragweed, followed by allergenic challenge.

The intradermal injection of numerous inflammatory mediators results in responses which mimic the LCR to antigen. Leukotrienes \( \text{LTB}_4, \text{LTC}_4, \text{LTD}_4 \) and \( \text{LTE}_4 \) (Soter et al, 1983), PAF (see section 2.3.d.), the complement anaphylotoxins C3a and C5a (Stalenheim & Zetterstrom, 1979) and kallikrein (Juhlin & Michaelsson, 1969a) cause inflammation grossly similar to the LCR. Prostaglandin E and \( \text{F}_{2\alpha} \) induce immediate responses and long-standing erythema (Juhlin & Michaelsson 1969b) but not oedema.

Histamine and PGD\(_2\) are believed to play an important role in the ECR, but their duration of action is brief and intracutaneous
injection of histamine or PGD$_2$ does not lead to a LCR (Crunkhorn & Willis, 1971; Solley et al, 1976; De Shazo et al, 1979). Eosinophil derived proteins, MBP and EDN also induce an acute weal and flare response when administered intradermally (Leiferman et al, 1984).

Examination of skin blister fluid and skin chamber techniques have shown that histamine and PGD$_2$ are released during the ECR. Conflicting results have been reported regarding the release of histamine and PGD$_2$ during the LCR, whilst levels of LTC$_4$ are maximal 2 hours after challenge and remain elevated for at least 4 hours. PAF can be detected 5-9 hours after challenge (Shalit et al, 1989). Thromboxane levels are increased 0.5 to 6 hours after challenge, and a kallikrein activity has been demonstrated during the LCR (Dorsch, 1990). Application of molecular biological techniques has recently demonstrated the expression of the cytokine gene cluster for IL-3, IL-4 and IL-5 and GM-CSF in allergen induced LCRs (Kay et al, 1991).

The pharmacology of cutaneous allergen challenge is outlined in Table 2.

1.6.d. Correlation between cutaneous and airway allergen challenge.

The similarity in the time course, histology and pharmacology of the cutaneous and airway responses to allergen in sensitised subjects has led to the suggestion that similar mechanisms may underlie the
Table 2

**Pharmacology of cutaneous allergen challenge**

<table>
<thead>
<tr>
<th>Drug</th>
<th>ECR</th>
<th>LCR</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1 antagonists</td>
<td>*</td>
<td>-</td>
<td>Smith et al, 1980</td>
</tr>
<tr>
<td>H2 antagonists</td>
<td>-</td>
<td>-</td>
<td>Smith et al, 1980</td>
</tr>
<tr>
<td>H1 &amp; H2 ant.</td>
<td>*</td>
<td>*</td>
<td>Smith et al, 1980</td>
</tr>
<tr>
<td>Aspirin</td>
<td>-</td>
<td>-</td>
<td>Smith et al, 1980</td>
</tr>
<tr>
<td>Indomethacin (topical)</td>
<td>-</td>
<td>* (erythema)</td>
<td>Dorsch &amp; Baur, 1980</td>
</tr>
<tr>
<td>Dazoxiben (TX synthesis inhibitor)</td>
<td>+</td>
<td>* (oedema)</td>
<td>Dorsch et al, 1983</td>
</tr>
<tr>
<td>Glucocorticosteroids</td>
<td>-</td>
<td>*</td>
<td>Talbot et al, 1987</td>
</tr>
<tr>
<td>DSCG</td>
<td></td>
<td>*</td>
<td>Gronneberg &amp; Zetterstrom, 1985</td>
</tr>
<tr>
<td>Beta-agonist (i.d. not oral)</td>
<td>*</td>
<td>*</td>
<td>Gronnenberg et al, 1981</td>
</tr>
<tr>
<td>Ketotifen</td>
<td>*</td>
<td></td>
<td>Phillips et al, 1983</td>
</tr>
<tr>
<td>Theophylline</td>
<td>-</td>
<td>-</td>
<td>Galant et al, 1973</td>
</tr>
<tr>
<td>Cetirizine</td>
<td>*</td>
<td>*(eos.)</td>
<td>Michel et al, 1988</td>
</tr>
<tr>
<td>Tranexamic acid</td>
<td>-</td>
<td>*</td>
<td>Gronnenberg 1984 e</td>
</tr>
<tr>
<td>Onion extracts</td>
<td>*</td>
<td>*</td>
<td>Dorsch &amp; Ring, 1984 e</td>
</tr>
<tr>
<td>Eicosatetraynoic acid</td>
<td>-</td>
<td>*</td>
<td>Dorsch 1990 e</td>
</tr>
</tbody>
</table>

**KEY**

* = inhibition
- = no effect

ECR = Early cutaneous reaction
LCR = Late cutaneous reaction

* = anti-IgE not allergen challenge
responses in the skin and in the lung.

Furthermore, there is a correlation in individual allergic subjects between their reaction to cutaneous and inhaled allergens (see section 1.5.a.i.). Cutaneous allergen challenge has therefore been used frequently as a more convenient model for investigating the allergic response and for assessing the actions of novel drugs that may have an anti-allergic activity.
Katz & Cohen (1941) initially demonstrated the release of histamine following the addition of antigen to human blood from allergic subjects, whereas no histamine release was observed in non-asthmatic subjects. They were able to show that the histamine was released by blood cells not by plasma. The work of Lichtenstein & Osler (1964) provided evidence that the release of histamine is an active process in human leucocytes which can proceed in vitro in the absence of serum factors.

To further elucidate this phenomenon experiments with animal blood were conducted by several investigators. Similar histamine release was demonstrated from rabbit leucocytes in the presence of antigen, which was independent of complement or other serum factors (Schoenbechler & Barbaro, 1968; Henson, 1969). In 1969, Zvaifler & Robinson showed that the immunization of rabbits with egg albumin resulted in antibody production which has similar physico-chemical properties to human IgE, and Siriganian & Osler (1969) were able to show functional histamine release from leucocytes sensitised in this manner. The antibody dependency of the reaction was shown by Colwell et al (1971) who were able to induce leucocyte dependent histamine release in leucocytes from normal rabbits by transfer of serum from
sensitized animals. This was confirmed by Benveniste et al (1972) who showed the antibody to be of the IgE class.

However, it had previously been shown that the major source of histamine in the rabbit is the platelet (Humphrey & Jaques, 1954). Although platelets themselves do not release histamine when incubated with antigen in vitro, significant release of histamine is observed in the presence of leucocytes. In 1969, Henson postulated that leucocytes produce a labile soluble agent, and that this was an active process since it is inhibited by the cold or by chelation of calcium. The soluble mediator was produced rapidly (within two minutes of stimulation) and induced non-lytic platelet histamine release associated with the disappearance of granules and canaliculi. Although Henson (1970) implicated the monocyte in the leucocyte-platelet interaction, Siraganian & Osier (1971) provided evidence that the basophil was the major source of the labile material, which was similar in characteristics to that described by Henson, although they observed platelet cytotoxicity in addition to histamine release. Benveniste et al (1972) provided histological evidence of basophil degranulation and rapid and specific platelet aggregation in close association to the degranulated basophils. Furthermore, Benveniste et al (1972) were able to recover the soluble intermediate, which they termed platelet activating factor (PAF), and initiated characterization of the constituent responsible for its biological activity. The active substance was found to be a basic molecule with a molecular weight of 1,100, a hydrophobic moiety and high positive charge (Benveniste, 1974). Further studies of the susceptibility of PAF to lipolysis suggested that PAF was a
1-lyso-glycero-phospholipid (Benveniste et al, 1977).

Independent investigations demonstrated the anti-hypertensive activity of a renal extract, initially observed in dogs with renoprival hypertension (Muirhead et al, 1960) and subsequently extracted from the renomedullary interstitial cells of rats (Muirhead et al, 1975). The characterization of the polar nature of this lipid led to its name, antihypertensive polar renomedullary lipid (APRL) (Prewitt et al, 1979).

The APRL and PAF fields ultimately merged when it was realized that the hypotensive (Blank et al, 1979) and platelet activating (Demopoulos et al, 1979) properties of a specific phospholipid molecule shared an identical chemical structure – 1-O-alkyl-2-acetyl-sn-1-glyceryl-3-phosphorylcholine (Fig. 1).

Total synthesis of PAF was achieved (Godfroid et al, 1980) and a series of detailed gas-liquid chromatographic and mass spectrometric analyses of natural PAF led Hanahan et al (1980) to conclude that natural PAF does have the same chemical structure as the synthetic material. McManus et al (1980) showed that the in vivo effects of synthetic PAF are identical to those of natural PAF derived from antigen-stimulated IgE sensitised basophils (Pinckard et al, 1979).

Structure activity studies have revealed that the ether linkage at position 1 on the sn-glyceryl is an absolute requirement for biological activity. A short acyl chain at position 2 provides optimal activity, although 2-acetyl and 2-propionyl compounds also have substantial activity. Only the natural stereoisomer of PAF (i.e. the L form with phosphocholine at the sn-3 position) is active, and
Figure 1

Schematic representation of the structure of platelet activating factor.
changes in the length of the alkyl chain are less crucial although maximal activity is observed with C16-C18 analogues (reviewed by Braquet et al, 1987).
2.2 Criteria for a substance to be an inflammatory mediator

The criteria set out by Dale for a substance to be considered as a neurotransmitter have been modified for application to a mediator of inflammation, and state that:

1. The enzymes necessary for synthesis should be present at the appropriate site.

2. The substance should be generated or released in appropriate amounts during the response.

3. Receptors for the substance should be demonstrable on or in the relevant cells.

4. The substance should produce the effect specified both in vivo and in vitro.

5. A mechanism for stopping the action of the substance should exist.

6. Drugs which interfere with the action or with the synthesis, storage, release or breakdown of the substance should produce the predicted alteration of the response.
7. Experimental techniques or clinical conditions which result in deficiencies of the substance or of the synthesising or metabolizing enzymes involved should result in the appropriate increase or decrease in the response.

Florey has defined the characteristics of inflammation in mucus membranes as

1. Vascular dilatation
2. Increased blood vessel permeability and plasma exudation
3. Leucocyte infiltration
4. Mucus secretion
5. Shedding of epithelial cells

The extent to which PAF fulfils the modified criteria of Dale and Florey for a mediator of mucus membrane inflammation will be outlined.
2.3. **PAF as an inflammatory mediator**

2.3.a. **PAF synthesis**

PAF can be enzymatically synthesized by either a remodelling or a de novo pathway [Fig. 2] (reviewed by Lee & Snyder, 1989). The de novo route involves the conversion of an alkyl analogue of lysophosphatidic acid, which occupies a pivotal position in ether lipid biosynthesis, since it can be either acylated by and acyl-CoA acyltransferase or acetylated by an acetyl-CoA acetyltransferase to form the corresponding alkyl ether analogues of phosphatidic acid. The sn-2 acetyl phosphatidic acid can then be converted to 1-alkyl-2-acetyl GPC (PAF) or 1-alkyl-2-acyl-GPC (substrate for the remodelling route) respectively in reaction steps catalysed by a phosphohydrolase and two different cholinephosphotransferases. This route is virtually the exclusive pathway in the kidney and is thought to maintain the physiologic levels of PAF. Highest specific activities are observed in resting cells. This pathway is not stimulated by inflammatory agents and does not result in the generation of eicosanoid mediators.

In contrast, the remodelling pathway is stimulated by inflammatory agents, involves the simultaneous release of eicosanoid mediators and has low activity in resting cells. Studies to date indicate that PAF is not stored, but synthesised de novo in activated inflammatory cells. Studies in several types of cells have suggested that the preferred
Figure 2

The synthesis and degradation of platelet activating factor.
substrate for the initial reaction is a phospholipid that contains arachidonic acid at the sn-2 position (1-alkyl-2-acyl-GPC). Hydrolysis, catalysed by a calcium-dependent phospholipase A₂ of this fatty acid yields lyso-PAF. This substance has similar physico-chemical properties to PAF, but is not able to activate platelets.

Lyso-PAF is then acetylated in a reaction catalysed by a specific acetyltransferase to yield PAF. The activity of the acetyltransferase seems to be correlated with the degree of activation of the cell, and involves phosphorylation. The bulk of PAF synthesis occurs in the endoplasmic reticulum. However, translocation of PAF to the plasma membrane has been reported.

The regulation of PAF synthesis is still poorly understood. It is clear that crucial signals for turning on and off the production of PAF are metal ions, thiol compounds, fatty acids, pH, compartmentalisation and the phosphorylation and dephosphorylation of enzymes. Calcium not only inhibits the enzymes involved in the de novo pathway of PAF synthesis, but it can also inhibit the deacetylation and transacylation steps in the remodelling pathway both in vivo and in vitro. On the other hand, calcium is essential for PAF production by the remodelling pathway.

It has been established that the acetyltransferase activity in the remodelling pathway is regulated by an activation/inactivation mechanism involving phosphorylation/dephosphorylation possibly via a cAMP-dependent kinase or the calcium/calmodulin-dependent protein kinase. Whereas protein kinase C is not capable of activating the
acetyltransferase, it is thought that it might be involved in the activation of the phospholipase A₂.

Arachidonic acid also appears to play an important role in regulating the remodelling pathway of PAF synthesis. Stimulated cells depleted of arachidonate are unable to produce PAF and it is known that arachidonic acid supplements and 5-lipoxygenase products enhance the formation of PAF via the remodelling route, presumably by stimulation of phospholipase A₂.

Autocrine stimulation of its own synthesis by PAF has recently been demonstrated in neutrophils (Tessner et al, 1989) and human monocytes (Valone 1991).

Concurrent synthesis of PAF and other inflammatory mediators is now well recognised. Alkylacyl-GPC, the preformed precursor of PAF, is in part comprised specifically of alkylarachidonoyl-GPC. The first step in PAF synthesis, the cleaving of arachidonate from alkylarachidonoyl-GPC therefore liberates both lyso-PAF and arachidonic acid. It is postulated that further metabolism of PAF and lipoxygenase and/or cyclooxygenase products will be determined by cell type or in the case of transcellular biosynthesis, on the presence of neighbouring cells. The best studied examples are the concurrent synthesis of PAF and LTB₄ in human neutrophils (Sisson et al, 1987) and PAF and PGI₂ in human endothelial cells (McIntyre et al, 1986).
2.3.b. PAF generation and release in inflammation

IN VITRO

A variety of human inflammatory cell types have been shown to release PAF in response to diverse stimuli. In some cells, notably neutrophils (Lynch & Henson, 1986), monocytes (Jouvin Marche et al, 1984) and endothelial cells (MacIntyre et al, 1986) only a small proportion of PAF is released. The majority remains cell-associated, regardless of the agonist used, the dose of agonist, the cell isolation technique, the presence of plasma (Lynch & Henson, 1986), the concentration of albumin or cell priming (Sisson et al, 1987).

PAF was initially identified as a product of rabbit basophils but the release of PAF by human basophils is uncertain. Camussi et al (1977) reported the generation of PAF by human basophils, but this has not been confirmed by two other groups (Betz et al, 1980; Sanchez-Crespo, 1980).

Mononuclear cells also produce PAF whereas purified lymphocyte populations do not (Camussi et al, 1981; Jouvin Marche et al, 1984). Elstad et al (1988) showed that human peripheral monocytes are able to synthesise PAF following stimulation with A23187, PMA or opsonised zymosan and that the majority of the PAF is released extracellularly. These authors also demonstrated that PAF synthesis was dependent on the stage of maturation, as monocyte derived macrophages release considerably less PAF than the parent peripheral blood monocyte. Valone and Epstein (1988) have recently demonstrated that IL-1 beta,
TNF and IFN can stimulate human peripheral blood monocytes to synthesise PAF in a novel biphasic pattern with an early (1-2 hours) and late (6-8 hours) peak of PAF synthesis. Pretreatment with cyclohexamide inhibited the late peak of PAF synthesis suggesting that these cytokines could synthesise a protein which was the stimulus for PAF synthesis. Interestingly, while the majority of the early PAF released was retained intracellularly, greater than 80% of the late peak was released extracellularly. The functional significance of these observations are unknown.

Human neutrophils produce large amounts of PAF (up to 100 pmol/10^6 cells) in response to a variety of stimuli including the calcium ionophore A23187, opsonised zymosan, C5a, FMA (Betz & Henson, 1980), FMLP (Sisson et al, 1987), immune complexes (Sanchez-Crespo et al, 1980), neutrophil cationic protein (Camussi et al, 1981), chymotrypsin, elastase, cathepsin G, IL-1, TNF (Camussi et al, 1987) and LPS (Worthen et al, 1988). This process is calcium dependent (Betz & Henson, 1980) and inhibited by cAMP (Bussolino & Benveniste, 1980).

Early experiments measured total PAF production but separate analysis of cell and supernatant fractions have now been carried out. The majority of PAF formed by neutrophils is found to remain associated with the cell pellet (Lynch & Henson, 1986). Extracellular metabolism of released PAF does not appear to account for the cell association of PAF and release and readsorption of PAF is not sufficient to account for the apparent retention of PAF (Lynch & Henson, 1986). Preliminary data indicate that intracellular PAF is membrane
associated (Riches et al, 1985). Membrane-associated PAF is reported to be protected from inactivation by acetylhydrolase, yet retains platelet activating activity, so that the release of only small amounts of the total PAF formed by cells into the extracellular fluid, together with the known potency of the molecule, would appear unlikely to prevent it from acting as a mediator of cell-cell interactions (Lynch & Henson, 1986). It has also been postulated that PAF may play a role in the control of receptors, (lateral migration, down regulation) or possibly in membrane fusion events (liposome and phagolysosome formation) (Riches et al, 1985) and hypotheses regarding an intracellular role for PAF have been proposed (Sisson et al, 1987; Henson, 1987; Worthen et al, 1988).

Whilst the molecular species of PAF reported to be produced by stimulated neutrophils differ, there appears to be general agreement that the predominant species formed is C16 PAF. It has also been suggested that the composition of the alkyl side chains of the PAF generated on stimulation of these cells differs from that of the precursor phosphocholines (Ramesha & Pickett, 1987).

Human eosinophils release PAF when stimulated with A23187, zymosan, ECF-A and C5a (Lee et al, 1984; Jouvin Marche et al, 1984) but the amount released has not been determined.

Human platelets will produce PAF following stimulation with A23187 (Chignard et al, 1979) and thrombin (Touqui et al, 1985) but PAF is not released by platelets aggregated by exposure to PAF (Chignard et al, 1980). Relative to other cells, the quantities of
PAF released (< 5 fmol/10^6 cells) is small, which may be due to a relative deficiency of acetyltransferase in the platelet. Lyso-PAF is released however, which may be used by other cells, such as neutrophils, to result in heightened PAF production via transcellular biosynthesis (Coeffier et al, 1987).

In addition to release by a number of blood cells, PAF release has been demonstrated in lung mast cells and alveolar macrophages. Human lung mast cells, collected by enzymatic digestion and purified to 98% homogeneity release PAF. However, PAF can only be recovered in the culture supernatant after destruction of these cells, suggesting that the PAF is retained intracellularly (Schleimer et al, 1986).

PAF has been obtained from human alveolar macrophages collected by bronchial lavage in patients with asthma but could not be demonstrated in cells taken from normal volunteers (Arnoux et al, 1987). Studies also suggest that human alveolar macrophages produce and release considerable quantities of lyso-PAF following stimulation with ionophore, or in the case of cells from asthmatics, specific antigen (Arnoux et al, 1987). Release of lyso-PAF may place this cell in a unique position to participate in transcellular biosynthesis of PAF by supplying other cells with the immediate precursor of PAF.

Isolated perfused lungs from sensitised guinea pigs release PAF on allergen challenge (Fitzgerald et al, 1986). Rabbit lung fragments challenged with specific antigen or monospecific antibody to rabbit IgE also release PAF, in a calcium dependent manner, with maximal
release 5-12 min. after challenge (Kravis & Henson, 1975).

It is also of interest that human endothelial cells release PAF in response to A23187, thrombin, angiotensin II, anti-human factor VIII, vasopressin (Camussi et al, 1983), \( \text{H}_2\text{O}_2 \) (Lewis et al, 1988) elastase (Camussi et al, 1988) and LPS (Lynch & Henson, 1986) as well as in response to mediators such as histamine, bradykinin or the leukotrienes (McIntyre et al, 1985, 1986) and cytokines such as IL-1 (Bussolino et al, 1986) and TNF (Camussi et al, 1987).

The amount of PAF released has been estimated at 100 to 170 pmol/10^6 cells.

Most physiological stimuli of PAF synthesis induce a response which peaks and decays within the first hour of stimulation in most cells. In contrast, PAF synthesis by endothelial cells in response to LTC\(_4\) and LTD\(_4\) is prolonged over 2 hours (McIntyre et al, 1986) while that with IL-1 and TNF is considerably delayed. Onset of PAF synthesis in response to the latter two stimuli is at 2 hours and peak production at 8-12 hours. Inhibition experiments led Bussolino et al (1988) to suggest that in endothelial cells de novo synthesis of acetyltransferase may be responsible for the delayed response. Activation of PLA\(_2\) also presumably follows this time course, and prostanoid regulatory effects have been implicated (Bussolino et al, 1988). Furthermore, it has recently been shown that when endothelial cells are activated to synthesise PAF, it rapidly appears on the external surface of the cell where it may signal neutrophil binding to the surface (McIntyre et al, 1985; Zimmerman et al, 1985).

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In addition, human epidermal cells (Cunningham et al, 1987; Michel et al, 1990) and dermal fibroblasts (Michel et al, 1987) have been reported to produce picogram quantities of PAF in vitro following stimulation with A23187.

The mechanism by which PAF is released is poorly understood, but may proceed in a monomeric fashion, or via an exocytotic process, or from shed membranes of disrupted or lysed cells. Whether albumin or other transport proteins (Matsumoto & Miwa, 1985) extract PAF from the cell surface, or whether PAF is released by one of the above mechanisms and then becomes protein-bound is unclear. Finally, whether release is a passive process or whether it is an active process is also not known (Elstad et al, 1988).

IN VIVO

Reports of the measurement of PAF in vivo are limited and have been hampered by the rapid conversion of PAF to lyso-PAF which is both its precursor and breakdown product, but which is biologically inactive. Although application of mass spectrometry to PAF analysis has now been described (Mallet & Cunningham, 1985), much of the information available regarding the generation of PAF during inflammatory responses was achieved by use of bioassay techniques, following thin layer chromatographic purification of samples. The action of PAF on platelets to cause aggregation by a cyclooxygenase and ADP independent mechanism and subsequent desensitization to PAF (Henson & Pinckard, 1977) has frequently been used (Thompson et al,
PAF release in vivo was first demonstrated by Pinckard et al (1979). A substance recovered from the plasma of rabbits undergoing IgE induced systemic anaphylactic shock was shown to have identical physico-chemical and functional properties to PAF released in vitro from antigen stimulated, washed buffy coat cells containing IgE-sensitised basophils. PAF release occurred within 60 sec. of antigen challenge and was maximal by 2-3 min.

In man, PAF (levels ranging from 2 fmols/ml to 90 nmols/ml) has been detected in the skin blister fluid of allergic subjects 5-9 hours after allergen administration to blister base sites in 8 allergic subjects (Shalit et al, 1989). PAF-like activity has also been demonstrated in whole blood taken from ragweed sensitive patients following challenge with ragweed (Cox et al, 1980) and from human lung fragments (Bogart & Stetschulte, 1974). PAF has been detected in the sputum of asthmatic subjects (Grandel et al, 1985) and in the lavage fluid of asthmatics but not in that of patients with emphysema and without bronchial hyperresponsiveness (Stenton et al, 1990a).

Recently, the concentration of lyso-PAF was reported to increase in parallel with the late-onset response to allergen challenge in atopic asthmatics (Nakamura et al, 1987), but this does not necessarily reflect the release of PAF.
2.3.c. PAF receptors

Receptors for PAF have been characterised by studies of the binding of $^3$H-labelled PAF, by analysis of the structure-activity relationships of synthetic analogues of PAF and by the characteristics of antagonism by various synthetic and naturally occurring selective receptor antagonists (Braquet & Godfroid, 1987; Parnham, 1988). In addition the transduction of the signal induced by the interaction of PAF with its receptor has recently been investigated.

Binding studies

A variety of binding experiments with $^3$H PAF have shown that high affinity binding sites exist in human platelets (Valone et al, 1982), human neutrophils (Valone & Goetzl, 1983), human lymphocytes (Ng & Wong, 1988), human endothelial cells in culture (Korth et al, 1989) and human lung membranes (Hwang et al, 1985).

Binding studies carried out at low temperatures have determined the number of specific PAF binding sites to be 250 per platelet (Kloprogge & Akkerman, 1984) and 1100 per neutrophil (Valone & Goetzl, 1983).

Eosinophils harvested from guinea pigs also have a high density of PAF receptors on their surface with which PAF interacts to induce an increase of intracellular calcium via receptor operated channels (Kroegel et al, 1989).

Binding studies with $[^3H]$ PAF have often proved difficult because of the high level of non-specific binding and the metabolism
and uptake of the radioligand. The development of antagonist radioligands has confirmed the receptor density on human platelets (Ukena et al, 1988a & b), human neutrophils (Marquis et al, 1988; Ukena et al, 1988b; Dent et al, 1988; Dent et al, 1989a) and human lung homogenates (Dent et al, 1989b). Furthermore, a common binding site for PAF and radiolabelled WEB 2086 has been demonstrated on human eosinophils from patients with the hypereosinophilic syndrome (Dent et al, 1988).

Exposure of human platelets (Chesney et al, 1985) and human neutrophils (O’Flaherty et al, 1981) to subaggregating concentrations of PAF causes a desensitization of these cells to subsequent levels of PAF that would normally induce their aggregation. Indeed, this phenomenon formed the basis for early investigations of binding sites for PAF in platelets (Henson, 1976). In most cells and tissues investigated, the high affinity binding correlates closely with the potency of PAF in stimulating a biological response (Parnham, 1988).

In several cells, including human platelets and neutrophils, low affinity binding has also been observed, but this appears to be associated with non-receptor uptake of the labelled PAF into membranes and other cellular structures (Parnham, 1988).

Receptor conformation

Although PAF contains a choline moiety within its structure, binding is unrelated to the acetyl-choline receptor, since platelet aggregation induced by PAF is not inhibited by hexamethonium.
Specific inhibitors of esterase and lipoxygenase enzymes did not affect the binding of PAF to the platelet membrane, implying that these enzymes are not associated with the PAF binding site (Shaw & Henson, 1980).

Studies based on structure-activity relationships for synthetic PAF agonists using rabbit platelet aggregation as an assay, led Braquet and Godfroid (1987) to propose a model for the putative conformation of the PAF binding site in the platelet. In this model, the C16-C18 fatty chain is inserted deeply into the membrane in a hydrophobic area probably changing membrane fluidity. Membrane activation is proposed to result from electron transfer from oxygen doublets of the ethoxide function to an unknown target in the membrane.

Valone (1984) reported the isolation of a human platelet membrane protein with an apparent molecular weight of 180,000 Da which may be the high affinity receptor for PAF. Speculation that a G-protein might co-exist with the PAF receptor within a solubilised PAF receptor complex led Chau et al (1989) to use a photoaffinity probe, which demonstrated that the PAF receptor has a molecular weight of 52,000 in rabbit platelets, which corresponds to the 65,000 Da molecular weight of the PAF receptor from human platelets reported by Shen et al (1989).

Honda et al (1991) have recently reported that the PAF receptor belongs to the superfamily of G protein-coupled receptors by using a ligand specific cloning strategy. Using the gene expression system in
the Xenopus caevis oocyte they concluded that the PAF receptor has a small in-frame tripeptide (similar to the beta_2 and dopamine_2 receptors), seven hydrophobic putative transmembrane segments (characteristic of G protein coupled receptors) and that the cytoplasmic tail contains possible phosphate acceptors (which might account for desensitisation).

Receptor sub-types.

Differences in the effects of ions and GTP on the binding of[^3H] PAF and[^3H] PAF antagonists have led to the hypothesis that ions and GTP modulate the conformation of the PAF receptor, and that multiple conformational states can exist in a single type of PAF receptor (reviewed by Hwang, 1990). Furthermore, binding of[^3H] PAF and PAF analogues is potentiated by wheat germ agglutinin or erythroagglutinin, and binding experiments suggest that WGA transforms PAF receptors from a low affinity state to a high affinity state (Hwang & Su, 1989). It is postulated that either the PAF receptor is itself a glycoprotein (so that WGA would induce a change in the conformation of the receptor) or that WGA binds not only to PAF receptors but also to an intrinsic inhibitor of the PAF receptor (Hwang & Wang, 1989).

The existence of intracellular PAF receptors (Hwang & Lam, 1986) has been confirmed by studies with PAF antagonists (Stewart et al, 1989) and is thought to mediate the effects of PAF synthesised and retained intracellularly.

Human platelets possess both intra and extra cellular PAF receptors, but these are identical, and are similar to the PAF
receptor on human lung tissue (taking into account possible affinity changes associated with conformational changes in the receptor) (Hwang & Lam, 1986; Hwang & Wang, 1989). In contrast, human neutrophil PAF receptors differ from those on human platelets in their binding characteristics under identical ionic conditions, to a variety of PAF agonists and antagonists (Hwang, 1988). It has also been suggested that the receptor on human eosinophils differs from that on human platelets (Numao et al, 1989).

Furthermore, human platelets and neutrophils differ in the G-proteins coupled to their receptors and in the effectors coupled to the receptors. PAF receptors in human platelets appear to be coupled to both adenylate cyclase and phospholipase C but not phospholipase A2, whilst PAF receptors in human neutrophils may be coupled to phospholipase A2 and phospholipase C (Hwang, 1990).

Post receptor events.

Experimental evidence suggests that the PAF receptor is coupled to a guanine nucleotide-binding regulatory protein (G-protein). PAF stimulates GTPase activity in human neutrophils and platelets, although the alpha subunit of the G protein appears to be different in these cells. The PAF receptor in platelets is thought to be linked to adenylate cyclase via an inhibitory G protein although this does not seem to be the case in human neutrophils (Hwang, 1988).

PAF has also been shown to stimulate an early degradation of phosphatidylinositol-4,5-biphosphate (PIP2) in human platelets and neutrophils via the activity of phospholipase C (PLC) (Mauco et al, 1983; O'Flaherty & Rossi, 1989). This yields two second messenger
molecules, inositol 1,4,5-trisphosphate (PIP$_3$) and 1,2-diacylglycerol (DAG). In neutrophils, PAF receptors seem to be coupled to phospholipase $A_2$ and phospholipase C but not adenylyl cyclase (Hwang, 1990).

PAF has been shown to increase intracellular calcium ion concentration in human platelets (Valone & Johnson, 1985), eosinophils (Kroegel et al, 1991) and endothelial cells (Bussolino et al, 1985) in a dose-dependent manner. These changes are largely dependent on extracellular calcium, and in human neutrophils appear to be via the activation of non-voltage dependent calcium channels (Merrit et al, 1989).

The putative mechanism of signal transduction on PAF stimulation, based on studies on human and rabbit platelets, is that PAF receptor occupation induces changes in membrane fluidity, leading to the activation of a guanyl nucleotide regulator protein with GTP hydrolysis. Phospholipase C is then stimulated initiating the phosphatidylinositol (PI) cycle of PI hydrolysis, generating PIP$_3$ which mobilizes calcium from internal pools. Diacyl glycerol, which is also produced activates protein kinase C, which together with increased calcium concentrations initiates the phosphorylation of a 20 KD myosin light chain and 40 KD protein which are essential for the secretion reaction in platelets (Yamada et al, 1988a; Hwang, 1990).
2.3.d. Biological actions in vivo and in vitro

Extensive investigation of the biological actions of PAF in vivo and in vitro have led to the suggestion that PAF may be a mediator of inflammation. The actions of PAF of relevance to its putative role as a mediator of allergic inflammation, together with its effects on airway smooth muscle will be outlined in this section.

2.3.d.i. Vasodilatation

In experimental animals, one of the most pronounced effects of PAF is systemic hypotension (Feuerstein, 1989). This hypotensive effect is instantaneous and profound and can be shown with extremely small doses of PAF (picomoles). This effect has been attributed to a direct effect on vascular smooth muscle, since substantial vasodepressor responses are observed in the rat, whose platelets are insensitive to PAF. Anti-platelet antisera also fail to affect the hypotensive response in guinea pigs and rabbits. Furthermore, vasodilatation has been observed directly in isolated perfused tissues following injection of low doses of PAF, although a constrictor response is seen in the pulmonary circulation (Goldstein et al, 1986) and in the hamster cheek pouch (Bjork et al, 1983).

In man, intradermal injection of 10 ul of $10^{-7}$ PAF resulted in instantaneous blanching of the skin followed 30-60 seconds later by peripheral erythema (10-30 mm) (Pinckard et al, 1980). The
vasodilator response to PAF is mediated by histamine, as evidenced by studies with histamine H₁ antagonists (Archer et al, 1985a; Fjellner & Hagermark, 1985; Scibberas et al, 1991).

The intratracheal installation of PAF (0.12, 0.4, 1.2 mg/kg) in humans presenting with cerebral death caused a marked fall in mean arterial blood pressure and cardiac index (Gateau et al, 1984).

Inhalation of PAF (6.35 ug initially followed by doubling doses to achieve >40% fall in Vp₃₀ or a cumulative dose of 400 ug) resulted in facial flushing 2-3 min. after inhalation, and in some subjects a drop in diastolic blood pressure was noted although this was not significant in a group of 8 subjects. The changes were short lived and returned to baseline levels within 10 min. (Cuss et al., 1986). These findings have been confirmed by Spencer et al (1990) who observed no significant changes in blood pressure, heart rate or electrocardiogram recordings following the inhalation of PAF at doses which induced a 35% fall in sGaw.

PAF has not been administered intravenously to man, but PAF antagonists which have been investigated in man to date have not induced an increase in blood pressure over a period of 4 weeks (Adamus et al, 1989).
2.3.d.ii Vascular permeability

The endothelium is a continuous layer of squamous epithelial cells. Post-capillary venules have the loosest functional organisation of the entire vascular system and a peculiar sensitivity to vasoactive substances. Changes in permeability are thought to result from ultrastructural reorganization of the inter-endothelial junctional region (Hulstrom & Svenjo, 1979) which increase the permeability to large molecules with little change in the permeability for small molecules (Curry & Michel, 1980). This change in gap junctions is thought to be the result of endothelial cell contraction (Majno et al, 1969). Three types of contractile fibres have been found within endothelial cells (Drenckhahn & Wagner, 1986). Sub-plasmalemmal and junctional webs of actin filaments are present in virtually all endothelial cells, but only the endothelial cells lining arterioles and vein valves contain the so-called stress fibres. The shape change of endothelial cells has been attributed to contraction of the actin-myosin complexes (Northover, 1988).

In this context it is of interest that contracted endothelial cells have been observed by electron microscopy in post-capillary venules of guinea pig lungs after ovalbumin anaphylaxis (Ryan & Ryan, 1984). Furthermore, in vitro studies have recently demonstrated that PAF elicits reorganization of the microfilaments and causes the endothelial cells to lose intimal contact with each other (Bussolino et al, 1986) and induces marked changes in endothelial cell shape both in culture (Grigorian & Ryan, 1987) and in intact guinea pig vessels (Northover, 1989). PAF antagonists have been shown to inhibit
the changes induced by PAF in cultured endothelial cell cytoskeletal structure (Bussolino et al, 1987) and the shape change in intact vessels (Northover, 1989).

In addition to changes in gap junctions, protein leakage across the vessel wall will be determined by blood supply, the hydrostatic pressure gradient across the vessel wall [so that vasodilators will enhance leakage (Williams & Morley, 1973)] and will vary with cell accumulation, determined by chemical stimuli in the small venules.

In vivo, in experimental animals, PAF has been shown to induce increased vascular permeability, as revealed by extravasation of vital dye or radiolabelled plasma protein, in a number of tissues. The findings in the skin and bronchial circulation will be outlined as they are of relevance to the potential role of PAF in allergic inflammation. The discussion is confined to the guinea pig, although similar data has been accumulated in the rabbit, rat and in primates. Changes have also been observed in the pulmonary circulation (Heffner et al, 1983) and the hamster cheek pouch (Bjork et al, 1983).

PAF has been shown to cause plasma protein extravasation in guinea pig skin (Stimler et al, 1981; Paul & Page, 1983) in a dose related manner. The response is complete within 15 to 30 minutes except with high doses where extravasation persists for 90 minutes (Morley et al, 1983). The response is associated with platelet accumulation (Paul & Page, 1983) but the weal induced by PAF is platelet independent, since the response is not abrogated by anti-platelet antisera in guinea pigs (Paul et al, 1984) and occurs
in rat skin, a species where platelets lack PAF receptors (Pirotzky et al, 1984). Furthermore, agents such as prostacyclin or stable analogues of prostacyclin such as ZK36374 (Iloprost) potentiate PAF induced extravasation whilst inhibiting platelet function (Archer et al, 1984a). The plasma protein extravasation response is also enhanced by vasodilator prostaglandins and verapamil, although these drugs concomitantly reduce platelet accumulation (Morley et al, 1983; Paul et al, 1984). Pretreatment with H₁ antagonists, 5-HT antagonists, cyclo-oxygenase inhibitors and cytotoxic drugs which deplete neutrophils fail to modify the response, suggesting a direct effect of PAF on the vascular endothelium (Archer et al, 1985a; Fjellner & Hagermarker, 1985; Sciberras et al, 1987).

The PPE response is inhibited by PAF antagonists (Hwang et al, 1985), beta agonists and alpha agonists (Morley et al, 1983) and by heparin (Paul et al, 1984). These observations are consistent with the interpretation that PAF acts directly at the level of the vascular endothelium to cause increased vessel wall permeability. The inference that PAF produces dysfunction of endothelial cells has been confirmed by transmission electron microscopy which demonstrates such separation associated with passage of colloidal carbon particles across the endothelial lining of post capillary venules at sites of intradermal PAF administration (Dewar et al, 1984).

PAF also induces PPE in the bronchial circulation of the guinea pig (Evans et al, 1987; O'Donnell & Barnett, 1987). The response is dose related, maximal 5 minutes after i.v. injection of PAF, and
independent of platelets as demonstrated by experiments with anti-platelet antisera (Evans et al, 1987). The response is unaltered by H1 antagonists and inhibitors of cyclooxygenase and lipoxygenase (Evans et al, 1987) but can be inhibited by PAF antagonists (Evans et al, 1987; Evans et al, 1988) and adrenaline (Boschetto et al, 1989).

PAF (8 nmol) superfused onto guinea pig tracheal mucosa in vivo produced both an acute (Persson & Erjefalt, 1986) and a late phase vascular leakiness 5 hours after provocation (Persson et al, 1987) which has not as yet been reported with any other mediator.

In human skin, the intradermal injection of PAF also induces increased vascular permeability. Nanogram doses of PAF produce a weal and hence PAF may be assumed to induce increased vascular permeability of dermal vessels (Pinckard et al, 1980; Basran et al, 1983, 1984; Dewar et al, 1984; Archer et al, 1984a & b; 1985a; Henocq and Vargaftig, 1986; Chung et al, 1987; Michel et al, 1987; Sciberras et al, 1987, 1991). Certainly, histological examination of biopsied tissues removed at the time of the weal and flare response reveals stasis of dermal capillaries, together with expansion of interstitial spaces, which are characteristic of oedema (Archer et al, 1985b).

The weal induced by PAF is enhanced by vasodilator prostaglandins (Archer et al, 1984a), but unaffected by prednisolone or cyclooxygenase inhibitors (Pinckard et al, 1980; Archer et al, 1985a). However, PAF induced weal responses can be inhibited by PAF antagonists (Chung et al, 1987) and by the H1 antagonists, azelastine (Iai et al, 1991), ketotifen (Chung et al, 1988) and terfenadine (Sciberras et al, 1991) but not by chlorpheniramine (Archer et al, 1985a).
2.3.d.iii. Activation of inflammatory cells

PAF has been found to activate numerous inflammatory cells, including neutrophils, eosinophils, platelets, monocytes, T-lymphocytes, basophils and macrophages as well as vascular endothelial cells and smooth muscle cells. In addition, PAF stimulates cells to release mediators that can activate other cells to release further mediators which in turn activate more inflammatory cells.

IN VITRO

a) Neutrophils

PAF has been shown to induce adhesion of neutrophils to endothelial cells in culture (Ingraham et al., 1982; Valone & Goetzl, 1983). PAF induces human neutrophil chemotaxis and chemokinesis (Czarnetzki & Benveniste, 1981) and stimulates neutrophil aggregation (Goetzl et al., 1980). Furthermore, PAF induces degranulation of both azurophil granules (Jouvin Marche et al., 1982) and specific granules (Dewald & Baggiolini, 1986) and stimulates the release of oxygen free radicals (Jouvin Marche et al., 1982).

Interaction with other neutrophil stimulants has been reported. Thus, PAF increases the superoxide generation induced by FMLP (Ingraham et al., 1982) as well as enhancing the chemotaxis produced by ECF-A (Czarnetzki, 1982). The latter effect is thought to be via the stimulation of LTB₄ synthesis in the neutrophil by PAF (Lin et al., 1982) since the enhancement is abolished by a dual cyclooxygenase
and lipoxygenase inhibitor (BW755C) (Czarnetzki, 1982).

PAF also stimulates the synthesis of other products of arachidonic acid metabolism, such as 5-HETE, hydroxy and dihydroxy eicosatetraenoic acids (Ingraham et al, 1982).

b) Eosinophils

PAF stimulates the adherence of human eosinophils to human vascular endothelial cells in concentrations as low as $10^{-10} \text{M}$ (Kimani et al, 1988; Lamas et al, 1988). Pretreatment with a monoclonal antibody directed against the Mac-1 complex reduced the PAF induced adherence suggesting that PAF acts at least in part by increasing the expression of these membrane surface receptors (Kimani et al, 1988).

PAF is the most potent chemotactic and chemokinetic factor for eosinophils reported to date inducing eosinophil movement at concentrations as low as $10^{-9} \text{M}$ (Wardlaw et al, 1986; Czarnetzki & Rosenbach, 1986; Tamura et al, 1987).

PAF will also induce degranulation of eosinophils with the release of proteins such as eosinophil peroxidase (Kroegel et al, 1988), glucuronidase and alkaline phosphatase from the specific granules and aryl sulphatase and acid phosphatase from the small granules (Kroegel et al, 1989) as well as stimulating the release of oxygen free radicals (Bruijnzeel et al, 1986).

Finally, PAF has been shown to induce the release of LTC$_4$ from human eosinophils (Verhagen et al, 1984), whilst other eosinophil chemotactic factors are ineffective in this regard (Bruijnzeel et al, 1987; Tamura et al, 1988).
PAF causes a greater activation of eosinophils from asthmatic patients than other atopic patients (Chanez et al, 1990). PAF has been reported to activate eosinophils in vitro by inducing a change in the density of eosinophils from normodense to hypodense (Kloprogge et al, 1989) and preliminary investigations in man indicate that the inhalation of PAF induces a change in the profile of the eosinophil of normal volunteers from normodense to hypodense in vivo (B O’Connor, personal communication).

c) Platelets.

The ability of PAF to aggregate human platelets was first described by Benveniste et al (1975). It is the most potent platelet aggregating agent identified to date, with a threshold of 0.8 nM in washed human platelets (Valone et al, 1982) and 2 nM in human platelet rich plasma (McManus et al, 1981a). PAF also induces the release reaction in platelets, the release of 5-HT (Henson & Oades, 1976), ATP (Vargaftig et al, 1980), PF4 (McManus et al, 1979) and thromboxane (Cazenave et al, 1979) have been described. Cyclo-oxygenase products are released if platelets are stimulated with doses of PAF that are sufficient to cause irreversible aggregation (MacIntyre et al, 1982).

However, the release reaction is not a prerequisite for aggregation in response to PAF (Cazenave et al, 1979) and thromboxane release does not accompany the aggregation response (Vargaftig et al, 1980). The aggregatory and release reactions to PAF exhibit selective desensitisation following exposure to PAF (Lalau-Keraly & Benveniste, 1982). Experiments using tritiated PAF have shown that exposure of
platelets results in the internalisation of PAF and its proposed receptor thus reducing the effective number of sites available for binding further PAF (Valone et al, 1982).

d) Macrophages

PAF stimulates superoxide generation in macrophages and has been shown to enhance prostanoid release (Hartung et al, 1983). Furthermore, PAF has delayed effects on macrophages. Several groups have demonstrated modulation of monocyte cytokine production (IL-1 and TNF alpha) by PAF (Barrett et al, 1987; Valone et al, 1988; Salem et al, 1990). A positive feedback loop is thus set up, since macrophages, endothelial cells and neutrophils will synthesise PAF when stimulated with these cytokines in vitro (Camussi et al, 1987; Bussolino et al, 1986; Valone et al, 1988).

e) Lymphocytes

Unlike other leucocytes, lymphocytes are unable to generate PAF and the literature on the effect of PAF on lymphocytes is conflicting, although this may be because mixed cell populations have been studied since Rola-Pleszczynski et al (1988) have recently demonstrated that PAF induces a decrease in CD 4+ T-cells accompanied by a marked increase in CD 8+ T cells.

IN VIVO

The administration of PAF by i.v. injection results in peripheral blood neutropenia and thrombocytopenia in a variety of experimental animals including the guinea pig (Vargaftig et al, 1980; Demopoulos...
et al, 1981), rabbit (McManus et al, 1980) and baboon (McManus et al, 1981b). Similar changes in peripheral blood cells have been reported after i.p. administration of PAF in the guinea pig (Morley et al, 1988).

The fall in peripheral blood leucocytes and platelets is accompanied by the accumulation of neutrophils, eosinophils and platelets in the lungs. By using a technique for continuously monitoring radio-labelled platelets and neutrophils in vivo, the transient nature of the accumulation of both platelets and neutrophils in the thoracic region of experimental animals following the i.v. administration of 10-100 ng PAF could be examined. The response was dose related, and the kinetics varied with dose, with maximal accumulation occurring between 18 and 48 seconds and returning to baseline within 1-2 to > 10 minutes with increasing doses of PAF (Page et al, 1984). Furthermore, both the platelet and neutrophil accumulation in response to i.v. PAF can be potentiated by pretreatment with the NO synthesis inhibitor, L-NAME, suggesting that NO derived from the pulmonary circulation physiologically regulates the extent of cell trapping within the lung vasculature (May et al, 1991).

The kinetics of cell accumulation are in agreement with the time course of the thrombocytopenia observed following intravenous PAF and with the histological studies which have confirmed platelet accumulation in the pulmonary vasculature following PAF treatment. Aggregates of platelets and neutrophils were noted within one minute of PAF injection (Dewar et al, 1984) and recently evidence of

Injection of PAF into the skin of experimental animals (i.d.) results in intravascular neutrophil accumulation within 15 minutes and these cells emigrate to the extravascular space over a period of hours. Aggregates of platelets have also been noted (Dewar et al, 1984; Humphrey et al, 1984).

In man, peripheral blood thrombocytopenia was noted in a preliminary study in subjects presenting with cerebral death following i.t. PAF administration (Gateau et al, 1984). However, thrombocytopenia has not been noted in studies in normal volunteers following the inhalation of PAF, although platelet activation may accompany PAF inhalation, since von Willebrand Factor (vWF) expression by platelets is induced (Wilson et al, 1990). Transient but profound neutropenia has been reported following the inhalation of PAF (Kioumis et al, 1988; Chung et al, 1988, 1989 a & b; Chung & Barnes, 1989; Spencer et al, 1990) and this is accompanied
by the emigration of neutrophils into the lungs, as evidenced by increased cell numbers in BAL fluid (Wardlaw et al, 1990).

I.d. injection of PAF in human skin results in the infiltration of neutrophils into the dermis. 24 hours after injection there is a mixed cell infiltrate predominantly consisting of histiocytes and activated mononuclear cells (Archer et al, 1985b). In contrast, in atopic volunteers, intradermal injection of PAF induced an eosinophil rich infiltrate at 24 hours, comparable to that observed following antigen administration in the same subjects (Henocq & Vargaftig, 1986, 1988; Fadel et al, 1990).

2.3.d.iv. Mucus secretion

PAF has been demonstrated to increase the output of bronchial mucus and to alter the physical properties of mucus. In anaesthetised ferrets, i.v. PAF (10^{-7} M/kg) causes an increased secretion of radiolabelled sulphate within 10 minutes of injection (Hahn et al, 1986a). In vitro studies have shown that this effect of PAF is indirect, because PAF is only active when applied to the luminal side of tracheal preparations, having no effect when administered to the submucosal side. In this preparation addition of leucocytes or platelets did not enhance the secretion induced by PAF, suggesting that secretion is independent of these cells (Wirtz et al, 1986). Histological examination of ferret trachea following PAF exposure has shown an increase in the volume and density of the gland ducts (Hahn
et al, 1986b). In vitro experiments have also shown increased mucus secretion from porcine tracheal explants which is dose-dependent and inhibited by a selective PAF antagonist but not affected by antagonists of acetylcholine, histamine, LTD$_4$ or by inhibitors of cyclooxygenase and lipoxygenase suggesting that it may be a direct receptor-mediated effect (Steiger et al, 1987).

Reduced mucociliary clearance has also been reported following administration of PAF to guinea pigs (Aursudkij et al, 1987) and more recently following inhalation of PAF by human volunteers (Nieminen et al, 1991a).

2.3.d.v. Shedding of epithelial cells

PAF, administered in vitro to guinea pig ciliated tracheal rings induces epithelial damage. This is greatly enhanced if eosinophils are also present (Read et al, 1989). In vivo, the epithelial damage induced by intravenous PAF in guinea pigs is reduced in the presence of the PAF antagonist, WEB 2086, which also reduces eosinophil infiltration (Lellouch Tubiana et al, 1988).
2.3.d.vi. Bronchoconstriction

IN VITRO

PAF does not generally induce contraction of human isolated tracheal or bronchial smooth muscle (Cerrina et al, 1983; Jancar et al, 1987). However, Johnson et al (1990) have reported that bronchi isolated from 7 out of 40 patients undergoing thoracotomy responded poorly to PAF.

In experimental animals, bronchial tissue from rabbits did not respond to PAF (Spina et al, 1991), whilst conflicting results have been reported in the guinea pig. Malo et al (1987) reported contraction of the trachea whilst Cerrina et al (1983) found no contraction of bronchi in response to PAF. Contraction of peripheral lung strips has been reported in the guinea pig (Lefort et al, 1984) and rabbit (Halonen et al, 1990) although such parenchymal preparations are known to consist largely of vascular smooth muscle.

IN VIVO

In view of the almost total lack of activity on airway smooth muscle in vitro, it is perhaps surprising that PAF is one of the most potent inducers of bronchoconstriction in vivo, described to date. Administered i.v., it can induce bronchoconstriction at concentrations as low as 10 ng/kg in experimental animals including guinea pigs (Vargaftig et al, 1980) and rabbits (Halonen et al, 1980).

The i.t. administration of PAF to rabbits (Page et al, 1985) and baboons (Denjean et al, 1983) has also been described to induce bronchoconstriction.

PAF induced bronchoconstriction was first described in man by Gateau et al (1984) in a preliminary report of the biological effect of PAF in comatose, ventilated patients. An increase in peak inspiratory pressure was observed following i.t. administration of PAF. This observation was extended by Cuss et al (1986) who reported dose-dependent bronchoconstriction following the administration of an aerosol of PAF to healthy volunteers. The effect was rapid in onset (1 min.) and resolved within two hours. No late onset changes in lung function were observed.

The effect of PAF inhalation in asthmatic patients has been studied by Chung & Barnes (1989) and Rubin et al (1987). Rubin et al failed to demonstrate a difference in the sensitivity of normals and asthmatic patients to the bronchoconstrictor effect of PAF, whilst Chung & Barnes reported similar changes in Vp30 in asthmatics following half the dose of PAF giving a similar response in normal subjects.

The mechanism of PAF induced bronchoconstriction remains unclear. Platelets or platelet products have been implicated, and products of
the platelet release reaction, rather than aggregation of platelets is thought to be important. Human bronchi will contract, in vitro, in the presence of platelets activated with PAF (Schellenberg et al, 1983). Furthermore, in vivo, the bronchoconstriction induced by i.v. PAF is abrogated by prior treatment of experimental animals with sulphipyrazone (Chignard et al, 1982) or prostacyclin (Vargaftig et al, 1980). Inhibition of the platelet release reaction with a combination of methysergide, chlorpheniramine and aspirin (Vargaftig et al, 1982; Leff et al, 1986) also inhibits PAF induced bronchoconstriction without inhibiting concomitant platelet aggregation.

In contrast, platelets do not seem to be necessary for the bronchoconstriction induced by inhaled PAF in guinea pigs (Lefort et al, 1984) or sheep (Christman et al, 1988) although they may contribute to PAF induced bronchospasm in the rabbit (Coyle et al, 1990b).

Pharmacological modulation of the PAF bronchoconstrictor response in experimental animals and in man has revealed conflicting results.

Histamine is thought to mediate, at least in part, the bronchoconstriction in experimental animals (Halonen et al, 1985; Leff et al, 1987). In man, Smith et al (1988a) showed that chlorpheniramine (8 mg 1 hour pre-challenge) caused a three fold shift of the PC35SGaw to PAF, but ketotifen (Chung et al, 1988) and azelastine (Lai et al, 1991) failed to inhibit PAF induced bronchoconstriction.

Cyclooxygenase inhibitors such as aspirin and indomethacin,
thromboxane synthetase inhibitors such as imidazole derivatives, or
the selective thromboxane receptor antagonist, EP092, have either no
effect or produce a slight to moderate reduction in the
bronchoconstriction induced by i.v. PAF (Vargaftig et al, 1980;
Bonnet et al, 1983; Lefort et al, 1984; Lewis et al, 1984). When PAF
is administered by inhalation cyclooxygenase inhibition does afford
some protection in the guinea pig (Lefort et al, 1984) and the
generation of cyclooxygenase products has been implicated in the
rabbit (McManus et al, 1983) and dog (Chung et al, 1986) although in
the baboon aspirin failed to protect against the bronchoconstriction
induced by i.t. PAF (Denjean et al, 1988).

In man, indomethacin (50 mg three times a day for 3 days and one dose
2 hours pre-challenge) provided no protection against the
bronchoconstriction induced by PAF (Smith et al, 1988a).

Although Bonnet et al (1983) suggested that lipoxygenase products
may be important, Vargaftig et al (1980) found a dual inhibitor
ineffective, Lewis et al (1984) reported that FPL55712 was effective
only at non-specific doses. Selective inhibitors of 5-lipoxygenase
failed to alter PAF induced bronchoconstriction in the guinea pig
(Barton et al, 1987). In contrast in the sheep, pretreatment with
FPL55712 abrogates the bronchoconstriction to inhaled PAF and in the
rabbit, PAF induced bronchoconstriction is inhibited by the
5-lipoxygenase inhibitor, PF-5901 (Herd et al, 1992). Two recent
clinical studies (Spencer et al, 1991; Kidney et al, 1991) have
reported that PAF induced bronchoconstriction is partially inhibited
by antagonists of LTD4.

The bronchoconstriction induced by PAF is only partially
inhibited by the beta-agonist, salbutamol, at a dose which completely inhibited a similar degree of bronchoconstriction induced by methacholine (Chung et al, 1989a). This has led to the suggestion that part of the airways obstruction following the inhalation of PAF may be via events unrelated to airway smooth muscle contraction such as airway oedema.

2.3.d.vii. Smooth muscle thickening and fibroblast deposition

Chronic administration of PAF has recently been shown to induce smooth muscle hyperplasia (Touvay et al, 1991). Guinea pigs were administered 20 ug/kg/hr PAF by subcutaneous osmotic mini-pumps for a period of 2 weeks. The lungs of these animals were congested, the bronchi and bronchioles were contracted, the epithelium showed metaplasia and the smooth muscle was hyperplastic. In addition, a significant number of eosinophils were observed infiltrating the bronchi. Furthermore, instillation of PAF into the trachea of rabbits has been shown to induce fibrosis one month later (Camussi et al, 1983a).

2.3.e. PAF inactivation

The primary route for the intracellular inactivation of PAF is catalyzed by a cytosolic acetylhydrolase (Blank et al, 1981) that hydrolyzes the acetate moiety to produce lyso-PAF. Acetylhydrolase
activity also occurs in the plasma (Blank et al, 1983; Farr et al, 1983; Stafforini et al, 1987; Sugiura et al, 1987) but this seems to be active only when associated with low density lipoproteins (containing about 70% of the total plasma acetylhydrolase activity); the remaining acetylhydrolase in plasma is bound to the high density lipoprotein fraction in an inactive form (Stafforini et al, 1987). The extracellular acetylhydrolase appears to have the same catalytic properties as the intracellular enzyme and it is thought that the serum acetylhydrolase originates from the intracellular enzyme pool (Blank et al, 1983). Catabolism of PAF also occurs in inflammatory cells, and has been documented in human neutrophils, platelets, endothelial cells (reviewed by Lee & Snyder, 1989). Interestingly, recent evidence has suggested that a group of Japanese asthmatic children are genetically deficient of the acetylhydrolase enzyme, and that this deficiency correlates with the severity of their asthma (Miwa et al, 1988).

Once the acetate is hydrolyzed from PAF by the intracellular acetylhydrolase, a microsomal transacylase rapidly acylates the lyso-PAF intermediate (Kramer et al, 1984; Chilton et al, 1987). The transacylase is highly specific for arachidonic acid and other polyenoic acids that appear to originate from phosphatidylcholine (Sugiura et al, 1987).

Direct inactivation of PAF by a phospholipase C has also been proposed (Nishihara & Ishibashi 1986; Okayasu et al, 1986) but the hydrolytic rates are considerably lower than the acetylhydrolase and, therefore the relevance of phospholipase C in PAF catabolism is questionable.
2.3.f. PAF antagonists

Numerous competitive, specific antagonists of PAF have been discovered, both synthetic and natural in origin. Three types of PAF receptor antagonists now available have been extensively reviewed elsewhere (Hosford et al, 1989), and only the PAF antagonists utilized in this thesis will be discussed.

Naturally occurring PAF antagonists may be amongst the first natural drugs utilized by man. The Chinese in particular, have always placed great importance on herbal medicines and one of the oldest of these natural therapeutic agents is the leaf-extract of the 'fossil tree', Ginkgo biloba. This tree is referred to in the medicinal book Chen Noung Pen T'sao, published in 2800 BC. In the modern Chinese pharmacopoeia, Ginkgo is still recommended as 'beneficial for the heart and lungs' and inhalation of a decoction of the leaves is used to alleviate asthma. Another plant of therapeutic value is Piper futokadsurae, which is widely used in southern China for its anti-rheumatic and anti-allergic properties.

Ginkgo biloba leaves provide the only natural source of the unique \( \text{C}_{20} \) cage molecules, the ginkgolides, while kadsurenone and other lignans with interesting pharmacological properties have been isolated from Piper futokadsurae. These two groups of chemicals, together with some gliotoxin-related compounds produced by various fungi and bacteria constitute the three groups of naturally-occurring PAF antagonists.
Ginkgolides are unique C\textsubscript{20} molecules incorporating a t-butyl group and six 5-membered rings A to F including a spiro[4.4]nanone, a tetrahydrofuran cycle and three lactone rings. These compounds were first isolated in 1932 by Furukawa from the bitter principles of Ginkgo biloba, but their structures have only been resolved recently. Ginkgolides A, B, C, and M were described by Nakamishi in 1967, and more recently ginkgolide J has been identified (Weinges et al, 1987). These structures have been renamed BN 52020, BN 52021, BN 52022, BN 52023 and BN 52024 (Institut Henri Beaufort), and differ only in the number and position of hydroxyl groups which may be present on C1, C3 or C7 of the spirononane framework.

Recent experiments using nuclear magnetic resonance and X-ray crystallography has confirmed the structures of these compounds deduced by chemical methods, although the stereochemistry of the H/R2 arrangement at the 1 position of the A ring is the reverse of that previously described (Dupont et al, 1986).

The pharmacology of the ginkgolides in vitro has been extensively studied and reviewed (Hosford et al, 1989). In vivo, the ginkgolide BN 52021 has been most extensively studied. In the guinea pig, BN 52021 inhibits PAF induced bronchoconstriction (Touvay et al, 1985), increased vascular permeability in the lung (Evans et al, 1987), and the thrombocytopenia and leucopenia (Desquand et al, 1987) which follows the administration of PAF.

The majority of PAF antagonists are synthetic in origin, and many of the early antagonists were modified derivatives of the PAF
molecule. However the lipid-like structure of these compounds resulted in limited oral absorption. More recently, new PAF antagonists of synthetic origin unrelated to the PAF structure have been developed. The benzodiazepine derivatives (alprazolam, triazolam, brotizolam) show potent PAF antagonistic properties both in vitro and in vivo. This effect is unrelated to the action as benzodiazepines, as they are specific inhibitors of PAF induced platelet aggregation, which is not blocked by the benzodiazepine receptor antagonist Ro 15-1788. Such results clearly indicated that the PAF antagonistic and central nervous system properties of these molecules could be separated (Casals-Stenzel and Weber, 1987). Modification of the structure of these widely used psychotropic drugs led to a series of compounds which retained the PAF antagonistic activity but was devoid of central effects - the prototype being the hetrazopine compound, WEB 2086. Further modification of the structure, restricting the rotation of the alkylene side chain in WEB 2086 led to WEB 2170, and the $\text{CH}_2$ analogue STY 2108. These compounds are all potent, competitive antagonists of PAF. The order of potency of the hetrazopines studied to date is WEB 2347 > WEB 2170 > WEB 2086. WEB 2086 has completed phase I clinical trials and has entered phase II trials (Heuer, 1992). To date, WEB 2086 has proved to be a potent and well tolerated PAF antagonist whether given by the oral, i.v. or inhaled route (Adamus et al, 1988, 1989, 1990; Brecht et al, 1991).

The availability of radiolabelled compounds has facilitated studies on the receptor binding characteristics of these molecules.
Radiolabelled WEB 2086 binds to human platelets with high affinity, and unlabelled PAF competes for this binding. The calculated number of binding sites for WEB 2086 per platelet (260) agrees well with the number of binding sites calculated for PAF (240; Ukena et al, 1988a). WEB 2086 binding sites have also been reported in human neutrophils (Dent et al, 1989a) and eosinophils (Ukena et al, 1989). Furthermore, WEB 2086 has been demonstrated by autoradiography in human lung sections in the peripheral airways, vessels and parenchyma but not on the segmental airways (Dent et al, 1989b).

In vitro, WEB 2086 competitively inhibits PAF induced aggregation of human platelets (Casals-Stenzel, 1987a). Inhibition of human neutrophil aggregation, beta-glucuronidase release and chemotaxis and eosinophil degranulation, superoxide production, calcium influx and chemotaxis has been described (Kroegel et al, 1989; Fukuda & Gleich, 1989). The in vitro contraction of human bronchi (Johnson et al, 1990) and isolated guinea pig lungs (Pretolani et al, 1987) is also inhibited by WEB 2086.

WEB 2086 inhibits human platelet aggregation ex vivo when given orally, i.v. and by inhalation (Adamus et al, 1988, 1989). The effect is maximal 1-2 hours after ingestion of WEB 2086 and persists for 10 to 12 hours (Adamus et al, 1988). No sedation was observed at doses of WEB 2086 up to 400 mg, and with treatment for 7 days.

In vivo, WEB 2086 and WEB 2170 administered orally, i.v. or by inhalation inhibit the bronchoconstriction induced in the guinea pig by the i.t. or i.v. administration of PAF (Casals-Stenzel et al,
PAF causes both an acute and a late-onset bronchoconstriction, and WEB 2086 blocks both these phases (Abraham et al, 1989). Recently oral WEB 2086 was shown to inhibit the bronchoconstriction induced by inhaled PAF in man (Adamus et al, 1990).

The effects of PAF on vascular permeability in the lungs of experimental animals are inhibited when this is assessed by the extravasation of Evans Blue dye (Evans et al, 1987), carbon particle deposition (O'Donnell & Barnett, 1987) or radiolabelled fibrinogen (Goldie et al, 1988). In contrast, WEB 2086 does not affect the extravasation induced by histamine or leukotriene D₄ (O'Donnell & Barnett, 1987).

In guinea pigs, intravenous WEB 2086 will inhibit eosinophil infiltration into the bronchial wall which occurs 6 hours after the inhalation of PAF (Lellouch-Tubiana et al, 1988) and prevents the accumulation of ¹¹¹ Indium-labelled platelets into the thoracic cavity (Casals-Stenzel et al, 1987). Furthermore, inhaled PAF is associated with the formation of mucus plugs and a decrease in tracheal mucus velocity associated with mucociliary dysfunction. These effects are also inhibited by WEB 2086 (Homolka et al, 1987; Lellouch-Tubiana et al, 1988).

Finally, PAF induces increased responsiveness of the airways to spasmogens, and WEB 2086 will inhibit the hyperresponsiveness to acetylcholine in guinea pigs (Dixon et al, 1989) and to carbachol in sheep (Soler et al, 1990).
2.3.g. Other drugs inhibiting the synthesis or action of PAF

Inhibitors are being sought for specific enzymatic steps involved in PAF biosynthesis. Although inhibitors of the acetyltransferase in the remodelling (inflammatory) pathway of PAF synthesis have been described (Shen et al, 1987) they are not particularly specific. Nevertheless, the fact that the enzyme activities in the de novo (physiological) and remodelling pathways for PAF synthesis are different offers great promise for the development of enzymatic inhibitors.

Interestingly, the acetyltransferase enzyme which catalyses the synthesis of PAF from lyso-PAF appears to be "switched on" in eosinophils of atopic individuals suggesting a possible abnormality of PAF production by eosinophils in allergic subjects (Lee et al, 1984).
CHAPTER 3

AIMS

As outlined in section 2, PAF fulfils the criteria for an inflammatory mediator, and mimics many of the features of asthma. PAF is the most potent inducer of bronchoconstriction and increased vascular permeability described to date, and is able to recruit eosinophils into the lungs of a variety of experimental animals. It has therefore been proposed that PAF may play a role in asthma (Morley et al, 1984). Since the BHR which follows allergen challenge is believed to result from the inflammatory reaction induced in the lung, if PAF does mediate the inflammatory events characteristic of asthma pathology, it would be predicted that PAF would induce BHR.

The first reports of PAF induced BHR came from the laboratory of Morley and co-workers, who observed whilst investigating the bronchoconstrictor properties of PAF, that repeated injections of PAF resulted in a marked sensitising effect. Formal investigation of this possibility showed that a 1 hr. infusion of PAF increased the response elicited to a fixed dose of several spasmogens including histamine, bombesin and substance P (Mazzoni et al, 1985 a,b). This observation was subsequently extended to a full dose-response curve to the spasmogen pre and post PAF, where the shift in responsiveness was found to be maximal at lower doses of PAF and the maximum contraction remained unaltered (Barnes et al, 1987; Robertson & Page, 1987; Robertson et al, 1988).
PAF has also been shown to induce BHR following aerosol administration in a variety of experimental animals including guinea pigs (Fitzgerald et al, 1987; Coyle et al, 1988a; Seeds et al, 1991) rabbits (Nieminen et al, 1991b; Spina et al, 1991) sheep (Christman et al, 1987) and dogs (Chung et al, 1986). BHR has also been reported in man (Cuss et al, 1986; Rubin et al, 1987; Townley et al, 1988a & b).

The BHR induced by intravenous PAF in guinea pigs is not due to an increase in the number or affinity of histamine or muscarinic receptors (Robertson et al, 1988) but is secondary to platelet activation (Mazzoni et al, 1985a). However this is a property of PAF not shared by other platelet agonists such as ADP, collagen and thrombin (Robertson & Page, 1987). On the basis of in vitro observations, it has been postulated that PAF may be acting to diminish the number of beta-adrenoceptor receptors on airway smooth muscle (Agrawal & Townley, 1987). However, in vivo administration of PAF did not lead to a decrease in sensitivity to beta-adrenoceptor agonists when estimated ex vivo from animals exhibiting BHR (Barnes et al, 1987).

If PAF is one of the endogenous mediators responsible for the airway inflammation which underlies the pathology of asthma, and which is now widely believed to underlie the functional increased sensitivity of asthmatic airways to spasmogens, then one would predict that PAF antagonists should modulate the response to allergen, and ultimately should be of benefit in the treatment of asthma.
The aims of this thesis were to evaluate clinically available PAF antagonists, assessing their efficacy in inhibiting PAF induced responses in human skin and human airways, and determining their effect on antigen induced cutaneous and airway responses in atopic subjects.

In addition, since the contribution of oedema to bronchial effects of PAF and antigen cannot easily be determined in man, the role of oedema in the changes in lung function induced by PAF and allergen were investigated in experimental animals.
3.1. Clinical studies

A number of diverse chemical entities have been identified which possess antagonistic activity against the actions of PAF in vitro and in vivo. At the time of commencing this thesis, only one study had been performed in man (Chung et al, 1987). In this thesis, studies designed to confirm the activity of potential antagonists against PAF in the skin and the lungs of normal volunteers are described. In addition, studies were planned where the active PAF antagonist would be used to evaluate the role of PAF in allergen induced responses in the skin and lungs of allergic subjects.


PAF is the most potent mediator of increased vascular permeability known to date (see section 2.3.d.ii.) and is a potent vasodilator (see section 2.3.d.i). The construction of a dose-response curve to PAF in human skin, measuring maximal flare areas (as an index of vasodilatation) and weal volumes (as an index of increased vascular permeability) provides a simple means of assessing the activity of potential PAF antagonists in man following oral administration.

Using this model, Chung et al (1987) were able to demonstrate that the ginkgo!ide mixture, BN52063 inhibits the weal and flare responses to PAF in a dose-dependent manner.

The triazolodiazepines brotizolam, triazolam and alprazolam have been found to selectively inhibit PAF induced human platelet
aggregation in vitro (with IC\textsubscript{50} values of 0.54, 7.6, 13.7 \text{\mu}M respectively), and to inhibit in vivo effects of PAF in guinea pigs, at high concentrations (Kornecki et al, 1984; Casals-Stenzel, 1987a).

The effect of triazolam, which is in clinical use as a sedative, on PAF induced weal and flare responses has been assessed to determine whether these PAF antagonistic activities are of relevance at therapeutic concentrations in man.

3.1.b. Investigation of the role of PAF in antigen induced cutaneous responses in man, using the PAF antagonist, BN 52063.

A double blind, placebo controlled study of the effect of BN52063 on allergen induced acute and late responses in atopic subjects, compared with its effects of PAF induced acute and late responses was performed to determine the role of PAF in the cutaneous allergic response.


A double blind placebo controlled study of the effect of the ginkgolide PAF antagonist, BN52063 on PAF induced bronchoconstriction and BHR was designed to assess the activity of this drug against the airway effect of PAF, given that the skin study of Chung et al (1987) had shown that this drug is active against PAF in the skin when administered orally 2 hr. before challenge.
3.1.d. Investigation of the role of PAF in antigen induced airway responses in man, using the PAF antagonist, BN 52063.

A double blind, placebo controlled study of the effect of BN52063 on allergen induced acute and late response in the airways of atopic subjects, and on the subsequent increase in BHR was planned, but was not performed in view of the results of experiments outlined in 3.1.b.
3.2. Experimental animal studies

3.2.a. Evaluation of PAF antagonists in guinea pig airways.

An animal model was set up to mimic the clinical challenge with PAF as closely as possible in order to further investigate the ability of PAF to induce BHR when administered by inhalation, and to determine the activity of the PAF antagonist, WEB 2086 in this experimental system.

3.2.b. Investigation of the role of PAF in antigen induced airway responses in the guinea pig, using the PAF antagonist, WEB 2086.

An animal model was set up which mimicked the clinical procedure for allergen challenge as closely as possible. The effects of such a challenge on bronchial tone, and responsiveness to inhaled methacholine were determined, and their modulation by the PAF antagonist, WEB 2086 investigated.

3.2.c. Investigation of the role of oedema in PAF and antigen induced bronchoconstriction and BHR in the guinea pig.

The extravasation of Evans Blue dye into airway tissues was determined as a measure of oedema. The degree of oedema was assessed at times of maximal bronchoconstriction and BHR after challenge with PAF or allergen, and compared with oedema formation in animals
challenged with suitable controls (lyso-PAF, BSA, allergen challenge of sham sensitised animals) and other bronchoconstrictor agents (bradykinin, formamide).
SECTION 2

MATERIALS AND METHODS
CHAPTER 4

MATERIALS

4.1 Reagents and drugs

4.1.a. Reagents

Platelet activating factor

C16 synthetic PAF and its precursor lyso-PAF were obtained from Novabiochem, Laufelfingen, Switzerland and stored at -80°C in absolute ethanol. PAF was stored at a concentration of 10 mg/ml for clinical airway studies and animal experiments, and at a concentration of 1 mg/ml (prepared at the beginning of the study) for skin studies.

For clinical studies, the stock solution was diluted in sterile saline on the day of study (and used only for 4 hr. once diluted).

For the EN52063 airway study, human serum albumin (Immuno, UK) was added to the solution to give a final concentration of 0.25%, but this was not used for subsequent experiments in view of the improbable but nonetheless possible contamination of such human blood products with the HIV virus.

For animal experiments, PAF was diluted in saline containing 0.25% (w/v) bovine serum albumin (BSA fatty acid free Fraction V, Sigma Chemical Co., Poole, Dorset, UK).
Ovalbumin

Ovalbumin (Grade V) was obtained from Sigma UK and stored at -20°C until required. Dilutions were made in sterile saline.

Methacholine

Methacholine chloride was obtained from Sigma UK and aliquoted on arrival to 128 g packets which were stored at -20°C. Each week, the aliquots were dissolved in sterile saline to a concentration of 128 mg/ml and then serial dilutions to 0.125 mg/ml were prepared in the clinical laboratory. For animal experiments, an aliquot of the 64 mg/ml solution was taken as stock solution, again used for 1 week, and was further diluted in sterile saline on the day of study.

Histamine

Histamine acid phosphate was obtained from Sigma UK and dissolved in sterile saline.

Allergens

House dust mite (Pharmalgen Dermatophagoides pteronyssinus, lot number KB 31374, Pharmacia, Uppsala, Sweden) was diluted in antigen diluent (human albumin 0.3 mg and phenol 4 mg in 1 ml saline) to a concentration of 20,000 BU *.

* Footnote: BU = biological unit equivalent to one thousandth 'histamine-equivalent-prick'.

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The stock solution was diluted in sterile saline on the day of study to the concentration required for each individual.

Grass pollen mix (Spectralgen 4 grass mix, g5, g6, g12, g13; lot number KA 31359, Pharmacia, Sweden) was diluted in antigen diluent (human albumin 0.3 mg and phenol 4 mg in 1 ml saline) to a concentration of 50,000 BU. This stock solution was diluted in sterile saline on the day of study to the concentration required for each individual.

Evans Blue dye

Evans Blue dye (Sigma UK) was diluted in sterile saline to a concentration of 30 mg/ml. The solution was filtered (5.0 um, Millipore filter) prior to use.

100% formamide was obtained from Sigma UK, and 0.9% sodium chloride for intravenous infusion from Travenol Laboratories (Thetford, UK).

4.1.b. DRUGS

CLINICAL STUDIES

BN52063.

Identical capsules of 120 mg BN52063 (which is a mixture of BN52020, BN52021, BN52022 [1:2:1]) and placebo were prepared and
randomised by Ipsen International, UK. A copy of the randomisation code was kept in a sealed envelope in the clinical pharmacology laboratory throughout the study, so that the code could be broken in an emergency.

Triazolam.

Triazolam tablets (0.25 mg) were concealed in capsules, and matched with identical lactulose placebo capsules. Although the ideal control for triazolam would have been a sedative, it was not possible to insert the short acting sedative drug temazepam in capsules.

Methoxamine hydrochloride.

Methoxamine hydrochloride (Sigma, UK) was diluted in sterile saline to a concentration of 20 umol on the day of study.

EXPERIMENTAL STUDIES

All animal experiments were conducted under diazepam premedication (5mg/ml, Roche Pharmaceuticals, UK) and Hypnorm (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml, Janssen Pharmaceuticals, UK) neuroleptanalgesia. Both were stored in sterile ampoules at 4°C.

WEB2086.

WEB2086 was obtained from Boehringer Ingelheim. A stock solution of 10 mg/ml was prepared using the recommended solvent, 1:20 HCl:saline. Further dilutions were made in sterile saline.
4.2. Equipment for monitoring lung function

4.2.a. Partial flow volume loops

Lung function was assessed using volume standardised partial expiratory flow volume curves. This method has the advantage of sensitivity (Bouhuys et al, 1969), so that small doses of constrictor agents are sufficient to produce measurable changes in lung function. The cardiovascular effects of PAF were thus minimized. The reproducibility of this parameter is of the order of 9-12% in normal subjects (Barnes et al, 1981) and about 14% after bronchoconstriction has been induced (Michoud et al, 1982).

Flow volume curves were measured using a wedged spirometer (Vitalograph, UK) and were analysed using a Hewlett-Packard microcomputer (Collingwood Measurements, Leicester, UK). Vital capacity (VC) was determined before each challenge and subsequent measurements were made from total lung capacity which does not change significantly during induced bronchoconstriction. The peak expiratory flow volume manoeuvre consists of expiration from 50-60% of VC, followed by a full inspiration to total lung capacity and determination of the expiratory flow at 30% of VC ($V_{p30}$). At least one minute was allowed between inspiration to TLC and the next partial flow volume manoeuvre, since this allows recovery of bronchial tone (Barnes et al, 1981).
4.2.b. Forced oscillation technique

Total respiratory resistance was measured by the forced oscillation technique with an Oscillaire (Jones Instruments Company, Chicago) according to the method of Landser et al (1976). The seated subject was asked to breathe quietly on a mouthpiece connected to a screen pneumotachograph while supporting the cheeks with the palms of the hands. Oscillations at regular frequencies were generated by a loudspeaker and superimposed during tidal breathing. Two identical differential pressure transducers (Validyne PM5) were used to measure mouth pressure and flow across the pneumotachograph. A Fourier analysis of the pressure and flow signals at the mouth yielded mean resistance values measured over 16 sec. periods. The superimposed signal consisted of a frequency spectrum of 4-52 Hz in steps of 2 Hz. As resistance measured at 6 Hz appeared to be the most sensitive for recording change in airway calibre in normal subjects, the mean of three consecutive measurements at this frequency was taken as a measure of airway calibre.

4.2.c. Pulmonary inflation pressure

In all animal experiments, pulmonary inflation pressure was assessed by an air filled pressure transducer (Druck Ltd, PDCR 75) connected to the afferent limb of the ventilator circuit by a lateral port. This was taken to be an index of bronchoconstriction as described by Dixon and Brodie (1903).
4.3. **Equipment for generating aerosols**

4.3.a. **Dosimeter**

All clinical studies were carried out using a breath activated dosimeter (Mefar, Brescia, Italy). All aerosols were generated using compressed air at a pressure of 22 psi. The median mass aerodynamic diameter was 4\(\mu\)m. Subjects were instructed to inhale from functional residual capacity to total capacity and breath hold for seven sec. before exhaling.

4.3.b. **Volumatic**

In order to expose conscious guinea pigs to aerosols generated by a compressed air nebuliser (Inspiron, UK) guinea pigs were placed in a volumatic (Glaxo, UK) with a perspex extension ring (Medical Physics Dept., King’s College, London) placed between the two commercially available parts to adjust the size of the container. This allowed the guinea pig some movement but did not allow the guinea pig to turn away from the aerosol jet. The nebuliser was connected to a modified inhaler which provided a tight conducting tube to the volumatic. The normal mouthpiece acted as an exit, and was connected to a long length of exhaust tubing, which was placed inside a fume cupboard.
4.3.c. Aerosol chamber

2 conscious guinea pigs were placed in a continuous flow chamber, separated from each other by a plastic panel. Aerosols were generated by a Collison atomizer using a pressure of 21 psi. The particles generated by this atomizer has been reported to be less than 0.5 um (Beijer et al, 1987). Animals were exposed for 1 hr. periods, and showed no signs of distress.

4.3.d. Ultrasonic nebuliser

Two Pulmosonic nebulisers (model 2512) [Devilbiss Health Care U.K.] were adapted as described by Lees and Payne, (1986) and inserted into the afferent limb of the ventilator circuit and arranged so that inspired air passed through one of the nebuliser chambers before entering the lungs of anaesthetised animals. This aerosol generates particles of less than 2 um. For all experiments a volume of 5ml of solutions to be nebulised was used, to ensure adequate contact with the ultrasonic membrane throughout the experiments. The availability of 2 nebulisers in parallel ensured that for most experiments only one solution was placed in a nebuliser per experiment. In experiments necessitating changes of solution, and daily at the end of experiments, the nebulisers were cleaned with glacial acetic acid in saline, followed by ethanol in saline and finally at least 10 ml saline. Each solution was nebulised to dryness to ensure that all the tubing was also cleaned.
CHAPTER 5

ANIMALS

All animal experiments were carried out using male Dunkin Hartley guinea pigs. The guinea pigs used naively weighed (350 to 500g). Animals were sensitised at 200-250g and weighed 350 to 500g by the time of challenge.

All animals were housed in the Brompton Hospital Animal house, in well ventilated steel cages with sawdust bedding which was changed every alternate day. The room was maintained at 20-22°C with a 12 hr. light dark cycle. Animals were fed a diet of chow and were allowed water ad libitum.

All experiments were carried out under anaesthesia. The animals were premedicated with 0.3 ml diazepam and anaesthetised with 0.5 ml Hypnorm i.m. Further diazepam and hypnorm were administered as determined by the return of the corneal reflex, the withdrawal response to paw pinching, and increases in blood pressure. For time course experiments, hypnorm was administered i.v. by infusion, using a Harvard infusion pump.
Volunteers were recruited from the Brompton Hospital and King's College, London. All gave written informed consent to participate in these studies, which were approved by the Brompton Hospital Ethics Committee.

6.1. Normal subjects

Subjects were excluded if they had overt diseases or clinically significant abnormalities in haematology or clinical biochemistry. Smokers were excluded from drug studies but were included in some of the pilot studies. Subjects were required to have been free of respiratory tract infections for 4 weeks prior to study, and were on no medication other than the oral contraceptive pill.

6.2. Atopic subjects

All subjects underwent intradermal injections of house dust mite or grass pollen, starting with a concentration of 0.01 units/site, to determine the amount of antigen required to produce a weal of >8 mm diameter, since it has been reported that weal diameter determines the likelihood of development of a late response (Dolovich et al,
1973). If subjects did not respond to 10 U/site they were considered unsuitable for allergen skin test studies.

Some of the subjects had allergic rhinitis and some had mild asthma not requiring any regular medication. All medication (except the oral contraceptive pill) was withheld for 24 hr. before the study.
7.1. **SKIN STUDIES**

7.1.a. **Effect of triazolam on PAF induced weal and flare responses**

Seven healthy, non-smoking male subjects, aged 18 to 35, were recruited. Subjects were randomised to receive coded capsules of triazolam (0.25 mg) or placebo in a double blind crossover fashion 1 hr. prior to skin testing. Six sites were marked on the volar aspect of both forearms according to a Latin square design. A low tension, spring loaded thickness gauge (Mitutoyo MFG Co. Ltd., Tokyo, Japan) was used to measure skin-fold thickness. Coded solutions of histamine (1 ug), PAF (50, 100, 200 and 400 ng) and saline were injected in a fixed volume of 50 ul i.d. using a 27G needle.

Cutaneous inflammatory responses were assessed by outlining the weal and flare perimeters onto transparent sheets 5, 15, 30 and 60 min. after injection. Weal and flare areas were subsequently calculated by planimetry using an Apple microcomputer (Apple Macintosh, Cupertino, California, USA) and expressed in square cm. Skin-fold thickness was measured 15, 30 and 60 min. after injection, and weal volume was calculated according to the formula weal area x 0.5 x increase in skin-fold thickness.
Statistical analysis

All values are given as mean ± s.e.m. The effect of triazolam on the responses to PAF and histamine were assessed by the Student’s paired t test with correction for multiple comparisons; a p value of < 0.05 was considered significant.

7.1.b. Effect of BN52063 on PAF and antigen induced weal and flare responses and late-onset cutaneous responses in atopic subjects

10 atopic but otherwise healthy volunteers aged 23 to 40 years (5 males and 5 females) with positive skin prick tests to common allergens were recruited. Five of the subjects were mild asthmatics requiring no maintenance therapy, 3 had allergic rhinitis and 2 had no symptoms of atopic disease. All were non-smokers.

The concentration of either grass pollen or house dust mite extract which would produce a late response following intradermal administration was determined for each individual one month prior to commencing the study, which was performed outside the grass pollen season.

Subjects were randomized to receive capsules of either placebo or BN52063 (120 mg as a single dose) in a double blind fashion 2 hr. prior to skin testing.

Study days were separated by at least 3 weeks to avoid the well documented refractory period to cutaneous antigen challenge Shaikh et al, 1977). A single batch of reagents was used throughout the study. Histamine acid phosphate was diluted to a concentration of 200 ug/ml,
PAF was diluted to a concentration of 40 ug/ml and the antigens were diluted to a concentration of 0.2 - 200 BU/ml.

Identical tubes were prepared, each containing 1 ml of each solution, and an independent investigator coded the solutions A – F according to a previously designed code. The three doses of antigen were designated as low, medium and high dose antigen.

Intradermal injections (using 27G needles) of a fixed volume of 50 ul of the coded solutions were administered to 6 marked sites on the flexor aspects of the forearms (3 on each arm) using an incomplete balanced Latin Square design to minimize bias. All subjects received histamine (1 ug) and PAF (200 ng). Three doses of allergen were administered to each subject, the medium dose being that which had previously been shown to elicit a late response. The low and high doses were 5 - 10 fold dilutions more or less dilute to compensate for any change in responsiveness during the 3 months of the study. Seven subjects were sensitive to house dust mite and received 0.01-0.1 BU (low dose), 0.5-5 BU (medium dose) and 1-10 BU (high dose). The remaining 3 subjects were sensitive to grass pollen and received 0.01 BU (low dose), 0.1 BU (medium dose) and 0.5-1.0 BU (high dose). Each subject also received a control solution consisting of the antigen diluent diluted in sterile saline to achieve the final concentration equivalent to the highest concentration of antigen in each individual.

Cutaneous inflammatory responses were assessed by outlining the weal and flare perimeters onto transparent sheets and subsequent calculation of area by planimetry using an Apple microcomputer (expressed in cm²).
A low tension, spring-loaded thickness gauge (Mitutoyo, Japan) was used to measure skin-fold thickness and weal volume was calculated according to the formula weal area x 0.5 x increase in skin-fold thickness.

Responses were assessed 5, 15, 30 and 60 min. after injection and again at 2, 4, 8 and 24 hr. after injection.

Statistical Analysis

All values are given as mean ± s.e.m. The effect of BN52063 on the responses to antigen, PAF and histamine were assessed by the Wilcoxon signed-rank test; a p value of < 0.05 was considered significant.
7.2. AIRWAY STUDIES

7.2.a. Double blind crossover trial of the effect of BN52063 on PAF induced bronchoconstriction and BHR

8 normal, non-atopic healthy non-smoking volunteers, aged 21-33, who were taking no medication other than the oral contraceptive pill (one subject), were recruited. Atopy was excluded by skin prick testing to a variety of common allergens. Baseline responsiveness was determined by measuring responsiveness to methacholine 3 times over the course of a week, using $V_{p30}$ as a measure of lung function. Subjects were randomised to receive matched capsules of either placebo or BN52063 (120 mg as a single dose) in a double-blind fashion 2 hr. prior to PAF challenge. Study days were separated by at least one month. PAF was administered at a fixed dose of 24 ug 5 times at intervals of 15 min. A 19G intravenous cannula was inserted in an antecubital vein for collecting blood samples (in 7 of the 8 subjects), for evaluation of the effects of BN52063 ingestion on routine haematological and biochemical parameters prior to commencing the study, and for full blood counts and differential white cell counts 5 and 15 min. after the first three inhalations of PAF and after completing the PAF challenge.

BHR to methacholine was measured 1, 3 and 7 days after the PAF challenge.

Methacholine inhalation challenge

Doubling concentrations of methacholine chloride (2-128 mg/ml)
were prepared in 0.9% sterile saline. Following 3 satisfactory flow volume manoeuvres, subjects inhaled 4 breaths of sterile saline from the dosimeter. \( V_{p30} \) was measured at 90 and 150 sec. after inhalation and the mean \( V_{p30} \) calculated. Starting at 2mg/ml, doubling concentrations of methacholine (4 breaths) were administered until the 90 and 150 sec. values of \( V_{p30} \) were approximately 50% below post-saline values. The provocative concentration of methacholine needed to cause a 40% fall in \( V_{p30} \) (PC\(_{40}\)) was then computed.

**PAF inhalation challenge**

After 3 satisfactory measurements of \( V_{p30} \), aerosols of PAF (1.5 mg/ml in 0.9% sterile saline containing a final concentration of 0.03% heat-treated human serum albumin) were generated from the dosimeter (nebuliser output = 12 ug/breath). Subjects were instructed to inhale for 3 sec. from functional residual capacity to total capacity and breath hold for 7 sec. before exhaling. This was repeated twice, and measurement of \( V_{p30} \) repeated 1, 3, 5, 10 and 15 min. after inhalation. The effects of repeated inhalations of PAF was studied, by repeating this procedure 5 times.

**Measurement of circulating cells**

Samples of venous blood (2ml) were taken prior to and 5 and 15 min. after the first 3 inhalations of PAF. These were collected into tubes containing disodium EDTA. Total white cell and platelet counts were measured on a Coulter Counter 880 (Coulter Electronics, Hialeah, Florida, USA). Blood smears were made from each sample and stained
with May-Grunwald-Giesma stain. Differential cell counts were performed on 100 cells from each smear by an independent observer unaware of the experimental protocol. The number of neutrophils, eosinophils, and lymphocytes per cubic mm of blood were estimated by multiplying the percentage of each cell type by the total white cell count.

Statistical analysis

All values are given as mean ± s.e.m. To assess the effect of BN52063 on PAF induced bronchoconstriction the mean percentage change in \( V_{p30} \) over the course of the study was calculated for each subject and a student t test of the differences between the means on the 2 study days calculated. Similar analyses were performed for the time course of each inhalation. This analysis avoids the problems of performing repeated t tests on a large number of time points. In addition the area under the curve was calculated using the trapezoid method for the first inhalation of PAF, and analysed using the Wilcoxon test for paired differences. A value of \( p < 0.05 \) was considered significant.

Pre challenge \( PC_{40} \) values were compared using a paired t test, after logarithmic transformation.

Changes in blood cell counts were analysed using an analysis of variance, followed by Student's t test for paired data.
7.2.b. **PAF induced bronchoconstriction and BHR, as assessed by changes in airway resistance.**

i. **Determination of PC₄₀ for PAF**

Baseline resistance was measured 3 times using the forced oscillation technique. Aerosols of PAF (1.06 mg/ml in sterile saline) were generated from the dosimeter (output 7.5 ug per breath). The subjects were instructed to inhale for 3 sec. from functional residual capacity to total capacity and breath hold for 7 sec. before exhaling. Resistance was measured 1, 3 and 5 min. after a single inhalation of PAF.

Provided a 40% increase in resistance had not occurred, subjects inhaled 2 puffs of PAF, and resistance was again measured 1, 3 and 5 min. after inhalation. This procedure was repeated, inhaling 4 puffs then 8 puffs of PAF. If a 40% increase in resistance had not been observed by this point a final 8 puffs were administered followed by lung function measurements 1, 3, and 5 min. after inhalation.

ii. **Reproducibility of PC₄₀ to methacholine using the oscillaire**

Doubling concentrations of methacholine chloride (2-128 mg/ml) were prepared in 0.9% sterile saline. Following 3 satisfactory airways resistance (RL) measurements, 21 normal volunteers inhaled 4 breaths of sterile saline from the dosimeter. RL was measured at 90 and 150 sec. after inhalation and the mean RL calculated. Starting at 2 mg/ml, doubling concentrations of methacholine (4 breaths) were
administered until the 90 and 150 sec. values of RL were approximately 50% above post-saline values. The provocative concentration of methacholine needed to cause a 40% increase in RL (PC\textsubscript{40}) was then computed. Measurements of PC\textsubscript{40} were repeated 3 times over the course of the next 14 days.

iii. Comparison of PC\textsubscript{40} to methacholine by resistance and by V\textsubscript{p}30 measurements

In 5 subjects, changes in RL and V\textsubscript{p}30 induced by methacholine were determined simultaneously. The above protocol was followed, with measurements of RL at 90 sec., V\textsubscript{p}30 at 110 sec., RL at 170 sec. and V\textsubscript{p}30 at 190 sec.

7.2.c. Effect of PC\textsubscript{40} dose of PAF on BHR

Responsiveness to methacholine (PC\textsubscript{40} RL) was measured 3 times over the course of a week to determine baseline responsiveness. Subjects inhaled PAF until a 40% increase in RL was observed as described above (PC\textsubscript{40} RL). Responsiveness to methacholine (PC\textsubscript{40} RL) was measured 1, 3 and 7 days after PAF challenge.

7.2.d. Effect of PC\textsubscript{40} dose of PAF in divided doses on BHR

4 weeks after the initial PAF challenge to determine individual PC\textsubscript{40} for PAF, the same subjects returned to the laboratory. Following 3 satisfactory baseline measurements of RL, subjects were
instructed to inhale PAF as before. However, on this occasion, the concentration of PAF in the nebuliser was individually tailored to be 20% of their PC40 dose. After measuring lung function 1, 3 and 5 min. after the initial inhalation, the same dose was administered repeatedly every five min., again measuring lung function at 1, 3 and 5 min.

Responsiveness to methacholine (PC40 RL) was determined in triplicate in the week prior to PAF challenge. Responsiveness to methacholine was determined 1 hr. prior to PAF challenge and again as soon as RL returned to baseline after challenge. Responsiveness to methacholine was repeated on days 1, 3 and 7 after challenge.

7.2.e. Effect of PC40 dose of PAF on BHR in atopic subjects

In 3 atopic subjects, a similar protocol was followed. Responsiveness to methacholine was determined 3 times over the course of a week. PAF (7.5 ug/puff) was administered every 5 min., doubling the number of puffs until a 40% increase in resistance was observed. Responsiveness to methacholine was determined 1, 3 and 7 days after challenge. Although the challenges were terminated when PC40 RL had been achieved changes in FEV1 were also monitored.

Statistical analysis

All variables derived from logarithmic dose-response curves were logarithmically transformed before statistical analysis. Changes in methacholine responsiveness, expressed in terms of doubling doses of
methacholine, were calculated using the following formula:

\[ \log_{10} \left( \frac{\text{post-treatment } PC_{40}}{\text{pre-treatment } PC_{40}} \right) \log_{2}10. \]

The expected minimum \( PC_{40} \) was calculated as described by Cartier et al (1982) and Cuss et al (1986). The difference between the expected and observed minimum were compared. Linear regression analysis was used to compare the changes in \( VP_{30} \) and \( PC_{40} \) after administration of PAF. Statistical significance was determined by two-way analysis of variance and group differences tested by Student’s t test for paired data. P values of less that 0.05 were considered significant.
7.3. **Role of oedema in PAF induced bronchoconstriction.**

A pilot study was conducted in two normal, non-smoking subjects. The alpha adrenoceptor agonist, methoxamine hydrochloride has been shown to be devoid of bronchoconstrictor properties in normal subjects (Black et al, 1982). Methoxamine hydrochloride (20 umol) or its diluent (saline) was inhaled 15 min. prior to the inhalation of PAF, in a single blind trial. Doubling doses of PAF were administered, starting with 10 µg, at 5 min. intervals, until resistance had increased by >50%.
CHAPTER 8

METHODS FOR EXPERIMENTAL ANIMAL STUDIES

Guinea pigs were weighed, anaesthetised and maintained at a constant temperature of 38°C using a Harvard small animal heating blanket with a rectal thermostat. The trachea was exposed as close to the larynx as possible, taking care not to disturb the circulation to the trachea more distally. A portex cannula (OD 1.25 mm, length 0.8 cm) was then inserted in the trachea whilst the animal breathed spontaneously.

The carotid artery was cannulated with portex cannula (OD 0.75 mm; Portex Ltd., Hythe, Kent), containing heparinised saline. The jugular vein was cannulated with a portex cannula (OD 1.02 mm) in experiments requiring the administration of drugs. In the experiments to determine the effects of inhaled PAF on vascular permeability a shortened cannula (dead space 0.2 ml) was inserted into the jugular vein for the administration of Evans Blue (EB) dye.

The carotid cannula was connected to a pressure transducer (Transamerica) which was in turn connected to a 2-channel recorder (Devices, Ormerod Ltd., Welwyn Garden City, UK) for monitoring blood pressure (BP).

The tracheal cannula was connected to a Harvard small animal ventilator via the nebuliser circuit, and a side arm connected to a pressure transducer which was in turn connected to the 2-channel recorder for monitoring pulmonary inflation pressure (PIP).
Animals were ventilated at a fixed volume of 5 cm$^3$ regardless of weight since the ventilator volume determined the output of the nebuliser. For short experiments, animals breathed room air, but for long experiments (greater than 30 min.) a mixture of oxygen and air (50:50 w/v) was used. All animals were ventilated at a rate of 60 strokes/min. Animals were allowed to stabilise for 30 min. prior to commencing experiments.

Sensitisation procedure

Animals were sensitised to ovalbumin as soon as they were weaned (weighing 200-250 g). The procedure consisted of a single 0.5 ml i.p. injection of ovalbumin (20 ug) and aluminium hydroxide (100 mg) in saline. This has been reported to induce mainly IgE antibodies (Anderson, 1980).

Control animals were injected with the same volume of aluminium hydroxide (100 mg) in saline.
8.1. AIRWAY STUDIES WITH PAF

8.1.a. Establishment of method for measuring responsiveness to inhaled methacholine

i. Concentration response curve to methacholine

Animals were weighed and anaesthetised prior to surgery for measurement of PIP and BP and ventilated for 30 min. before commencing the experiments. Aerosols of methacholine were generated every 5 min. Doubling concentrations of methacholine from 1 to 256 ug/ml were administered for 16 sec. The nebuliser was disconnected and cleansed with saline between each concentration. The PIP response was measured continuously.

ii. Time response curve to methacholine compared with saline

Animals were weighed and anaesthetised and prepared for the measurement of PIP and BP. After 30 min. stabilization on the ventilator, animals were exposed to a fixed concentration of methacholine (128 ug/ml) or saline for doubling times from 1 sec. to a maximum of 64 sec. or until a 100% increase in PIP was observed. Aerosols were administered at 1 min. intervals from 1 to 32 sec. and for 64 sec. after a 2 min. interval. The PIP response was monitored continuously. The time of exposure resulting in a 50% increase in PIP was then calculated by interpolation ($FT_{50}$).
8.1.b. **Effect of inhaled PAF on PIP and BHR to inhaled methacholine**

Animals were anaesthetised and weighed. Tracheal and carotid cannulae were inserted and the animal ventilated for 30 min. prior to commencing the experiment. A time response curve to methacholine was constructed. 15 min. later, when the PIP had returned to baseline, the animal was exposed to an aerosol of PAF (0.1 - 100 ug/ml), lyso-PAF (100 ug/ml) or BSA (0.25%) for 1 min. and the PIP response monitored. Cumulative time response curves to methacholine were the repeated at 15, 30 and 60 min. following exposure to PAF or its controls. In some animals, methacholine cumulative time response curves were also performed at 2, 4 and 6 hr. after exposure.

8.1.c. **Effect of the PAF antagonist, WEB 2086 on PAF induced bronchoconstriction**

Animals were weighed, anaesthetised and prepared for measurement of PIP and BP, and the jugular vein cannulated for the administration of drugs. After 30 min. stabilisation, WEB 2086 (1 mg/kg) or its vehicle were administered either i.v. (dilutions were prepared to give a ml/kg volume in each case; followed by 0.5 ml saline to flush the line) or i.p. Aerosols of PAF (50 - 100 ug/ml) were generated for 1 min., 5 min. after the i.v. injections, and 1 hr. after the i.p. injections. The PIP response was monitored for 5 min.
8.2. **AIRWAY STUDIES WITH ALLERGEN**

8.2.a. **Effect of inhaled allergen on PIP and BHR to inhaled methacholine**

**i. Acute Responsiveness**

Animals were sensitised (or sham sensitised) 3 weeks prior to use as described above. Animals were weighed, anaesthetised and prepared for measurement of PIP and BP. The jugular vein was cannulated. After 30 min. stabilisation on the ventilator, animals were exposed to methacholine. A cumulative time response curve was constructed, until PIP had increased by >200%. $P_{T50}$, $P_{T100}$, $P_{T150}$ and $P_{T200}$ were then calculated by interpolation from the appropriate readings. 15 min. later, when the PIP had returned to baseline, an aerosol of ovalbumin (100 ug/ml, 1 mg/ml) was generated for doubling times every 30 sec. until 100% increase in PIP was observed. Any further increase in PIP was monitored. Cumulative time response curves to methacholine were repeated 30 and 60 min. after challenge, and again in some animals 2, 4 and 6 hr. after challenge.

**ii. 24 hour Responsiveness**

Animals were sensitised 3 weeks prior to use as described above. Conscious animals were challenged with ovalbumin (100 ug/ml) for 1 hr. in pairs (1 sensitised and 1 sham sensitised animal) in the aerosol chamber. 24 hr. after challenge, animals were anaesthetised...
and cumulative time response curves to methacholine were constructed as outlined previously.

8.2.b. **Effect of the PAF antagonist, WEB 2086 on allergen induced bronchoconstriction**

Animals were sensitised 3 weeks prior to use as described above. Animals were weired, anaesthetised and prepared for measurement of PIP and BP. The jugular vein was cannulated for the administration of drugs. After 30 min. stabilisation, WEB 2086 (1 mg/kg; 10 mg/kg) or its vehicle was administered intravenously (1 ml/kg injection volume, flushed with 0.5 ml saline). 5 min. later, ovalbumin aerosol (100 ug/ml, 1 mg/ml) was generated for 1 min. and the PIP response monitored continuously for 5 min.

8.2.c. **Effect of the PAF antagonist, WEB 2086 on allergen induced BHR**

i. **Acute BHR**

Animals were weighed and anaesthetised. After surgical preparation for the measurement of PIP and BP and stabilisation on the ventilator, a cumulative time response curve to methacholine was constructed. 10 min. later, WEB 2086 (1 mg/kg, 10 mg/kg) or its vehicle were administered i.v. via the jugular vein (1 ml/kg injection volume, 0.5 ml saline flush). 5 min. later, ovalbumin aerosol (100 ug/ml) was generated for doubling times until 100%
increase PIP was observed. 1 hr. after challenge the cumulative time
response curve to methacholine (12.8 ug/ml, 128 ug/ml) was repeated.

ii. 24 hour BHR.

Animals were sensitised 3 weeks prior to use as described above.
Animals were challenged with ovalbumin (100 ug/ml) for 1 hr. in pairs
(1 sensitised and 1 sham sensitised animal) in the aerosol chamber
daily for 5 days. 1 hr. prior to each challenge, animals received 1
mg/kg WEB 2086 or its vehicle i.p.
24 hr. after the last challenge, cumulative time response curves to
methacholine were constructed.

Statistical analysis.

All variables derived from logarithmic dose-response curves were
logarithmically transformed before statistical analysis. All other
results are expressed as mean ± s.e.m. The effect of WEB 2086 on PAF
and antigen induced bronchoconstriction was determined by unpaired t
tests. The effect of WEB 2086 on antigen induced changes in PT_{50},
PT_{100}, PT_{150} and PT_{200} were determined by paired t tests.
8.3. VASCULAR PERMEABILITY STUDIES

8.3.a. Effect of intravenous PAF on bronchial oedema

Bronchial oedema was assessed by the extravasation of Evans Blue (EB) dye, which binds to serum albumin (Udaka et al, 1970; Saria & Lundberg, 1983). Guinea pigs were weighed and anaesthetised. The jugular vein was exposed and EB (30 mg/kg) was administered directly using a 27G needle, passing the needle through the pectoralis major muscle to minimize bleeding on withdrawal. 1 min. later, BSA (0.25% in saline) or PAF (1-100 ng/kg) were administered directly to the contralateral jugular vein. 5 min. after the administration of PAF or BSA, the thorax was opened and a blunt ended 13 G cannula inserted into the aorta via a left ventriculotomy. The heart was clamped, the right atrium incised to allow outflow of perfusate, and the animal was perfused with 100 ml saline (pH 5.5, 21°C) at a pressure of 100 mmHg to remove intravascular dye. The trachea, main bronchi and lungs were then removed. The main bronchi were separated from the trachea and the remaining intrapulmonary airways stripped of parenchyma. Wet weights of all tissues were recorded. EB was extracted by incubating tissues in 2 ml of 100% formamide at 37°C for 16 hr. and its concentration determined by light absorbance at 630 nm wavelength (SP 1750 spectrophotometer, Pye Unicam, Cambridge, UK) and by interpolation on a standard curve of EB dye concentrations (0.5 to 10 ug/ml). EB content of each tissue was expressed as ng per mg wet weight tissue.
8.3.b. **Effect of inhaled PAF on bronchial oedema and PIP**

For studies of the effect of inhaled PAF on bronchial oedema the above protocol was modified as follows.

Guinea pigs were anaesthetised, weighed and prepared for measurement of PIP and BP. The jugular vein was cannulated with a portex cannula (OD 0.75mm; dead space 0.2 ml) for the administration of EB dye. After 30 min. on the ventilator, EB dye (30 mg/kg + 0.2 ml to account for dead space) was administered via the jugular cannula. 1 min. later the animals were exposed to an aerosol of PAF (50 ug/ml, 1 min.), lyso-PAF (50 ug/ml, 1 min.) or BSA (0.25%, 1 min.). The PIP response was monitored continuously. The animals underwent thoracotomy and preparation for the quantification of EB dye as described above 5, 15, 30 or 60 min. after exposure to PAF.

8.3.c. **Effect of inhaled allergen on bronchial oedema and PIP**

3 weeks after sensitisation, animals were weighed and anaesthetised. They the underwent surgical preparation for the measurement of PIP and BP and the administration of EB dye as described above. After 30 min. stabilisation, animals were exposed to ovalbumin (1 mg/ml). For acute experiments, animals were exposed to ovalbumin continuously until a 100% increase in PIP was observed. Tissues were harvested for analysis of EB extravasation 5 min. after exposure.

To determine extravasation over 1 hr., the time of exposure was doubled every 30 sec. until 100% increase in PIP was achieved, or
until a maximum of 64 sec. Tissues were prepared for quantification of EB extravasation 1 hr. after exposure to the allergen.

To determine whether extravasation occurred 24 hr. after exposure to ovalbumin, guinea pigs were exposed whilst conscious to an aerosol of ovalbumin, generated by the Inspiron nebuliser. 5 ml of a 40 ug/ml solution was administered to sensitised and sham sensitised animals. 24 hr. later the animals were anaesthetised, EB dye was injected directly into the jugular vein and the tissues removed 6 min. later for quantification of EB dye extravasation.

8.3.d. Effect of inhaled bradykinin and formamide on bronchial oedema and PIP

The effects of inhaled PAF on bronchial oedema were compared with those of bradykinin and formamide. These substances were administered at concentrations which produced comparable (100% increase) changes in PIP to PAF. The protocol for the quantification of EB extravasation was identical to that for PAF at 5 min.

Statistical analysis

Data for the concentration of EB dye extractable from tissue do not approximate a Gaussian (normal) distribution but show positive skewness (Evans et al, 1987). Differences between EB extravasation were therefore compared using the Mann-Whitney U test.
SECTION 3

RESULTS
9.1. SKIN STUDIES

9.1.a. Double blind, crossover trial of the effect of triazolam on PAF induced weal and flare responses

The intradermal injection of doses of 50-400 ng PAF to normal volunteers 1 hr. after ingestion of placebo induced dose-related weal and flare responses [Fig. 3,4]. The flare area was maximal 5 min. after intradermal injection [Fig. 5] and weal volume was maximal 15 min. after intradermal injection [Fig. 6].

The response to PAF 200 ng was comparable to that induced by lug histamine, so that PAF is five times more potent than histamine in human skin [Fig. 7, 8].

Triazolam (0.25 mg) taken orally 1 hr. prior to skin testing, failed to inhibit the weal and flare responses to PAF [Fig. 7, 8], although all subjects reported a sedative effect.
Figure 3
Dose related weal response to intradermal PAF in human skin (n=7).
Figure 4
Dose related flare response to intradermal PAF in human skin \((n=7)\).
Figure 5

Time course of PAF induced flare response in human skin (n=7).
Figure 6

Time course of PAF induced weal response in human skin (n=7).
Figure 7
Effect of triazolam (●) and placebo (○) on weal response to PAF in human skin (n=7).
Figure 8
Effect of triazolam (●) and placebo (○) on flare response to PAF in human skin (n=7).
9.1.b. **Double blind crossover trial of the effect of BN 52063 on PAF and antigen induced weal and flare responses and late-onset cutaneous responses in atopic subjects**

The intradermal injection of 200 ng PAF to atopic subjects 1 hr. after ingestion of placebo induced a weal and flare response comparable to that seen in normal subjects [Fig. 9, 10].

BN52063 (120 mg) taken orally 2 hr. prior to skin testing, significantly inhibited the weal and flare response to PAF in the atopic subjects, and had no effect on the weal and flare response to histamine [Fig. 11].

The inflammatory response to PAF persisted for 4 hr. in 6 subjects. The mean weal volume was 0.025 ± 0.016 cm³, which although small, was significantly greater than that observed at the histamine and antigen diluent sites (p < 0.05, Wilcoxon signed rank test). The response had virtually disappeared by 8 hr. (mean weal volume 0.003 cm³).

Pretreatment with BN52063 attenuated the late response to PAF. The mean weal volume at 4 hr. was 0.007 ± 0.004 cm³. The response was abolished in 3 individuals and reduced from 0.134 to 0.004 cm³ in another individual, but persisted in the other 2 subjects so that statistical significance was not attained for the group as a whole.

The intradermal injection of antigen (house dust mite or grass pollen) to atopic subjects resulted in dose-related weal and flare responses [Fig. 12,13]. The weal response was maximal at 30 min. following injection of the low dose of antigen and at 60 min.
Figure 9

Weal responses to PAF and histamine (HA) in normal (open bars, n=7) and atopic (shaded bars, n=10) volunteers.
Figure 10

Flare responses to PAF and histamine (HA) in normal (open bars, n=7) and atopic (shaded bars, n=10) volunteers.

Flare Area (cm²)

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Comparison of PAF induced weal and flare with that induced by histamine. Effect of pretreatment with BN 52063 (shaded bars) compared with placebo (open bars) (n=10).

Figure 11
Figure 12

Time course and dose dependency of antigen-induced inflammatory swelling of human skin (n=10).
[diluent (●), low dose (△), medium dose (○) and high dose (▲) antigen].
Figure 13

Time course and dose dependency of antigen-induced erythema of human skin (n=10) [Low dose (△), medium dose (○) and high dose (▲) antigen].
following the injection of the medium and high dose [Fig. 12]. The flare response was maximal 15 min. after injection [Fig. 13]. Dose related late responses were also observed, and were maximal 8 hr. after injection [Fig. 12].

Pretreatment with BN52063 had no effect on the flare response to antigen [Fig. 14]. The acute weal response to the low and high dose was unaffected, and although the response to the medium dose was attenuated in some subjects this was not statistically significant (paired Students’ t test) [Fig. 15, 16]. The late response to all three doses was attenuated, at 8 hr. the responses induced by the low and medium dose of antigen were significantly reduced [Fig. 17]. The effect of BN52063 on the entire time course of the responses to the medium dose of antigen are depicted in Fig. 18 and 19.
Figure 14

Effect of BN 52063 (shaded bars) and placebo (open bars) on flare responses to antigen (n=10).
Figure 15

Effect of EN 52063 (shaded bars) and placebo (open bars) on weal response to antigen (n=10).
Figure 16

Individual weal and late responses to medium dose antigen after pretreatment with BN 52063 (BN) or placebo (P).
Effect of BN 52063 (shaded bars) and placebo (open bars) on late inflammatory responses to antigen (n=10).

Figure 17
Figure 18

Time course of swelling induced by medium dose of antigen after pretreatment with EN 52063 (•) or placebo (O) (n=10).
Figure 19

Time course of flare induced by medium dose of antigen after pretreatment with BN 52063 (●) or placebo (○) (n=10).
9.2. AIRWAY STUDIES

9.2.a. Double blind crossover trial of effect of BN52063 on PAF induced bronchoconstriction and BHR

Following pretreatment with placebo, PAF (24ug) administered by inhalation induced marked changes in $V_{P30}$ evident within 1 min. of inhalation, and maximal 5 min. after inhalation [Fig. 20]. Repeated administration of the same dose of PAF resulted in less marked bronchoconstriction [Fig. 21].

The bronchoconstrictor response to PAF was accompanied by symptoms of chest tightness, facial flushing and throat irritation, sometimes with coughing. These symptoms were also less marked with subsequent inhalations of PAF.

Pretreatment with BN52063 (120 mg) orally 2 hr. prior to challenge provided only partial protection against the bronchoconstrictor effects of PAF. The bronchoconstrictor response to the first and second inhalation of PAF were significantly reduced when analysed by the differences between the means, and the area under the curve following the initial inhalation of PAF. However, when the whole time course was analysed there was no significant difference between the responses following pretreatment with placebo and BN52063 [Fig. 22].

Bronchial responsiveness to methacholine was determined at least four times prior to PAF challenge, and on days 1, 3 and 7 after
Figure 20

Time course of PAF induced bronchoconstriction in normal volunteers (n=8).
Figure 21

Tachyphylaxis to repeated inhalation of PAF by normal volunteers, as assessed by changes in $Vp_{30}$ ($n=8$).
Figure 22

Effect of BN 52063 (○) and placebo (●) on PAF induced bronchoconstriction in normal volunteers (n=8).
challenge in 8 healthy volunteers. Mean $PC_{40}$ methacholine determined by changes in $V_{P30}$ prior to placebo was 11.2 (GSEM 1.3) mg/ml and prior to BN52063 was 10.3 (GSEM 1.4) mg/ml. The coefficient of variation for baseline $PC_{40}$ values was 22.5 ± 3.4%.

The effect of exposure to PAF (24ug, repeated 5 times at 15 min. intervals) on BHR to methacholine following pretreatment with placebo or BN52063 was assessed by comparing the group mean $PC_{40}$ on days 1, 3 and 7 after PAF challenge, and by calculating the lowest expected $PC_{40}$ based on pre-PAF values. Mean $PC_{40}$ on days 1, 3 and 7 post PAF did not differ from the pre-PAF mean $PC_{40}$ following placebo or BN52063 (2 way analysis of variance [Fig. 23]. The expected minimum $PC_{40}$ value for each subject was calculated as described by Cartier et al (1982). Using this method 5 out of the 8 subjects became "hyperreactive" on at least one occasion in the week following PAF challenge when pretreated with placebo, and six of the eight subjects were "hyperreactive" following BN52063. However the two subjects who did not become hyperreactive following placebo became hyperreactive after BN52063 so that they do not constitute PAF "non-responders". Furthermore the shift in $PC_{40}$ was less than one doubling dose in all except one subject following placebo, and in three of the six subjects following BN52063 [Table 3].

Five min. after PAF inhalation, a marked neutropenia was observed ($1.13 \pm 0.47 \times 10^9 \, l^{-1}$ compared with $3.69 \pm 0.53 \times 10^9 \, l^{-1}$ prior to the administration of PAF). This was followed by a rebound increase in neutrophil counts ($5.35 \pm 0.77 \times 10^9 \, l^{-1}$) which
Figure 23

Effect of PAF inhalation on bronchial responsiveness to methacholine in normal volunteers, after pretreatment with BN 52063 (•) or placebo (○) (n=8).
Table 3

**BHR induced by PAF as assessed by expected minimum \( PC_{40} \).**

(using \( Vp_{30} \) as a measure of lung function)

<table>
<thead>
<tr>
<th>PRE-PAF</th>
<th>EXPECTED minimum</th>
<th>OBSERVED minimum</th>
<th>BHR +ve</th>
<th>&gt;1 doubling dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>BN52063</td>
<td>Placebo</td>
<td>BN 52063</td>
<td>Placebo</td>
</tr>
<tr>
<td>1.449</td>
<td>1.3784</td>
<td>1.574</td>
<td>1.216</td>
<td>-</td>
</tr>
<tr>
<td>0.943</td>
<td>0.8724</td>
<td>0.726</td>
<td>0.483</td>
<td>+</td>
</tr>
<tr>
<td>0.555</td>
<td>0.4844</td>
<td>0.352</td>
<td>0.618</td>
<td>+</td>
</tr>
<tr>
<td>1.435</td>
<td>1.3644</td>
<td>1.451</td>
<td>0.902</td>
<td>-</td>
</tr>
<tr>
<td>0.827</td>
<td>0.7564</td>
<td>0.662</td>
<td>0.481</td>
<td>+</td>
</tr>
<tr>
<td>1.810</td>
<td>0.7394</td>
<td>0.956</td>
<td>1.141</td>
<td>-</td>
</tr>
<tr>
<td>1.340</td>
<td>1.2694</td>
<td>1.175</td>
<td>1.144</td>
<td>+</td>
</tr>
<tr>
<td>0.925</td>
<td>0.8544</td>
<td>0.600</td>
<td>0.860</td>
<td>+</td>
</tr>
</tbody>
</table>
persisted for at least 1 hr. No significant changes were observed in lymphocyte, eosinophil, red cell or platelet counts. After pretreatment with BN52063 there was a similar neutropenia, which was less marked, but not significantly different from control studies [Fig. 24].

9.2.b. Effect of PAF on bronchoconstriction assessed by changes in airways resistance

PAF, at a dose of 7.5 ug administered by inhalation induced an increase of 16.2 ± 4% in airways resistance in 9 healthy volunteers. 10 ug administered by inhalation resulted in a 58.5 ± 20% increase in resistance in a separate group of 7 healthy subjects. In a pilot study in atopic subjects 7.5 ug PAF induced a 18.5 ± 22% increase in RL in 3 subjects.

i. Determination of PC_{40} for PAF.

A formal dose response curve was not performed due to the difficulty of performing such a study in view of the tachyphylaxis observed to PAF induced bronchoconstriction. However, the effect of doubling doses of PAF on resistance was assessed in 7 normal subjects and 3 atopic subjects. Doubling numbers of puffs of a fixed concentration of PAF delivering 7.5 ug per puff were administered at 5 min. intervals. A 40% increase in RL was observed in 6 of the seven normal subjects. The limited dose response curves are depicted in Fig. 25.
Figure 24

Effect of PAF on peripheral blood neutrophil count, following pretreatment with BN 52063 (O) or placebo (●) (n=7).
Partial dose-response curves to PAF induced bronchoconstriction in normal volunteers, as assessed by changes in RL (n=7).
Mean $PD_{40}$ for the 6 subjects in whom >40% increase in RL was observed was 27.5 (GSEM 1.3) ug.

ii. Reproducibility of $PC_{40}$ to methacholine using the oscillaire.

The reproducibility of $PC_{40}$ measurements determined by changes in RL was determined in 21 subjects. Responsiveness was determined at least twice over the course of no less than one week. The coefficient of variation was $15.7 \pm 1.7\%$ for values ranging from 3.3 to 239 mg/ml.

iii. Comparison of $PC_{40}$ to methacholine by resistance and $Vp_{30}$ measurements.

In 5 subjects undergoing methacholine challenges to determine $PC_{40}$ as measured by $Vp_{30}$, the degree of change in RL, measured using the oscillaire, was also monitored. A 40% increase in RL was observed within 1 doubling dose of that required to give a 40% decrease in $Vp_{30}$ (mean difference $PC_{40}$ 0.9 mg/ml (GSEM 1.7)).

The mean $PC_{40}$ for methacholine in the 7 normal subjects was 21.38 (GSEM 1.6) ug/ml. No association was observed between $PC_{40}$ methacholine and $PC_{40}$ PAF. Thus, using the least squares method to describe the line of best fit for the data, the regression constants are $a = 0.884$ ($p > 0.05$) and $b = 0.503$ ($p > 0.05$) where $PC_{40} \text{ PAF} = a + b \times PC_{40} \text{ methacholine}$. 

-180-
9.2.c. Effect of PD$_{40}$ dose of PAF on BHR

In 7 subjects, methacholine responsiveness was determined 3 times prior to PAF challenge. PAF was given at an initial dose of 7.5 ug and doubling doses administered until a 40% increase in RL or a maximum of 120 ug PAF were given. The mean dose administered was 49.3 ± 20 ug PAF. BHR to methacholine was measured 1, 3 and 7 days after this challenge. Mean PC$_{40}$ RL to methacholine was unaltered [Fig. 26]. The expected minimum PC$_{40}$ was also calculated for each subject. Values below this minimum were observed on at least one occasion in 3 of the 7 subjects, but in each case this was less than 1 doubling dilution below the mean pre-challenge PC$_{40}$ [Table 4].

9.2.d. Effect of PD$_{40}$ dose of PAF in divided doses on BHR

At least one month after the initial study, bronchial responsiveness to methacholine was determined 3 times over a period of at least one week in the seven subjects for whom the PD$_{40}$ dose of PAF had been determined. The subjects were then re-challenged with PAF. 20% of the individual PD$_{40}$ dose of PAF was administered initially, and this was repeated 5 times at 15 min. intervals so that the PD$_{40}$ dose of PAF was administered in total. The mean bronchoconstriction observed following the initial inhalation was 30.2 ± 6% increase in RL. Tachyphylaxis was observed with subsequent inhalations [Fig. 27]. Bronchial reactivity to methacholine was repeated 1 hr. and 1, 3 and 7 days after challenge with PAF. The mean PC$_{40}$ was unaltered [Fig.
Figure 26

Effect of PAF inhalation ($PC_{40}$ PAF) on bronchial responsiveness to methacholine ($n=7$).
Table 4

BHR induced by PAF as assessed by expected minimum $PC_{40}$
(Using RL as a measure of lung function).

<table>
<thead>
<tr>
<th>Log $PC_{40}$</th>
<th>BHR +ve</th>
<th>&gt; 1 doubling dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRE-PAF</td>
<td>EXPECTED</td>
<td>OBSERVED minimum</td>
</tr>
<tr>
<td>minimum</td>
<td>minimum</td>
<td></td>
</tr>
<tr>
<td>$PC_{40}$</td>
<td>$PC_{40}/5$</td>
<td>$PC_{40}$</td>
</tr>
<tr>
<td>1.012</td>
<td>0.9733</td>
<td>0.845</td>
</tr>
<tr>
<td>1.266</td>
<td>1.2273</td>
<td>1.298</td>
</tr>
<tr>
<td>1.068</td>
<td>1.0293</td>
<td>0.849</td>
</tr>
<tr>
<td>1.351</td>
<td>1.3123</td>
<td>1.269</td>
</tr>
<tr>
<td>0.688</td>
<td>0.656</td>
<td>0.690</td>
</tr>
<tr>
<td>2.257</td>
<td>2.2183</td>
<td>2.538</td>
</tr>
<tr>
<td>1.668</td>
<td>1.6293</td>
<td>1.747</td>
</tr>
</tbody>
</table>
Figure 27

Tachyphylaxis to repeated inhalation of PAF by normal volunteers, as assessed by changes in RL (n=7).
A PC$_40$ lower than the expected minimum was observed in 5 of the 7 subjects on at least one occasion but a change of greater than one doubling dilution was observed in only 1 subject [Table 4].

9.2.e. Effect of PC$_40$ dose of PAF on BHR in atopic subjects

In a pilot study in 3 atopic subjects the effect of PAF, administered in doubling doses starting with 7.5 ug until a maximum of 60 ug had been administered or until a 40% increase in RL was observed, on bronchial responsiveness to methacholine was determined. Only one of the 3 atopic subjects achieved >40% increase in RL after 60 ug of PAF had been administered.

The mean of three determinations of baseline reactivity and the values obtained on days 1, 3 and 7 after challenge are depicted in Fig. 29. A convincing shift in responsiveness was observed in one subject on day 7 and this had returned to baseline 28 days after challenge. The maximal bronchoconstriction observed in this subject was 22% increase in RL.
Figure 28

Effect of PAF inhalation (PC_{40} PAF in divided doses) on bronchial responsiveness to methacholine in normal volunteers (n=7).
Figure 29

Effect of PAF inhalation (PC_{40} PAF) on bronchial responsiveness to methacholine in atopic volunteers (n=3).
9.3. Role of oedema in PAF induced bronchoconstriction

In a pilot study in two volunteers, the inhalation of methoxamine prior to PAF inhalation failed to inhibit PAF induced bronchoconstriction. The responses to PAF following the inhalation of methoxamine and its vehicle are illustrated in Fig. 30.
Figure 30

Effect of methoxamine hydrochloride (O) and placebo (●) on PAF induced bronchoconstriction in normal volunteers (n=2).
CHAPTER 10

EXPERIMENTAL ANIMAL STUDIES

10.1. AIRWAY STUDIES WITH PAF

10.1.a. Effect of inhaled PAF on PIP and BHR to inhaled methacholine

i. Establishment of method for measuring responsiveness to inhaled methacholine

Methacholine, administered by inhalation for 16 sec. at concentrations ranging from 1 - 256 μg/ml induced concentration related increases in PIP [Fig. 31]. Using a fixed concentration of 128 μg/ml and doubling the time of exposure from 1 to 64 sec., time related increases in PIP were observed, which were not observed following exposure in a similar manner to saline [Fig. 32]. The duration of exposure required to induce a 50% increase in PIP (designated PT50) was calculated from the individual time-response curves after log transformation. Mean PT50 was 5.7 sec. (GSEM 0.06) with 95% confidence intervals of 3.96 - 8.24 sec.

ii. PAF induced bronchoconstriction

PAF, administered at a concentration of 50 μg/ml for 1 min. induced a significant increase in PIP (99.5 ±17%) compared to its
Figure 31
Concentration response curve to methacholine induced bronchoconstriction in the guinea pig (n=4).
Figure 32

Time response curve to methacholine (•, n=50) and saline (○, n=8) induced bronchoconstriction.
precursor, lyso PAF and vehicle 0.25% BSA [Fig. 33]. The bronchoconstriction was maximal 5 min. after challenge but persisted for 30 min. [Fig. 34]. The percentage increase in PIP in the first 5 min. following the administration of PAF at concentrations ranging from 0.1 - 100 ug/kg were concentration dependent [Fig. 35].

The effect of 50 ug/ml PAF on PIP was comparable to that induced by exposure of anaesthetised ventilated guinea pigs to 5 mg/ml bradykinin for 1 min. (91.0 ± 9% increase in PIP, n=4) and to 410 ug/ml methacholine for 16 sec. (92.2 ± 18% increase in PIP, n=4). Histamine (100 ug/ml for 1 min.) induced a 188.4 ± 5% increase in PIP (n=4).

iii. PAF induced BHR

Methacholine responsiveness was measured in anaesthetised guinea pigs prior to and 0.5, 1, 2, 4 and 6 hr. after the inhalation of PAF (10 ug/ml) in 5 animals. A reduction in the mean log $PT_{50}$ was observed following PAF inhalation, with the most marked reduction 1 hr. after the administration of PAF [Fig. 36]. However, when a further 6 animals were studied measurements of log $PT_{50}$ 1 hr. after PAF were not significantly different from pre-challenge values [Fig. 37-39]. 1 hr. after PAF challenge PIP had returned to pre-PAF values. The time-response curve to methacholine was shifted to the left. This was largely due to a reduction in the threshold exposure time needed to induce bronchoconstriction.

PAF, at a concentration of 100 ng/ml administered for 1 min. had little effect on the time-response curve to methacholine assessed 1
Figure 33

Effect of exposure for 1 minute to PAF (50 ug/ml) on PIP, as an index of bronchoconstriction, compared with lyso-PAF (50 ug/ml) and BSA (n=5-14).
Figure 34

Time course of PAF induced bronchoconstriction (n=14).
Figure 35
Concentration response curve to inhaled PAF induced bronchoconstriction (n=5-14).
Figure 36

Time course of PAF induced BHR (n=5).
Figure 37

Time response curve to methacholine before (○) and 1 hour after (●) the inhalation of PAF (10 ug/ml, 1 min; n=11).
Figure 38

Individual guinea pig responses to methacholine before (●) and after (○) exposure to PAF (0.1-100 μg/ml).
Figure 39

Methacholine responsiveness before (○) and 1 hour after 1 min inhalation of: A. BSA (0.25%)  B. lyso PAF (10 μg/ml)  C. PAF (100 ng/ml)  D. PAF (1 μg/ml)  E. PAF (10 μg/ml)  F. PAF (100 μg/ml)  (n=3-11).
hr. after challenge [Fig. 40] and there was no significant change in the mean log $PT_{50}$ [Fig. 39]. The $PT_{50}$ after PAF was reduced in 2 of the 3 animals [Fig. 38].

PAF, at a concentration of 1 ug/ml administered for 1 min. induced a leftward shift in the time-response curve to methacholine assessed 1 hr. after the PAF challenge. Baseline PIP was similar, the threshold exposure time required to induce bronchoconstriction was reduced, and a parallel shift was observed between 8 and 64 sec. [Fig. 41]. The log $PT_{50}$ was reduced after PAF exposure in each of the three animals [Fig. 38], but whilst the mean log $PT_{50}$ was reduced the mean log $PT_{100}$, log $PT_{150}$ and log $PT_{200}$ were unchanged [Fig. 42].

PAF, at a concentration of 100 ug/ml administered for 1 min. induced a leftward shift of the time response curve to methacholine assessed 1 hr. after PAF challenge. However, this shift was largely due to the fact that this concentration induced persistent bronchoconstriction, although there was a slight increase in the response to methacholine administered for 1 sec. [Fig. 43]. Log $PT_{50}$ was reduced in 4 of the 5 animals studied after exposure to PAF [Fig. 38].

Exposure of guinea pigs to lyso-PAF [Fig. 44] and 0.25% BSA [Fig. 45] resulted in much less marked leftward shifts of the time response curve to methacholine. Log $PT_{50}$ values were not significantly altered 1 hr. after exposure to lyso-PAF 10 ug/ml or 0.25% BSA [Fig. 39].
Figure 40

Time response curve to methacholine before (●) and 1 hour after (○) the inhalation of PAF (100 ng/ml, 1 min.; n=3)
Figure 41

Time response curve to methacholine before (●) and 1 hour after (○) the inhalation of PAF (1 μg/ml, 1 min.; n=3)
Figure 42
Responsiveness to methacholine before (●) and 1 hour after (○) inhalation of PAF (1 μg/ml, 1 min) (n=3).
Figure 43

Time response curve to methacholine before (O) and 1 hour after (★) the inhalation of PAF (100 ug/ml, 1 min.; n=5)
Figure 44

Time response curve to methacholine before (●) and 1 hour after (○) the inhalation of lyso-PAF (100 μg/ml, 1 min; n=5).
Figure 45

Time response curve to methacholine before (●) and 1 hour after (○) the inhalation of BSA (0.25% 1 min; n=6).
10.1.b. Effect of the PAF-antagonist, WEB 2086 on PAF induced bronchoconstriction

WEB 2086, administered i.v. 5 min. prior to PAF challenge significantly inhibited the bronchoconstriction induced by inhaled PAF (100 ug/ml for 1 min.) when compared to that induced following pretreatment with 0.05% HCl, the vehicle for WEB 2086 [Fig. 46]. WEB 2086 administered i.p. 1 hr. before challenge with PAF (50 ug/ml) significantly inhibited the bronchoconstriction induced by PAF when compared with its vehicle [Fig. 47].
Figure 46

Effect of WEB 2086 (1 mg/kg i.v.) and its vehicle on PAF (100 ug/ml, 1 min) induced bronchoconstriction (n=3).
Effect of WEB 2086 (1 mg/kg i.p.) and its vehicle on PAF (50 ug/ml 1 min) induced bronchoconstriction (n=3).
10.2. AIRWAY STUDIES WITH ALLERGEN

10.2.a. Effect of inhaled allergen on PIP and BHR methacholine

Animals were sensitised according to the method of Anderson (1980) which has been reported to induce predominantly IgE antibodies. The sensitisation procedure was well tolerated by the majority of animals. Occasional deaths occurred within hr. of the sensitisation injection, presumably due to bowel perforation. However, the majority of animals gained weight normally and showed no overt signs of respiratory distress or increased secretions over the 3 week sensitisation period.

Challenge of sensitised animals with ovalbumin (100 ug/ml for 1 min.) by inhalation resulted in bronchoconstriction as assessed by an increase in PIP. The bronchoconstriction was maximal 5 min. after challenge and gradually resolved over 1 hr. [Fig. 48]. Exposure of sensitised animals to 1 ug/ml ovalbumin for 1 min. resulted in less severe bronchoconstriction [Fig. 49]. Exposure of sensitised animals to doubling exposure times of aerosol of a fixed concentration of 100 ug/ml resulted in dose related increases in PIP [Fig. 50, 51]. 100% increase in PIP was reached in 20 of 23 animals after exposure to ≤ 64 sec. ovalbumin [Fig. 51]. The mean exposure time was 25.2 ± 6.3 sec. This protocol was therefore followed in all subsequent experiments. Challenge of "sham" sensitized animals resulted in no or only slight increases in PIP [Fig. 52].

Sensitisation did not alter responsiveness to methacholine. Thus,
Figure 48

Time course of antigen (OVA 100 ug/ml, 1 min) induced bronchoconstriction (PIP) (n=8).
Figure 49

Effect of i.v. WEB 2086 [1mg/ml (†); 10 mg/ml (++)] and its vehicle (open bars) on antigen induced bronchoconstriction (n=3-6).
Figure 50

Time response curve of antigen (OVA, 100 ug/ml) induced bronchoconstriction (n=22).
Figure 51

Cumulative bronchoconstrictor response to antigen, determination of PC\textsubscript{100}.
Figure 52

Effect of i.v. WEB 2086 [1mg/kg, (○); 10 mg/kg (□)] and its vehicle (open bars) on antigen induced bronchoconstriction, and on the exposure time required to induce bronchoconstriction (n=3-6).
3 weeks after sensitisation or "sham" sensitisation, time response curves to methacholine did not differ significantly from those in untreated animals [Fig. 53].

i. Acute Responsiveness.

Bronchial responsiveness to methacholine was increased after exposure to ovalbumin. BHR was maximal at 1 hr., but persisted for up to 6 hr. [Fig. 54]. 1 hr. after challenge with ovalbumin as described above, the mean $PT_{100}$ was reduced from 8.7 sec. to 0.5 sec. in sensitised animals, but remained unchanged in "sham" sensitised animals [Fig. 55a & b]

ii. 24 hour Responsiveness.

The effect of antigen on BHR 24 hr. after challenge was assessed in groups of sensitised and sham sensitised animals by 2 methods.

The group of sensitised animals challenged with ovalbumin in the "volumatic" chamber showed signs of bronchoconstriction, whilst those challenged in the aerosol chamber with ovalbumin (100 $\mu$g/ml for 1 hr.) did not show any obvious tachypnoea or respiratory distress.

24 hr. after challenge the "volumatic" group showed significant differences in responsiveness to methacholine compared to "sham" sensitised animals challenged in a similar manner with saline [Fig. 56]. Following exposure of sensitised and "sham" sensitised animals in pairs to ovalbumin in the aerosol chamber there was no difference in the threshold exposure time inducing a response. However, the
Figure 53

Time response curve to methacholine in untreated (○, n=50), sham sensitised (○, n=18) and sensitised (●, n=64) animals.
Figure 54

Time course of antigen induced BHR (n=6).
Figure 55a

Effect of allergen challenge on methacholine responsiveness in sham sensitised (●●●) and sensitised (▲▲▲) animals compared to responsiveness in untreated animals (open bars) (n=18-26).
Figure 55b

Time response curves to methacholine before (open) and 1 hour after (closed) exposure of sham sensitised (triangles) and sensitised (circles) animals to antigen (n=18-26).
Figure 56

Methacholine time response curves 24 hours after antigen challenge in sensitised (●) and sham sensitised (○) animals (n=5).
response induced was larger in sensitised animals than in "sham" sensitised animals. A statistically significant increase in response was observed for exposure times of 1-8 sec. of methacholine 128 ug/ml.

The effect of repeated antigen exposure on BHR was assessed by performing a time-response curve to methacholine 24 hr. after the 5th of daily challenges with ovalbumin 100 ug/ml for 1 hr. to sensitised and "sham" sensitised animals in an aerosol chamber. There was no difference between sensitised and "sham" sensitised animals pretreated with the vehicle for WEB 2086 i.p. 4 hr. prior to each challenge. However, the responsiveness to methacholine was greater than that observed in sensitised and "sham" sensitised unchallenged animals [Fig. 57a & b].

10.2.b. Effect of the PAF-antagonist, WEB 2086 on allergen induced bronchoconstriction

WEB 2086, 1mg/kg administered intravenously 5 min. prior to antigen challenge partially inhibited the bronchoconstriction induced by ovalbumin 1 ug/ml for 1 min. A further reduction in the bronchoconstrictor response was achieved by pretreatment with WEB 2086, 10 mg/kg [Fig. 49]. WEB 2086, 10 mg/kg also partially attenuated the response to ovalbumin 100 ug/ml for 1 min.
Figure 57a

Methacholine time response curves 24 hours after repeated antigen challenge, following pretreatment with WEB 2086 (●) or its vehicle (○), in sham sensitised animals (n=4-5).
Figure 57b

Methacholine time response curves 24 hours after repeated antigen challenge, following pretreatment with WEB 2086 (•) or its vehicle (○), in sensitised animals (n=4-5).
10.2.c. **Effect of the PAF-antagonist, WEB 2086 on allergen induced BHR**

i. **Acute BHR.**

Pretreatment of sensitised animals with 1:20 HCl in saline, the vehicle for WEB 2086, i.v. 5 min. prior to ovalbumin challenge did not affect the BHR observed 1 hr. following antigen challenge [Fig. 58, 59].

Pretreatment with WEB 2086 1 mg/kg 5 min. prior to antigen challenge with ovalbumin 100 μg/ml for doubling times of exposure until 100 % increase in PIP was observed failed to protect against the BHR induced by antigen challenge [Fig. 60, 61]. In contrast, pretreatment with WEB 2086, 10 mg/kg intravenously 5 min. prior to ovalbumin challenge significantly reduced the magnitude of the shift in the methacholine time response curve to methacholine [Fig. 62, 63]. Log PT$_{100}$, PT$_{150}$ and PT$_{200}$ were not significantly different after ovalbumin challenge (paired t test, p > 0.05) although a significant shift was observed in the PT$_{50}$ (paired t test, p < 0.05). The bronchoconstriction induced by ovalbumin in the presence of WEB 2086 was not significantly different from that induced in the presence of its vehicle (by nature of the design of the experiment), however WEB 2086 did provide partial protection against the bronchoconstriction induced by antigen, since the exposure time required to induce 100% increase in PIP was significantly prolonged (Mann Whitney U test, p < 0.05) [Fig. 52].
Figure 58

Time response curve to methacholine before (O) and 1 hour after (●) exposure to antigen, following pretreatment with WEB 2086 vehicle (n=7).
Figure 59
Effect of WEB 2086 vehicle on antigen induced BHR (n=6).
PT<sub>50-200</sub> PIP before (●) and 1 hour after (○) challenge.
Figure 60

Time response curve to methacholine before (●) and 1 hour after (○) exposure to PAF, following pretreatment with WEB 2086 (1 mg/kg; n=6).
Figure 61

Effect of WEB 2086 (1 mg/kg) on antigen induced BHR (n=6).
PT$_{50-200}$ PIP before (●) and 1 hour after (○) challenge.
Figure 62

Time response curve to methacholine before (○) and 1 hour after (●) exposure to PAF, following pretreatment with WEB 2086 (10 mg/kg; n=6).
Figure 63
Effect of WEB 2086 (10 mg/kg) on antigen induced BHR (n=7).
PT$_{50-200}$ PIP before (●) and 1 hour after (○) challenge.
ii. 24 hour BHR.

Time-response curves to methacholine 24 hr. after the 5th of daily challenges with ovalbumin 100 μg/ml for 1 hr. were not different in sensitised and "sham" sensitised animals pretreated with WEB 2086 (1 mg/kg), or its vehicle, i.p. 4 hr. prior to each challenge [Fig. 57a & b].
10.3. VASCULAR PERMEABILITY STUDIES

10.3.a. Effect of intravenous PAF on bronchial oedema

PAF administered i.v. to anaesthetised guinea pigs at doses of 1-100 ng/kg induced dose-related bronchial oedema. Oedema was assessed by the extravasation of Evans blue dye, which binds to serum albumin, over 5 min. after PAF challenge. Responses in the trachea [Fig. 64], main bronchi [Fig. 65] and intrapulmonary airways [Fig. 66] were compared with responses to lyso-PAF and vehicle (0.25% bovine serum albumin). Significant increases in the extravasation of dye were produced by 5 ng/kg in the trachea and main bronchi, and 10 ng/kg in the intrapulmonary airways (Mann Whitney U test, p < 0.05).

10.3.b. Effect of inhaled PAF on bronchial oedema and PIP

PAF, administered by inhalation at a concentration of 50 μg/ml for 1 min. to anaesthetised, ventilated guinea pigs failed to induce significant extravasation of EB dye determined by the same technique [Fig. 67]. Furthermore, the extravasation of EB dye between 10 and 15 min., 25 and 30 min. and 55 and 60 min. after challenge with PAF was comparable to that seen in the first five min. [Fig. 68].

10.3.c. Effect of inhaled allergen on bronchial oedema and PIP

The contribution of oedema to the bronchoconstriction and BHR
Figure 64

Dose related extravasation of Evans Blue dye induced by intravenous PAF in the trachea of the guinea pig (n=3-16).
Dose related extravasation of Evans Blue dye induced by intravenous PAF in the main bronchi of the guinea pig (n=3-16).
Figure 66

Dose related extravasation of Evans Blue dye induced by intravenous PAF in the intrapulmonary airways (n=3-16).
Figure 67

Effect of inhaled PAF (50 ug/ml, 1 min; ▲), lyso-PAF (50 ug/ml, 1 min; ○) and BSA (0.25%, 1 min; □) on the extravasation of Evans Blue Dye in guinea pig airway tissues (n=5).
Figure 68

Time course of Evans Blue extravasation after inhalation of PAF (50 ug/ml 1 min) in the guinea pig (n=3-5).

- **Trachea**
- **Main Bronchi**
- **Intrapulmonary Airway**
- **Bladder**

![Graphs showing time course of Evans Blue extravasation](image-url)
induced by antigen challenge in sensitised animals was assessed by measuring the extravasation of EB dye, which binds to albumin. Extravasation of dye was not significantly different in sensitised animals challenged with ovalbumin to achieve a 100% increase in PIP from that observed in "sham" sensitised animals when assessed over the first 5 min. following challenge [Fig. 69], and between 55 and 60 min. after challenge, when the animals demonstrated maximal BHR [Fig. 70].

In addition, sensitised animals were challenged consciously in the "volumatic" chamber with an aerosol of ovalbumin (approximate dose aerosolised 200 ug). This procedure induced tachypnoea in all animals and some became cyanosed. 24 hr. later, there was no obvious increase in secretions, nor respiratory distress. The guinea pigs were anaesthetised and the extravasation of EB dye over a 5 min. period measured. The extravasation was not significantly different from that observed in a group of "sham" sensitised animals challenged in the same manner with saline [Fig. 71].

10.3.d. Effect of inhaled bradykinin and formamide on bronchial oedema and PIP

The effect of PAF was compared with that of bradykinin and formamide. Formamide administered by inhalation for 1 min. induced significant bronchoconstriction that was accompanied by an increase in the extravasation of EB dye when compared with saline in the trachea, main bronchi and intrapulmonary airways (Mann Whitney U test, p < 0.05). Bradykinin at a concentration of 1 ug/ml induced
Figure 69

Extravasation of Evans Blue dye into guinea pig airway tissues 5 minutes after antigen challenge of sensitised (shaded bars; n=7) and sham sensitised (open bars; n=6) animals.
Figure 70

Extravasation of Evans Blue dye into guinea pig airway tissues 60 minutes after antigen challenge of sensitised (shaded bars) and sham sensitised (open bars) animals (n=4).
Figure 71
Extravasation of Evans Blue dye into guinea pig airway tissues 24 hours after antigen challenge of sensitised (shaded bars, n=10) and sham sensitised (open bars, n=9) animals.
minimal bronchoconstriction (% increase in PIP 12.7 ± 9%) and no significant increase in the extravasation of EB dye compared to saline (Mann Whitney U test, p >0.05) in any airway tissue. Bradykinin at a concentration of 100 ug/ml induced a 41.3 ± 14% increase in PIP, and an increase in the extravasation of EB dye into the trachea (P<0.05) and intrapulmonary airways (P<0.05). The extravasation of EB dye into the main bronchi was increased but failed to attain statistical significance (P=0.06) [Fig. 72, 73].
Figure 72

Bronchoconstriction induced by inhaled saline (open bars, n=5), bradykinin (0.1 mg/ml ( ), 1 mg/ml ( ), and 5 mg/ml (+); n=5) and formamide ( n=4) in the guinea pig.
Extravasation of Evans Blue dye into guinea pig airway tissues 5 minutes after inhalation of saline (open bars), bradykinin [(0.1 mg/ml (X), 1 mg/ml (□))] or formamide (Δ) (n=4-6).
DISCUSSION

SECTION 4
In human skin, the potent pro-inflammatory effects of PAF in normal volunteers have been confirmed and extended to atopic volunteers. The activity of the PAF antagonists, triazolam and BN52063 have been evaluated, and the ability of BN52063 to influence the response to cutaneous allergen challenge in atopic subjects studied in a double blind, placebo controlled, cross over study.

The intra-dermal injection of PAF resulted in a weal and flare response in normal and atopic subjects. Following pre-treatment with placebo, dose related increases in weal volume and flare area were observed in healthy volunteers, which were comparable to the results of Basran et al (1983; 1984), Archer et al (1984 a & b) and Chung et al (1987). The time course of the cutaneous responses were similar to those reported by Archer et al (1984b). In addition to a comparable acute response to PAF, some atopic subjects also had a more persistent inflammatory response similar to that described by Basran et al (1984) and Archer et al (1984b) in normal subjects.

The triazolobenzodiazepine, triazolam, which is an effective short acting hypnotic (Pakes et al, 1981), has been shown to possess PAF antagonistic activity both in vitro and in vivo in experimental animals (see section 2.3.f.). Its activity as a PAF antagonist in man had not previously been determined. Triazolam, at a dose of 0.25 mg failed to modify the weal and flare response to PAF in normal subjects. The discrepancy between this study and the findings in experimental animals may be a dose effect,
since Casals-Stenzel (1987a) reported that a much higher dose, 200 mg/kg of triazolam, administered orally 1 hr. prior to challenge provided complete protection against the reduction in respiratory flow induced by an infusion of PAF (30 ng/kg/min.) in guinea pigs.

It therefore remains possible that triazolam may possess PAF antagonistic activity if administered at higher doses, but such a study was precluded by the marked sedation experienced at a dose of 0.25 mg. These results indicate that antagonism of endogenous PAF is unlikely to contribute to the clinical effects of triazolam.

Recently, another triazolobenzodiazepine, alprazolam, which has weaker PAF antagonistic activity in vitro and in vivo than triazolam (Casals-Stenzel, 1987a), was also reported to be devoid of PAF antagonistic activity in man, when administered orally at the therapeutic dose for sedation 1 hr. prior to the intra-dermal injection of PAF (Ormerod et al, 1989).

It is possible that analogues of triazolam rejected on the basis of a lack of sedative effect may retain PAF antagonistic activity. In addition to such compounds, systematic modification of the triazolobenzodiazepine structure led to the development of WEB 2086, which has recently been reported to be a non-sedating, selective, orally active PAF antagonist in man (Adamus et al, 1989; Brecht et al, 1991).

The ginkgolide family of naturally occurring PAF antagonists (see section 2.3.f.) have been prepared for clinical use as a mixture of BN 52020, BN 52021 and BN 52022 referred to as BN 52063. BN52063 has previously been shown to inhibit the cutaneous response to PAF in

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normal subjects in a dose-related manner (Guinot et al, 1986; Chung et al, 1987). The observations of Chung et al (1987) have been extended to atopic volunteers. A single oral dose of BN52063 (120 mg) was found to reduce the weal and flare response to 200 ng PAF without significantly affecting the response to histamine. The late response to PAF was abolished in 3 subjects by pre-treatment with BN52063, but the reduction in the group as a whole failed to attain statistical significance. This may be explained partially by the small number of subjects studied (only 6 subjects had a late response) and by the fact that the visible response is small so that reduction is difficult to quantify. Nonetheless, definite cellular changes have been observed in human skin following the administration of PAF both histologically (Archer et al, 1985b; Markey et al, 1990) and using the skin window technique (Henocq & Vargaftig, 1986; 1988; Fadel et al, 1987). It has also recently been reported that the novel anti-allergy drug cetirizine inhibits PAF-induced eosinophil infiltration in human skin, although this is not via PAF antagonistic activity (Leprevost et al, 1988; Fadel et al, 1990).

The PAF antagonist BN52063 was used to evaluate the role of PAF in the cutaneous response to allergen in atopic subjects.

The acute and late responses to 3 doses of antigen (either house dust mite or grass pollen) were measured over 24 hr. in 12 atopic subjects. Dose related increases in the flare area, weal volume and the magnitude of the late response were observed. BN52063, administered as a single oral dose of 120 mg 2 hr. prior to
skin testing had no effect on the flare response, in agreement with previous observations that this component of the response is largely mediated by histamine and a cutaneous axon reflex (Smith et al., 1980; Phillips et al., 1983; Bedard et al., 1983).

Pre-treatment with BN52063 reduced the acute weal response to the medium dose of allergen in half of the subjects but this did not achieve statistical significance, and no reduction of the weal response to the low or high doses of allergen were observed. This suggests that PAF is not an important mediator of allergen induced cutaneous microvascular permeability. The fact that BN52063 did not significantly inhibit the early response to allergen and did not affect histamine-induced responses also indicates that this drug is not having any effect on cutaneous mast cell histamine release or acting as an $H_1$ antagonist.

The volume of the late-onset inflammatory swelling following both the low and medium dose of antigen were significantly inhibited by pre-treatment with BN52063. This suggests that PAF plays an important role in mediating the late response to antigen. The high dose of antigen resulted in an extensive late response, and whilst this was attenuated by BN52063, this failed to reach statistical significance. The high antigenic load may have resulted in the activation of other inflammatory mechanisms, which cannot be accounted for by PAF alone. However, in the absence of data with a higher dose of BN52063, it is plausible that this dose of BN52063 was not able to completely antagonise all the PAF released. Recent studies using the skin chamber technique have demonstrated the presence of nanomolar concentrations of PAF in antigen treated sites 3 to 9 hr. after
incubation with antigen (Michel et al, 1988; Shalit et al, 1989) and skin window experiments have shown that PAF is equi-potent to antigen at recruiting eosinophils to cutaneous challenge sites in atopic subjects (Henocq & Vargaftig, 1986; 1988). It is therefore possible that the role of PAF in the late response is related to its potent chemo-attractant effects on eosinophils (Wardlaw et al, 1986; Henocq & Vargaftig, 1986). In this context, it is interesting to note that following pre-treatment with cetirizine, PAF is no longer detectable in the chamber media and eosinophil infiltration at sites of allergen challenge is significantly reduced (Michel et al, 1988).

PAF administered by inhalation to normal subjects has been reported to induce dose-dependent decreases in $V_{p30}$ (Cuss et al, 1986). $V_{p30}$, like $FEV_1$ is thought to be a measure of the flow-resistive properties of small airways (Mead et al, 1967). The precursor and metabolite of PAF, lyso-PAF, and the diluent for PAF (ethanol, saline and HSA) had no effect on pulmonary measurements. Tachyphylaxis to the pulmonary effects of multiple doses of PAF was observed as previously reported by Cuss et al (1986).

These findings have been confirmed in a double blind cross over study of the effect of BN 52063 on the airway effects of PAF. Thus, following pre-treatment with placebo, subjects inhaling a fixed dose of PAF of 24 ug experienced acute changes in $V_{p30}$, which were evident at 1 min. and maximal at 5 min. after inhalation. The bronchoconstrictor response to PAF was associated with feelings of chest tightness, tracheal irritation and transient facial flushing.
Repeated administration of the same dose of PAF was associated with a lesser reduction in $V_{p30}$ and fewer symptoms.

These observations have recently been confirmed by Chung et al (1989) in a study of similar design and Lai et al (1990) demonstrated a mean fall of $V_{p30}$ of $65.9 \pm 8.94\%$ following 100 ug PAF. In contrast, Rubin et al (1987) failed to demonstrate a significant change in $V_{p30}$ following the administration of increasing concentrations of PAF from 0.1 to 1000 ug/ml at ten min. intervals, although a decrease in $SGaw$ of 35% or more was observed in 5 of the 6 subjects following the highest dose of PAF. The dose of PAF administered to the airways in this study cannot be calculated from the data given. However, only 2 of the subjects complained of flushing and warmth, suggesting that the starting dose was smaller than that used in our $V_{p30}$ studies. Furthermore, albumin was not added to the PAF solution. It is known that PAF binds to albumin, and that this increases the effect of PAF in human skin (Archer et al, 1985a). It is therefore possible that tachyphylaxis developed before doses of PAF which would alter $V_{p30}$ were administered. It is likely that this explains the disparate results, since in the reproducibility study, Rubin et al (1987) also observed that a single inhalation of 1000 ug/ml produced a significant decrease in $V_{p30}$ of 19.5%.

Increases in airways resistance were also observed in normal subjects following inhalation of PAF, indicating an effect on large airways (Macklem & Mead, 1967) as well as small airways. Increasing the dose of PAF inhaled by normal volunteers from 7.5 ug to 10 ug...
resulted in a marked increase in the degree of bronchoconstriction, suggesting that the response is concentration dependent. Further evidence for the dose-dependency of the effect of PAF on airways resistance was obtained by administering doubling doses of PAF at 5 min. intervals. An incremental increase in RL was observed by doubling the dose of PAF, although the response may be blunted by the presence of tachyphylaxis. Tachyphylaxis of the RL response was evident when a fixed dose of PAF was administered repeatedly every 15 min., as observed when $V_{D30}$ was used as the index of bronchoconstriction.

More recently, Spencer et al (1990) have reported the effects of inhaled PAF on specific airway conductance ($SGaw$) and flow at 30% of VC above RV ($Vmax30$). In 6 healthy volunteers PAF induced a fall in $SGaw$ of 69% and of $Vmax30$ to 72% of control values after the inhalation of diluent.

In a pilot study of non-asthmatic atopic subjects, although PAF induced a similar mean increase in RL, the response was very variable and a $PD_{40}$ was only reached in 1 of the 3 subjects. In contrast, in normal subjects a $PD_{40}$ was achieved in 6 of the 7 subjects despite tachyphylaxis to the bronchoconstrictor effects of PAF by commencing with a dose of 7.5 ug and doubling the dose every 5 min. This suggests that atopic subjects are less sensitive to PAF than normal subjects or that they develop tachyphylaxis to PAF more rapidly.

Rubin et al (1987) observed a similar variability in the bronchoconstrictor response to PAF in mild asthmatic subjects. Dose-related decreases in $SGaw$ and $FEV_1$ but not $V_{D30}$ were
observed following repeated inhalations of PAF at concentrations from 0.1 to 1000 ug/ml. However, 3 of the 6 subjects failed to respond to the highest dose of PAF, whereas in a similar group of 6 normal subjects there was only one non-responder.

This is in contrast to the findings of Chung & Barnes (1989) who found that atopic asthmatic subjects consistently bronchoconstricted to PAF with a 28.9 ± 4.2% fall in Vp30 after 12 ug and a 44.5 ± 8.5% fall in Vp30 with the subsequent inhalation of 24 ug. It is difficult to compare these results with the data obtained in normal subjects, because the initial dose of PAF was halved, but it would appear from the magnitude of the response to 12 ug that these subjects may have been more sensitive to the bronchoconstrictor effects of PAF.

No relationship was found between the sensitivity of normal airways to PAF and the sensitivity to methacholine. This is consistent with the findings of Cuss et al (1986) measuring changes in Vp30, and Rubin et al (1987) measuring SGaw, although it is difficult to interpret such results in view of the tachyphylaxis to the pulmonary effects of PAF. Chung et al (1989a) reported a lack of correlation between the sensitivity to methacholine and PAF in atopic asthmatics.

Lung function was not monitored beyond the duration of PAF challenge, since Cuss et al (1986) failed to observe a late response to PAF when lung function was monitored hourly for 8 hr. and again at 24 hr. Rubin et al (1987) have confirmed these observations. 6 normal
subjects were challenged with PAF at concentrations ranging from 0.1 to 1000 ug/ml at ten min. intervals. The challenge was discontinued when a 35% decrease in SGaw or a 30% decrease in Vp30 was achieved or after the inhalation of the highest dose. A fall in SGaw of 35% or more was observed in 5 of the 6 subjects. No late responses were observed 0.5, 1, 2, 4, 6 and 24 hr. after a PAF challenge when changes in SGaw, Vp30 and FEV1 were monitored.

Analysis of peripheral blood changes associated with the bronchoconstriction induced by 24 ug of PAF in normal volunteers revealed a profound neutropenia in association with the initial bronchoconstriction, but there was no significant fall in the platelet count. The neutropenia was maximal 5 min. after challenge and was followed by a rebound neutrophilia by 15 min. Similar changes in neutrophil counts have been observed by other investigators (Chung et al, 1988; 1989 a & b; Kioumis et al, 1988; Spencer et al, 1990) in normal subjects and Chung & Barnes (1989) in asthmatic subjects. Thrombocytopenia has been observed following the intra-tracheal instillation of large doses of PAF (0.12 - 1.2 mg/kg) to humans exhibiting cerebral death (Gateau et al, 1984) and in the baboon, the intra-tracheal administration of PAF resulted in a fall in the peripheral platelet count in association with bronchoconstriction (Denjean et al, 1983). The dose used in this study is much lower than that used by Arnoux et al (1988) which may explain the lack of thrombocytopenia, especially since it has been shown in experimental animals that although only modest amounts of PAF are needed to induce thrombocytopenia in anaesthetised guinea pigs (Vargaftig et al, 1980)
much higher doses are required in conscious animals (Demopoulos et al, 1981) and in other species such as rabbits (McManus et al, 1980) and baboons (McManus et al, 1981b).

It is also possible that this dose did induce a fall in platelet count which may have recovered by the time of sampling since animal studies have suggested that platelet accumulation in the thoracic region of experimental animals in response to i.v. PAF is maximal at 1 min. (Page et al, 1983) i.e. at a time prior to maximum bronchoconstriction. Perhaps of more relevance than platelet numbers is platelet activation, and the recent demonstration of an increase in the expression of von Willebrand factor by platelets after PAF inhalation in man is of interest (Wilson et al, 1990).

The mechanisms of the neutropenia and platelet activation remain unknown. Human neutrophils possess high affinity binding sites for PAF (Valone et al, 1982) and PAF is chemotactic for neutrophils both in vitro (Czarnetzki & Benveniste, 1981) and in vivo in human skin (Archer et al, 1985b). PAF promotes the adhesion of neutrophils to vascular endothelium in culture (Ingraham et al, 1982) and the administration of PAF to experimental animals is associated with the infiltration of the lung by platelets and neutrophils. Furthermore, in human volunteers BAL fluid obtained following PAF challenge is associated with an increase in neutrophil numbers compared to challenge with the precursor lyso-PAF (Wardlaw et al, 1990) (see section 2.3.d.iii). Recently, neutrophil accumulation in human lungs exposed to PAF has been documented (Tam et al, 1991) so that the peripheral neutropenia may reflect increased margination and adhesion of neutrophils in the pulmonary circulation and their migration into
Although it is possible that the changes in neutrophil counts are secondary to mechanical changes in the pulmonary vasculature consequent upon bronchoconstriction this seems unlikely since the kinetics of the responses differ, especially with repeated PAF exposure. Repeated challenge with the same dose of PAF resulted in smaller changes in lung function when assessed by changes in RL and Vp30. This phenomenon of tachyphylaxis to the bronchoconstrictor response to PAF has previously been described in guinea pigs (Lefort et al, 1984) and in man (Cuss et al, 1986). Similarly, the neutrophil changes were tachyphylactic, but in contrast to the bronchoconstrictor response which was still detectable after 5 inhalations of PAF, neutropenia was observed only after the first inhalation, and was followed by a rebound neutrophilia. Further evidence for the dissociation between the bronchoconstrictor and neutropenic effects of PAF are provided by the findings of Spencer et al (1991) who showed that whilst the cysteiny1 leukotriene antagonist SK&F 104353-Z partially inhibits the bronchoconstrictor response to PAF in man, the neutropenia was unaffected. Conversely, the infusion of prostacyclin inhibited the neutropenia but failed to inhibit the bronchoconstriction induced by inhaled PAF (Kioumis et al, 1988).

The response of blood elements to PAF in vitro has been extensively studied. Following exposure to PAF, rabbit platelets neither release nor aggregate when subsequently challenged with PAF (Lalau-Keraly and Benveniste 1982) yet retain their reactivity to other agonists. Similar desensitisation has been described in human platelets (Chesney et al, 1985) and human neutrophils (O’Flaherty et
Although the mechanism of desensitisation is uncertain, exposure of human platelets to $^3$[H] PAF results in internalisation of PAF (and its proposed binding site) thus reducing the effective number of binding sites available for binding further PAF (Valone et al, 1982). If PAF induced bronchoconstriction in man is platelet dependent, as has been described in guinea pigs and rabbits in vivo (Vargaftig et al, 1980; Halonen et al, 1981) it is plausible that internalisation of platelet PAF receptors results in desensitisation to the bronchoconstrictor response to PAF.

BHR is a descriptive term for the sensitivity of the airways to a wide variety of non-sensitizing bronchoconstricting stimuli of chemical or physical origin. In asthma, the dose response curve to methacholine or histamine is not only shifted to the left but also differs from normal dose response curves in slope and maximal response (Michoud et al, 1981; Woolcock et al, 1984). It has therefore been suggested that the term hypersensitivity be used to describe the leftward shift (e.g. changes in $PC_{20}$) and that the term bronchial reactivity be reserved for changes in slope (Orehhek et al, 1977). The specificity and sensitivity of $PC_{20}$ in the objective measurement of BHR in asthmatic subjects compared with that in normal subjects have been described (Hopp et al, 1984) and this index has been shown to reflect the clinical state of asthma (Hargreave et al, 1981).

Although Orehek advocated measurement of the slope of the dose-response curve, subsequent studies have either failed to show
any differences in slope (Hargreave et al, 1981) or shown a poor relationship between the slope of the log dose-response curves and the clinical state of asthma (Beaupe & Malo, 1981). Malo et al (1985) whilst able to distinguish subjects with symptoms of bronchial hyperexcitability from normal subjects on the basis of the slope of the dose-response curves to methacholine or histamine found that, using the standardised method of doubling histamine and methacholine concentrations from one nebulization to the other, satisfactory assessments of curves could be done in only 30% of the subjects. Furthermore, they found that an extra dose (beyond that necessary to achieve PC$_{20}$) had to be given in some asthmatics.

In normal subjects the dose response curve reaches a reproducible maximal response plateau to both histamine (Woolcock et al, 1984) and methacholine (Sterk et al, 1985a). This phenomenon is not due to tachyphylaxis to methacholine, and is unlikely to be due to a change in the dose caused by a change in site of deposition of the higher concentrations of methacholine. It is also unrelated to the bronchodilator effect of a deep breath (Sterk et al, 1985b). In contrast asthmatic patients are capable of reaching more severe obstruction with higher or immeasurable plateau levels (Woolcock et al, 1984; Sterk et al, 1986). It has been suggested that this should be referred to as hyperresponsivity (Ariens, 1987).

Preliminary investigations in our laboratory failed to demonstrate convincing plateau responses to methacholine using RL in normal subjects (G. Nicholl, personal communication), despite administering doses of methacholine which produced palpitations,
flushing and salivation. Measurements of BHR in human subjects have therefore been limited to measurements of hypersensitivity i.e. changes in $PC_{40}$ methacholine measured by either $VP_{30}$ or RL.

The inhalation of PAF did not result in BHR to methacholine, measured by $PC_{40}VP_{30}$ 1, 3 and 7 days after the PAF challenge.

Morley et al put forward the hypothesis in 1984 that "it is the inflammatory sequelae of exposure to Paf-acether including an effect of platelets or their products on airway smooth muscle, that causes a reduced threshold to activation stimuli and also induces hypertrophy of smooth muscle". Numerous investigators have shown that PAF administered intravenously or by inhalation to a variety of experimental animals will induce a shift in the dose response curves to histamine, acetylcholine, bombesin, substance P and 5-HT (see chapter 3).

The ability of PAF to induce an increase in non-specific bronchial responsiveness in non-asthmatics subjects was first demonstrated by Cuss et al (1986) and provided the first evidence of persistent changes in bronchial responsiveness in man following the administration of an inflammatory mediator.

In order to determine the effect of BN52063 on PAF induced bronchoconstriction and BHR the protocol described by Cuss et al (1986) was modified slightly. In the Cuss study subjects inhaled doubling doses of PAF every 5 min. starting with 6.25 ug until $VP_{30}$ had dropped by 40% or a cumulative dose of 400 ug had been delivered. In subjects who did not achieve 40% decrease $VP_{30}$ the test was repeated on another day starting with a higher initial dose.
The PD_{40} of PAF was calculated only from those response curves where a >40% drop was induced by two or three doses of PAF. This design could not be followed in a double blind drug trial without the inclusion of a third and possibly fourth (if PD_{40} PAF were not achieved) limb to the study. At the time of commencing the study of the effect of BN52063, a study of the effect of ketotifen on PAF induced bronchoconstriction and BHR was in progress in the laboratory, where a fixed dose of PAF was administered 5 times to each subject over 1 and a half hr. This protocol resulted in BHR (Chung et al, 1988) and was therefore adopted for the BN52063 study.

However, the mean PC_{40} methacholine in the 8 subjects studied did not change after PAF inhalation, in contrast to the findings of Cuss et al (1986) who reported a change in PC_{40} from 10.8 to 4.3 mg/ml on day 3. Although the minimum PC_{40} in the week following PAF was below the expected minimum in 5 of the 8 subjects on at least one occasion, the magnitude of the change was small, and well within the variability of the measurement, given that the coefficient of variation for baseline PC_{40} in our laboratory was 22.5%. These results are at variance with the findings of Chung and colleagues who have reported a decrease in PC_{40} in 3 days after PAF challenge (5 x 18 ug, from 62.9 (GSEM 2.69) to 23.3 (GSEM 2.34) mg/ml, Chung et al, 1988; 6 x 18 ug, from 17.1 (GSEM 1.40) to 8.7 (GSEM 1.44) mg/ml, Wardlaw et al, 1990; 5 x 24 ug, 18.2 (In sem 0.285) to 6.25 (In sem 0.198) mg/ml, Chung et al, 1989a; 55 x 18 ug, from 12.8 (GSEM 1.98) to 7.9 (GSEM 1.79) mg/ml, Chung et al, 1989b) using identical methodology to that adopted in this thesis.

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There are numerous possible explanations for the failure to reproduce the findings of Cuss et al (1986). Firstly, the partial flow volume loop manoeuvre is difficult to execute consistently, and failure to inspire to TLC can cause significant changes in the measurements. Although all subjects received training in the technique, it is possible that the variability in the measurement of $PC_{40}$ was partly due to errors of technique. The simpler oscillaire method of determining lung function was therefore adopted. Using this method, the concentration of methacholine required to induce a 40% decrease in $V_{P30}$ induced approximately the same degree of increase of RL. The $PC_{40}$ methacholine was therefore used as an index of responsiveness for all studies using the oscillaire. The method was found to be more reproducible than the $V_{P30}$ method (coefficient of variation 15.3%).

Adhering strictly to the protocol of Cuss et al (1986) but using RL as an index of lung function, PAF administered repeatedly at 15 min. intervals at a dose of 20% $PD_{40}$ failed to modify the mean $PC_{40}$ methacholine measured 1 hr. or 1, 3 or 7 days. Again small, clinically insignificant deviations of the $PC_{40}$ outside the expected minimum range were observed in some subjects. In addition, administration of PAF to induce a 40% increase in RL also failed to modify the responsiveness to methacholine significantly.

Another difference between these studies and those of Cuss, is that 3 atopic subjects were included in the study of Cuss et al (1986). A preliminary study of the effect of challenge with PAF (to achieve a 40% increase in RL) in 3 atopic subjects did show a convincing shift in the $PC_{40}$ methacholine in 1 subject on day 7.
which recovered by day 14.

In addition, 2 of the subjects who took part in the bronchoconstrictor study of PAF did not take part in the BHR study, and a further subject did not become hyperresponsive, so that in fact 3 out of the 8 subjects studied by Cuss et al (1986) were also potential or actual "non-responders".

Rubin et al (1987) reported that a single inhalation of PAF at a concentration of 100 ug/ml increased the sensitivity of the airways to methacholine as determined by the PC\textsubscript{35} SGaw. However, the authors do not indicate that the statistical analysis was performed on log transformed data, so that the significance of the data presented is uncertain. The degree of sensitisation was 2.5 fold, which may be within the error of the measurement, which is not evaluated. Furthermore, this change in sensitivity was assessed within 1 hr. of PAF challenge, and its duration was not monitored. The most interesting feature of the data of Cuss et al (1986) was the similarity in the time-course of the BHR to that observed in asthmatics following allergen challenge.

Numerous other investigators have now disputed the findings of Cuss et al (1986). Lai et al (1990) found that the inhalation of 3 single doses of PAF (100, 200 and 400 ug) and a control solution consisting of a single PC\textsubscript{40}Vp30 dose of methacholine administered in a double blind fashion on four separate occasions to 9 normal subjects (4 atopic) failed to alter methacholine responsiveness (PC\textsubscript{20} FEV\textsubscript{1} methacholine) measured 3, 7 and 24 hr. and 2, 3, 4, 7, 10 and 14 days after each challenge. In all groups PAF caused bronchoconstriction with a dose-response relationship. Spencer et al
(1990) also failed to demonstrate any significant increase in BHR to methacholine (PC_{30} V_{max30}; PC_{35} S_{Gaw}) in any of their 6 subjects (1 atopic) on two separate occasions over a 2 week period following PAF challenge. Furthermore, in a study designed to investigate the role of thromboxane in mediating PAF induced bronchoconstriction and PAF induced BHR, Stenton et al (1990b) found that BHR after PAF was poorly sustained, poorly reproducible and not readily detectable. 12 subjects (2 atopic) underwent challenges with increasing doses of PAF from 0.09 to 290 μg of PAF until a greater than 40 % fall in V_{p30} or a maximum cumulative dose of 422 μg had been administered. 10 of the 12 subjects underwent acute bronchoconstriction to PAF, but whilst the geometric mean PD_{40} V_{p30} methacholine 24 and 72 hr. following PAF challenge was significantly reduced the changes were small in individual subjects. Although 7 subjects showed some BHR, 5 of these subjects were re-studied after placebo premedication, and only 3 underwent increases in methacholine sensitivity on a second occasion, so that the group mean was not significantly different to the pre-PAF mean PD_{40} V_{p30} methacholine. In contrast, the bronchoconstrictor response to PAF was reproducible in the subjects re-studied.

Four other groups have reported negative results in abstract form (Yamada et al, 1988b; Townley et al, 1988 a & b; Gebre-Michael & Leuenberger 1989; Di Maria et al, 1990). Thus, using a variety of methods for measuring lung function and administration of PAF, at least 4 groups have been unable to demonstrate PAF induced BHR in human volunteers.
Furthermore, both Chung & Barnes (1989) and Rubin et al (1987) have failed to demonstrate heightened BHR in asthmatic subjects following the administration of PAF by inhalation, although this has been interpreted as reflecting desensitisation to the effects of exogenous PAF due to the endogenous release of PAF by inflammatory mediators.

BN52063, administered orally 2 hr. prior to PAF challenge at a dose of 120 mg had only a partial inhibitory effect on the bronchoconstrictor response to inhaled PAF and did not significantly alter the neutropenia or rebound neutrophilia induced by inhaled PAF.

The dose of BN52063 used is the maximum for which toxicological data is available and has previously been shown to inhibit the response to 200 ng PAF intra-dermally by 60% when administered orally two hr. prior to skin testing (Chung et al, 1987). Data regarding the bio-availability of BN52063 after oral administration are difficult to obtain since no specific assay is available at present. The inhibition of PAF induced cutaneous responses in normal human skin is maximal 2 hr. after pre-treatment with BN52063 but is still evident 8 hr. after pre-treatment (Guinot et al, 1987a). It therefore seemed reasonable to administer the drug 2 hr. prior to challenge with PAF.

Following pre-treatment with BN52063 the responses to repeated challenges with PAF were partially inhibited. In view of the multiple measurements made following each PAF challenge, the data has been analysed using a t test for the differences between the means for each subject following pre-treatment with placebo and BN52063,
providing a simple measure of the differences between the time courses. This analysis reveals a significant difference in the response to the first and second inhalation but not in the responses to subsequent inhalations. Although the trend is maintained in the latter half of the study, and given the small number of volunteers studied may have achieved statistical significance had a larger number of subjects been studied, the clinical significance of such changes are questionable since there is marked tachyphylaxis to the PAF response by this time and the observed differences in Vp30 (about 10%) would not be associated with symptomatic improvement.

In addition the area under the curve has been calculated following the first inhalation, but not for subsequent inhalations, since the analysis of subsequent inhalations is complicated by the fact that pre-challenge Vp30 is not basal, nor equal on each study day. This analysis confirms a significant inhibition of the response to the first inhalation of PAF. Although this confirms the activity of this compound in man, the degree of protection afforded is insufficient to allow this drug to be used as a tool in the investigation of the role of PAF in allergen induced responses in the lung.

The failure of BN52063 to inhibit the bronchoconstriction induced by the inhalation of PAF in normal volunteers precluded its use to evaluate the role of PAF in the airway response to allergen in atopic subjects. At the time of completion of the practical aspects of this thesis, no other PAF antagonist was available for clinical use.
An animal model was therefore set up to assess the effect of the PAF antagonist, WEB 2086 (see section 2.3.f.) which was the most potent PAF antagonist available when the animal studies were commenced, on allergen induced bronchoconstriction and allergen induced BHR to methacholine, if this were observed. In order to confirm the activity of WEB 2086 in this animal model, the effects of pre-treatment with WEB 2086 on PAF induced bronchoconstriction and any ensuing BHR was first assessed.

A technique was described by Lees & Payne (1986) for adapting ultrasonic nebulizers for use in the ventilator circuit normally used for the measurement of PIP in anaesthetised ventilated guinea pigs by the method of Dixon & Brodie (1903).

Using this technique, PAF (0.1 to 100 ug/ml for 1 min.), administered by inhalation to anaesthetised guinea pigs induced dose-related bronchoconstriction which was not observed with its precursor lyso-PAF or vehicle 0.25% BSA. The response was maximal 5 min. after inhalation and persisted for 30 min. These data are consistent with those of Lefort et al (1984) who first reported the effects of aerosolized PAF in guinea pigs. The administration of 115-330 ug/ml for 2 min. via a medical aerosolator apparatus to anaesthetised animals paralysed with pancuronium and artificially ventilated resulted in bronchoconstriction within 2 to 3 min. which peaked at 5 to 8 min. and persisted for 15 to 20 min. This response was accompanied by systemic hypotension but no thrombocytopenia was observed. No significant fall in blood pressure was noted with the lower concentrations of PAF used in these studies, as was observed by
WEB 2086, administered either i.v. 5 min. prior to an aerosol of PAF, or i.p. 1 hr. prior to challenge significantly inhibited the bronchoconstrictor response to PAF. These results are consistent with the findings of Casals-Stenzel et al (1987). WEB 2086 was shown to inhibit PAF (0.05 uM) induced platelet aggregation in vitro with an IC$_{50}$ of 0.17 uM and to inhibit PAF induced platelet accumulation in the lungs of guinea pigs in vivo. Furthermore i.v. (0.005-1 mg/kg), oral (0.05-2 mg/kg) or inhaled WEB 2086 (0.25 - 10 mg/ml for 3 min.) protected guinea pigs from death and inhibited in a dose dependent manner the decrease in respiratory flow and blood pressure induced by the i.v. infusion of PAF (30 ng/kg/min.). Similar results were obtained when PAF (300 ug/kg) was administered locally by intra-tracheal instillation (Casals-Stenzel et al, 1987). The ability of WEB 2086 to inhibit the bronchoconstriction induced by inhaled PAF has not previously been reported in guinea pigs, but has recently been demonstrated in man (Adamus et al, 1990).

In this thesis, the experimental techniques described by Fitzgerald et al (1987) were used to determine the effect of PAF on responsiveness to methacholine. Fitzgerald et al determined the effect of PAF on responsiveness to 5-HT 15 min. after an aerosol of PAF. Methacholine was used in preference to 5-HT since 5-HT is a weak bronchoconstrictor in man (Barnes et al, 1988) and methacholine is used routinely in clinical practice for the measurement of
responsiveness. Methacholine was used in preference to histamine because methacholine is thought to act directly on the smooth muscle, whereas histamine also has indirect effects via its effects on vascular permeability and irritant receptors (Raymond et al, 1980).

It was considered inadvisable to measure BHR 15 min. after challenge since PIP had not returned to baseline so that changes in airway calibre could complicate the interpretation of the responses to methacholine. BHR was therefore assessed 1 hr. after PAF challenge. The design of the experiments allowed comparisons to be made between the responsiveness to methacholine before and after challenge in each animal, so that changes could be seen despite the inter-animal differences in sensitivity to methacholine.

Methacholine administered by inhalation induced concentration dependent bronchoconstriction in anaesthetised guinea pigs. However, because of the nature of the experimental set-up, changing the methacholine solutions and cleaning the nebuliser chamber was cumbersome and occasionally resulted in changes in the baseline PIP readings. A fixed concentration of methacholine was therefore used, increasing the time of exposure to increase the dose delivered to the airways. By this means, time-related increases in PIP were observed, which were not due to a hydration effect since they were not observed with similar exposure to saline, the vehicle for methacholine. The responses were considered to be cumulative, since a small but significant cumulative dose effects has previously been described when methacholine was administered at 5 min. intervals (Juniper et al, 1978).
Although there was a greater leftward shift of the dose-response curve to methacholine following PAF than that observed following lyso-PAF and BSA the PT\textsubscript{50} did not differ significantly before and after PAF challenge.

The lack of BHR induced by PAF may in part be explained by the choice of anaesthesia. Recently, Gentil et al (1989) have shown that intravenous fentanyl (10 and 20 ug/kg) induces BHR to infused 5-HT and histamine in ventilated, paralysed guinea pigs. These agents are known to induce bronchoconstriction partly due to a direct effect on the bronchial smooth muscle, and partly due to the modulating effects exerted by the cholinergic and adrenergic systems (Douglas et al, 1973). Gentil et al (1989) suggest that fentanyl acts by reducing the adrenergic response to these bronchoconstrictor agents since the effect is suppressed by pretreatment with naloxone and propranolol but unaffected by atropine.

It is possible that the guinea pigs in the PAF studies were hyperresponsive after the administration of fentanyl, so that further increases in BHR were not observed following PAF. However, there are several differences in methodology between the study of Gentil et al (1989) and the studies reported in this thesis. In addition to the different indices of lung function measured, the use of muscle relaxants, the route of administration of fentanyl (i.v. versus i.p.) and the bronchoconstrictors (infusion versus inhalation), the time of assessment of BHR (immediate versus 1 hr.) and the use of the direct acting methacholine versus histamine and 5-HT may all have a bearing on the resulting BHR. The observation that further increases in BHR were observed following allergen challenge with the same anaesthesia
would tend to suggest that the use of fentanyl is not a major reason for the lack of BHR in PAF treated animals.

A number of animal models have been developed to study the effects of allergen in the airways. Large animals including sheep (Ahmed et al, 1983), dogs (Chung et al, 1985) and monkeys (Kelly et al, 1974; Hamel et al, 1986; Wegner et al, 1991) have been studied. Techniques have also been developed for sensitising rabbits to preferentially produce IgE antibody from birth. Challenge of these animals with an aerosol of antigen results in an immediate and late bronchoconstrictor response followed by a period of hyperresponsiveness to inhaled histamine (Shampain et al, 1982; Marsh et al, 1985). Preliminary pharmacological analyses of these responses is similar to that observed in human allergen challenges (Larsen et al, 1984). Furthermore, this model allows repeated study of the same animal on several occasions. This model has several advantages, but it was considered preferable to set up a guinea pig model of asthma if possible, since experiments were planned to determine the contribution of oedema to the allergic response, necessitating sacrifice of the animals after challenge.

A technique for the challenge of anaesthetised guinea pigs with antigen by inhalation was described by Daffonchio et al (1987). Animals sensitized by the i.p. and s.c. injection of ovalbumin were challenged with a supra-maximal dose of antigen (anaphylactic macroshock). However, the resulting bronchoconstriction was almost maximal, necessitating the use of a bronchodilator, isoprenaline infused intravenously, before changes in airway reactivity could be

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assessed. This technique was subsequently modified and animals were challenged with a low dose of antigen (anaphylactic microshock) to avoid the need for bronchodilator treatment (Daffonchio et al, 1989).

The methodology used in this thesis is based on the techniques of Daffonchio et al (1987) with several modifications. Firstly, the animals were sensitised by a technique which has been shown to induce predominantly IgE antibodies (and represents the only guinea pig model where this method of sensitisation has been used in studies of BHR). Secondly, the allergen challenge was standardized to produce a given response, as is used in clinical practice. Thirdly, the degree of BHR was assessed by constructing dose response curves to methacholine using the same methodology as for the PAF studies. This agonist was chosen because of its clinical relevance and lack of effects on vascular permeability, and was administered by inhalation in contrast to the studies of Coyle et al (1988a) and Sanjar et al (1990b) where the agonists were administered intravenously. Furthermore, all challenges were performed without pre-treatment of the guinea pigs with H_1 antagonists (as used by Hutson et al, 1988a) or propranolol (as used by Lagente et al, 1988a) and in the absence of muscle paralysis (unlike Lagente et al, 1988a). The degree of bronchoconstriction induced resolved spontaneously within 1 hr., avoiding the need for bronchodilator therapy (unlike Daffonchio et al, 1987).

The sensitisation procedure consisted of a single i.p. injection and was generally well tolerated. The animals remained well during the 3 week sensitisation period. Responsiveness to methacholine was not altered by the sensitisation procedure. This is in contrast to
the findings of Sanjar et al (1990b) who found that the sensitivity to histamine of animals sensitised to 10 μg ovalbumin in aluminium hydroxide and pertussis vaccine was 3 fold greater than that of naive guinea pigs. In rabbits, sensitisation of newly born rabbits followed by booster injections at intervals afterwards (Pinckard et al, 1972) also results in BHR compared to non-sensitised animals, which is increased further by antigen challenge (Metzger et al, 1988). It is possible that with repeated antigen injections low grade inflammatory reactions occur as the antibody titres increase. The single i.p. injection was considered preferable to an inhalation sensitisation procedure since sensitisation of guinea pigs by exposure to an aerosol of ovalbumin on two occasions separated by 7 days has been shown to induce airway inflammation. A 3.3 fold increase in the number of eosinophils recovered from the lungs of sensitized animals was noted (Featherstone et al, 1988). The technique described by Hutson et al (1988a) involves multiple boosters and again the question of when a sensitisation procedure becomes a challenge remains unanswered. Furthermore, since it is well established that antigen challenge results in the infiltration of inflammatory cells and oedema formation, it seemed preferable to use a method where the lungs were as normal as possible prior to challenge with antigen if the basic mechanisms of bronchoconstriction and BHR were to be explored.

Challenge of sensitised guinea pigs with inhaled ovalbumin resulted in prolonged bronchoconstriction in a dose-related manner. In order to study the effect of antigen challenge on BHR the animals were exposed to ovalbumin at a fixed concentration for doubling times
of exposure every 30 sec. until 100% increase in PIP was observed. This allowed standardisation of the bronchoconstrictor response, avoided severe bronchoconstriction which had not resolved by 1 hr., and enabled the effect of small doses of antigen to be observed before further antigen was administered. This was found to minimize the changes in blood pressure accompanying the response, and hence the mortality associated with the procedure.

The exposure of guinea pigs to methacholine at a concentration of 128 ug/ml resulted in increasing bronchoconstriction with increasing time of exposure. Short exposure periods elicited no or only slight bronchoconstriction. However, once a threshold dose was delivered the curve became steep. The concentration-response curve of human airways to methacholine is also steep. The exposure time required to induce a 50% increase in PIP was found to be reasonably consistent between animals (5.7 sec., (GSEM 0.06), 95% CL 3.96-8.24 sec.). No convincing plateau could be demonstrated with doses of methacholine inducing >200% increase in PIP. Higher doses induced hypotension and were not administered in EHR experiments.

Challenge of sensitised guinea pigs with an aerosol of antigen resulted in acute EHR. The most marked change was an increase in sensitivity of the airways, evidenced by a leftward shift of the time response curve to methacholine, and a reduction in the exposure time required to induce 100% increase in PIP. The effect on PT100 methacholine was maximal at 1 hr. and persisting for at least 6 hr. Since the antigen-induced bronchoconstriction had resolved by
1 hr., this time point was used for subsequent measurements of BHR. One hr. after challenge, the curve was parallel to the steep portion of the pre-challenge curve, but there was not an initial shallow part to the curve despite challenging the animals with a 10-fold lower concentration of methacholine. Although it is possible that this represents an effect of antigen on reactivity it is also possible that less marked increases in PIP would have been observed following exposure to a more dilute solution of methacholine. "Sham" sensitised animals did not become hyperresponsive after ovalbumin challenge.

The effect of WEB 2086, administered i.v. at a dose of 1 mg/kg and 10 mg/kg, on the acute (1 hr.) hypersensitivity and hyperrreactivity induced by antigen was investigated.

At a dose of 1 mg/kg WEB 2086, administered intravenously 5 min. prior to antigen challenge, failed to modify the bronchoconstriction induced by antigen or the BHR which followed 1 hr. after challenge. After completion of this study, other investigators found that WEB 2086, and a related but more potent PAF antagonist, WEB 2170 inhibited antigen induced bronchoconstriction at a dose of 10 mg/kg (H. Heuer, personal communication). The effect of this dose of WEB 2086 was therefore investigated.

At a dose of 10 mg/kg administered intravenously 5 min. prior to antigen challenge WEB 2086 provided partial protection against the bronchoconstrictor response to antigen in that the exposure time required to induce 100% increase in PIP was significantly prolonged. This effect was confirmed by determining the effect of WEB 2086 on
antigen induced bronchoconstriction in groups of animals exposed to ovalbumin 1 ug/ml and 100 ug/ml for 1 min. WEB 2086 10 mg/kg, administered i.v. 5 min. prior to challenge, partially attenuated the response to 100 ug/ml and virtually abolished the response to 1 ug/ml. Furthermore, 1 mg/kg WEB 2086 partially attenuated the response to the lower dose of ovalbumin showing a dose-dependent inhibition of the bronchoconstriction by WEB 2086.

In the guinea pig, the in situ generation of large amounts of PAF has been demonstrated following the administration of antigen directly to the trachea (Fitzgerald et al, 1986). Furthermore, the role of PAF in acute bronchoconstriction induced by aerosolized ovalbumin in passively sensitised animals was implied by the observation that repeated PAF inhalation, inducing desensitization to PAF, resulted in a reduction in the bronchoconstrictor response to ovalbumin (Lagente et al, 1988a).

In addition, the inhibition of the acute bronchoconstriction induced by aerosolized antigen in passively sensitised animals has been reported following pre-treatment with the PAF antagonists, BN52021 (Lagente et al, 1987) and Ro 193704 (Lagente et al, 1988b). In passively sensitised animals challenged with i.v. administered ovalbumin, i.v. WEB 2086 (0.01 to 5 mg/kg 15 min. prior to challenge) afforded significant protection against the bronchoconstriction and hypotension induced by the challenge (Casals-Stenzel, 1987b; Pretolani et al, 1987).

In actively sensitised animals, Pretolani et al (1987) failed to demonstrate a protective effect of WEB 2086 but in the presence of mepyramine (5 ug/kg i.v.) oral WEB 2086 (0.05 to 5 mg/kg 1 hr. prior
to challenge) afforded significant protection against the bronchoconstriction induced by the challenge of animals with i.v. ovalbumin (Casals-Stenzel, 1987b).

The data presented in this thesis represent the first demonstration of the effect of a PAF antagonist on the acute bronchoconstriction induced by aerosolized antigen in actively sensitised animals, in the absence of H1 antagonists.

The fact that more WEB 2086 was needed to block antigen induced bronchoconstriction than to block the effects of PAF itself is probably not because of non-specific properties of the antagonist additional to PAF antagonism, since the doses of other antagonists needed to inhibit antigen-induced bronchoconstriction are also at least 10 fold above those needed to block the effects of PAF (Lagente et al, 1987; Lagente et al, 1988b). The increase may be secondary to the poor penetration of the antagonists into cells generating PAF, as much of the PAF generated endogenously is thought to act at the intracellular level (Lynch & Henson, 1986).

In the guinea pig studies no late-onset airways obstruction was noted after allergen challenge. However, in the model of Hutson et al (1988a) guinea pigs undergo 2 late phase reactions peaking at 17 and 72 hr. after challenge, which are inhibited by WEB 2086 (Hutson et al, 1988b). Furthermore, in rabbits sensitised from birth to produce IgE antibodies challenge with an aerosol of the antigen results in an immediate fall in dynamic lung compliance which returns to baseline after about 30 min. and is followed by a second fall approximately 2 hr. after challenge which persists for up to 6 hr. (Shampain et
al, 1982). Two independent groups have shown that different PAF antagonists BN 52021 (Coyle et al, 1988b) and L-659,989 (Smith et al, 1988b) inhibit the late-onset response induced by allergen challenge. In the same model, the acute response was not attenuated by these PAF antagonists. Similarly in the sheep, animals with a natural cutaneous and respiratory sensitivity to Ascaris suum antigen undergo a dual response to inhaled antigen. WEB 2086 administered i.v. 20 min. prior to antigen challenge inhibited the late but not the acute response to antigen (Stevenson et al, 1987).

The BHR induced 1 hr. after antigen challenge was inhibited by the higher dose of WEB 2086. Thus the hypersensitivity to methacholine was markedly reduced. Indeed when the PT_{50-200} were calculated, a significant difference in sensitivity was found only for PT_{50}. The slope of the time-response curve, and hence reactivity was reduced. In this particular group of animals a plateau was observed prior to challenge, and this was also reduced, suggesting an effect of WEB 2086 on hyperresponsivity.

Dixon et al (1989) have provided evidence that WEB 2086 does not possess muscarinic receptor antagonistic activity in vivo in guinea pigs when administered at a dose of 1 mg/kg. The ability of WEB 2086 to inhibit all three components of BHR induced by antigen challenge provides evidence to support the role of PAF in the generation of BHR, at least in the guinea pig.
The paradox between the implication that PAF is important in the induction of BHR whilst PAF administered in the same manner cannot itself induce BHR could be interpreted as evidence that the PAF antagonists are non-selective. Against this is the fact that several structurally unrelated PAF antagonists, in several different animal models have been shown to inhibit the antigen induced BHR. WEB 2086 provides partial protection against the BHR induced by antigen in allergic sheep (Soler et al, 1989) and in the rabbit both L-659,989 (Smith et al, 1988b) and BN52021 (Coyle et al, 1988b) inhibit antigen induced BHR.

In the guinea pig, Coyle et al (1988a) demonstrated the protective effect of EN 52021 on antigen induced BHR to intravenous agonists 24 hr. after challenge. and similar findings were reported by Seeds et al (1991) using the antagonist WEB 2170. However, guinea pigs sensitised to produce IgE antibodies, and challenged by a single exposure to ovalbumin whilst conscious using the same technique as that subsequently used by Coyle et al (1988a), did not develop BHR when compared with a group of "sham" sensitised animals. These animals received a bronchoconstrictor dose of ovalbumin, as evidenced by tachypnoea, cyanosis and transient respiratory distress. Other investigators found that BHR to inhaled antigen was maximal if a non-bronchoconstrictor dose of antigen was administered for a period of 1 hr. (S. Sanjar, personal communication).

The effect of a single 1 hr. challenge with ovalbumin (100 μg/ml in the aerosol chamber) which did not obviously cause any respiratory distress on the time response curve to methacholine was therefore evaluated. The curve was shifted slightly to the left in sensitised
animals compared to "sham" sensitised animals. In an attempt to amplify this shift, the effect of WEB 2086 was investigated in animals repeatedly challenged in this manner daily for five days. However, in this series of experiments no difference was detected between sensitised and "sham" sensitised animals treated with the vehicle for WEB 2086 or WEB 2086 (1mg/kg) i.p. 1 hr. prior to each challenge. These animals did however show increased responsiveness to methacholine when compared to sensitised but not challenged animals. It is therefore possible that the repeated exposure of non-sensitised animals to a foreign protein does induce some non-IgE mediated increase in responsiveness. The lack of protection by WEB 2086 is difficult to interpret since the dose used also failed to modify the acute BHR induced by antigen. Thus it remains possible that a higher dose of WEB 2086 might have afforded some protection against the BHR in these animals. Alternatively, this BHR may be different to the acute BHR, in that it is observed in both "sham" sensitised and sensitised animals, and may be related to the cold air which generates the aerosol. It is also difficult to exclude an effect of contaminants in the aerosol chamber and the aerosol generating apparatus, since despite the fact that every effort was made to clean and maintain the apparatus, the induction of BHR in "sham" sensitised animals may indicate contamination with a substance such as endotoxin which has been reported to induce BHR (Hutchinson et al, 1983). However, endotoxin inhalation results in PAF release (Rylander & Beijer, 1987) and endotoxin-induced pulmonary platelet recruitment is inhibited by the PAF antagonists, CV 3988, BN52021 and brotizolam (Beijer et al, 1987). Casals-Stenzel (1987c) reported a protective
effect of WEB 2086 in endotoxic shock, and WEB 2086 has been shown to inhibit the pulmonary microvascular leakage induced by endotoxin (Chang et al, 1987). WEB 2086 might therefore be expected to inhibit the effects of any contaminating endotoxin, whereas no difference was seen in WEB 2086 versus vehicle treated animals.

Despite the discrepancy between the prolonged BHR findings in this thesis and those reported in the literature, in view of the numerous reports of the effects of PAF antagonists on BHR in numerous species, the study of the efficacy of WEB 2086 in man would seem warranted. Although such a study was planned, at the time of completion of this thesis, WEB 2086 had not been awarded a Certificate of Toxicological Investigation. The only study of the effect of a PAF antagonist on antigen inhalation in man was therefore the study of Guinot et al (1987b). The effect of BN 52063, 40 mg tds for 3 days and 80 mg 2 hr. pre-challenge was investigated in a double blind crossover trial in 8 atopic asthmatics. BN 52063 was reported to afford significant protection against the acute bronchoconstrictor effect of antigen. However, the washout period was only 1 week, and as outlined in this thesis, BN 52063 lacks potency against PAF in human airways. In addition, the responsiveness to acetylcholine was determined 6 1/2 hr. after challenge, at a time when several subjects had significant bronchoconstriction (i.e. a late response) so that these results are difficult to interpret. The question of the role of PAF in the BHR induced by antigen inhalation in atopic subjects therefore awaits further clinical studies.
The mechanisms underlying the putative role of PAF, as implied by the data in this thesis with BN52063 in normal and atopic subjects and with WEB 2086 in guinea pigs, in the late response to cutaneous allergen challenge, in antigen induced acute bronchoconstriction and antigen induced BHR are largely unknown.

As outlined in section 1, a number of inflammatory cells which bear receptors for IgE are able to release PAF. Perhaps the most likely source of PAF following inhalation challenge is the alveolar macrophage since it is the predominant free inflammatory cell in the lung. Whatever the source of PAF, it is clear that PAF is a potent bronchoconstrictor in experimental animals and in man. The mechanism by which PAF induces bronchoconstriction is unknown. Evidence from in vitro and in vivo animal experiments suggest an indirect mechanism. Pharmacological data suggest that the bronchoconstriction is mediated at least in part by histamine and leukotrienes in man, and the role of platelets remains controversial (see section 2.3.d.vi). Recently the interaction of PAF with epithelial cells, stimulating the release of the bronchoconstrictor 15-HETE has been implicated (Salari & Schellenberg, 1991).

In view of the potent effects of PAF on vascular permeability in the airways of experimental animals (Evans et al, 1987, O'Donnell & Barnett, 1987, Persson et al, 1987) it has been suggested that oedema may contribute to the bronchoconstriction induced by PAF. Townley et al (1988a) and Chung et al (1989a) observed that beta agonists such as salbutamol and isoprotenerol, afforded only partial protection against the bronchoconstrictor effect of PAF and suggested that the residual effect of PAF on $Vp_{30}$ may reflect oedema of the airways.
However, under experimental conditions where the inhalation of PAF resulted in a 100% increase in PIP, the extravasation of EB dye was not different to that observed following the inhalation of its precursor, lyso-PAF or its vehicle, 0.25% BSA. Furthermore, the extravasation of EB dye was not increased at later time points up to 1 hr. In contrast, using the same technique for measuring oedema, dose related extravasation of EB dye could be detected following the administration of PAF i.v., confirming the findings of Evans et al (1987). Furthermore, animals treated surgically in the same manner did develop oedema following the inhalation of bradykinin or formamide so that the failure to detect oedema following PAF administration is unlikely to result from possible damage to the tracheal circulation during surgery. In contrast to these studies, Persson et al (1987) reported that the superfusion of guinea pig trachea with PAF (4 nmol, 0.04 ml over 2 min.) resulted in an immediate and a late phase (5 hr.) leakage of plasma into the airway lumen and tissue which had resolved by 7-18 hr., a process which is inhibited by WEB 2086 (O’Donnell et al, 1990). The reasons for this discrepancy are unclear. Direct delivery of PAF to the tracheal mucosa, and its prolonged administration may account for greater penetration than that achieved by aerosolization.

These data suggest that, at least in this guinea pig model, the bronchoconstrictor response is not due to oedema formation. The role
of oedema is difficult to examine in man. However, in a pilot study methoxamine failed to protect against PAF induced bronchoconstriction, suggesting that oedema is unlikely to be the major underlying mechanism in man.

Exudation of plasma into the airway lumen has been postulated to contribute to the pathogenesis of asthma (Persson, 1988). In addition to the exudation of proteins which may give rise to mediators such as the complement fragments C3a and C5a and kinins, and proteins which may react with mucus glycoproteins to form a tenacious mucus plug, microvascular leakage may result in impairment of mucociliary clearance and promote shedding of the airway epithelium. Furthermore, the resultant swelling of the submucosa would have profound effects on airway calibre (Hogg et al, 1987) and may thus contribute to BHR. However, in the experimental model described in this thesis, the extravasation of EB dye was similar in sensitised and "sham" sensitised animals when measured 5 min., 60 min. and 24 hr. after antigen challenge, i.e. at times of bronchoconstriction and BHR. These results are at variance with those reported by Erjefaelt and Persson (1986; 1991) and Persson et al (1986). Although the guinea pigs were sensitised in a similar manner and challenged 4-5 weeks later, the ovalbumin was delivered by tracheal superfusion (ovalbumin 3 pmol at a constant rate of 0.02 ml/min. for 2 min.). This resulted in significant extravasation of a circulating plasma tracer \( ^{131}\text{I}\)-albumin, into airway tissues and the airway lumen 10 min. after provocation. In contrast, there was no change in the absorption of \( ^{131}\text{I}\)-albumin from the lumen into the circulation under similar challenge conditions, implying maintenance of an intact mucosal
barrier to macromolecules. Technical differences between this study and the effect of aerosol antigen on EB extravasation may explain the different results.

The mechanism whereby PAF released as a consequence of allergen challenge may influence responsiveness to contractile agonists is unclear. PAF itself has been shown to induce BHR to i.v. and inhaled agonists in a variety of species (see chapter 3) and although there is now some doubt as to the ability of a single challenge with PAF to induce such changes in human volunteers, this does not preclude its involvement in antigen induced BHR.

It is possible that the failure of PAF to induce BHR may be related to its rapid destruction, particularly following inhalation (Haroldsen et al, 1987). Whilst the fact that PAF administered by inhalation to man results in flushing and a fall in the peripheral blood neutrophil count may be taken as evidence of systemic absorption this does not preclude the possibility that PAF is rapidly taken up by airway cells and/or enzymatically destroyed before it is able to influence the cell(s) which induce BHR. In contrast following antigen challenge, a variety of cells bearing IgE receptors may release PAF such as the alveolar macrophage, the eosinophil and the platelet. Furthermore, PAF can itself activate these cells, so that further PAF release may ensue. Thus it is possible that very high concentrations of PAF may be released locally, and/or that PAF release persists over a longer period of time (e.g. there is a delay before eosinophils and platelets are recruited into the airway lumen).
It is interesting to note in this context that chronic infusion of PAF (15 days) to guinea pigs resulted in increased BHR to histamine and hyperplasia of the Reisseissen muscles of some small bronchi, together with eosinophil infiltration and muciparous metaplasia of the epithelium (Touvay et al, 1991), which are all features of asthma.

The ability of PAF to recruit eosinophils into the airways may be of crucial importance in antigen-induced BHR. In vitro PAF is the most potent eosinophil chemotactic agent described to date, and in vivo the administration of PAF i.v. or by inhalation to guinea pigs, rabbits and baboons results in lung eosinophilia measured histologically or by BAL cell counts. At least in the rabbit, evidence has been gathered which suggests that this effect of PAF is platelet-dependent and it has been suggested that PAF initiates the release of platelet factors which can influence eosinophil migration (Coyle et al, 1990a).

Recently, PAF antagonists have been used to demonstrate the role of PAF in the infiltration of eosinophils into the airways of experimental animals following antigen challenge. In the guinea pig, WEB 2086 is able to inhibit the eosinophil infiltration observed 6 hr. after i.v. ovalbumin challenge of passively sensitised animals (Lellouch-Tubiana et al, 1988) and in actively sensitised animals, WEB 2086 administered 1 hr. prior to aerosol challenge of ovalbumin is able to block the allergen induced rise in eosinophils and neutrophils in the fluid collected by BAL at 17 hr. post challenge
(Hutson et al, 1988b). Similar results were reported by Coyle et al (1988a) using the PAF antagonist BN 52021. However, other groups have failed to inhibit antigen-induced eosinophil infiltration with PAF antagonists (Sanjar et al, 1990b; Ishida et al, 1990) even though the latter group did observe an inhibition of antigen induced BHR.

The role of PAF in cell migration is supported by the findings in human atopic skin. Thus the ability of PAF to reduce the late response to antigen without significantly affecting the early weal response could be interpreted as an effect of the PAF antagonist on the cellular component of the late response since it does not seem to have a large effect on the oedematous reaction to antigen.

In conclusion, the clinical studies in this thesis provide further evidence that PAF plays a role in allergic reactions in the skin by providing the first human data on a PAF antagonist in allergic subjects, but refute the claim that PAF is capable of producing prolonged BHR in normal subjects.

The animal experiments have shown that whilst PAF itself fails to produce BHR by inhalation, this does not exclude its role in antigen-induced BHR, but that neither PAF induced bronchoconstriction nor PAF-mediated component of antigen induced BHR are secondary to its effects on vascular permeability.
SECTION 5

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