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Expression of eicosanoid pathway enzymes in inflammatory cells.

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ABSTRACT

FACULTY OF MEDICINE HEALTH AND BIOLOGICAL SCIENCES
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EXPRESSION OF EICOSANOID PATHWAY ENZYMES IN INFLAMMATORY CELLS.

By Michelle Louise Seymour

Eicosanoids including leukotrienes (LT) and prostaglandins (PG) are derived from arachidonic acid, and have important inflammatory and immunomodulatory roles in human airway disease. 5-lipoxygenase (5-LO) pathway derived cys-LTs are critical bronchoconstrictor mediators and eosinophil-specific chemoattractants, while LTB_4 is a non-specific leukocyte chemoattractant. Prostanoids produced by cyclooxygenase have varied actions on bronchial and vascular smooth muscle, and may also modulate the synthesis and activity of other mediators including the LTs.

We hypothesised that abnormal levels of expression and/or altered cellular localisation of 5-LO and COX pathway enzymes in the airway may predict the susceptibility of human subjects to common precipitants of asthma, and/or that these triggers may modify eicosanoid pathway enzyme expression in a way that promotes chronic inflammation. Immunohistochemistry was used to quantify and localise the expression of eicosanoid enzymes in GMA embedded bronchial biopsies before and during exposure to (a) seasonal allergen, (b) human rhinovirus (HRV), and (c) ozone, and combined with immunoassays of eicosanoids in BAL fluid and with clinical measures of disease severity. The results showed diverse cellular and enzymatic changes in response to these common triggers of asthma. A link was demonstrated between poor lung function during seasonal allergen exposure and increased expression of 5-LO pathway, but not COX pathway, enzymes. This was a product both of leukocyte influx (eosinophils and macrophages) and of the induction of their expression within infiltrating and resident cells (eosinophils and mast cells). Ozone exposure produced inflammatory changes centred around neutrophil migration, while HRV infection altered eicosanoid enzyme expression and inflammatory cell counts in such a way that the normal airway resembled the airway of persistent asthmatics.

The bronchial biopsy studies indicated that individual eicosanoid enzymes are expressed by subpopulations of leukocyte phenotypes. This was confirmed by immunocytochemical and FACS analysis of their expression within immunomagnetically purified mast cells from human lung and leukocytes from venous blood. In eosinophils, upregulation of 5-LO pathway enzymes was demonstrated in response to eosinophilopoietic cytokines (IL-5, GM-CSF) that are generated by mast cells and T-cells within the asthmatic lung. The capacity of mast cells to generate cys-LT may be dependent on transcellular synthesis with accessory cells.

Preliminary efficacy data is presented on a compound (XR9173) that may be the precursor of a new class of leukotriene modifier drug which inhibit the proteins (MRP-1 and MRP-2) thought to export LTC_4 from cells. These drugs have potential applications in combating airway inflammation in asthma, and also enhancing the efficacy of cytotoxic drugs in cancer chemotherapy. The study highlighted the relative lack of selectivity for 5-LO pathway proteins not only of this compound but also of other anti-leukotriene drugs.

A combination of techniques including GMA immunohistochemistry, FACS analysis, immunocytochemistry, immunomagnetic purification, short-term cell culture, and immunoassays, was used to show that the influx of stimulus-specific leukocyte cell-types and induction of eicosanoid enzyme expression by NF-kB and other pathways, underlie abnormal metabolism of eicosanoids and may vary with diverse phenotypes of asthma. Better understanding of these mechanisms at the genetic, biochemical, cellular, pharmacological and clinical levels may lead not only to novel therapies but also to better targeting of current and new therapies to those patients most likely to demonstrate clinical benefit.

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Abbreviations

12-HETE	12-hydroxyeicosatetraenoic acid
12-HPETE	12-hydroperoxy-eicosatetraenoic acid
12-LO	12-lipoxygenase
15-HETE	15-hydroxyeicosatetraenoic acid
15-HPETE	15-hydroperoxy-eicosatetraenoic acid
15-LO	15-lipoxygenase
5-HETE	5-hydroxyeicosatetraenoic acid
5-HPETE	5-hydroperoxy-eicosatetraenoic acid
5-LO	5-lipoxygenase
A23187	Calcium ionophore (Calcimycin)
AA	Arachidonic acid
ADP	Adenosine diphosphate
AEC	Aminoethyl carbazol
AIA	Aspirin intolerant asthma
APC	Antigen presenting cell
ATA	Aspirin tolerant asthmatic
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BMMC	Mouse bone marrow derived mast cells
BPI	Bacterial permeability inducing protein
BSA	Bovine serum albumin
C3a	Complement fraction
cAMP	cyclic adenylate monophosphate
COX-1	cyclooxgenase-1 (PGH_2 synthase-1)
COX-2	cyclooxygenase-2 (PGH_2 synthase-2)
CPE	Cytopathic effects
$cPLA_2$	cytosolic phospholipase A_2
Cys-LT	cysteinyl leukotriene
DAG	Diacyl glycerol
DMEM	Dulbecco's modified Eagles medium
DNA	Deoxyribose nucleic acid
DTT	Dithiothreitol
EAR	Early allergic response
EBV	Epstein Barr virus
ECP	Eosinophil cationic protein
EDN	Eosinophil derived neurotoxin
EGF	Epidermal growth factor
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbence assay
EPO	Eosinophil peroxidase
ER	Endoplasmic reticulum
FACScan	Fluorescence activated cell scan
FcεRI	High affinity IgE receptor
FBS	Foetal bovine serum
FEV_1	Forced expiratory volume in 1s
FLAP	5-lipoxygenase activating protein
fMLP	formyl-methionyl-leucyl-phenylalanine
FSC	Forward scatter

FVC	Forced vital capacity
GMA	Glycolmethacrylate
GM-CSF	Granulocyte-macrophage colony stimulating factor
Gro-α	Growth related oncogene-α
HBSS	Hank's balanced salt solution
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HRV	Human rhinovirus
HS	Human serum
I-κB	Inhibitory subunit of NF-κB
IC50	Inhibitory concentration
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP_3	Inositol triphosphate
IP_4	Inositol tetraphosphate
LAR	Late allergic reaction
LFA	Lymphocyte function-associated antigen
LRT	Lower respiratory tract
LT	Leukotriene
LTA_4H	Leukotriene A_4 hydrolase
LTC_4 S	Leukotriene C_4 synthase
MBP	Major basic protein
MC_T	Mast cell containing tryptase
MC_{TC}	Mast cell containing tryptase and chymase
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MRP-1	Multidrug resistance associated protein-1
NF-κB	Nuclear factor-κB
NLS	Nuclear localisation sequence
NSAID	Non-steroidal anti-inflammatory drug
PAF	Platelet activating factor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
$PC_{20}FEV_1$	Provocative concentration of agent required to reduce FEV_1 by 20%
PCR	Polymerase chain reaction
PECAM	Platelet-endothelial cell adhesion molecule
PEF	Peak expiratory flow
PG	Prostaglandin
PGD_2S	Prostaglandin D_2 synthase
PGHS-1	Prostaglandin H synthase-1 (COX-1)
PGHS-2	Prostaglandin H synthase-2 (COX-2)
PIP_2	Phosphatidylinositol diphosphate
PKC	Protein kinase C
PL	Phospholipid

PLA_2	Phospholipase 2
PLC	Phospholipase C
PMA	Phorbol-12 myristate 13-acetate
PPARα	Peroxisomal proliferator-activated receptor-α
ppm	Parts per million
RAST	Radio allergosorbent test
RSV	Respiratory syncitial virus
SABC	Streptavidin-biotin complex
SCF	Stem cell factor
sICAM	Soluble intercellular adhesion molecule
$sPLA_2$	Soluble phospholipase A_2
SRS-A	Slow reactive substance of anaphylaxis
SSC	Side scatter
TCID	Tissue culture infective dose
TCR	T-cell receptor
TGF	Transforming growth factor
TLC	Total lung capacity
TNF	Tumour necrosis factor
TX	Thromboxane
TXS	Thromboxane synthase
URT	Upper respiratory tract
VCAM	Vascular cell adhesion molecule
VIP	Vasointestinal peptide

For Steve, my inspiration.

CHAPTER 1

Introduction

1.1 Asthma.

Asthma is a common respiratory disease manifesting itself in sufferers by reversible airflow obstruction caused by bronchoconstriction, bronchial inflammation, hyperresponsiveness and hypersecretion of mucus (Ciba Foundation Guest Symposium, 1959; American Thoracic Society Committee on Diagnostic Standards., 1962). Asthma is increasing in prevalence despite intensive research efforts and improved treatments, and now affects approximately 10% of the population of Western Europe and the USA. The numbers of children diagnosed with asthma are high, making asthma the most common chronic disease in children in the western world.

Atopy (allergy) is frequently associated with asthma but triggers other than inhaled allergens including respiratory viral infection, aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs), exercise, cold air, emotional stress, smoke or fumes (including ozone) induce many asthma exacerbations in patients who are sensitive to these factors. Not all asthmatics are sensitive to all of these triggers, and it appears that the pathophysiology of these disease phenotypes may vary. Hence, diagnosis and subsequent effective prevention of asthma is difficult and use of β_2-agonist bronchodilators a common drug regime in the treatment of asthma are directed only at the symptoms and not the underlying cause.

1.1.2 Diagnosis of asthma.

Diagnosis of asthma is difficult due to the lack of an accepted definition, reflecting varied concepts of the pathophysiology of the disease. Diagnosis is firstly based upon the appearance of symptoms. Asthmatics typically present with an intermittent wheeze that is worse on expiration, chest tightness, shortness of breath and cough, which may be associated with sputum production. Expiratory rhonchi (lung sounds) and hyperinflation are more physical signs. Patients commonly undergo lung function tests to give measurements of forced expiratory volume in 1second (FEV_1) and peak expiratory flow (PEF) which are often reduced by 20% of predicted values. Asthmatic patients should show >15% increases in both FEV_1 and PEF within 15 min of treatment with salbutamol (a β_2 agonist bronchodilator) and within 2 weeks of treatment with oral prednisolone (a steroid targeting inflammation). Total lung capacity (TLC) and residual volume are measured by plethysmography and are also reduced. Asthmatics classically experience non-specific bronchial hyperresponsiveness to inhalation challenges with histamine or

methacholine even when lung function tests appear normal.

A large proportion of patients experience asthma as a result of allergy and have a family history of allergy. The identification and avoidance of the offending allergen may be an effective treatment in some cases, the allergen in question can often be identified using skin prick testing with a range of common aeroallergens, and radioallergosorbent tests (RAST) for allergen specific IgE in blood. Exercise is a common trigger for asthma, and patients may present with reduced lung function measurements recorded during a period of exercise. Rarely, challenges with non-steroidal anti-inflammatory drugs (NSAIDs) or allergens may be useful for diagnosis of the particular trigger of asthma, but these tests are not routine.

1.1.3 Causes of asthma.

It has long been recognised that asthma and atopic disease run in families, which strongly suggests that development of the disease is linked to a genetic factor. Analysis of allergic patients has shown that 50% of children with one allergic parent and 80% of those with two allergic parents develop atopic disease (Cooke and Vander Veer, 1916). Chromosomal regions 5q, 6p, 11q, 12q, 13q and 14q are highly implicated in the susceptibility for asthma (The Collaborative Study on the Genetics of Asthma (CSGA). 1997).

However, the considerable increase in the prevalence of asthma and related allergic diseases over the last 25 years suggests that environmental factors must have an influence. It is considered that early exposure to allergens when the infant immune system is immature might be a predetermining factor in the development of allergic disease. This risk is increased with Western life-style. Houses with gas central heating and double-glazing have high levels of domestic allergens, for example, house dust mite (Dermatophagoides pteronyssinus), as a result of a warmer more humid atmosphere and allergens from domestic pets build up in carpets and soft furnishings (Sporik et al. 1990). Evidence suggests that viral respiratory infection may be instrumental in the onset of asthma (Pullian and Hey, 1982) with viral infections cause inflammation of the airways that might facilitate allergic responses to allergens in the environment, and hence promoting the sensitisation that leads to asthma (Glezen, 1998). However, Martinez (1994) suggests a protective role for viral infections in the onset of asthma, this is supported by the observation that asthma is most likely to develop in a first born child

who is not exposed to older siblings' infections (Glezen, 1998). Evidence also suggests that the onset of asthma might be influenced by parental smoking (Hanrahan et al. 1992) and air pollution, in particular ozone (Molfino et al. 1991), nitrogen dioxide (Devalia et al. 1994) and diesel fumes (Wade and Newman, 1993).

1.1.4 Treatment of asthma.

There are a number of medications available for the treatment of asthma, targeted at both the symptoms and the underlying inflammation. The most commonly used are short-acting inhaled β_2 **adrenergic receptor agonists** (e.g. salbutamol and terbutaline); these are used in the event of bronchoconstriction as a rescue remedy, but do not treat the underlying inflammation. Stimulation of the adrenergic β_2-receptor causes rapid bronchodilation by raising cAMP levels within the bronchial smooth muscle cell and hence inhibiting contraction (Reviewed in Rang and Dale 1992). β_2-agonists also inhibit release of histamine and leukotrienes from mast cells by a similar mechanism (Butchers et al. 1979). The inhaled route allows rapid access to the lungs and reduces systemic effects, but access of the drug to constricted airways is low, greater than 90% of the inhaled drug is swallowed and compliance with inhalers is poor (Kelloway et al. 1994).

The cromones, e.g. cromolyn sodium and nedocromil sodium, are directed at the underlying airway inflammation but are not directly bronchodilatory (Reviewed in Rang and Dale 1992). These drugs, when used on a regular basis, can reduce bronchial hyperreactivity. Taken via the inhaled route, the cromones are thought to stabilise the membrane on mast cells and other cells and hence reduce mast cell degranulation and mediator release in response to activation by allergen (Assem and Mongar, 1970). However, this stabilisation does not occur at clinically relevant doses. It is now thought that the cromones may modulate noncholinergic, noradrenergic neural control of the airways, or they may affect membrane chloride channels (Reviewed in Wenzel 1998).

Inhaled and oral corticosteroids control inflammation in the bronchi and hence improve symptoms and lung function and reduce bronchial hyperreactivity (reviewed in Busse, 1995). In 1992, Djukanovic et al. showed a reduction in numbers of mast cells and eosinophils in bronchial mucosal biopsies after asthmatics were treated for 6 weeks with inhaled beclomethasone. Steroids reduce margination of leukocytes from the blood, reduce bone marrow production of leukocyte progenitors, and reduce local recruitment of leukocytes (Reviewed in Rang and Dale 1992). Treatment of allergic asthmatics with oral prednisolone reduced lavage fluid eosinophil numbers, and numbers of cells

expressing IL-4 and IL-5 (Robinson et al. 1993). This suggests that steroids reduce airway cells' ability to generate cytokines such as IL-4, IL-5 and TNF-α, which cause influx of inflammatory cells in particular eosinophils into the airway. It is likely that this effect of steroids results from an inhibition of nuclear gene transcription. Similarly, they may have negative regulatory effects on lipoxygenase, endothelin and cyclooxygenase gene transcription as these are steroid responsive, hence steroids modulate synthesis of leukotrienes (Riddick et al. 1997). Steroids also reduce mucosal oedema by decreasing vascular permeability caused by vasoconstriction, and increase the synthesis and sensitivity of β-adrenergic receptors such that the effect of β-agonists are increased.

Histamine is not implicated in the development of asthma. This theory is further substantiated by the lack of efficacy of oral **anti-histamine drugs** in alleviating the characteristic bronchoconstriction experienced by asthmatics. These drugs are effective in the treatment of allergic rhinitis, allergic conjunctivitis and allergic skin conditions by blocking the H_1 receptor and hence blocking the vasodilator, secretory, and nociceptive actions of histamine (Reviewed in Rang and Dale 1992). Long-acting, non-sedating antihistamines include cetirazine and astemizole.

The **methylxanthine** theophylline is used for the reduction of recurrent attacks of bronchospasm. It inhibits cyclic AMP degradation by phosphodiesterases and is a competitive antagonist of adenosine receptors (Fredhol and Persson, 1982). Adenosine, when inhaled by asthmatic subjects causes profound dose-related bronchoconstriction, which is reversed by low doses of inhaled theophylline (Cushley et al. 1985). There is evidence that theophylline has anti-inflammatory actions reducing bronchial eosinophil counts in the submucosa and epithelium (Sullivan et al. 1994), and reducing epithelial $CD8^+$ cells, and submucosal IL-4^+ and IL-5^+ cells (Finnerty et al. 1996).

Recently a new class of anti-asthma drug has been developed, the first new class to enter clinical practice for over 25 years. These are **leukotriene-modifier drugs,** which may be inhibitors of enzymes of the leukotriene pathway, or specific cys-LT_1 receptor antagonists. These drugs are discussed in more detail in sections 1.3.2.10-11.

Non-steroidal anti-inflammatory drugs (NSAIDs) are inhibitors of prostanoid-producing cyclooxygenase enzymes (Reviewed in Rang and Dale 1992). Prostanoids have both pro- and anti-inflammatory actions and have subtle modulatory actions on the leukotriene pathway (see section 1.3.4.3-1.3.3.5). NSAIDs are not recommended for use in asthma management as 5-10% of asthmatic patients experience severe, sometimes life threatening asthma exacerbations after ingesting NSAIDs (British Thoracic Society et al.

1990; National Institutes of Health, 1995). Aspirin-sensitive asthma is discussed further in section 1.3.4.4.

1.2 Basic mechanisms of allergic disease.

1.2.1 Allergen exposure.

The first exposure to an allergenic substance can lead to sensitisation of the immune system to that allergen and result in the production of immunoglobulins IgM or IgD, and in a class switch to allergen specific IgE synthesis by B-lymphocytes. A subsequent exposure to the allergen may then provoke an allergic response in the form of an allergic disease such as asthma, rhinitis or urticaria with symptoms centred around the lungs, nose, eyes, gut and skin, which are prime sites for allergen to access the immune system. Concomitant exposure to pollutants, cigarette smoke or viral infections may enhance the immunogenicity of allergens by increasing epithelial permeability and hence, increasing allergen access. Some people have a tendency to produce an excess of IgE and this is correlated with a genetic predisposition to respond to the antigens that elicit an IgE response characteristic of atopy.

1.2.2 Antigen presenting cells.

In order for an allergen to elicit an allergic response an antigen presenting cell (APC) ingests/phagocytoses the antigen which is then processed into short polypeptide fragments by lysosomal enzymes. These fragments are then expressed on the cell surface by major histocompatibility complex (MHC). There are two forms of MHC. The first (MHC I) is expressed on all cells in the body and is involved in the humoral immune response which allows host cells to recognise cells expressing non-self MHC I and reject those which are foreign. The second, MHC II presents the processed antigen fragment to the T lymphocyte, which has, on its surface, an antigen specific T-cell receptor (TCR). The T lymphocyte cannot directly recognise antigens; without first being processed by an APC for recognition in association with MHC II molecules.

B Lymphocytes: B lymphocytes may be the initial sites of contact for allergen but are only one of many APCs. However, only the B-lymphocyte can produce antigen specific IgE molecules which elicit type I immediate hypersensitivity reactions. The IgE molecules synthesised by the B-lymphocyte are either secreted or expressed on the surface of the cell.

Monocytes, macrophages, Langerhan's cells and dendritic cells: In addition to B lymphocytes, cells of the monocyte/macrophage system and the Langerhan's cell/dendritic cell system also perform antigen presentation as a result of MHC II expression on their surfaces. These cells all originate from the same stem cell in the bone marrow (Reid et al. 1990b) but differentiate in response to colony-stimulating factors (Reid et al. 1990a). Blood monocytes migrate to become tissue macrophages, which express more MHC II when activated. The dendritic cell is an interdigitating cell found in the lymph nodes, spleen, skin and below the bronchial epithelium (Holt et al. 1989). The Langerhan's cell is a dendritic cell subtype. On processing an antigen, dendritic cells are thought to travel to the lymph node (Hill et al. 1990) where they interact with T-cells specific for the same antigen from a recirculating pool.

1.2.3 T Lymphocytes.

T-lymphocytes are classified into subtypes by their expression of surface markers. All T-lymphocytes express the markers CD2 and CD3 and most $CD3^+$ T-cells either express CD4 or CD8 markers. The $CD4^+$ T-lymphocytes are T-helper cells that interact with B-lymphocytes, while $CD8^+$ cells are cytotoxic T-cells that recognise and kill malignant or virally infected cells. They also kill cells expressing non-self MHC antigens. T-helper ($CD4^+$) lymphocytes recognise the antigen fragment-MHC II complex on APCs. This stimulates the T-helper cell to produce a range of cytokine messengers. The T-helper cell population can further be subdivided into two functional subsets Th1 and Th2 characterised by their cytokine profile. Activation of Th1 cells causes the release of interleukin (IL)-2, interferon gamma (IFNγ), and TNF-β, while activation of Th2 cells results in the synthesis of IL-4, IL-5, IL-10 and IL-13. Both cell-types also produce IL-3 and granulocyte-macrophage-colony-stimulating-factor (GM-CSF). The Th2 cytokines are predominantly involved in regulating the allergic inflammation seen in allergic diseases.

It has been shown that opposing signals from Th1 cells producing IFN-γ (which inhibits the class switch to IgE) (Coffman and Carty, 1986), and Th2 cells producing IL-4 and IL-13 (which promote the class switch to IgE) (Gauchat et al. 1993; Zurawski and de Vries, 1994) influence IgE synthesis by B cells (Vercelli et al. 1990). $CD4^+$ T cell production of IFN-γ is significantly lower in babies born to families with an allergic history (Holt et al. 1992). Warner and colleagues (1994) found raised T-cell

proliferative responses and defective IFN-γ production to stimulation with specific allergens in babies who have subsequently developed allergic problems in relation to those allergens (Warner et al. 1994). They have also proposed that there is a maternal and placental influence on the foetal immune response that promotes Th2-like activity (Warner et al. 1997).

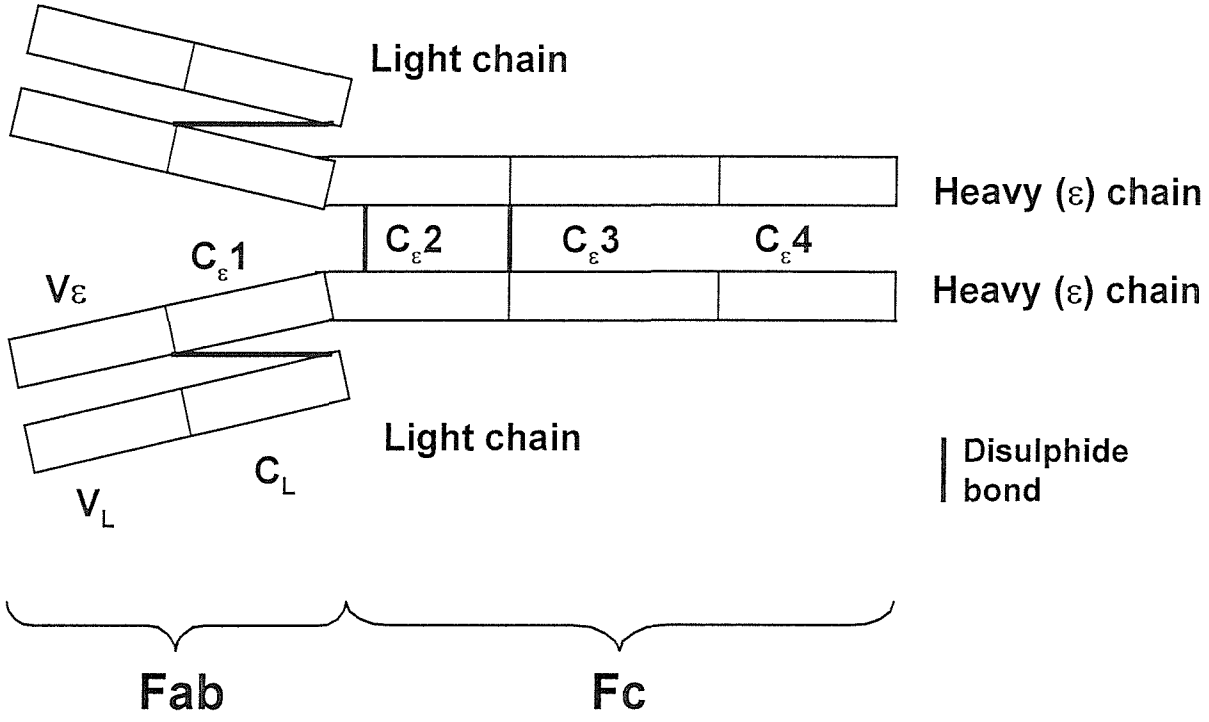
1.2.4 Immunoglobulins and hypersensitivity reactions.

Human immunoglobulins belong to five classes: IgG, IgA, IgM, IgD and IgE. IgG is the dominant immunoglobulin in blood. It binds to and causes attack of invading microorganisms by activation of the complement cascade that results in an inflammatory response. Secretory IgA is dominant in external secretions; it covers the mucosal surface and protects the body against invading microorganisms by inhibition of their attachment at the mucosal surface. If the intruding microorganism overcomes this first line of defence, the IgG and IgM systems in the mucous membrane are stimulated. The principal function of IgM is agglutination of foreign particles and molecules; it can also participate in some adverse reactions to drugs. Both IgD and IgM are surface markers on naïve B lymphocytes before they are switched to IgE for example (see section 1.2.5).

Immune responses in the body can take many forms presenting as a variety of disease states such as hayfever, transplant and transfusion reactions and autoimmune diseases. Gell and Coombs classified these reactions into 4 types of hypersensitivity. Type I hypersensitivity occurs in the form of acute allergy to an antigenic material such as pollen. This response is mediated by IgE and/or IgG and mast cells, an example being hay fever. Type II hypersensitivity (blood transfusion reactions, and autoimmune diseases) involves elimination of seemingly non-self antigens mediated by IgM and IgG with complement, killer cells and phagocytes. Type III hypersensitivity occurs when antibody reacts with soluble antigen, an example being the Arthus reaction or serum sickness. Type IV is cell mediated hypersensitivity and involves T cell mediated recruitment of macrophages, eosinophils etc. It is thought that the late phase of asthma is an example of type IV hypersensitivity.

The immunoglobulins have two functional regions: an Fc region by which they may attach to effector cells, and an Fab region (**Fig 1.1**), which is responsible for interactions with antigens. Immunoglobulins comprise two heavy chains (κ) and two light chains

Fig. 1.1 Structure of human IgE



(λ), each with variable (V) and constant (C) regions. The C regions of the heavy chain determine the class of the immunoglobulin such that IgE has 4 C_ε domains whereas IgM has C_μ domains. The two identical light chains consist of one variable region (V_L) and one constant region (C_L). Cleavage of immunoglobulin IgE between the first two C regions of the heavy chains ($C_\varepsilon1$ and $C_\varepsilon2$) yields two Fab fragments consisting of the V_L and C_L regions of the light chain bound to the V_ε and $C_\varepsilon1$ regions of the cleaved heavy chain. The remaining regions of the heavy chains, which in IgE are $C_\varepsilon2$, $C_\varepsilon3$ and $C_\varepsilon4$, bound together with disulphide bonds, form the Fc region. (**Fig1.1**)

1.2.5 IgE

IgE is the most important immunoglobulin in allergy and is present in the smallest quantities. It represents 0.001% of total circulating immunoglobulin but is raised between 2 and 10-fold in allergic patients. IgE is produced by B-lymphocytes as a result of a 'class switch' in response to IL-4 and IL-13 (Gauchat et al. 1993, Zurawski and de Vries, 1994). Immature B-lymphocytes synthesise μ and δ heavy chains for IgM and IgD production respectively. For the switch to synthesis of an ε heavy chain, the B-lymphocyte must receive a signal from an antigen-specific T-helper cell, and Th2 cell generated IL-4 stimulation is also required. The Th1 cytokine IFNγ can inhibit the IL-4 induced IgE class-switch. The ratio of Th1 and Th2 cells and hence the balance between IFNγ and IL-4 is critical in the regulation of an allergic response.

There are two receptors for IgE: a high affinity receptor ($Fc_\varepsilon RI$), present on mast cells and basophils, and a low affinity receptor ($Fc_\varepsilon RII$, CD23) expressed on a number of inflammatory cells including lymphocytes, eosinophils and macrophages. The FcεRI receptor plays an important role in the development of the allergic response and is upregulated in atopic subjects with high levels of serum IgE. The receptor present on the cell surface binds the Fc region of the IgE molecule leaving the antigen-specific Fab region free for binding of allergen particles (Reviewed in Church and Holgate 1993). Each Fab fragment of the IgE molecule has a site that binds antigen, and hence each IgE molecule can bind two allergen particles. This allows an allergen particle to form a bridge between two IgE molecules. This cross-linking of IgE on the surface of the mast cell or basophil causes the cell to degranulate, release granule associated mediators including histamine and tryptase, and mediators synthesised *de novo* such as eicosanoids (Reviewed in Church and Holgate 1993).

1.2.6 Epithelial cells.

Epithelia are the first line of defence against invading organisms or allergens. In the lung, bronchial epithelium is superimposed upon a basement membrane and lines the airways from the nose to the alveoli. Airway epithelium is ciliated pseudostratified. Cilia serve to propel a surface layer of mucus produced by resident goblet cells and submucosal glands towards the pharynx to remove entrapped particles. In asthmatic patients, the respiratory epithelium may become damaged due to the combined effects of toxic eosinophil-derived proteins, viral infection, chemical damage, excessive stickiness of mucus, and shear forces resulting from bronchoconstriction. Clusters of sloughed epithelial cells called Creola bodies are regularly found in asthmatic sputum. Epithelial damage of this kind permits increased access of allergens to the submucosa and impairs mucociliary clearance of allergens.

The epithelium is not only a passive barrier but is also an active participant in the recruitment of inflammatory cells from the blood into the tissue in response to allergic or other stimuli. Epithelial cells produce the cytokines IL-6, IL-8, GM-CSF (Marini et al. 1992), SCF (Wen et al. 1996), RANTES (Stellato et al. 1995), MCP-1 (Paine et al. 1993), IL-1 (Maestrelli et al. 1995), and epidermal growth factor (EGF) which attract leukocytes and promote airway remodelling by myofibroblast deposition of collagen and proliferation of smooth muscle.

1.2.7 Recruitment of inflammatory cells and adhesion molecules.

In order for blood leukocytes to respond to an inflammatory stimulus in the tissue they must migrate from the circulation, through the vascular endothelium, and into the affected tissue this migration requires the expression of adhesion molecules on the surface of the leukocyte and endothelium (Huttenlocher et al. 1995). Cytokines including the interleukins 1-8 and GM-CSF, produced by the affected tissue may upregulate adhesion molecule expression on blood leukocytes and on the endothelium (Reviewed in Burke et al. 1993). Blood flow causes shear forces to be applied to circulating leukocytes, and in response the cells reversibly attach to or 'roll' along the endothelium. Interactions between the selectin family of adhesion molecules (E-selectin, P-selectin, and L-selectin) and their sialylated ligands mediate this rolling and the cells become flattened against the endothelium. E-selectin is expressed exclusively on activated endothelium, P-selectin on both activated platelets and activated endothelium,

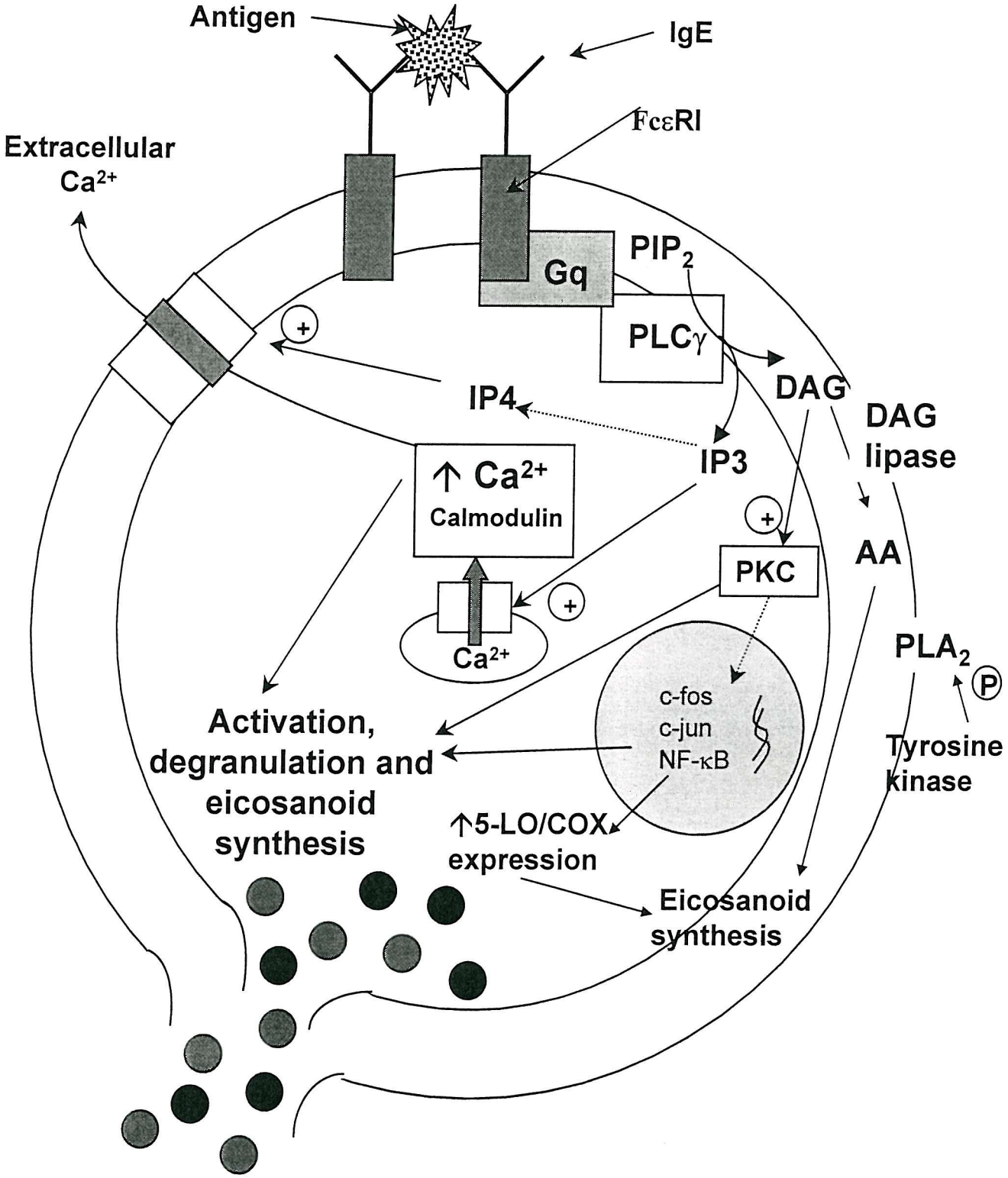
and L-selectin is expressed on circulating leukocytes. Inflammatory mediators, including histamine, leukotrienes and PAF, rapidly induce P-selectin expression (Pedersen et al. 1997). The induction of E-selectin expression on cells activated by endotoxin, IL-1 or TNF may take hours. It has been suggested that the integrin VLA-4 can also participate in rolling of cells such as eosinophils and lymphocytes that express this molecule (Sriramarao et al. 1994; Alon et al. 1995). Leukocytes are then activated as a result of exposure to chemokines produced by endothelial cells or by interactions with endothelial adhesion molecules.

Activation of leukocytes upregulates the expression of integrins e.g. very late antigen (VLA-4), Mac-1 and lymphocyte function-associated antigen (LFA-1). These integrins are ligands for intercellular adhesion molecules–1 and 2 (ICAM-1 and ICAM-2) and vascular cell adhesion molecule-1 (VCAM-1) expressed on the endothelium which mediate firm leukocyte-endothelial adhesion (Springer, 1995). Flattened leukocytes then migrate through the tight junctions between endothelial cells (diapedesis) as result of PECAM-1 that is constitutively expressed on endothelial cells and most leukocytes (DeLisser et al. 1994), although integrins, selectins and their ligands may also participate. The passage of leukocytes through the tissue to the area of inflammatory stimulus is mediated by chemotactic factors (e.g. cytokines) and interactions between the CAMs and integrins (Clark and Brugge, 1995), which are expressed both on the cell and the extracellular matrix.

1.2.8 Mast cells and basophils.

Mast cells and basophils are the primary initiating cells of IgE-mediated allergic reactions; the membrane high affinity IgE receptors ($Fc_{\varepsilon}RI$) are unique to these cell types although recent reports suggest that $Fc_{\varepsilon}RI$ may be induced on eosinophils (Sullivan et al. 1998) and neutrophils (Soussi-Gounni et al. 1998). The mast cell contains granules bound by a thin perigranular membrane. In allergic diseases allergen causes degranulation in mast cells and basophils. When the allergen cross-links IgE the $Fc_{\varepsilon}RI$ receptor is activated, this stimulates a G-protein linked to phospholipase C which converts phosphatyidylinositol-4,5-biphosphate (PIP_2) into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). IP_3 mediates the release of calcium from intracellular stores and the influx of extracellular calcium, which binds to its receptor calmodulin which, thus activated can stimulate a variety of enzymes. DAG activates protein kinase

Fig. 1.2 Signalling pathway for mast cell degranulation and eicosanoid production.



C (PKC), the increase in intracellular calcium and activation of PKC are essential for degranulation where the granule membrane fuses with the cell membrane and the granule contents are released (**Fig 1.2**). Degranulation results in the release of pre-formed biochemically active mediators stored in the granules into the surrounding tissue causing an inflammatory response. Activation of PKC by DAG also leads to the modulation of gene expression by causing activation of the protooncogene products c-fos and c-jun that regulate transcription though the AP-1 binding site (Razin et al. 1994).

The basophil, eosinophil and mast cell all originate from the same bone marrow stem cell and differentiate into individual progenitor cells in the bone marrow under the influence of the haematopoietic growth factors GM-CSF and IL-3. Some stem cells circulating in the blood migrate into tissues where they reach phenotypic maturation under the influence of cytokines in the microenvironment to become mast cells. Stem cell factor (SCF) or *c-kit* ligand, a haematopoietic growth factor, is essential for the differentiation of these progenitor cells to mast cells (Besemer, 1991) and promotes mast cell survival by suppressing apoptosis (Iemura et al. 1994). IL-9 and IL-10 have also been reported to influence mast cell proliferation and granule protease phenotype (Thompson-Snipes et al. 1991).

The mast cell is found in the connective tissue of all organs, excluding the brain but particularly in the skin, airways and gastrointestinal tract. Mast cell populations can be subdivided into two subtypes by the protease content of the granules (Irani et al. 1986). MC_{TC} (containing tryptase and chymase) are the predominant mast cell subtype in the skin, whereas the MC_T subtype (containing tryptase but not chymase) predominates in the lungs and gastrointestinal mucosa (Irani et al. 1986), both MC_T and MC_{TC} cells develop from the same progenitor cell. MC_T mast cells are important in allergic reactions of the respiratory tract. Some studies show that asthmatic subjects have a higher percentage of mast cells in BAL fluid than normal control subjects (Flint et al. 1985; Tomioka et al. 1984).

The basophil is a circulating cell most closely related to the eosinophil, but sharing with the mast cell the capacity to release histamine on activation of $Fc_{\varepsilon}RI$. Like the eosinophil, it has a bilobed nucleus but fewer granules of variable size. The basophil will migrate to affected tissue sites and take part in local allergic reactions such as IgE mediated allergic-airways disease. Unlike the mast cell, basophils do not contain tryptase or synthesise PGD_2 (Naclerio et al. 1996)

1.2.8.1 Mast cell derived mediators.

The mast cell has the capacity to generate a wide range of preformed and newly generated mediators, causing both immediate and long-term effects on their target organs.

Preformed mediators.

Proteoglycans. Heparin and chondroitin are proteoglycans comprising the major structural unit of the mast cell granule. Interactions between protein and proteoglycan cause the unique scroll, lattice and grating patterns observed in the granules under the electron microscope. During mast cell degranulation proteins dissociate from the proteoglycan by ionic exchange, initially with Ca^{2+} and then with Na^{+}. Histamine and the acid hydrolases dissociate rapidly due to their relatively neutral isoelectric points, whereas the more highly charged neutral proteases (tryptase, chymase and carboxypeptidase) dissociate more slowly and only remain active while in proteoglycan complexes.

Histamine: Of the preformed mediators, histamine (β-imidazolethylamine) is probably the most rapidly acting, causing contraction of smooth muscle, stimulation of irritant receptors and an increase in vascular permeability within a few minutes of release. Histamine is a basic amine formed from histidine by the removal of a carboxyl group by histidine decarboxylase. It has an imidazole ring with a basic side chain. Histamine associates by ionic bonds with carboxyl groups of heparin, with which it is stored in the mast cell granule. The mast cell contains about 1-2 μg histamine per 10^6 cells and is the major source of tissue histamine, whereas the basophil is the major source of humoral histamine. Release of histamine by mast cell degranulation occurs by cation exchange with extracellular ions; histamine is then metabolised by histamine-N-methyltransferase to methylhistamine and then by monoamine oxidase to methylimidazole acetic acid. Alternatively, histamine may be metabolised by diamine oxidase to imidazole acetic acid, then sequentially by imidazole acetate phosphoribosyl transferase, and phosphoribotidylimidazole acetyl phosphatase to riboside-N-3 imidazole acetic acid (Schwartz and Austen, 1984).

Histamine binds to one of three types of histamine receptor in the tissue, H_1, H_2 and H_3, which can be distinguished using specific antagonists and agonists. Stimulation of H_1 receptors found on human bronchial smooth muscle causes contraction of the

muscle, but although bronchial inhalation of histamine causes cough and smooth muscle contraction leading to bronchoconstriction, histamine is not a major bronchoconstrictor mediator in asthma. Histamine is widely implicated in the development of symptoms associated with allergic rhinitis, skin wheal and flare responses, urticaria and anaphylaxis. H_2 receptors are found in the acid secreting cells of the stomach and in the heart, with stimulation causing gastric acid secretion and increased heart rate respectively. H_3 receptors are associated with neural tissue.

Neutral proteases.

Tryptase: Tryptase is a tetrameric serine protease of about 130kDa that has trypsin-like activity. Tryptase has 40% sequence identity with trypsin. This enzyme is stored and released in association with heparin and chondroitin sulphate within the granules of all mast cells (Schwartz and Bradford, 1986). The four equivalent monomers are arranged in a square flat ring, and the active sites of each monomer is facing inwards into an oval central pore restricting access to macromolecular substrates (Pereira et al. 1998). The tetramer is inactivated when not in association with heparin by irreversible conversion into inactive monomers (Schwartz and Bradford, 1986).

Human lung mast cells typically contain 10pg of tryptase per cell (Schwartz et al. 1987). Tryptase rapidly inactivates fibrinogen and activates collagenases. In the lung, tryptase may cause an imbalance between the bronchodilator neuropeptide VIP (vasoactive intestinal peptide) and the bronchoconstrictor neuropeptide substance P by selectively metabolising VIP and hence tryptase could be implicated in asthmatic bronchoconstriction. Tryptase is released into BAL fluid within minutes of inhaled allergen challenge (Wenzel et al. 1988), but not after exercise challenge. The concentration of tryptase is elevated in the BAL fluid of asthmatics, indicating persistent mast cell degranulation (Broide et al. 1991). The use of inhaled steroids decreases levels of tryptase in the BAL in parallel with a decline in mast cell numbers in the BAL and bronchial mucosa (Djukanovic et al. 1992). The role of tryptase in asthma and allergic disease is at present unclear. However, a study using the tryptase inhibitor APC 366 reduced the late allergic reaction but not the early allergic reaction or bronchial hyperresponsiveness to histamine in allergen-challenged mild-moderate asthmatics suggesting a role for tryptase in the late phase response (Krishna et al. 1998b).

Chymase: Chymase is a monomeric serine protease of approx. 30 kDa, stored in mast cell granules in association with heparin, but in a complex distinct from that of tryptase.

It is secreted in a fully active form. Chymase may have a role in inflammation as it may cleave cytokines to activate IL-1β and degrade IL-4; it promotes vascular permeability, and can participate in neutrophil recruitment amongst other functions.

Carboxypeptidase: Human mast cell carboxypeptidase is an approx. 34.5 kDa monomeric metalloprotease stored and released in association with heparin in the same macromolecular complex as chymase (Goldstein et al. 1992). Carboxypeptidase is likely to act in association with other mast cell proteases to degrade peptides and proteins.

Acid hydrolases. The acid hydrolases including β-hexosaminidase, β-glucuronidase and β-galactosidase present in the granules of the mast cell are also present in the lysosomes of other inflammatory cells.

Newly synthesised mediators.

The lipid derived mediators: leukotrienes, prostaglandins, thromboxanes and platelet activating factor (PAF) are synthesised *de novo* upon activation of the mast cell and also by other inflammatory cell types, including eosinophils, basophils, macrophages and T-lymphocytes. Lipid mediators are discussed in detail in section 1.3.

1.2.9 Eosinophils.

The eosinophil, identified by its bilobed nucleus and cytoplasmic granules that stain red with the acidic dye eosin, is an important pro-inflammatory leukocyte which contributes to allergic inflammation. Numbers of peripheral blood eosinophils are increased in asthmatics, and correlate with clinical severity of asthma and inversely with pulmonary function (Bousquet et al. 1990).

The eosinophil and basophil are derived from the same progenitor cell and are sustained by GM-CSF and IL-3. Final differentiation of eosinophils occurs under the influence of IL-5 (Tagari et al. 1993), the basophil progenitor cell will also differentiate into an eosinophil under the influence of IL-5 (Bagely et al. 1997). Eosinophil granules contain four basic proteins: major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO) (Gleich and Loegering, 1984; Olsson et al. 1977). These are helminthotoxic but also damage mammalian cells particularly the bronchial epithelium (Frigas et al. 1980; Hastie et al. 1987).

In addition to the toxic cationic proteins, eosinophils synthesise the lipid mediators LTC_4 and PAF (see sections 1.3.4 and 1.3.2 respectively). There are two sub-

populations of eosinophils in patients with eosinophilia associated diseases such as asthma and allergy, distinguishable by their density. In normal persons more than 90% of eosinophils are normodense (1.095-1.105 g/ml) with the remaining few being hypodense (<1.095 g/ml) (Kloprogge et al. 1989). Eosinophilic patients have a higher proportion (up to 80%) of hypodense eosinophils and this correlates with their degree of eosinophilia (Fukuda et al. 1985). Hypodense eosinophils may be primed and, hence, partially degranulated (Connell, 1968), they are also more metabolically active (Pincus et al. 1981) and have increased LTC_4 production (Kajita et al. 1985; Moqbel et al. 1990).

1.2.10 Neutrophils

Neutrophils are polymorphonuclear leukocytes of myeloid lineage with a characteristic multilobed nucleus and many granules. The larger (azurophil) granules differ from the smaller (specific) granules by their content and can be differentiated by the presence of myeloperoxidase only found in azurophil granules, while the specific granules contain lysozyme, lactoferrin, vitamin B_{12} binding protein, collagen and the dipeptidase that converts LTD_4 into LTE_4 (Lee et al. 1983b). The contents of the granules are essential for the role of the neutrophil in host defence, particularly in the eradication of bacteria.

Like the eosinophil, the neutrophil is a short-lived circulating leukocyte that migrates to inflamed tissue in response to chemotactic factors such as the complement component C5a, the bacterial product formyl-methionyl-leucyl-phenyalanine (FMLP), LTB_4 (Spada et al. 1994), PAF (Wardlaw et al. 1990), IL-8 (Kunkel et al. 1991) and TNFα (Munro et al. 1989).

When the neutrophil reaches the inflamed tissue site it phagocytoses bacteria. The granules then fuse with the phagosome and release their contents including lysozyme, collagenase and bacterial permeability inducing protein (BPI) (Henson et al. 1995). The compartmentalisation of the antimicrobial agents within the granules prevents damage to the host cell but allows the contents to be directed to the target particle within the phagosome. Following phagocytosis, the neutrophil undergoes apoptosis and is ingested by macrophages before being cleared by the spleen. During the process of host defence to infection it is inevitable that there will be some damage to the host cells caused by the neutrophil, with endothelial and epithelial cells being most at risk.

Bronchoalveolar neutrophilia is associated with the late (but not early) asthmatic response to allergen (Metzger et al. 1986; Diaz et al. 1986, De Monchy et al. 1985). Neutrophil counts are raised in bronchial biopsies and BAL from severe

glucocorticosteroid-dependent asthmatics compared to mild-moderate asthmatics or normal controls (Metzger et al. 1997). In patients who experience nocturnal asthma, the overnight fall in PEF is related to an increase in neutrophil counts in BAL (Martin et al. 1991). Frequently, neutrophils make up more than 75% of cells in sputum from asthmatic subjects with acute exacerbation (Fahy et al. 1995), and in the submucosa of sudden-onset fatal asthma patients neutrophils are raised in comparison to slow-onset fatal asthma patients (Sur et al. 1993). In addition, neutrophil chemotactic factors are increased during early and late asthmatic reactions induced by allergens (Nagy et al. 1982). The neutrophilia (Boschetto et al. 1987) and enhanced plasma chemotactic activity (Gin and Kay, 1985) are prevented by both steroids and cromolyn. Neutrophilia associated with increased bronchial responsiveness is also evident after PAF inhalation (Wardlaw et al. 1990) and exposure to ozone (Seltzer et al. 1986), however the airway hyperresponsiveness induced by ozone develops before neutrophil infiltration of the airway mucosa (Murlas and Roum, 1999). Therefore, neutrophils undoubtedly are involved in acute exacerbations of asthma, but their role in the pathological and physiological features of persistent asthma is probably limited (discussed in Boschetto et al. 1989).

1.2.11 Cytokines

Cytokines control cell-to-cell communication. They are multifunctional glyco-proteins that regulate growth and differentiation of cells, and regulate the immune response by T-cells. Some are pro-inflammatory and others have a cytotoxic or inhibitory role.

Cytokine	Cellular source	Actions
Interleukin-1	Monocyte/macrophage	Proliferation and differentiation of T and B cells. A proinflammatory cytokine
IL-2	Th1 cells ($CD4^+$)	Proliferation and differentiation of T and B cells
IL-3	Th2 cells Th1 cells Mast cells	Growth and differentiation of stem cells of all lineages including eosinophils, basophils and mast cells. Activation of eosinophils and basophils.
IL-4	Th2 cells Mast cells	Proliferation and differentiation of B-cells. Promotes IgE class switch. Growth of rodent mast cells (with IL-3)
IL-5	Th2 cells Eosinophils Mast cells	Growth and differentiation of eosinophil stem cells. Activates eosinophils and prolongs eosinophil survival.

IL-6	Macrophages Fibroblasts T cells	Proliferation and differentiation of B-cells. A proinflammatory cytokine.
IL-8	Macrophages Mast cells Neutrophils	Neutrophil chemotaxis.
IL-10	T cells B cells Mast cells	Inhibits synthesis of cytokines from Th1 cells. Enhances expression of MHC II on B cells. Mast cell growth and differentiation (with IL-3& IL-4) and T cell growth.
IL-13	T-cells	Similar actions to IL-4 Promotes IgE class switch.
GM-CSF	Th1 and Th2 cells. Epithelium. Endothelium Fibroblasts Macrophages Eosinophils	Proliferation and differentiation of haematopoietic stem cells. Activates mature granulocytes.
Interferon (IFN)-γ	Th1 cells	Inhibition of viral replication. Macrophage activation. Inhibition of B cell differentiation and the IgE class switch
Tumour necrosis factor (TNF)-α	Mast cells Macrophages	Proinflammatory and cytotoxic
Stem cell factor (SCF) / c-kit ligand	Stromal cells Epithelial cells	Growth and differentiation of mast cells.

Table 1.1 Cellular sources and actions of cytokines and haematopoietic growth factors important in asthma, allergy and inflammation.

1.2.12 IL-5 and GM-CSF

IL-5 is predominantly a T-cell product with more than 70% of IL-5 mRNA localised to T-cells and the remaining IL-5 mRNA localising to mast cells and eosinophils (Ying et al. 1997). IL-5 protein was not detected in large quantities in T- cells as it is rapidly secreted by T-cells, whereas IL-5 protein is easily detectable in mast cells and eosinophils due to storage of cytokines in granules (Ying et al. 1997). GM-CSF is produced by T-cells, bronchial epithelial cells, lung fibroblasts, macrophages and

eosinophils (Reviewed in Redington et al.1993). The production of IL-5 and GM-CSF by eosinophils suggests a possible autocrine effect. IL-5 is a cytokine that specifically acts on eosinophils with regard to stimulating production of eosinophils from progenitor cells (Clutterbuck et al. 1989), IL-3 and GM-CSF increase numbers of IL-5 responsive eosinophil colony forming cells and IL-5 acts later to generate mature eosinophils from these precursors (Clutterbuck et al. 1990). IL-5 and GM-CSF induce the production of hypodense eosinophils *in vitro* (Owen et al. 1987) and appear to have the same effect in vivo as serum from asthmatics has higher levels of these cytokines (Corrigan et al. 1993, Brown et al. 1991). IL-5 and GM-CSF increase the survival of eosinophils in vitro, with IL-5 this effect is due to inhibiting apoptosis (Owen et al. 1987, Stern et al. 1992). GM-CSF and IL-5 are chemotactic for eosinophils at high concentrations (Warringa et al. 1991, Wang et al. 1991), at lower concentrations they prime the cells for increased response to other chemotactic agents including leukotrienes and PAF. Eosinophils from asthmatic subjects exhibit enhanced increased responses to chemotactic agents suggesting that in vivo priming has already taken place (Warringa et al. 1992). IL-5 and GM-CSF also prime eosinophils for increased mediator release (Fujisawa et al. 1990, Lopez et al. 1988).

1.3 Lipid mediators.

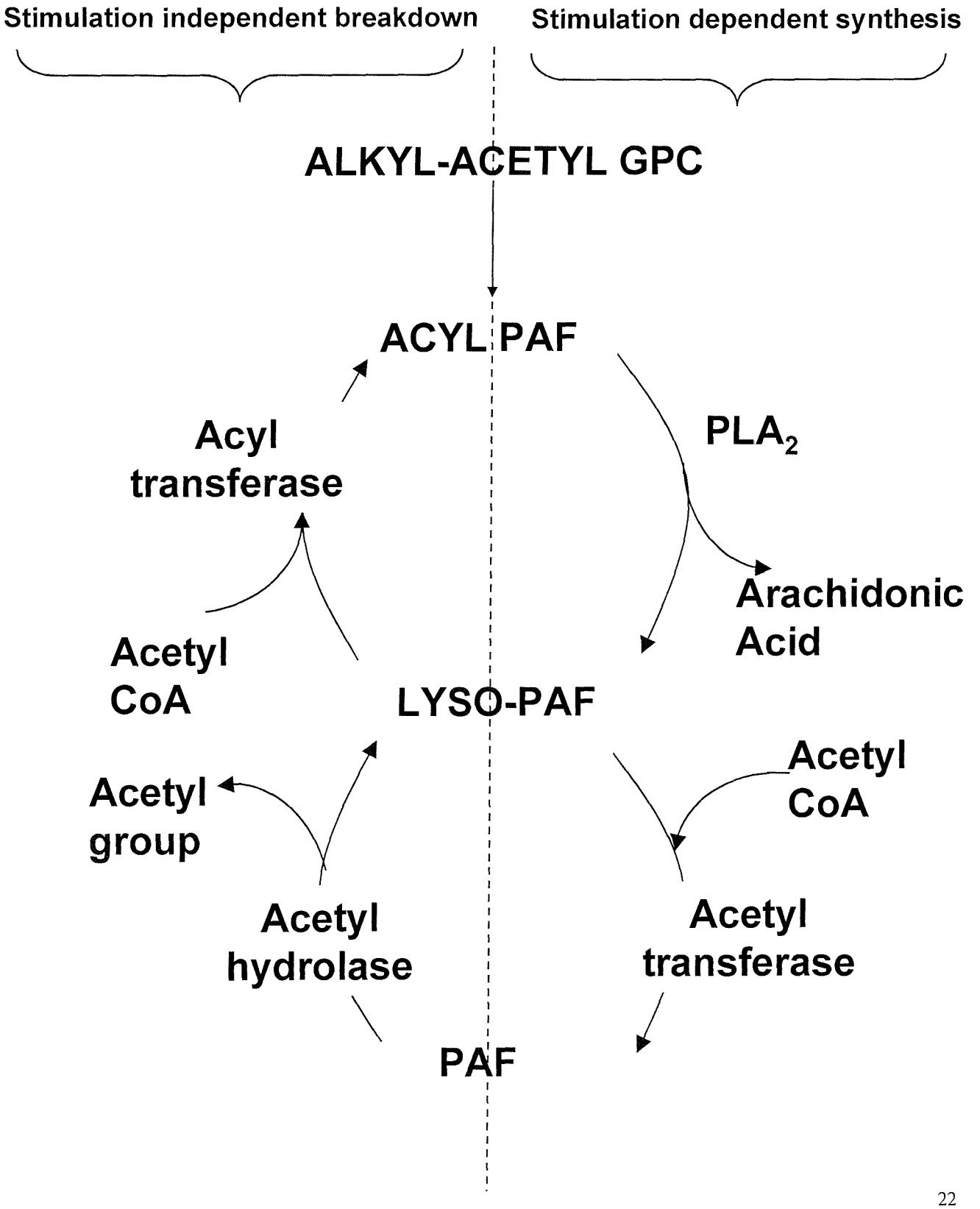
1.3.1 Platelet Activating Factor (PAF).

Platelet activating factor (PAF) is a potent biologically active lipid generated by most inflammatory cells. Macrophages and eosinophils produce PAF on activation, neutrophils produce PAF as a result of phagocytosis of opsonised particles, and PAF is also produced but not released by mast cells when stimulated with antigen (Spina et al. 1989) this may suggest that PAF has a role intracellularly. The intracellular role of PAF has not yet been determined however PAF-specific binding sites have been detected in the cytosol of neutrophils (Svetlov and Nigam 1993).

PAF is synthesised from acyl-PAF derived from alkyl-acetyl-glycerophosphorylcholine (alkyl-acetyl-GPC) a component of the cell membrane (Braquet et al. 1987) (**Fig 1.3**). Phospholipase A_2 (PLA_2) acts on acyl-PAF to liberate arachidonic acid and lyso-PAF which is acetylated to biologically active PAF by acetyl transferase (Braquet et al. 1987) (**Fig 1.3**). PAF is broken down to lyso-PAF by acetyl hydrolase and further to acyl-PAF by acyl-transferase (Braquet et al. 1987). (**Fig 1.3**)

PAF has many actions relevant to the pathophysiology of asthma including

Fig 1.3 The synthesis and breakdown of Platelet - activating factor



bronchoconstriction, bronchial hyperresponsiveness to methacholine, vascular permeability and accumulation of leukocytes especially eosinophils and neutrophils (Chung and Barnes, 1991). However the role of PAF in allergic asthma is seen to be limited by studies using PAF antagonists (Kuitert and Barnes, 1995). The specific oral PAF antagonist MK-287 given to allergic asthmatics 1 hr before antigen challenge inhibits neither the EAR nor LAR, or the increase in airway responsiveness (Bel et al. 1991). The role of PAF in increasing bronchial hyperresponsiveness is unclear. Inhaled PAF fails to induce airway hyperresponsiveness in normal human subjects (Lai and Holgate, 1990; Spencer et al. 1990; Cuss et al. 1986), however, bronchial responsiveness to methacholine was increased after administration of PAF compared to lyso-PAF (Cuss et al. 1986) and the PAF antagonist Y-24180 significantly reduced bronchial responsiveness to methacholine in asthmatic subjects (Hozawa et al. 1995). It is likely that PAF exerts some of its effects by stimulating the secondary production of leukotrienes (Kuitert and Barnes 1995), as PAF-induced bronchoconstriction is inhibited by a leukotriene receptor antagonist (Kidney et al. 1993).

1.3.2 Metabolism of Arachidonic acid.

Eicosanoids are derived from arachidonic acid (AA, eicosatetraenoic acid) released from the nuclear/cell membrane phospholipids. These consist of the leukotrienes (LT), prostaglandins (PG), and thromboxanes (TX) which are synthesised by almost every cell type in the body either constitutively or upon cell activation.

Upon stimulus-specific cell activation arachidonic acid is released from the sn-2 /A2 position of membrane phospholipids by phospholipase A_2 (PLA_2) (Clark et al. 1991). Release of arachidonic acid by the action of PLA_2 in the mast cell occurs within 30 seconds of IgE dependent activation (Nakamura et al. 1991).

Arachidonic acid can also be liberated as a result of phospholipase C (PLC) activation following cross linkage of the IgE receptor. PLC cleaves the membrane phospholipids at the C position to produce IP3 and DAG. Diacylglycerol lipase may then cleave DAG at the sn-2/A2 position to liberate AA directly without the need for downstream PKC activation and raised intracellular Ca^{2+} required for activation of PLA_2 (**Fig 1.2**).

1.3.2.1 Phospholipase A_2

Two classes of PLA_2 enzymes have been characterised in mammalian cells, the 14kDa secretory PLA_2 ($sPLA_2$) is subdivided into extracellular forms which are abundant in

pancreatic secretions and also in plasma, lymph and pulmonary alveolar secretions. Intracellular $sPLA_2$ is widely distributed in cells and tissues where it is located either in the cytosolic compartment associated with the plasma membrane or stored within secretory granules. This enzyme requires mM levels of Ca^{2+} for catalysis (Dennis et al. 1995). The 85kDa cytosolic PLA_2 ($cPLA_2$) is also widely distributed in cells and tissues. Cytosolic PLA_2 releases AA from membrane phospholipids (**Figs 1.2, 1.5, 1.7**), whereas $sPLA_2$ also hydrolyses phospholipid substrates to produce fatty acids other than AA. The catalytic activity of $cPLA_2$ increases sharply with an increase in calcium concentration (to μM levels) corresponding to the change in cytosolic calcium concentration when cells are activated (Clark et al. 1991) (**Fig 1.2**). Increased phosphorylation by protein kinases and tyrosine kinases is also associated with an increase in activity of $cPLA_2$ (Goldberg et al. 1990) (**Fig 1.2**). This increase in activity is accompanied by the enzyme translocating to the nuclear membrane fraction of the cell where it may be more closely associated with 5-LO, FLAP (**Fig 1.5**) and the COX iso-enzymes (**Fig 1.7**)(Clark et al. 1991).

1.3.3 Leukotrienes.

The leukotrienes were formerly known as 'slow reacting substance of anaphylaxis' (SRS-A) in 1938 when they were described by Feldberg and Kellaway. Early experiments showed that smooth muscle contractions caused by snake venom was not mediated by histamine or directly by a substance within the venom, but by a substance formed within the tissue. Shortly after, SRS-A was recognised as a product of an in vitro anaphylactic reaction in guinea pig lung by Kellaway and Trethewie in 1940 and by Brocklehurst in 1960. When human lung tissue from allergic individuals was stimulated with an appropriate antigen, two contractile patterns were seen, a slow and sustained smooth muscle contraction distinguishable from the fast twitch-like contraction caused by histamine (Schild et al. 1951). In 1971, it was shown that SRS-A was not a prostaglandin (Strandberg and Uvnäs 1971) and in the late 1970's, SRS-A was established to be a combination of the three cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4. (Murphy et al. 1979; Morris et al. 1980; Lewis et al. 1980).

1.3.3.1 5-lipoxygenase pathway of leukotriene synthesis.

Following release from phospholipids by PLA_2, arachidonic acid is thought to be presented to 5-lipoxygenase by 5-LO activating protein (FLAP) (Mancini et al. 1993;

Dixon et al. 1990) an 18kDa protein resident in the nuclear membrane (**Fig 1.5**). The interaction between AA and the 5-LO is a two-stage process via the intermediate 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) before the formation of the unstable and highly reactive leukotriene (LT) A_4 (Rouzer et al. 1986) (**Fig 1.4**). Depending on the cell type, LTA_4 may either be converted to the 5,12-dihydroxy acid LTB_4 by LTA_4 hydrolase (Radmark et al. 1984) or to the parent cysteinyl leukotriene LTC_4 by the action of LTC_4 synthase, which conjugates LTA_4 to reduced glutathione (Yoshimoto et al. 1988, Lam et al. 1994) (**Fig 1.4**). Extracellular transport of LTC_4 occurs by the ATP dependent multidrug resistance associated protein-1 (MRP-1) (Leier et al. 1994; Lam et al. 1992; Bartosz et al. 1998) (**Fig 1.5**). This transport precedes sequential cleavage of glutamic acid by γ-glutamyl transpeptidase and of glycine by dipeptidase enzymes ubiquitous to the tissues and the circulation, yielding the cysteinyl leukotrienes LTD_4 and LTE_4 (Anderson et al. 1982; Lee et al. 1983b). (**Fig 1.4**).

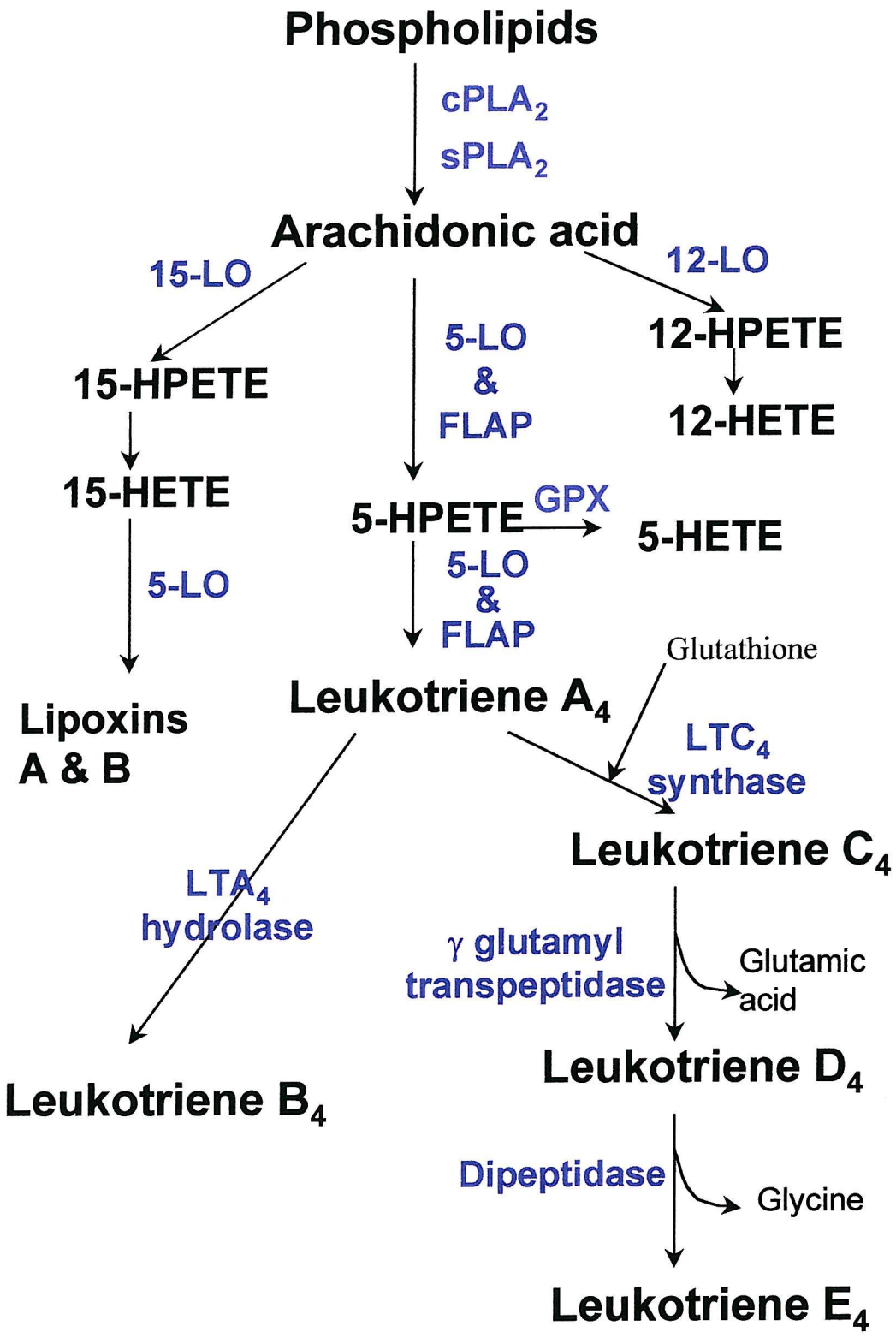
1.3.3.2 Enzymes of the leukotriene pathway.

Upon activation of 5-LO, the enzyme undergoes a Ca^{2+}-dependent redistribution from a soluble intracellular compartment to a nuclear membrane compartment (Rouzer and Kargman, 1988; Woods et al. 1993) (**Fig 1.5**). The 5-LO activating protein FLAP is permanently resident in the nuclear membrane (Miller et al. 1990; Woods et al. 1993) and hence the translocation of 5-LO to the nuclear membrane facilitates the interaction between 5-LO and FLAP necessary for leukotriene synthesis (**Fig 1.5**). There is also a second pool of 5-LO in the nucleus of some resting cells, which is associated with euchromatin and again translocates to the nuclear membrane upon cell activation (Wilborn et al. 1996; Woods et al. 1995) (**Fig 1.5**).

LTA_4H belongs to a family of zinc metallohydrolases. The subcellular localisation of LTA_4H has not yet been confirmed but it is considered that the hydrolytic activity resides in the cytosol although some studies suggest that it may be membrane bound (Gut et al. 1987; Haeggstrom, 1997) (**Fig 1.5**).

During the hydrolysis of LTA_4 to LTB_4, LTA_4 hydrolase is covalently modified and inactivated by LTA_4; a process referred to as suicide inactivation. (Orning et al. 1990). A mutation of the peptide segment in LTA_4 hydrolase to which LTA_4 binds, by the exchange of a tyrosine residue (Tyr 378) for either a phenylalanine or glycine residue renders the enzyme virtually resistant to suicide inactivation (Mueller et al. 1996) but still allows the hydrolysis of LTA_4 to LTB_4 and an isomer of LTB_4. (Mueller et al.

Fig.1.4 Lipoxygenase pathway for leukotriene synthesis.



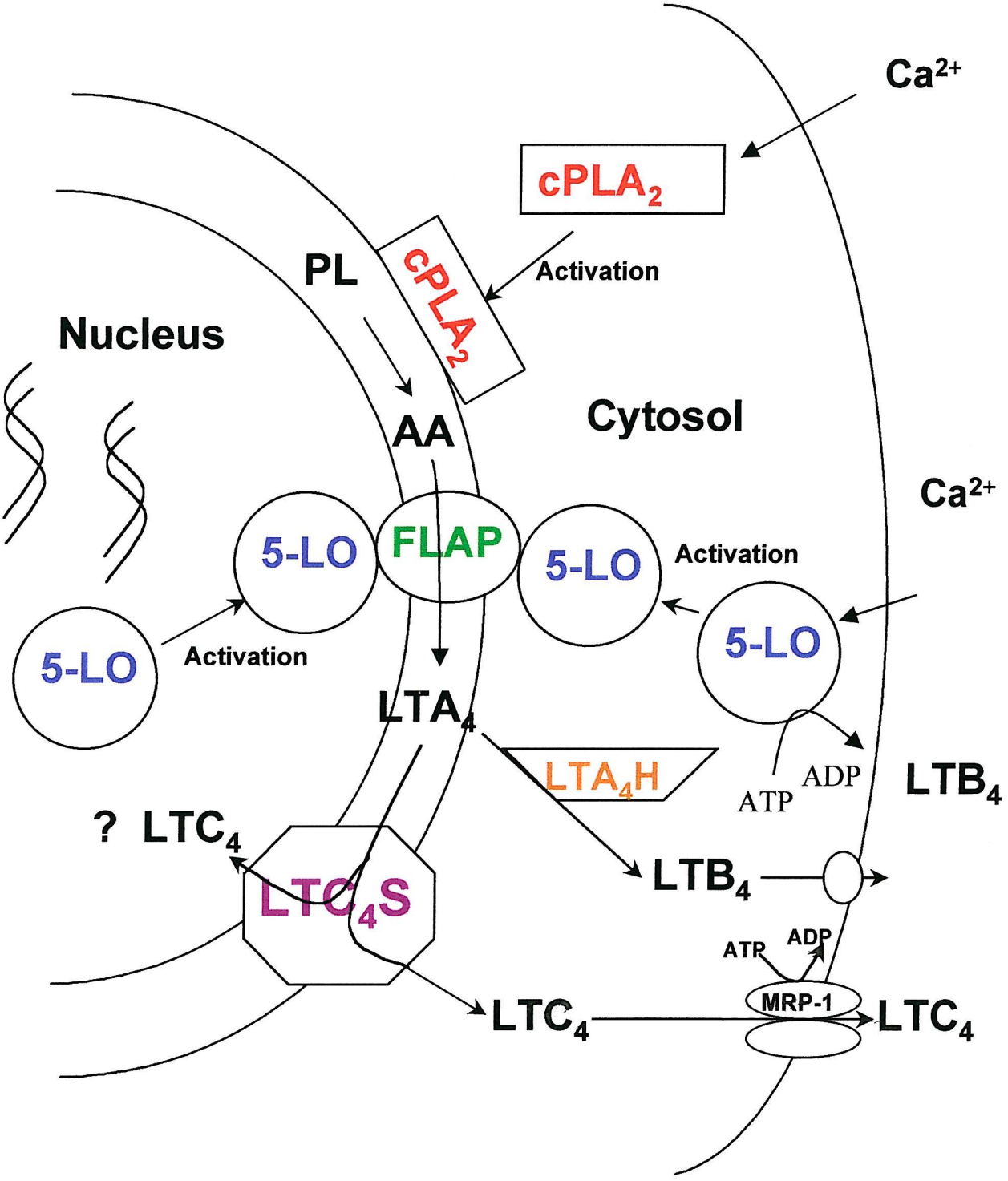
1996). Tyr 378 therefore plays an important role, perhaps by assisting in the proper alignment of LTA_4 in the substrate-binding pocket. Leukotriene A_4 hydrolase also has peptidase activities (Wetterholm and Haeggstrom, 1992) which may be abrogated by a mutation of glutamic acid-296 a residue conserved in several zinc hydrolases (Wetterholm et al. 1992).

Expression of LTC_4 synthase is predominantly limited to cells of myeloid lineage: eosinophils (Weller et al. 1983), mast cells (MacGlashan et al. 1982) and monocyte/macrophages (Williams et al. 1984), but it has also been detected functionally in platelets (Maclouf and Murphy, 1988) and vascular smooth muscle cells (Feinmark and Cannon, 1987). The latter lack 5-lipoxygenase, suggesting that these cells take up extracellular LTA_4 for enzymatic conversion to LTC_4 within the cell (Pace-Asciak et al. 1986). This is an example of transcellular leukotriene synthesis.

LTC_4 synthase is a dimer of 16.6kDa proteins (Penrose et al. 1992). Staining using immunofluorescence on fixed sections of human lung using a polyclonal IgG against purified LTC_4 synthase from human lung showed LTC_4 synthase expression in macrophages (Penrose et al. 1995). In human bronchial tissue from asthmatic subjects LTC_4 synthase was predominantly localised to eosinophils, mast cells and macrophages (Cowburn et al 1998). Staining also demonstrated a predominantly perinuclear distribution of LTC_4 synthase (Penrose et al. 1995) (**Fig1.5**) as is seen with FLAP to which LTC_4 synthase has a high degree of homology. Despite the glutathione conjugation of the protein the cDNA for LTC_4 synthase shows no outstanding homology to glutathione S-transferases, but shares substantial (44%) amino acid identity in its consensus N-terminal two-thirds with FLAP. This region of FLAP is where it binds AA for presentation to 5-LO for the synthesis of LTA_4; the homologous domains in LTC_4 synthase might accept LTA_4 for conjugation with GSH. This is also the binding site of the FLAP inhibitor MK-886, which also inhibits LTC_4 synthase, but with 1000-fold lower efficacy. (Lam et al. 1994).

The location or translocation of PLA_2, 5-LO, FLAP, and LTC_4 synthase at the nuclear membrane suggests that the nuclear membrane is the site at which AA release, LTA_4 synthesis and conversion to LTC_4 all occur (**Fig 1.5**). Leukotrienes synthesised in the nucleus may have important nuclear signalling actions other than the classical extracellular ones. The observation that LTB_4 can bind to the peroxisomal proliferator-activated receptor-α (PPARα) which is a transcription factor that regulates the oxidative degradation of fatty acids and their derivatives indicates that intranuclear actions of

Fig. 1.5 Localization of the lipoxygenase pathway within the cell.



leukotrienes are feasible (Devchand et al. 1996). There is evidence to suggest that 5-LO products may modulate mitogenesis and regulation of *fos* and *egr* expression. Lipoxygenase inhibition reduced platelet-derived growth factor (PDGF)- induced mitogenesis in hepatic Ito cells and suppressed the resultant *fos* and *egr* expression (Beno et al. 1995). Apoptosis may also be modulated by lipoxygenase derived products, prolonged exposure of neutrophils to LTB_4 dose-dependently inhibited neutrophil apoptosis (Hebert et al. 1996). Lipoxygenase products may also be involved in the regulation of NF-κB (Baeuerle and Henkel, 1994). Alternatively these effects may depend on reactive oxygen species which are a by-product of the 5-LO pathway and have been implicated as important second messenger molecules (Los et al. 1995). The nuclear location of 5-LO and related enzymes suggest there may be interactions between 5-LO and intranuclear proteins or even genes (**Fig 1.5**). Recent work has demonstrated a nuclear localisation sequence (NLS) in the 5-LO protein that appears to facilitate 5-LO redistribution to the nucleus of activated cells (Lepley and Fitzpatrick, 1998; Chen et al. 1998).

Leukotriene C_4 is exported from the cell by an ATP dependent mechanism (Shaub et al. 1991), LTD_4 and LTE_4 are also substrates of this export pump (Shaub et al. 1991, Keppler et al. 1992). The multidrug resistance-associated protein (MRP) confers multidrug resistance when overexpressed in cells such as the human small cell lung cancer cell line H69AR (Loe et al. 1996, Grant et al. 1994). Transport of LTC_4 was 25-fold higher in HL60 cells overexpressing the MRP protein than the drug sensitive HL60 cells and [^{3}H]LTC_4 was detected bound to a 190kDa protein which was identified by immunoprecipitation with antiserum against MRP (Jedlitschky et al. 1994). MRP is a member of the ATP-binding cassette transporter superfamily (Cole et al. 1992). Experiments using membrane vesicles from MRP transfected HeLa cells have shown ATP-dependent transport of LTC_4, D_4 and E_4 and other GSH S-conjugates by MRP with LTC_4 having the highest affinity for this transporter (Leier et al. 1994b). This ATP-dependent carrier system is distinct from the related ATP-dependent P-glycoprotein, which also transports cytotoxic agents but is not involved in transport of cys-LTs (Ishikawa et al. 1990). The leukotriene D_4 receptor antagonist MK-571 which is structurally similar to LTD_4 effectively inhibited LTC_4 transport by MRP and inhibited binding of [^{3}H]LTC_4 to a 190kDa protein in the MRP transfectants (Leier et al. 1994b). Both transport and binding of [3H]LTC_4 can also be inhibited by an MRP-specific monoclonal antibody (Loe et al. 1996). Together this provides compelling evidence for

the involvement of MRP in cysteinyl leukotriene transport. MRP has been detected functionally in eosinophils (Lam et al. 1992). MRP mRNA and protein are present in the mast cell line HMC-1(Bartosz et al. 1998) and bronchial epithelial cells (Brechot et al. 1998). Analysis of subcellular fractions of HL60 cells overexpressing MRP has localised MRP primarily to the endoplasmic reticulum with lower levels also present in plasma membranes of drug resistant HL60 cells (Krishmanary and Center 1993).

Chapter 7 of this thesis aims to determine the effect of MRP inhibition on A23187 stimulated LTC_4 transport in eosinophils, and characterise compound XR9173 (Xenova) as an MRP inhibitor by comparison with MK-886 (a leukotriene synthesis inhibitor) and MK-571 (a leukotriene receptor antagonist and MRP inhibitor).

1.3.3.3 Genetics of 5-LO pathway.

The human 5-LO gene is located on chromosome 10, containing 14 exons , 13 introns and a single transcriptional start site with the general characteristics of a constitutive gene (Funk et al. 1989). The 5-LO gene promoter, however, contains numerous consensus binding sites for many known transcription factors including Sp1, Sp3, Egr-1, Egr-2, AP-2, glucocorticoid receptors and NF-κB (Hoshiko et al. 1990) suggesting that expression of 5-LO may be inducible. Polymorphisms are present in the 5-LO gene promoter region, inducing a variation in the number of tandem zinc-finger (Sp1/Egr-1) binding sites in the transcription factor-binding region. There are 5 in the wild type, and 3,4,6 or 7 in the mutant forms. The mutant alleles are associated with reduced reporter gene activity and altered transcription factor binding suggesting their potential roles in regulation of 5-LO gene expression (In et al. 1997). In a recent study of mutant 5-LO alleles in asthmatics by Drazen and colleagues (1998), homozygotes for the normal 5-LO allele responded to a 12-week treatment with the 5-LO inhibitor ABT-761 with an increase in FEV_1 by 15%. The same was true for heterozygotes but homozygotes for the mutant 5-LO alleles responded to treatment with ABT-761 with only a 5% increase in FEV_1. This would suggest that the wild-type 5-LO allele is dominant. Only 4-5% of the population are homozygotes for mutant 5-LO alleles (Drazen, 1998).

The gene for FLAP has also been cloned and sequenced (Kennedy et al. 1991), its promoter sequence contains features characteristic of a highly inducible gene, including a TATA box (normally absent from constitutive genes) and response elements for AP-2 and glucocorticoids (Kennedy et al. 1991). Data from both eosinophils and neutrophils

confirm that FLAP expression is more highly inducible than 5-LO (Cowburn et al, 1999, Pouliot et al. 1994a, Pouliot et al. 1994b, Coffey et al. 1994).

The gene for LTC_4 synthase is located on chromosome 5q35 (Penrose et al. 1996), which is of interest because it is in close proximity to the genes encoding many proinflammatory cytokines including IL-3, IL-4, IL-5, IL-13, IL-9, and GM-CSF localised in the region 5q23-5q31 (Van Leeuwen et al. 1989). Other genes located in this region include those encoding the β_2-adrenergic receptor, the lymphocyte specific corticosteroid receptor, CSF receptors and platelet-derived growth factor. The long arm of chromosome 5 has also been linked to atopy and allergic disease by genome-wide scans for candidate genes. In contrast the gene for FLAP is on chromosome 13 (Ford-Hutchinson et al. 1994) and the gene for 5-LO is on chromosome 10 (Funk et al. 1989).

Aspirin intolerant asthmatics (AIA) over-express LTC_4 synthase in cells (the majority being eosinophils) of the bronchial submucosa and have associated increased levels of cys-LTs in BAL fluid (Cowburn et al. 1998). LTC_4 synthase is overexpressed in aspirin induced asthmatics attributed to a polymorphism in the LTC_4 synthase gene promoter of aspirin intolerant asthmatics. The polymorphism is in 5'-flanking region of the LTC_4 synthase gene promoter and creates a new recognition site for the nuclear transcription factor AP-1 (Sanak et al. 1997). Homozygotes for the LTC_4 synthase variant allele have higher urinary LTE_4 than heterozygotes (Szczeklik et al. 1998). Aspirin intolerant asthma is discussed further in section 1.4.

1.3.3.4 Cellular leukotriene synthesis and its regulation.

Calcium ionophore causes release of LTC_4 from peripheral blood eosinophils within 4-8 minutes of stimulation, reaching a peak of 60ng per 10^6 cells at 30 minutes and tailing off at 1hour (Silberstein et al. 1986), unstimulated, these cells produce less than 10ng per 10^6 cells.

Stimulation of human eosinophils with GM-CSF enhanced ionophore-induced generation of LTC_4 by 135% within 60 minutes (Silberstein et al. 1986). Stimulation of human eosinophils with IL-3 similarly enhanced ionophore-induced LTC_4 production 3-fold within 30 minutes (Rothenberg et al. 1988). Platelet activating factor (PAF) directly induces LTC_4 production from eosinophils (Tamura et al. 1988), and this PAF induced LTC_4 production is enhanced by approx. 600% by priming with IL-3 or IL-5 (Takafuji et al. 1991).

In mouse bone marrow mast cells (BMMC), IgE dependent activation induces

LTC_4 production of 8ng per 10^6 cells. This LTC_4 production was enhanced by culture over 2-5 weeks with IL-3 (Murakami et al. 1995c) and stem cell factor (SCF) (Murakami et al. 1995d) and was associated with increased expression of PLA_2 (Murakami et al. 1995a; Murakami et al. 1995d), 5-LO, FLAP, and LTC_4 synthase (Murakami et al. 1995d). The human mast cell line (HMC-1) has been shown to produce 5-HETE, LTC_4 and LTB_4 upon stimulation with arachidonic acid and calcium ionophore (Macchia et al. 1995). Human lung mast cells also produce LTC_4 on stimulation with anti-IgE (MacGlashan et al. 1982), but the factors regulating 5-LO pathway enzyme expression in human mast cells are unclear.

Treatment of monocytes with dexamethasone for 2 days upregulates 5-lipoxygenase and FLAP proteins and mRNAs and increases the calcium ionophore stimulated release of 5-LO pathway products (Riddick et al. 1997). Upregulation of 5-LO and FLAP expression also accounts for the marked increase in LTB_4 synthesis observed as blood monocytes differentiate into alveolar macrophages *in vivo* (Coffey et al. 1994). Coculture of monocytes with lymphocytes over 4 days enhances calcium ionophore-stimulated 5-LO activity in monocytes, if the supernatants from lectin stimulated lymphocytes are added to monocytes stimulated 5-LO activity is again enhanced (Ring et al. 1996). The supernatants from the lectin stimulated lymphocytes were found to contain GM-CSF and IL-3, and the effect of the supernatants could be mimicked by addition of these cytokines to the monocytes (Ring et al. 1996).

GM-CSF has also been shown to upregulate 5-LO and FLAP proteins in human neutrophils (Stanková et al. 1995, Pouliot et al. 1994a, Pouliot et al. 1994b)). This occurs by inducing gene transcription and *de novo* protein synthesis in human neutrophils as confirmed by pre-treatment of the cells with the transcription inhibitor actinomycin D or with the protein synthesis inhibitor cycloheximide inhibiting the GM-CSF induced increase in protein (Pouliot et al. 1994a, Pouliot et al. 1994b).

1.3.3.5 Leukotrienes in asthma.

The role of the cysteinyl leukotrienes in asthma has been clearly established by their potent biological activities, their presence and release in the airways, and the clinical effects of 5-LO inhibitors and LT receptor antagonists in asthma. The initial hypothesis that the cysteinyl-leukotrienes were important mediators in asthma was based on the facts that the cys-LTs are more potent contractors of smooth muscle than either acetylcholine or histamine (Hay et al. 1987), that they are released upon antigen

challenge in the human lung (Brocklehurst, 1960; Brocklehurst, 1953, Wenzel et al. 1990), and urinary and BAL fluid levels of cys-LTs are elevated in asthmatics compared to non-asthmatics (Lam et al. 1988; Taylor et al. 1989).

In vitro stimulation of bronchial tissue from asthmatic subjects with allergen or A23187 induced bronchoconstriction that correlated with cys-LT production by the tissue (Dahlen et al. 1982). The bronchoconstriction is mostly blocked by anti-leukotriene drugs with a contribution from antihistamines (Bjorck and Dahlen, 1993), a result confirmed in vivo using the leukotriene receptor antagonist zafirlukast and antihistamine loratadine (Roquet et al. 1997).

1.3.3.6 Biological effects of cysteinyl-leukotrienes.

The long-lived contractions of smooth muscle produced by cys-LTs have already been described (Orange and Austen, 1969). The cys-LTs are extraordinarily potent contractors of airway smooth muscle. Inhaled LTC_4 and LTD_4 are 1000 times, and LTE_4 10 times more potent than histamine at constriction of bronchial smooth muscle in normal subjects (Dahlen et al. 1980, Chagnon et al. 1985). In asthmatic subjects the cysteinyl leukotrienes are almost equipotent and more active than in normal subjects (Arm et al. 1990).

Microvascular permeability is an important effect of the leukotrienes as it allows exudation of plasma and contributes to oedema. Challenge with synthetic LTD_4 or ovalbumin causes microvascular leakage in sensitised guinea-pig airways that is inhibited by the leukotriene receptor antagonists FPL 55712 and ONO-1078 (Woodward et al. 1983; Evans et al. 1989; Obata et al. 1992). Electron microscopic studies on microvascular permeability indicate that LTE_4 induces hyperpermeability by opening tight junctions between cells of the vascular endothelium (Joris et al. 1987).

The cys-LTs also induce mucus secretion and reduce mucociliary transport. Leukotrienes C_4 and D_4 stimulate mucus secretion from cultured human airway mucosal explants, an effect that is blocked by FPL 55712 (Marom et al. 1982; Coles et al. 1983). The cys-LTs also decrease the beat frequency of human respiratory cilia and, hence, reduce mucus clearance from the airways (Bisgaard and Pedersen, 1987).

Leukotriene D_4 has been shown to induce an eosinophil chemotaxis *in vitro* that is abolished by SK&F 104353 (pobilukast) (Spada et al. 1994). Inhalation of LTE_4 by allergic subjects increases numbers of eosinophils in the lamina propria of bronchial mucosal biopsies, but there are no changes in numbers of mast cells, lymphocytes,

macrophages or plasma cells (Laitinen et al. 1993). Similarly, eosinophil counts are increased in induced sputum following inhaled LTD_4 challenge (Diamant et al. 1997). In ovalbumin sensitised guinea pigs, there was a similar four-fold increase in eosinophils 24 hours after ovalbumin challenge, and this was attenuated by a cys-LT receptor antagonist but not by an H_1 histamine antagonist or by a COX inhibitor (Foster et al. 1991). LTD_4 induced airway eosinophilia in guinea pigs is antagonised by the rat anti-mouse IL-5 monoclonal antibody derived from the TRFK-5 hybridoma cell line (Underwood et al. 1995). This indicates that leukotriene induced eosinophil recruitment is mediated by secondary release of IL-5.

The combined actions of histamine and the cys-LTs mediate the intrinsic tone of isolated human bronchi (Ellis and Undem, 1994).

Smooth muscle proliferation in the ovalbumin-challenged rat is attributed to the cys-LTs, as the subsequent increase in airway smooth muscle mass is reduced in animals pre-treated with MK 571 (Wang et al. 1993). LTD_4 was shown not to have a direct proliferative effect on human airway smooth muscle cells *in vitro* but to markedly augment proliferation induced by the mitogen, epidermal growth factor (EGF) (Panettieri et al. 1998). These studies suggest that cys-LTs may play a role in structural remodelling of the airways, an important feature of asthma.

It is possible that cys-LTs may influence pulmonary function by modulating the activity of the afferent nervous system. In the guinea pig, cys-LTs may directly stimulate local afferent nerves to release tachykinins, leading to bronchoconstriction and plasma exudation (McAlexander et al. 1998), but similar experiments have not been performed in man.

1.3.3.7 Leukotriene B_4

Leukotriene B_4 has chemotactic activities for neutrophils and eosinophils (Bray, 1983, Ford-Hutchinson 1980). Its other actions include increasing complement receptors on granulocytes (Nagy et al. 1982), release of lysosomal enzymes from neutrophils, increased adherence of neutrophils to endothelium (Hoover et al. 1984), and increased IL-6 release from monocytes. Neutrophils, macrophages and monocytes generate LTB_4 (Bray, 1983; Henderson et al. 1984) and both neutrophils and eosinophils express LTB_4 receptors. Although its role in asthma is unclear the presence of neutrophils in late phase asthma and sudden onset fatal asthma means that a role in asthma remains a possibility

(Christie and Barnes, 1996). The lack of effect of the LTB_4 receptor antagonist LY29311 on symptoms of asthma after allergen challenge although reducing counts of neutrophils in BAL (Evans et al. 1996) suggests that LTB_4 does not have a pivotal role in the pathophysiology of acute asthma.

1.3.2.8 Leukotriene receptors.

The classification of the cys-LT-receptors is based on functional data, obtained mainly with the use of cys-LT antagonists. Initial experiments in guinea-pig airway preparations using the cys-LT antagonist FPL 55712 suggested two distinct receptors for LTC_4 and LTD_4. When LTC_4 or LTD_4 were administered to guinea pig trachea FPL 55712 had comparable effects (Krell et al. 1983). If the metabolic conversion of LTC_4 to LTD_4 was inhibited the action of LTC_4 was as potent as LTD_4 but the action of LTC_4 could not be antagonised by FPL 55712 (Snyder and Krell, 1984). This suggested the existence of distinct LTC_4- and LTD_4-receptors, only the latter of which is antagonised by FPL 55712.

These distinct receptors were not found in the human airway where FPL 55712 antagonised the bronchoconstrictor effect of LTC_4 and LTD_4 to the same extent in the presence of serine-borate (Buckner et al. 1986) hence in humans the receptors were named *Cys-LT$_1$* and *Cys-LT$_2$* (Coleman et al. 1995). The *Cys-LT$_1$* receptor is found in the human bronchus, guinea pig trachea, ileum and gall bladder, and the rat lung, and is antagonised by a large number of pharmacological agents. Leukotrienes C_4 and D_4 are equipotent at this receptor and greater in potency than LTE_4. The *Cys-LT$_2$* receptor is not present in the human bronchus but is described in the human pulmonary vein, the guinea pig trachea and ileum, and in sheep and ferret airways. At *Cys-LT$_2$* LTC_4 is more potent than LTD_4 and of far greater potency than LTE_4. Apart from BAY u9773 there are few antagonists proven to block this receptor.

Of the cys-LT receptors only cys-LT_1 has been cloned (Lynch et al. 1999). It is the first marker of asthma to have been mapped to the X chromosome (q13-q21) (Lynch et al. 1999). Expression of the cys-LT_1 receptor mRNA has been localised to lung, spleen and peripheral blood leukocytes, (Lynch et al. 1999). In normal human lung expression of cys-LT_1 mRNA was seen only in smooth muscle cells and macrophages with very low expression in airway lumen epithelial cells (Lynch et al. 1999). Cys-LT_1 is G-protein coupled (Metters et al. 1993, Howard et al. 1992) and activation of this receptor elevates intracellular calcium (Chan et al. 1994).

The receptor for LTB_4 is designated the BLT-receptor and is found on human polymorphonuclear leukocytes and eosinophils. Cloning of the BLT-receptor shows that it belongs to the 7-transmembrane domain family linked to G-proteins and is coupled to phospholipase C (PLC) (Yokomizo et al. 1997).

1.3.3.9 ***Presence of enzymes to degrade active leukotrienes at the disease site.***

Leukocytes have a relatively limited capacity to catabolise the cys-LTs that they generate. Cys-LTs are eliminated mostly by diffusion into the circulation, from where they are rapidly removed and broken down by ω and β oxidation in the liver. (Keppler, 1992). Leukotriene E_4 undergoes ω oxidation in the liver directly to 20-OH-LTE_4 and to 20-COOH-LTE_4 (Orning, 1987), or it may be acetylated by N-acetyl transferase to the biologically active N-acetyl-LTE_4 (Maltby et al. 1990) before ω oxidation to 20-carboxy-N-acetyl-LTE_4 by a cytochrome P-450. The 20-carboxy products of ω oxidation then undergo β oxidation, which shortens the carbon chain sequentially by 2 carbons to products such as 16-carboxy-LTE_4 or the N-acetylated equivalent (Stene and Murphy, 1988).

Cys-LTs can be metabolised locally by the peroxidative pathway (Lee et al. 1983a). The cysteine residue of the cys-LTs contains a sulphur that is able to make further bonds with oxygen. When this sulphur has completed its valency with double bonds to two oxygen atoms it forms a sulphoxide. This oxygenation occurs when eosinophils and/or neutrophils produce EPO or MPO, as part of the respiratory burst. The product O_2^- (superoxide) combines with H^+ and Cl^- ions to produce hypochlorous acid (HOCl), acid that oxidises the cysteinyl sulphur of cys-LTs to less active sulphones and sulphoxides. Further oxidation cleaves the peptide side chain to produce 6-trans-LTB_4 or 12-epi-6-trans-LTB_4 that are metabolised in the liver by β-oxidation. LTE_4 and β-oxidation products are excreted in the urine. Approx. 5% of LTE4 is excreted in the urine (Maltby et al. 1990).

Leukotriene B_4 is metabolised faster in blood and tissues than the cys-LTs as the enzymes involved in ω oxidation are constitutively present in the neutrophil. LTB_4 undergoes ω oxidation to 20-OH-LTB_4 (which retains biological activity) (Clancy et al. 1984) by leukotriene B_4 ω-hydroxylase, a cytochrome P-450 (Shak and Goldstein, 1984). This is then converted to 20-oxo-LTB_4 and further to 20-COOH-LTB_4. Alternatively, this same degradation of 20-OH-LTB_4 may occur in hepatocytes by the action of alcohol dehydrogenase and aldehyde dehydrogenase. The 20-COOH-LTB_4 is then subject to β-

oxidation in the liver similarly to the cys-LTs resulting in chain shortening to products such as 16-COOH-LTB_3. β-oxidation products of LTB_4 are excreted predominantly in bile but also in urine. LTB_4 itself and its ω-oxidation products are rarely detected in either bile or urine because of local metabolism that occurs with a half-life of 3-5 minutes in purified neutrophils (Shak and Goldstein, 1984).

1.3.2.10 Anti-leukotriene drugs.

For preparations that target only one mediator, agents that inhibit the synthesis or antagonise the activity of the cys-LTs i.e. 5-LO and FLAP inhibitors and cys-LT receptor antagonists have demonstrated unexpectedly high efficacy in clinical trials. The production of leukotrienes can be inhibited by blocking the activity of 5-LO directly, or indirectly by preventing arachidonate binding to FLAP. 5-LO and FLAP inhibition reduces the synthesis of both the cys-LTs and LTB_4. Alternatively, the actions of the leukotrienes can be blocked by leukotriene receptor antagonists acting either at the *cys-LT_1* receptor or at the BLT receptor.

1.3.2.11 Efficacy of anti-leukotriene drugs in improving asthma symptoms.

Agents such as the anti-leukotrienes are devoid of intrinsic bronchodilatory activity but induce bronchodilation when administered to asthmatic individuals (Hui and Barnes, 1991; Gaddy et al. 1992). They can be administered in addition to patients existing β-agonist and/or corticosteroid treatment regime. An added advantage of anti-leukotriene drugs is that they are oral preparations, which confers greater patient acceptance and compliance than inhaled medications (Kelloway et al. 1994), although this precludes their use as 'rescue' medication.

Recent clinical trials with the *cys-LT_1* receptor antagonists montelukast (Singulair®, MK-0476), pranlukast (ONO 1078), and zafirlukast (Accolate™; ICI-204,219), the first anti-leukotriene drugs to be marketed for clinical use, have shown significant improvements in many key areas of asthma. Montelukast, zafirlukast, and pranlukast offer protection against LTD_4-induced bronchoconstriction (Schoors et al. 1993; Smith et al. 1993; O'Shaugnessy et al. 1997) and exercise induced asthma (Leff et al. 1998; Makker et al. 1993; Fujimura et al. 1993). Zafirlukast decreases both the early and late responses to allergen and attenuates the increased bronchial responsiveness that follows an inhaled allergen challenge (Taylor et al. 1991). After an 8-week 10mg daily treatment regime of montelukast, baseline forced expiratory volume in 1 sec (FEV_1) was

significantly improved (Knorr et al. 1998; Reiss et al. 1998; Leff et al. 1998; Noonan et al. 1998). This suggests that a broad range of patients with asthma would benefit from leukotriene receptor antagonist treatment.

The 5-LO inhibitors zileuton (A-64077) and ABT-761 compared with placebos, specifically inhibit leukotriene biosynthesis in asthmatics as measured by urinary excretion of LTE_4 and *ex vivo* blood LTB_4 synthesis (Dahlen et al. 1998; van Schoor et al. 1997). The addition of zileuton to existing medium to high doses of inhaled corticosteroids or the use of ABT-761 after discontinued corticosteroids also improves pulmonary function in asthmatics at baseline with increased FEV_1 compared to placebo (Dahlen et al. 1998; van Schoor et al. 1997). 5-LO inhibition blocks the fall in FEV_1 after allergen challenge (Hui et al. 1991; Rosenberg et al. 1995), cold dry air challenge (Israel et al. 1990; Strek et al. 1995; Fischer et al. 1995) and exercise challenge, but not after adenosine challenge (van Schoor et al. 1997). Zileuton reduces β-agonist and inhaled steroid use by 30% and 22% respectively in moderate asthmatics (Liu et al. 1994) and short courses of oral steroids by 80% in severe asthmatics (Israel et al. 1995). FLAP inhibition has shown similar effects to 5-LO inhibition on response to allergen challenge and urinary LTE_4 production. The FLAP inhibitors MK-0591 and BAYx 1005 blocked the immediate response to allergen by greater than 70% and blocked the associated rise in urinary LTE_4 also by greater than 70% (Diamant et al. 1995; Dahlen et al. 1997).

There are reports of decreased cellular inflammation with leukotriene receptor antagonism. A decrease in peripheral blood eosinophils has been observed after montelukast treatment (Knorr et al. 1998; Reiss et al. 1998; Noonan et al. 1998). Zafirlukast treatment reduced numbers of BAL fluid eosinophils, lymphocytes, basophils, and macrophages after segmental allergen challenge in allergic asthmatic patients (Calhoun et al. 1998). This suggests that these antagonists may also have anti-inflammatory actions. Anti-inflammatory actions of zileuton have been suggested by inhibition of eosinophil chemotaxis to fMLP in guinea pig tracheal explant preparations (Munoz et al. 1997).

MK-571 the leukotriene receptor antagonist has been shown to inhibit the LTC_4 export pump MRP (Leier et al. 1994b). MK-571 is structurally similar to LTD_4 and hence this overlap of effects is not unlikely. MK-886 is a FLAP inhibitor, it also acts as a LTC_4 synthase inhibitor (Leier et al. 1994b) and an inhibitor of MRP (Schaub et al. 1991). There are no specific MRP inhibitor drugs that have been clinically trialed but

this proposes a new class of leukotriene modifier drug that may be of benefit in the treatment of asthma.

The role of LTB_4 itself in asthma is yet to be confirmed so the anticipated effects of LTB_4 receptor antagonism on the symptoms of asthma are limited. Indeed, the LTB_4 receptor antagonist LY29311 had no effect on baseline lung function, the early or late asthmatic response to allergen or on airway responsiveness to histamine compared to placebo. There was, however, a reduction in the number of neutrophils in BAL fluid with LY29311 (Evans et al. 1996).

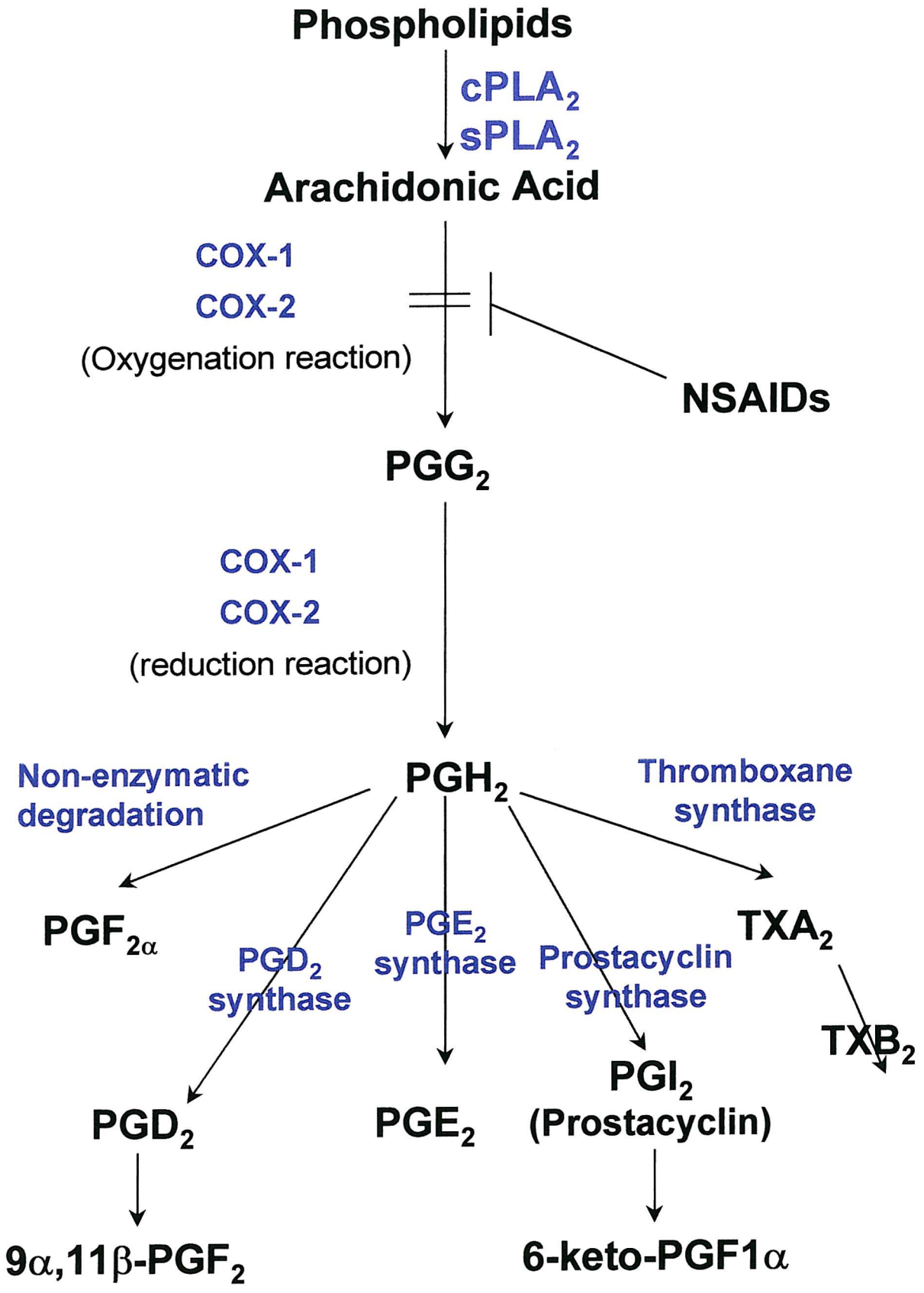
1.3.4 Prostanoids.

Virtually every cell type in the body has the capacity to generate prostanoids. The prostaglandins were first discovered in the 1930s, following the observation that human semen produced a marked contraction of human uterus both *in vitro* and *in vivo* (Kurzok and Lieb, 1930). Shortly after, human semen was seen to have smooth muscle contracting activity and was also hypertensive (Goldblatt, 1933; Goldblatt, 1935; Von Euler, 1934; Von Euler, 1935). This lipid soluble constituent was termed "prostaglandin" by von Euler. In 1966, Bygdeman and Samuelsson, and Hamberg and Samuelsson isolated 13 different prostaglandins from seminal fluid. Also in the 1960s, it became clear that prostaglandins were derived from essential fatty acids (Bergstroem et al. 1964; Van Dorp et al. 1964), and the production of prostaglandins from arachidonic acid by cyclooxygenase was described (Hamberg and Samuelsson, 1974).

1.3.4.1 Cyclooxygenase pathway for prostanoid and thromboxane synthesis.

Prostanoids are formed by the oxidative cyclization of the 5 central carbons within arachidonic acid. AA is converted initially to prostaglandin G_2 by the oxygenation action of the constitutive prostaglandin H synthase-1 (PGHS-1/cyclooxygenase-1 [COX]-1) and/or by the cytokine inducible PGHS-2/COX-2. PGG_2 is reduced to prostaglandin H_2 by the hydroperoxidase activity of the same enzymes (Vane, 1994) (**Fig 1.6**). Prostaglandin H_2 is the common substrate for both the prostanoid synthases and the thromboxane synthases (DeWitt and Smith, 1988; O'Banion et al. 1992) leading to the production of a variety of eicosanoids that includes PGE_2, PGD_2, thromboxane (TX) A_2 and prostacyclin (**Fig 1.6**). $PGF_{2\alpha}$ and to some extent PGE_2 are also generated from PGH_2 non-enzymatically by isomerisation. The array of prostanoids produced in a particular cell type varies depending on the expression of the terminal enzymes. For

Fig.1.6 Cyclooxygenase pathway for prostanoid synthesis



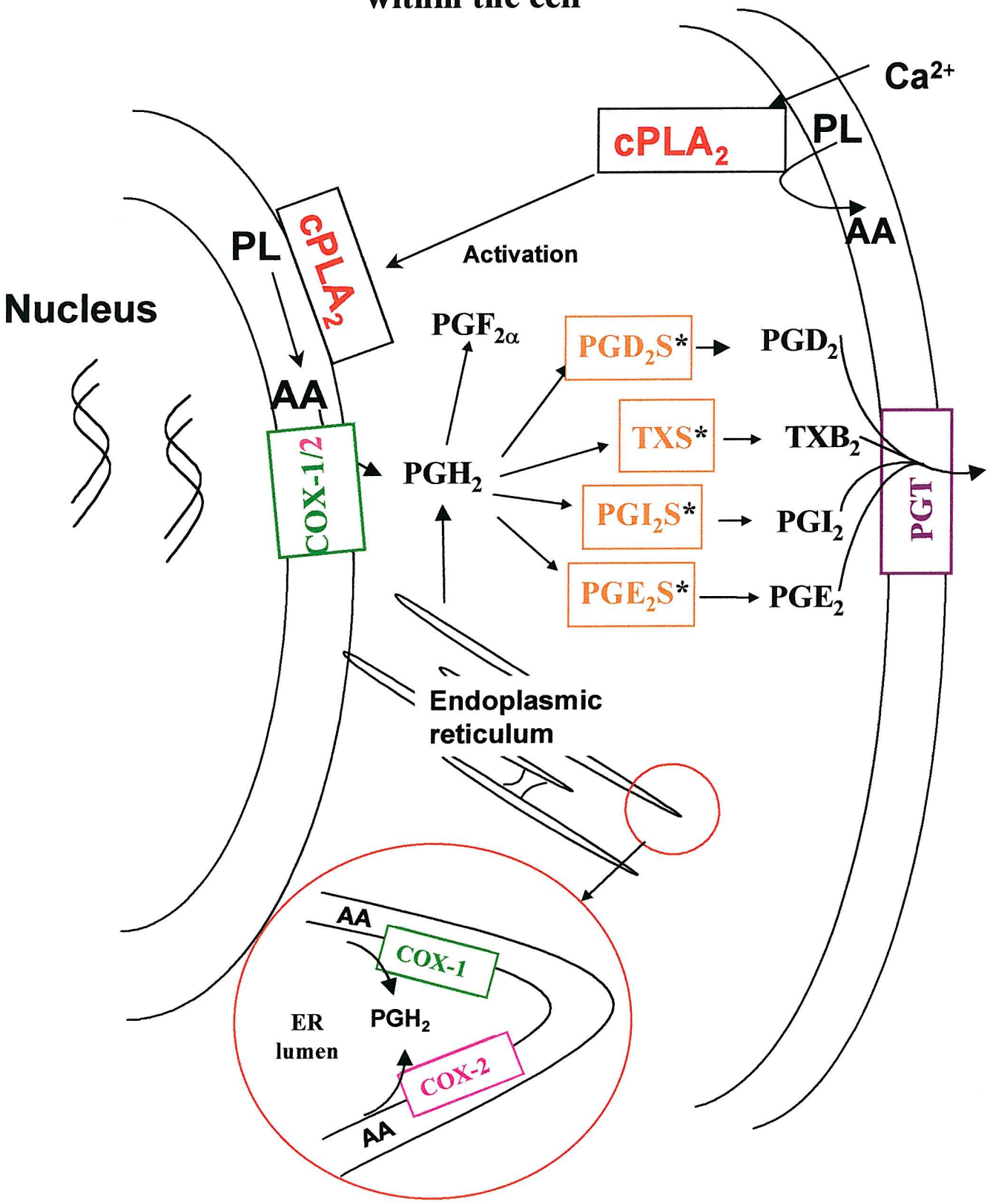
example, endothelial cells produce primarily prostacyclin (PGI_2), mast cells mainly produce PGD_2 and platelets produce predominantly TXA_2. Non-steroidal anti-inflammatory drugs all inhibit the action of COX and hence, reduce prostanoid synthesis.

A prostaglandin transporter (PGT) has been identified that is abundant in the lung and which might transport newly synthesised prostanoids across plasma membranes (Kanai et al. 1995) (**Fig 1.7**). The rank order of clearance of prostanoids from the lung ($PGE_1 \approx PGE_2 \approx PGF_{2\alpha} > TXB_2 > PGI_2$) is the same as that of transport by PGT, hence PGT may mediate vascular clearance of prostanoids in the lung (Kanai et al. 1995).

1.3.4.2 Enzymes of the prostanoid pathway.

There are two COX isoforms, both of 70kDa mass (Vane, 1994). COX-1 is expressed constitutively in most cells, while COX-2 (DeWitt and Smith, 1988), is rapidly induced in response to proinflammatory cytokines such as IL-1 and TNF (Jones et al. 1993), growth factors including TGF-β (Pilbeam et al. 1993), lipopolysaccharide (Jones et al. 1993) and phorbol esters including PMA (phorbol 12-myristate 13-acetate) (Hla and Neilson, 1992). The induction of COX-2 by PMA suggests involvement of the protein kinase C (PKC) pathway in transcriptional activation of the gene. Levels of COX-2 mRNA are decreased by glucocorticoids (O'Banion et al. 1992). The genes for COX-1 and COX-2 are located on human chromosomes 9 and 1 respectively. The COX-1 gene is 22kB compared to the 8kB COX-2 gene, due to the smaller size of the COX-2 introns (Smith et al. 1995). The COX-1 promoter does not contain the TATA box that is present in the COX-2 promoter, this absence is common in 'housekeeping' genes (Smith et al 1995), the COX-2 promoter also contains other transcriptional elements including NF-κB (Appleby et al. 1994). There is 60% homology between the COX-1 and COX-2 proteins (Xie et al. 1991; Fletcher et al. 1992; Jones et al. 1993), with all the amino acid residues required for catalytic activity being conserved. COX-2 contains an 18 amino acid insert at its C-terminal end which is absent from COX-1 and also contains a putative N-glycosylation site (Hla and Neilson, 1992). COX-2 also has a shorter N-terminal signal peptide. The aspirin acetylation site at Ser 516 is conserved between COX-1 and COX-2 and both proteins have a sequence resembling an endoplasmic reticulum-retention signal at their C-terminal end (Hla and Neilson, 1992). COX-1 and 2 are located on the luminal side of the endoplasmic reticulum (ER) and/or nuclear envelope (Otto and Smith, 1994) (**Fig 1.7**). COX-1 is equally distributed in the ER and nuclear envelope, whereas COX-2 is more concentrated in the nuclear membrane than

Fig. 1.7 Localization of the cyclooxygenase pathway within the cell



* The subcellular localisation of PGD_2S, PGE_2S, PGI_2S, TXBS is not known.

the ER (Morita et al. 1995) (**Fig 1.7**). Both COX isoforms undergo suicide inactivation following catalysis of about 400 arachidonate molecules (Kulmacz et al. 1994).

PGD_2 synthases (PG-H D-isomerases), which catalyse the conversion of PGH_2 to PGD_2 exist in two forms each with a molecular mass of 26 kDa (Nagata et al. 1991, Urade et al. 1987). The form found in haematopoietic cells such as mast cells is glutathione dependent (Urade et al. 1987; Urade et al. 1989) whereas the brain form is glutathione independent (Nagata et al. 1991). PG-H E-isomerase catalysing the formation of PGE_2 from PGH_2, is a glutathione S-transferase (Meyer et al. 1996). The subcellular localisation of the downstream enzymes of the COX pathway are not known (**Fig 1.7**).

1.3.4.3 *Prostanoids in asthma.*

There is some evidence that prostanoid production is increased in asthma and that these mediators exert many effects relevant to the pathophysiology of asthma.

Immunological stimulation of passively sensitised normal lung parenchyma results predominantly in PGD_2 generation, representing 50% of all arachidonate products (Vigano et al. 1988). Measurements of inflammatory mediators in BAL fluid show that PGD_2 is elevated in asthmatic subjects compared to normal subjects (Liu et al. 1990), and allergen challenge in allergic subjects further increases PGD_2 and TXB_2 (Wenzel et al. 1990). The thromboxane A_2 metabolite, 11-dehydro-TXB_2 is also increased in urine after inhaled allergen challenge (Sladek et al. 1990). Aspirin challenge significantly reduces BAL fluid PGD_2 and TXB_2 in both aspirin intolerant asthmatics and aspirin tolerant asthmatics (Szczeklik et al. 1996).

1.3.4.4 Biological effects of prostaglandins.

Prostaglandin production by the constitutive enzyme COX-1 is believed to be primarily involved in cellular housekeeping responses such as vascular homeostasis, control of water resorption by the kidney and attenuation of acid secretion by the stomach (Smith et al. 1996; Vane, 1994). COX-2 expression is induced by mitogenic stimuli (Xie et al. 1991; Hla and Neilson, 1992; Jones et al. 1993), and prostaglandins produced via COX-2 are implicated in inflammatory responses (O'Banion et al. 1992; Lee et al. 1992). COX-2 is, however, constitutively expressed in some tissues including the nasal mucosa (Picado, 1997), brain (Yamagata et al. 1994), testes (Simmons et al. 1991), tracheal epithelia (Walenga et al 1996), and macula densa of the kidney (Harris et al. 1994).

COX-1 expression can be down-regulated by fibroblast growth factor (Brannon et al. 1994) and upregulated in mast cell cultures with SCF and dexamethasone (Hla and Maciag, 1991). Therefore, it is likely that the purely inflammatory role of COX-2 and housekeeping role of COX-1 is not as straightforward as preciously described.

Thromboxane A_2 is synthesised predominantly in platelets and is a potent constrictor of vascular and airway smooth muscle. PGI_2 is responsible for AA-induced vasodilation and for an anti-aggregatory influence on platelets. PGD_2, the primary cyclooxygenase product of mast cells, inhibits platelet aggregation, causes peripheral vasodilation, pulmonary vasoconstriction, bronchoconstriction and airway hyperreactivity. It may also have neuromodulatory actions as suggested by high concentrations of PGD isomerase in the central nervous system. PGE_2 is the predominant AA product from a variety of cell types. It inhibits gastric acid secretion and is protective to the gastric and intestinal mucosa. Importantly, PGE_2 induces relaxation of airway smooth muscle by a direct effect on the muscle and by an inhibitory effect on acetylcholine release (Walters et al. 1984). PGE_2 reduces LTB_4 synthesis in human eosinophils and neutrophils, and in rat macrophages in vitro (Ham et al. 1983; Elliott et al. 1991). $PGF_{2\alpha}$ is a potent constrictor of airway smooth muscle (Hamberg et al. 1975), and may also cause hyperresponsiveness by sensitising airway nerve endings (O'Byrne et al. 1984).

Non-steroidal anti-inflammatory drugs (NSAIDs) e.g. aspirin and indomethacin inhibit the activity of both cyclooxygenase enzymes, thereby producing anti-pyretic, analgesic, anti-inflammatory, and anti-thrombogenic effects. COX inhibition by NSAIDs is irreversible in the case of aspirin, but is usually reversible (ibuprofen) (Laneuville et al. 1994), or time dependently reversible (flurbiprofen and meclofenamic acid) (Laneuville et al. 1994). Aspirin covalently acetylates the Ser^{530} residue of COX-1 and the Ser^{516} of COX-2 to produce an acetyl group that causes steric inhibition of the binding of arachidonic acid to the oxygenase active site (Loll et al. 1995; Lecomte et al. 1994). Acetylation of both COX-1 and COX-2 prevents PGG_2 generation, but COX-2 acetylation by aspirin leads to the formation of 15-HETE in its inactive 15R form (Meade et al. 1993; Lecomte et al. 1994). Aspirin and indomethacin are more selective for COX-1 than COX-2 and thereby produce side effects related to inhibition of COX-1 such as peptic ulceration and impaired renal function (Meade et al. 1993). Selective COX-2 inhibitors are being developed to have anti-inflammatory and analgesic activities with minimal gastric and renal toxicity, early examples including nimesulide and

meloxicam (Smith et al. 1994).

1.3.3.5 The biological effects of the prostanoids are determined by their receptors.

The original prostanoid receptor classification was devised by Kennedy and Coleman (1982) based on functional data obtained with a range of agonists and some antagonists. Receptors are designated by their specificity for the naturally occurring prostanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2, and are termed DP, EP, FP, IP and TP. However, considerable cross-reaction can occur. These receptors are 7-transmembrane domain proteins with some conservation between them. The DP, EP_2, EP_4 and IP receptors are G_s-protein linked and are coupled to adenylyl cyclase, activation therefore resulting in increased intracellular cAMP. The EP_1, EP_3, FP and TP receptors are linked to phospholipase C (PLC) via a G_q-protein, and activation therefore, increases both inositol triphosphate (IP_3) and free Ca^{2+} intracellularly. There are at least 4 types of EP receptor (EP_{1-4}), which differ in their G-protein coupling (Coleman et al. 1987).

PGD_2 acts primarily on the DP receptor to cause vasodilatation, inhibition of platelet aggregation, relaxation of gastrointestinal and uterine muscle and regulation of pituitary and hypothalamus hormone release. PGD_2, however, can also act on TP receptors to cause bronchoconstriction, and is the most potent bronchoconstrictor of the prostaglandins (Hardy et al. 1984). In man, the bronchoconstrictor effects of PGD_2 are mediated mostly by TP receptors (Hamid Broomfield et al. 1990). When TXA_2 acts on the TP receptor it primarily causes vasoconstriction and platelet aggregation, but it can also cause bronchoconstriction (an effect which is greater in guinea pigs than in man). $PGF_{2\alpha}$ also causes bronchoconstriction via the TP and FP receptors. In normal subjects inhaled $PGF_{2\alpha}$ causes a short acting bronchoconstriction, but in asthmatic subjects its effect is 8000 times more (Mathe and Hedqvist, 1975). PGI_2 is not a bronchoconstrictor, its main effects via the IP receptor being vasodilatation, inhibition of platelet aggregation and fibrinolysis. Inhaled PGE_2 in normal subjects causes bronchodilation but in asthmatic subjects the effect is more variable (Mathe and Hedqvist, 1975). Inhaled PGE_2 also protects against exercise- and allergen- induced bronchoconstriction (Pavord et al. 1993, Pavord et al. 1994) and prevents aspirin induced exacerbations in aspirin-sensitive asthmatics (Sestini et al. 1996). There are at least three forms of EP receptor, perhaps explaining the diverse effects of PGE_2 on lung function. Stimulation of the EP_1 receptor causes contraction of bronchial and gastrointestinal smooth muscle, whereas stimulation of the EP_2 receptor induces relaxation of bronchial, gastrointestinal and venous smooth

muscle. The EP_3 receptor has no function in the lung, but induces contraction of intestinal muscle and of the pregnant uterus. Cloning experiments have shown the existence of splice variants of the EP3 receptor in some species, and hence there may be EP_{3A}, EP_{3B}, and EP_{3C} receptors (Coleman et al. 1995).

Manning and co-workers (1991) put forward the theory that prostanoids, especially TXA_2 have an important role in the early bronchoconstrictor response to allergen, some studies have confirmed this observation. The NSAID flurbiprofen was shown to inhibit the EAR by 30% (Curzen et al. 1987) and GR3219 (a TP receptor antagonist) also blocks the EAR in some patients (Beasley et al 1989). There are also other studies that directly contradict these effects of NSAIDs on the response to allergen. Indomethacin, for example, does not effect allergen-induced bronchoconstriction in atopic asthmatics despite a reduction in prostanoid levels *in vivo* (Skoner et al. 1988). However, the LAR in allergen challenged asthmatics is reduced by indomethacin (Fairfax et al. 1983) but allergen challenge itself does not cause an increase in TXB_2 or PGD_2 in BAL fluid. Hence the role for prostanoids in the response to allergen is debatable, and suggests that they may only make a small contribution secondary to that of the cysteinyl leukotrienes.

1.3.3.6 Presence of enzymes to degrade the prostanoids at the disease site

The half-life of prostaglandins in the circulation is less than 1 minute due to initial uptake from pulmonary circulation. The prostaglandins are also extensively catabolised. The enzyme involved in the first stage of breakdown is 15-hydroxy-prostaglandin dehydrogenase, which targets the double bond between carbons 13, 14 and the 15-hydroxy group common to all prostanoids (except PGI_2). Reduction of the 13,14-double bond is catalysed by prostaglandin 13,14-reductase, producing 13,14-dihydro-prostaglandin that undergoes ω-oxidation by cytochrome P-450, followed by chain shortening by β-oxidation. The major urinary metabolites in man are 20-carboxy-15-keto-13, 14-dihydro-2,3,4,5-tetranor prostaglandins. PGI_2 is not taken up by transport mechanisms present in lung cells and is therefore hydrolysed to 6-keto $PGF_{1\alpha}$ giving it a half-life of 5 minutes. Thromboxane A_2 hydrolyses non-enzymatically to its inactive product TXB_2 within 30 seconds.

1.4 Aspirin intolerant asthma.

Aspirin intolerant asthma is a phenotype of asthma characterised by adverse respiratory reactions to aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs). Approximately 10-20% of asthmatics bronchoconstrict with aspirin challenge (Spector et al. 1979; Stevenson and Simon, 1993). Aspirin intolerant patients have 3- to 5-fold elevated levels of urinary LTE_4 when compared to aspirin tolerant patients or normal subjects implying a constitutive chronic activation of the 5-LO/LTC_4 synthase pathway. (Christie et al. 1991; Kumlin et al. 1992; Smith et al. 1992). Following challenge with aspirin, a further increase in cys-LTs can be measured in the BAL fluid and urine of aspirin intolerant patients but not in aspirin tolerant patients (Christie et al. 1991; Kumlin et al. 1992; Sladek and Szczeklik, 1993; Sladek et al. 1993; Szczeklik et al. 1996). The role of the cys-LTs in aspirin intolerant asthma is confirmed by the use of LT synthesis inhibitors and cys-LT receptor antagonists which markedly attenuate aspirin-induced respiratory reactions (Israel et al. 1993; Nasser et al. 1994; Christie et al. 1991; Dahlen et al. 1993; Yamamoto et al. 1994). At the same time that cys-LT levels rise with aspirin challenge, levels of prostanoids in BAL fluid and urine fall (Sladek and Szczeklik, 1993; Sladek et al. 1993; Szczeklik et al. 1996).

Pre-treatment of passively sensitised normal human lung parenchyma with indomethacin before immunological stimulation inhibits the cyclooxygenase production of PGD_2 by more than 90% but this occurs without a concomitant shift of arachidonate metabolism towards leukotriene formation (Vigano et al. 1988). This provides strong evidence against the idea that NSAIDs 'shunt' arachidonate along the 5-LO pathway leading to a surge in LT synthesis in aspirin intolerant asthmatic patients.

Prior inhalation of PGE_2, can completely abrogate the aspirin-induced rise in urinary cys-LTs and the associated bronchoconstriction in aspirin intolerant asthmatics (Sestini et al. 1996). Thus, it is thought that NSAIDs could cause respiratory symptoms in aspirin intolerant patients by reducing PGE_2-dependent suppression of cys-LT synthesis in the lung. This, however, does not explain why NSAIDs do not cause the same increase in cys-LTs in aspirin tolerant patients or normals (Kumlin et al. 1992; Sladek et al. 1990; Sladek et al. 1993; Szczeklik et al. 1996) and it does not explain the persistently elevated levels of urinary LTE_4 in aspirin intolerant patients (Christie et al. 1991; Kumlin et al. 1992; Smith et al. 1992).

More recently, it has been shown that the persistent overproduction of cys-LTs by aspirin intolerant asthmatics (AIA) and elevated levels of BAL fluid cys-LTs following

NSAIDs can be attributed to a unique overexpression, in eosinophils, of the first committed enzyme in the synthesis of the cys-LTs, LTC_4 synthase (Cowburn et al. 1998a). Counts of cells expressing LTC_4 synthase were 5 fold higher in AIA patients than in aspirin tolerant patients (ATA) and 18-fold higher than normal subjects Cowburn et al. 1998a). Closely related to the overexpression of LTC_4 synthase was elevated BAL fluid cys-LTs, which were increased further with lysine-aspirin challenge (Cowburn et al. 1998a). Bronchial responsiveness to lysine-aspirin correlated with LTC_4 synthase positive cell counts (Cowburn et al. 1998a). The removal of the PGE_2 'brake' mechanism by NSAIDs in patients with increased cys-LT production exacerbates the overactivity of 5-LO/ LTC_4 synthase such that NSAIDs will induce a cys-LT-dependent bronchoconstriction (Cowburn et al. 1998). The overexpression of LTC_4 synthase is attributed to an over-representation in aspirin intolerant patients of a polymorphism of the 5'-flanking region of the LTC_4 synthase gene that creates a new recognition site for the AP-1 nuclear transcription factor in the aspirin intolerant asthmatic population (Sanak et al. 1997). Expression of this LTC_4 synthase variant allele is linked to higher urinary LTE_4 in AIA patients with the polymorphism compared to those without it. (Sanak et al. 1997; Szczeklik et al. 1998). Whilst the polymorphism is a convincing mechanism for AIA, it cannot fully explain aspirin intolerance as only 75% of AIA patients are hetero- or homozygotes for polymorphic allele, and 42% of the normal population have the polymorphism. The frequency of the polymorphic allele is nevertheless doubled in the AIA population compared to the ATA and normal populations. The polymorphism may contribute to enhanced LT synthesis and asthma severity in AIA patients.

1.5 Seasonal allergen exposure, eicosanoids and asthma.

Allergen challenge results in an early bronchoconstrictor response, and a late response in approx. 50% of patients. The early airway response to allergen (EAR) involves the rapid release of preformed and *de novo* inflammatory mediators from mast cells e.g. histamine, leukotrienes and PGD_2 within minutes of allergen challenge. Three to six hours after the EAR, 50% of patients experience a second period of bronchoconstriction, the late asthmatic response (LAR). This bronchoconstriction is associated with influx of eosinophils, neutrophils, basophils and monocytes and increased non-specific bronchial responsiveness to agents such as histamine or methacholine. Both the EAR and LAR depend at least in part on leukotrienes (Fischer et al. 1995).

Allergen challenge increases LTE_4 in urine of allergic asthmatics and allergic nonasthmatics following the EAR (Taylor et al. 1989; Manning et al. 1990; Sladek et al. 1990; Smith et al. 1991). The thromboxane A_2 metabolite, 11-dehydro-TXB_2 is also increased in urine (Sladek et al. 1990). Stimulation of bronchial tissue from asthmatic subjects *in vitro* with allergen or A23187 induced bronchoconstriction that corresponded with cys-LT production by the tissue (Dahlen et al. 1982). Measurements of inflammatory mediators in BAL fluid after allergen challenge in allergic subjects showed increases in cys-LTs, PGD_2, TXB_2 and histamine after the EAR (Wenzel et al. 1990). The activity of $sPLA_2$ and arachidonate levels are also increased in BAL fluid after allergen challenge (Bowton et al. 1997).

The role of the cys-LTs in allergic asthma can be illustrated by the use of anti-leukotriene drugs. The *Cys-LT_1* receptor antagonist BAYx 7195 and the FLAP inhibitor BAYx 1005 significantly attenuated the bronchoconstriction associated with both the EAR and LAR (Boulet et al. 1997; Hamilton et al. 1997). The contribution of the cys-LTs to the EAR and the LAR can be demonstrated by MK-571, which blocked the EAR and LAR by 88% and 63% respectively (Rasmussen et al. 1991). Similarly, zafirlukast (ICI 204,219) reduced the early and late responses by 81% and 55% respectively (Findlay et al. 1992). Zafirlukast reduced both the EAR and LAR by approx. 60%, the anti-histamine loratadine reduced the EAR by 25% and the LAR by 40%, and when the two drugs were used in combination, the EAR and LAR were both reduced by 75%. This suggests that, together, leukotrienes and histamine mediate the major part of both the EAR and LAR following exposure to allergen (Roquet et al. 1997).

The increase in inflammatory mediators is accompanied by a number of cellular changes in the bronchial mucosa in allergic asthmatics after allergen challenge. The airways of mild asthmatics are inflamed even before allergen challenge, showing infiltration of activated eosinophils and T-lymphocytes, degranulation of mast cells, epithelial damage, deposition of collagen below the basement membrane, and goblet cell hyperplasia (Beasley et al. 1989; Djukanovic et al. 1990; Jeffery et al. 1989). Data obtained from immunostaining in bronchial biopsies has shown that local or inhaled allergen challenge increases counts of eosinophils (Bentley et al. 1993; Montefort et al. 1994; Woolley et al. 1995) and IL-2 receptor-positive activated T-cells ($CD25^+$) (Bentley et al. 1993; Frew et al. 1996) in the bronchial mucosa. The allergen-induced eosinophilia is confirmed in measurements of BAL fluid (Woolley et al. 1995; Frew et al. 1996) and patients who exhibit a LAR are seen to have greater increases in

eosinophils in BAL fluid 6 hrs after allergen challenge than those who do not show a late response (Silvestri et al. 1997).

Allergen challenge is a useful model for the study of allergic asthma, but it does not effectively mimic long-term, low-level exposure to allergen during a natural pollen season or with domestic exposure. Seasonal allergen exposure increases bronchial responsiveness to non-allergic stimuli such as methacholine (Boulet et al. 1983; Barbato et al. 1987) and induces eosinophilia in nasal lavage fluid of allergic rhinitics (Pipkorn et al. 1988), and in BAL fluid of allergic asthmatics (Rak et al. 1991). Djukanovic and colleagues were unable to detect significant increases in bronchial responsiveness or eosinophil counts in BAL or bronchial biopsies during the grass pollen season as a large proportion of their subjects were also house dust mite allergic and hence might have had higher baseline measurements (Djukanovic et al. 1996). There were, however, increases in mucosal activated ($CD25^+$) T-cells, and $CD4^+$ T-cells (Djukanovic et al. 1996).

No study has demonstrated increases in eosinophil counts, or examined eicosanoid enzyme expression in the bronchial mucosa of atopic asthmatic patients naturally exposed to allergen.

The present study hypothesised that allergic asthmatic subjects experience increased clinical symptoms of asthma due to eosinophil influx, and an increased expression of leukotriene pathway enzymes in inflammatory cells producing more cys-LTs. Chapter 3 of this thesis addressed this issue by using immunohistochemistry to study eicosanoid pathway enzyme expression and inflammatory cell markers in bronchial biopsies, and enzyme immunoassays to measure cys-LTs and PGD_2 in BAL fluid from patients before and during the birch pollen season in Gothenburg Sweden.

1.6 Rhinovirus infection.

Viral upper respiratory tract (URT) infections trigger wheezing in many asthmatic patients (McIntosh et al. 1973; Minor et al. 1976; Hudgel et al. 1979) but it is not only asthmatic patients who experience lower respiratory tract (LRT) symptoms with URT infection, as normal subjects may also experience cough and sputum production with a severe cold. The most frequent viral cause of respiratory disease is human rhinovirus (HRV) (Gwaltney et al. 1966). Epidemiologic studies have shown that 80% of exacerbations of asthma in children and 50% of those in adults are associated with viral URT infection, the majority of which could be identified as HRV (Beasley et al. 1988; Nicholson et al. 1993; Johnston et al. 1995; Johnston et al. 1996; Teichtahl et al.

1997). Further studies have shown that experimental HRV infection increases airway reactivity in allergic rhinitic or asthmatic subjects (Gern et al. 1997; Lemanske et al. 1989; Cheung et al. 1995; Grunberg et al. 1997). However, non-allergic, non-asthmatic subjects did not show this increased reactivity when infected with HRV (Skoner et al. 1996; Summers et al. 1992). In addition in allergic subjects there is an enhanced airway inflammatory response to allergen and a change in the pattern of this response (Lemanske et al. 1989; Busse et al. 1992; Calhoun et al. 1994; Calhoun et al. 1991). In a study by Busse et al. (1992), subjects underwent bronchial antigen challenge both during and after HRV infection. The resulting histamine release and eosinophilia in BAL fluid was enhanced during infection, and subjects who were not normally dual responders developed a late response to allergen (LAR).

The epidemiologic and experimental evidence to support an important role for HRV infection in the pathogenesis of lower airways symptoms and asthma is substantial, but there is still very little evidence to provide a mechanism by which these physiologic changes may occur. HRV is thought only to infect the nasal mucosa, but there is some evidence to suggest that HRV may also infect the mucosa of the lower airways (Halperin et al. 1983). ICAM-1 is a receptor for 90% of the more than 100 HRV serotypes including HRV 16 (Couch, 1985; Abraham and Colonno, 1984; Staunton et al. 1989). Increased expression of epithelial ICAM-1 may be induced by interferon (IFN), tumour necrosis factor (TNF) and other cytokines (Munro et al. 1989). Interferon is induced by HRV in macrophages (Roberts et al. 1979). Hence, HRV binding to ICAM-1 on the cell surface further increases ICAM-1 expression and facilitates the spread of HRV infection (Staunton et al. 1989). The upregulation of ICAM-1 also promotes an inflammatory antiviral response to viral infection by enhancing adhesion of inflammatory cells to vascular endothelium, the extracellular matrix, epithelium, and other inflammatory cells. ICAM-1 is upregulated on eosinophils, T-cells and epithelial cells in asthmatics compared to normals, which may account for asthma sufferers having more frequent colds (Hansel et al. 1991; Derose et al. 1994; Bentley et al. 1993; Vignola et al. 1993, Chanez et al. 1993).

There is only one study to date that suggests an association between naturally occurring URT viral infection and increased LTC_4 concentrations in nasopharyngeal secretions in both wheezing and non-wheezing children (Volovitz et al. 1988). However, exposure of human blood monocytes to the Epstein-Barr virus (EBV) causes rapid, transient priming of cells for LTB_4 and LTC_4 synthesis which involves stimulatory

events at both the level of 5-LO activation and arachidonate release (Gosselin and Borgeat, 1997). Early experiments also suggest that viruses may indirectly stimulate prostaglandin synthesis via interferon induction (Fitzpatrick and Stringfellow, 1980). It is possible that prostaglandins may restore the antiviral potency of interferon in animals desensitised by viral infection (Stringfellow, 1978). Hence, upregulation of prostaglandin and leukotriene synthesis may be involved in host defence mechanisms against virus infection.

The present study hypothesised that the reported increase in asthma symptoms associated with rhinovirus infections in asthmatics, and the respiratory symptoms of a rhinovirus cold in normal subjects might be caused by increased eicosanoid production. This might be initiated by an increase in inflammatory cells expressing the enzymes of the eicosanoid pathway and increased expression of these enzymes in inflammatory cells. Immunostaining for inflammatory cell markers and enzymes of the eicosanoid pathway was performed in bronchial biopsies, and enzyme immunoassays for the measurement of cys-LTs in BAL fluid from normal and asthmatic subjects before and during experimental infection with HRV 16.

1.7 Ozone exposure and lung function.

In the years preceding the 1950s the UK suffered a major health problem relating to air pollution derived from the burning of fossil fuels. Levels of air pollution correlated directly with mortality from respiratory disease (Ministry of Health. 1954). The major pollutants then were sulphur dioxide and large particulate matter, which have since been reduced by the introduction of the 'Clean Air Act' in 1956. However, over the last 30 years the use of motor vehicles has increased substantially causing an increase in primary pollutants including oxides of nitrogen, small particulate matter, carbon monoxide and volatile organic compounds such as benzene. Secondary pollutants which are derived from the interaction of the primary pollutants with each other and with environmental factors such as sunlight and humidity are also increased, the most important of these being ozone (O_3). This increase in vehicle derived pollutants has occurred in parallel with an increase in the morbidity and mortality of asthma which has led to the hypothesis that air pollution is a contributing factor to increases in asthma cases.

A number of studies have linked high levels of ozone to increased hospitalisation for asthma (Bates et al. 1990; Schimitzberger et al. 1993; Cody et al. 1992; Whittemore and Korn, 1980; White et al. 1994). Ozone increases bronchial responsiveness in both

healthy (Golden et al. 1978) and asthmatic subjects (Kreit et al. 1989). Changes in lung function and hyperresponsiveness are reported to occur following exposure to levels of ozone between 0.1ppm and 0.4ppm (Dimeo et al. 1981; Goldsmith and Nadel, 1969). However, it is unclear whether these changes occur at concentrations as low as those actually detected in large urban areas (0.1 ppm) (Koenig et al. 1985). Exposure to low levels of ozone (0.12 ppm) increases bronchial responsiveness to allergen in allergic asthmatics (Molfino et al. 1991) but has no effect on exercise-induced asthma (Fernandes et al. 1994).

Asthma is an inflammatory disease, and for ozone to be confirmed as having a role in causing/worsening asthma it must be shown to have an effect on inflammation associated with airway hyperresponsiveness. Initial experiments on ozone inflammatory effects on lung were carried out in dogs (Holtzman et al. 1983). Bronchial responsiveness and counts of epithelial neutrophils were increased after ozone exposure (Holtzman et al. 1983). Similar experiments were carried out in human volunteers. Inhaled ozone increased bronchial responsiveness (Seltzer et al. 1986; Koren et al. 1989), and counts of neutrophils were raised in BAL fluid (Seltzer et al. 1986; Koren et al. 1989; Aris et al. 1993; Basha et al. 1994) and in nasal lavage fluid (McBride et al. 1994). This was associated with increased levels of PGE_2, $PGF_{2\alpha}$ and TXA_2 (Seltzer et al. 1986; Koren et al. 1989) but not LTB_4 in BAL fluid (Koren et al. 1989). Histamine, LTB_4 and PAF were undetectable in nasal lavage fluid before and after ozone exposure and levels of IL-8 were unchanged but correlated well with leukocyte counts (McBride et al. 1994). Interleukin-8 and IL-6 levels increased in BAL fluid with ozone exposure (Basha et al. 1994). Lung function responses to ozone are not significantly different in asthmatic compared to normal subjects (Scannell et al. 1996). However, asthmatic subjects do present greater increases in inflammatory responses as regards neutrophil counts in BAL and total protein levels (Scannell et al. 1996) suggesting that asthmatics may be more at risk of ozone induced damage than non-asthmatics.

The present study hypothesised that the increase in expression of enzymes of the eicosanoid pathway associated with or independent of inflammatory cell influx might decrease lung function in normal subjects exposed to 0.2ppm ozone. Bronchial biopsies were taken from normal subjects exposed to ozone or filtered air in a blinded, placebo controlled, crossover study, and immunostaining for inflammatory cell markers and enzymes of the eicosanoid pathway was related to changes in clinical measures of cold severity.

CHAPTER 2

Materials and methods

2.1 Protocol for allergen exposure study.

2.1.1 Patient selection.

Ethical permission for the study was obtained from Sahlgrenska University Hospital Ethics Committee. Twelve non-smoking mild asthmatic adults (mean age 30.6 yr., range 23-43) with a clinical history of exacerbations during the birch pollen season were recruited by Dr Sabina Rak (Allergy Centre, Sahlgrenska University Hospital, Gothenburg, Sweden). Patients were confirmed as allergic to birch pollen by positive skin-prick tests (wheal >3mm). All patients had negative skin-prick tests to house-dust mite and moulds, six patients tested positive for cat or dog dander but had no daily contact with pets. Seven subjects allergic to grass pollen were not excluded as the grass pollen season (June) follows the birch pollen season (late April to early June) and is unlikely to influence either the pre-season results (Feb/Mar) or the in-season results (May/June). Before entering the study, patients had not experienced a respiratory infection in the previous two months. Patients had not used inhaled or systemic corticosteroids for at least one year before the study, but were using β_2-agonist bronchodilators for asthma symptoms and anti-histamines (oral acrivastine and topical levocabastine) for rhinoconjunctival symptoms as required.

2.1.2 Experimental protocol.

Patients were studied in February/March 1997 (pre-season) and in May 1997 (in-season). At pre-season and in-season screening sessions 2 days before each bronchoscopy, total serum IgE was assayed by Radioallergosorbent test (RAST), and bronchial responsiveness was measured as the provocation concentration of inhaled methacholine required to decrease FEV_1 by 20% from baseline (methacholine PC_{20} FEV_1). Pre-season and in-season monitoring of lung function with twice-daily peak expiratory flow (PEF) measurements was initiated, using Wright mini peak flow meters, and patients completed diary cards recording symptom scores on a scale where 0 = no symptoms, 1 = mild, 2 = moderate, and 3 = severe symptoms. A record of medication use (β_2 agonists and antihistamines) was also kept for airway-related and rhinoconjunctival symptoms.

2.1.3 Sample production.

On the pre-season and in-season days, patients underwent flexible fiberoptic bronchoscopy using an Olympus BF1 bronchoscope (Lake Success, NY, USA)

according to ATS guidelines (American Thoracic Society, 1985) from the middle or lower lobe bronchus. Bronchoscopy was performed by Dr. G. Riise (Gothenburg). Bronchoalveolar lavage (BAL) was performed using 100ml saline in 40 ml, 30ml, and 30ml aliquots, and a bronchial biopsy obtained from an upper lobe segment.

2.1.4 Sample processing.

Bronchial biopsies were processed into GMA and immunohistochemical analysis of the markers for mast cells, eosinophils, monocyte/macrophages, neutrophils, basophils and T-cells, the leukotriene pathway proteins (5-LO, FLAP, LTA_4 hydrolase, LTC_4 synthase, MRP-1 and MRP-2) and the prostanoid pathway enzymes (COX-1, COX-2 and PGD_2 synthase) was carried out as described below (sections 2.5 and 2.5.2). Bronchoalveolar lavage samples were assayed by enzyme immunoassay for the mediators LTC_4 and PGD_2 as described in section 2.7.

2.1 Protocol for HRV infection study.

2.2.1 Patient selection.

The combined Southampton and SW Hampshire Joint Ethics Committee gave ethical approval for the study, and all subjects gave written informed consent before participation in the study. Seventeen non-smoking subjects recruited by Dr S Johnston (University Medicine, Southampton General Hospital, UK.) took part. Nine were 'normal' (healthy, non-asthmatic, non-atopic) (4M, 5F; mean age 33.2 years range 20-50) and 6 were atopic asthmatics (5M, 1F; mean age 24.3 years, range 22-27). Subjects had not experienced any upper respiratory tract infection in the previous 6 weeks, and negative or low levels of neutralising antibodies (titre≤1:2) to HRV 16 in the nasal lavage were confirmed in nasal lavage.

The asthmatics patients presented with an appropriate clinical history with a diurnal peak expiratory flow (PEF) variability of 15%, five out of six subjects having a provocation concentration of inhaled histamine required to reduce baseline forced expiratory volume in one second (FEV_1) by 20% (PC_{20} FEV_1histamine) of <8mg/ml. One asthmatic patient had a baseline PC_{20} FEV_1 histamine of 22.9 mg/ml. Atopic status was confirmed by ≥2 positive skin prick tests producing a wheal of 3mm or greater to a battery of common aeroallergens (*D. pteronyssinus*, mixed grass pollen, cat dander, dog hair, feathers, *A. fumigatus*), with histamine and diluent controls (Bencard: Brentford, Middlesex, UK). Serum IgE levels were measured from venous blood samples using a

double-antibody solid-phase ELISA (geometric mean 366 U/ml; range 133-1627) (Enzognost IgE: Calbiochem-Behring Corp., La Jolla, CA).

Normal subjects had no clinical history of chronic respiratory disease, normal lung function, negative skin prick tests, and normal serum IgE (geometric mean 24.8, range 10-64 U/ml).

2.2.2 Experimental protocol.

After the initial screening day, subjects were required to attend on 2 occasions. Firstly, subjects underwent fiberoptic bronchoscopy to provide 'baseline' biopsy and bronchoalveolar lavage (BAL) samples. Two weeks later, to exclude any pre-existing infection, the subjects returned for admission to the hospital infectious diseases unit where subjects underwent 3 nasal lavages during the first two days. 5ml prewarmed Hank's Balanced Salt Solution (HBSS) with 0.5% gelatin were instilled into each side of the nasal cavity and after 5 seconds the fluid was expelled and collected into a sterile petri dish. On days 2 and 3 of admission subjects were inoculated with HRV 16 solution (a generous gift from Drs. E Dick and W. Busse, Madison, WI) as previously described (Fraenkel et al. 1995). 0.75ml of HRV 16 solution was placed into each nostril by aerosol insufflation using a hand held atomiser. The viral dose was in the range of 5,000 to 10,000 tissue culture infective doses 50% ($TCID_{50}$). Symptom scores were recorded and nasal lavage was performed daily throughout admission and a venous blood sample for viral titre measurements was taken to confirm successful infection. On day 6 of admission a second bronchoscopy was performed to provide 'during-infection' biopsy and BAL samples, with the biopsy being taken from the contralateral side to the baseline procedure. On day 7 of the admission, spirometry and a repeat histamine bronchoprovocation test were performed and subjects were discharged.

2.2.3 Bronchoscopy.

On two occasions (before infection and during infection) patients were premedicated with salbutamol (2.5mg) and atropine was administered intramuscularly. Patients were sedated throughout the procedure with intravenous midazolam and topical anaesthesia achieved using lignocaine gel and solutions (1-4%). Bronchoscopy was performed using an Olympus BF 1T20 flexible fiberoptic bronchoscope (Olympus Optical Co., Tokyo, Japan), and biopsies were taken from the lower lobe subcarinae (Olympus FB 15C). The asthmatic patients received further nebulised salbutamol after the procedure. None of the

patients experienced adverse effects. Bronchoalveolar lavage was performed with 6x20ml aliquots of isotonic saline, which were pooled and centrifuged at 400g at 4°C to remove cells. The supernatant was stored at -20°C until immunoassayed for eicosanoids.

2.2.4 Sample processing.

Bronchial biopsies were processed into GMA and immunohistochemical analysis of markers for mast cells, eosinophils, monocyte /macrophages and T-cells, enzymes of the leukotriene pathway (5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase) and the prostanoid pathway enzymes (COX-1 and COX-2) was carried out as described below (sections 2.5 and 2.5.2). Bronchoalveolar lavage fluid was assayed by enzyme immunoassay for the mediators LTC_4 and PGD_2 as described in section 2.7.

2.2.5 Virologic assessment

Nasal lavage fluid was inoculated into Ohio HeLa cell cultures, and HRV infection was diagnosed by means of the typical cytopathic effects (CPE) on daily examination by light microscopy. Cultures were reported as negative if they failed to show any CPE after two passes in cell culture. The presence of virus in nasal lavages was confirmed by neutralisation with HRV 16 serotype-specific antiserum (ATCC: Cat. No. V105-501-558: National Institute of Allergy and Infectious Diseases, Bethesda, MD). In order to quantify viral shedding, a scoring system for viral culture was devised such that the daily CPE scores (range 0-4) were summed over the 3-day postinoculation period of collection, with a maximum cumulative score of 12.

The measurement of serotype-specific antibodies was performed using a microneutralization test in 96-well tissue-culture plates. Subjects with a negative or low level titre (<1:2) against a 20 to 25 $TCID_{50}$/ml level of HRV 16 were considered eligible for the study. Serial doubling dilutions of sera (0.05ml) were made in diluent (HeLa maintenance, 2% foetal calf serum). This was added to an equal volume of virus (40 to 50 $TCID_{50}$/ml) and incubated at room temperature for 1.5 hours. Freshly stripped HeLa cells ($3x10^5$ cells/ml) were added to the serial dilutions, and the plates were incubated at 33°c. Appropriate serum, cell, and viral controls were included. Cytopathic effects were read microscopically after 2 to 4 days of incubation and confirmed by fixing and staining the plates. Positive infection was established by at least one identified viral isolate from nasal lavage and culture and confirmed where possible by 4-fold or greater rises in titre of neutralising antibody.

Symptom scoring used the previously validated criteria of Jackson et al. 1958. A value of 0-3 was assigned on each day for each of the symptoms of nasal discharge, nasal congestion, sneezing, cough, throat discomfort, headache, malaise, and fever. Individual symptom scores were aggregated over the 5-day period after inoculation. A cumulative score of 14 or greater was a positive criterion for infection, and a score of 20 or greater indicated a severe cold. The remaining criteria were either a subjective impression of a cold or rhinorrhea for at least 3 of the 5 days after inoculation. Subjects had to satisfy a minimum symptom score of 14 and at least one of the other two criteria (subjective cold or rhinorrhea) in order to demonstrate a cold on symptomatic grounds.

2.3 Protocol for ozone exposure study.

2.3.1 Patient selection.

Twelve non-smoking, healthy subjects were recruited, 10 males and 2 females, mean (±SEM) age 27.6 ± 6.2 yr. Written informed consent was obtained from all subjects and ethical approval was granted by the Southampton and Southwest Hampshire Joint Ethics Committee. Subjects underwent a physical examination and a medical history was taken to exclude those with a medical history of asthma or allergies. Subjects had not experienced a respiratory infection in the previous 6 weeks or taken any anti-inflammatory drugs (including NSAIDs), antihistamines or dietary antioxidant supplementation. Three of the twelve subjects were unable to tolerate the procedure and hence, the results presented here are from paired samples from nine subjects.

2.3.2 Experimental protocol.

Subjects were required to attend the laboratory on two study-days 6 weeks apart to undergo a randomised, double blind, crossover treatment with filtered air (placebo) or with filtered air containing 0.2 ppm ozone. This concentration is representative of peak ozone levels encountered during summertime pollution episodes (Gong et al. 1997). Lung function (FEV_1) was measured, and then subjects were exposed to the randomised treatment in a purpose-built ozone-exposure system that delivers filtered air with or without the addition of ozone though a facemask as described (Krishna et al. 1997). Water, oil, particles above 0.01μm, active gaseous pollutant species including ozone, oxides of nitrogen and sulphur dioxide, and any organic species were removed from compressed air by specialist filters. Exposures were carried out under controlled conditions at 25°c and 40-60% humidity. During the 2 hr

exposure to filtered air or filtered air with ozone, subjects were required to undertake 15 minute cycles of exercise and rest on a bicycle ergometer such that inspiration was raised to 30 l/min, to simulate mild outdoor activity. Lung function measurements were repeated 15 minutes after the last period of exercise and after a further 5 ½ hours as the response to ozone is reported to occur 6-10 hours after short-term exposures (Schelegle et al. 1991). Fiberoptic bronchoscopy was performed and bronchoalveolar lavage fluid and a bronchial biopsy were collected.

2.3.3 Bronchial biopsy and BAL fluid collection.

On both study days, patients were premedicated with salbutamol (2-10mg) and atropine (0.6mg) administered intravenously and with 4% lignocaine spray delivered to the nasal airways and postpharyngeal wall. Local anaesthesia of the upper and lower airways was achieved with 4% and 1% lignocaine respectively. The Olympus BF 1T20 bronchoscope (Olympus Optical Co., Tokyo, Japan) was introduced either via the nose or mouth at the choice of the patient. Bronchoalveolar lavage (BAL) was performed with 160ml prewarmed (37°C) saline instilled into contralateral upper lobes on the two study days. Bronchial mucosal biopsies were taken using fenestrated forceps from the contralateral lower lobe subcarinae.

2.3.4 Processing of bronchial biopsies and BAL fluid.

Bronchial biopsies were embedded into GMA and immunostained as described in sections 2.5 and 2.5.2. Immunostaining was carried out using monoclonal antibodies EG2 and AA1 as markers for eosinophils and mast cells respectively, monoclonal antibodies against enzymes of the prostanoid pathway (COX-1 and COX-2), and polyclonal antibodies against enzymes of the leukotriene pathway (5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase).

BAL fluid samples were passed through a 100μm filter (Becton-Dickenson, Oxford, UK), centrifuged at 300g for 20 min and the cells washed in sterile PBS. Cytospin preparations were obtained using a Shandon cytospin (Shandon Southern Instruments, Runcorn, UK). Slides were air-dried and differential cell counts were performed using rapid Giemsa stain (HemaGurr, BDH, Poole, UK). A minimum of 400 cells was counted. The BAL fluid supernatant was stored at -70°C for mediator analysis. Total protein was measured by spectrophotometery at 562nm (Lowry et al.1951, Smith et al. 1985). Myeloperoxidase, histamine and tryptase were measured using commercially available radioimmunoasssay kits (Pharmacia, Uppsala, Sweden) with sensitivities of 8μg/ml, 0.1ng/ml and 0.5ng/ml respectively. Total IL-8 and Gro-α were measured by

enzyme immunoassays (CLB, Amsterdam, The Netherlands, R&D systems, Abingdon, UK) with sensitivities of 1 pg/ml and 15 pg/ml respectively.

2.4 Purification of human cells.

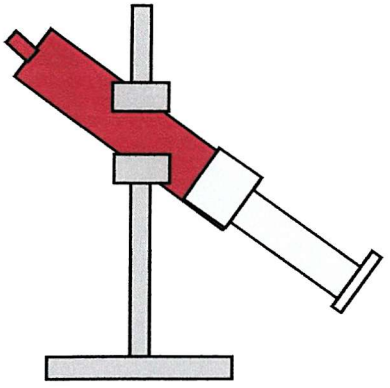
2.4.1 Dextran sedimentation of peripheral blood leukocytes.

50ml peripheral blood was collected in EDTA vacutainer tubes (Becton Dickinson) from normal or asthmatic donors. EDTA tubes are preferential as EDTA prevents clumping of mononuclear cells as was seen using heparin as an anticoagulant. The blood was added to 10ml 5% dextran (Sigma, Poole, UK) in calcium and magnesium free PBS (Gibco) + 2% heat inactivated FBS (Gibco). The blood dextran mixture was drawn up into a 50ml syringe, clamped at a 45° angle with the plunger downwards and left at room temperature for 45-60 min after which time the blood separates into two layers. An uppermost layer containing leukocytes and some contaminating red blood cells, and a lower red blood cell layer (**Fig 2.1**). This leukocyte layer was expelled from the syringe via a bent 21 gauge needle into a 50ml centrifuge tube (Triple Red) and an equal volume of PBS/ 2% FBS added before centrifuging at 300g for 10min at 20°c. The resulting pellet was subject to a hypotonic red cell lysis by resuspension in 5ml distilled water for 45 sec and flooded with PBS/2%FBS. Cells were centrifuged again at 300g for 10min at 20°c and the cells were resuspended in 1ml PBS/2%FBS for counting on an improved Neubauer Haemacytometer. Cells were fixed in 4% paraformaldehyde (Sigma. Poole, UK) for 2 hours rolling at 4°c and flooded with PBS/2%FBS before immunostaining of 5-LO, FLAP, LTA_4 hydrolase, LTC_4 synthase, MRP-1, MRP-2, COX-1, COX-2 and PGD_2 synthase for flow cytometry as described in section 2.6.

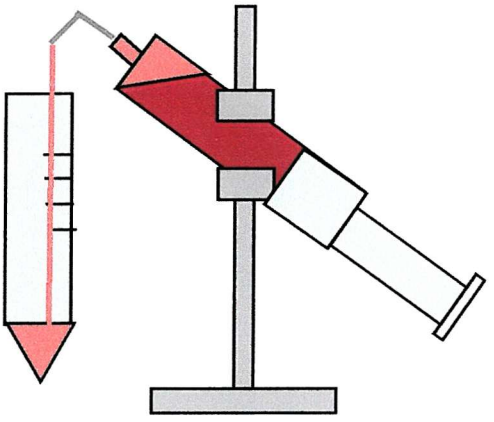
2.4.2 Isolation of peripheral blood eosinophils.

Peripheral blood eosinophils were isolated from peripheral blood as described previously (Hansel et al. 1991). 100ml blood was collected in vacutainer tubes containing EDTA. Anticoagulated blood was layered onto an equal volume of Lymphoprep (Life Technologies, Paisley) and centrifuged at 2000rpm for 30 min. Lymphoprep was used for eosinophil purifications in these experiments as opposed to Percoll (Hansel et al 1991) due to the ready availability of Lymphoprep-prepared PMN pellets, which would otherwise have been discarded.. A large number of blood preparations were carried out for the purification of peripheral blood mononuclear cells which were producing a pellet containing eosinophils and neutrophils of marginally inferior purity to that obtained with

Fig 2.1 Isolation of peripheral blood mixed leukocytes

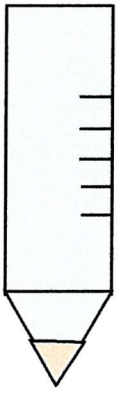


Blood-dextran mixture in 50ml syringe clamped at 45° for 45-60 min.



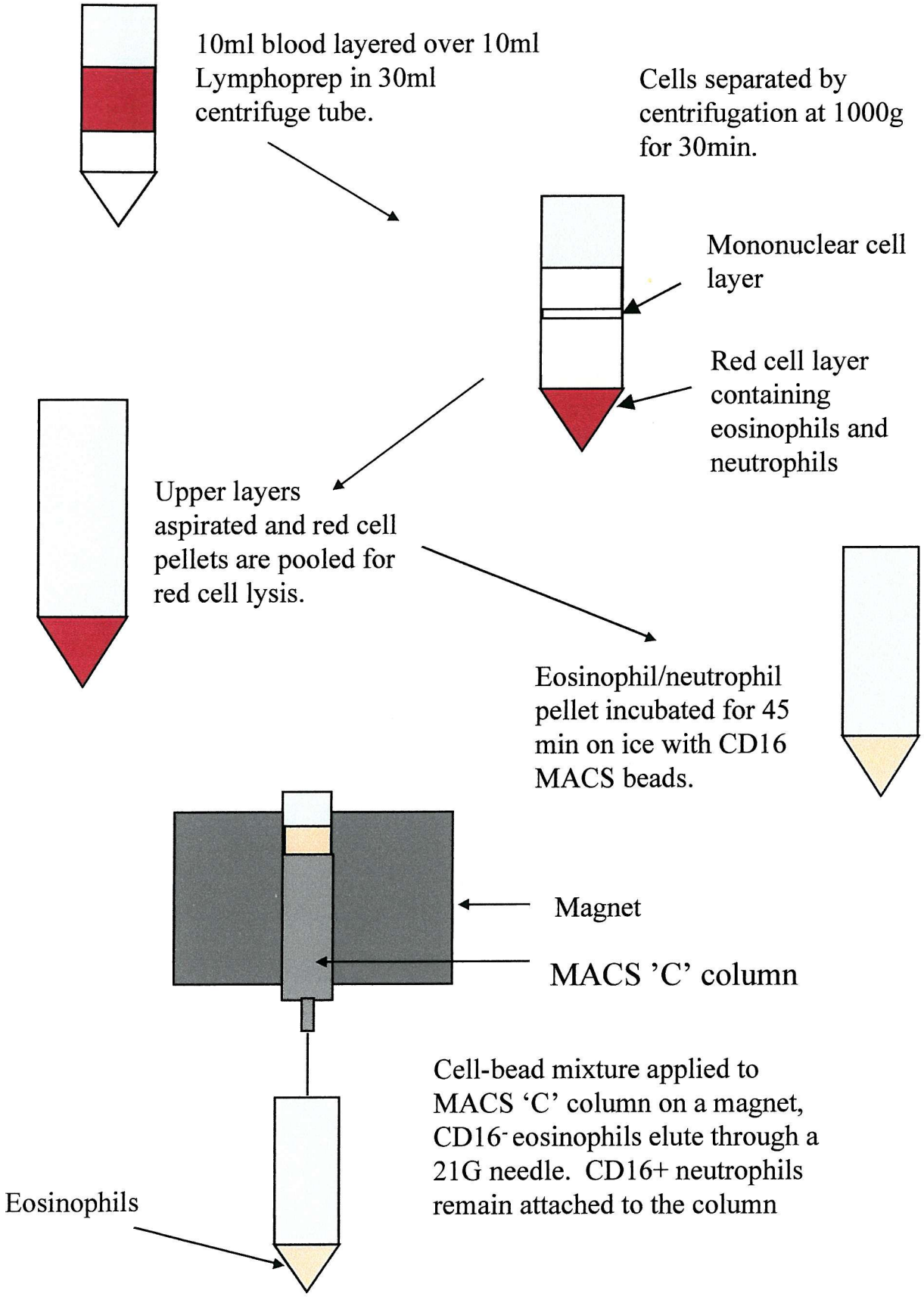
Mixed leukocyte layer expelled through bent 21G needle into 50ml centrifuge tube and mixed with an equal volume of PBS/2%FBS.

Tube centrifuged and pellet subjected to hypotonic red cell lysis with 5ml distilled water and quenched by flooding with PBS/2%FBS.



After centrifugation mixed leukocyte pellet is fixed in 4% paraformaldehyde at 4°for FACS staining.

Fig 2.2 Isolation of peripheral blood eosinophils

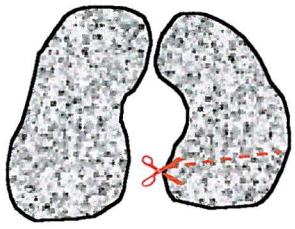


Percoll, the major contaminant being lymphocytes. This method recovers hypodense eosinophils that are lost during Percoll purification. Following centrifugation with Lymphoprep three layers are apparent (**Fig 2.2**). The upper two layers with mononuclear cells at the interface were discarded and the red pellet containing neutrophils and eosinophils was pooled and subjected to a hypotonic red cell lysis with 400ml reverse osmosis water for 45 seconds. 400ml of 1.8% NaCl was added to restore isotonicity and centrifuged at 770g for 10min at 20°c. The resulting pellets were pooled and washed in PBS/2%FBS and a second red cell lysis performed if required with 5 ml reverse osmosis water for 30 seconds and flooding with PBS/2%FBS. The pellet was resuspended in 1ml PBS/2%FBS and incubated on ice for 30mins with 100µl anti-human CD16 MACS microbeads (Mylteni Biotec, Camberley, UK). The CD16 antigen is the low affinity IgG receptor expressed on neutrophils, NK cells and macrophages and is used for the magnetic depletion of neutrophils. Following incubation cells were passed through a MACS column type C (Mylteni Biotec) attached to a strong magnet and unlabelled eosinophils eluted through a 21 gauge needle with 25ml PBS/2%FBS (**Fig 2.2**). Magnetically labelled neutrophils were retained on the column. Cells were assessed for viability by trypan blue exclusion and for purity by Kimura stain. This procedure typically yielded 1-10 million eosinophils per 100ml blood of >80% purity and >98% viability.

2.4.3 Purification of human lung mast cells

Human lung mast cells were purified using an enzymatic method described previously (Benyon et al. 1987) (Fig 2.3). Human lung parenchyma (10-50g) was obtained within 1 hour of lung resection surgery and stored in sterile HBSS containing 1% penicillin-streptomycin, 1% Fungizone and 2% FBS (Gibco) (HBSS/FBS) on ice for less than 6 hrs before cutting to a size approx. $1mm^3$. The cell suspension was washed in fresh HBSS/FBS twice by centrifuging at 770g for 10min at 4°c, before being resuspended in DMEM containing 1% penicillin-streptomycin, 1% non-essential amino acids, 1% L-glutamine and 2% FBS (DMEM/FBS) and stored rolling overnight at 4°C. Cells were digested at 37°C in a shaking water-bath for 90min using a DMEM digestion solution (4ml/1g tissue) containing 1.5mg/ml collagenase (Sigma), 0.75mg/ml hyaluronidase (Sigma), 15µg/ml deoxyribonuclease (Sigma), and 10% FBS. The digested cell suspension was filtered through tea strainers and nylon filter mesh. Tissue that did not filter though the system was retained and treated for 10 min with dithiothreitol (DTT)

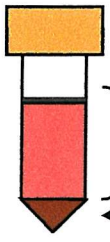
Fig. 2.3 Isolation of human lung mast cells.



Lung parenchyma obtained from resection surgery.

Tissue cut to <1mm^3 and connective tissue digested

Percoll cell separation

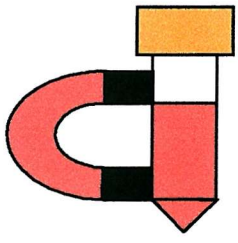


Upper layer retained

Red cell layer discarded

Washed and filtered

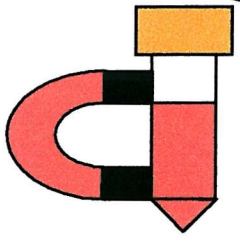
Incubation with CD2 Dynal beads



Magnetic selection for CD2$^+$ pan T-cells (discarded)

Incubation of remaining cells with mouse anti-human c-kit antibody YB5.B8

Incubation with sheep anti-mouse Dynal beads



Positive magnetic selection for c-kit$^+$ human lung mast cells

(1mM) (Sigma) to disperse mucus, before further filtration. The resulting cell suspension was filtered again using a 100μm cell strainer (Falcon, Becton Dickinson) and washed in HBSS/FBS.

The dispersed mast cells were further purified as previously described (Benyon et al. 1987; Okayama et al. 1994) (Fig. 2.3). The cell pellet was resuspended in DMEM/FBS (5ml/15g tissue) and mixed with a 65% continuous Percoll gradient (1.084g/ml) (25ml/15g tissue). For a 25ml 65% continuous Percoll gradient, 19.5ml Percoll (Sigma) was added to 2ml 10x HBSS (Gibco) and 3.4ml HBSS containing 25mM HEPES, 0.15M NaCl, and 0.3mg/ml deoxyribonuclease). Separation was performed by centrifugation at 1200g for 20min at 4°C such that erythrocytes were discarded in the pellet and the fraction containing the mast cells was uppermost. This layer was retained and diluted in six 30ml volumes of HBSS/FBS to dilute the Percoll. The cells were washed a second time in 30ml HBSS/FBS, filtered through a 100μm cell strainer and the pellet resuspended in 1ml DMEM/FBS. 10μl of cell suspension were removed for identification and counting of mast cells using Kimura's stain (Kimura et al. 1973), to calculate the number of beads required (approx. 3 beads per mast cell) for positive magnetic selection. 2ml PBS blocking buffer containing 1% FBS and 1% BSA was added with 50μg/ml mouse IgG serum (Calbiochem). This is a blocking step to prevent non-specific binding of antibodies used in this procedure. The cells were washed in 30ml HBSS/FBS at 430g for 8 min. at 4°C.

T-lymphocytes were removed from the enriched mast cell pellet by positive selection using a suspension of immuno-magnetic Dyna beads (Dynal) coated with mouse anti human CD2 antibody against pan T-cells in blocking buffer, which remove 99% of T-lymphocytes (Vartdal et al. 1987). The bead cell mixture was incubated rolling for 75min at 4°c on ice, and following incubation the cell suspension was made up to 20ml and placed on a magnet. The positively selected T-cells in the cell suspension were then discarded. The remaining cells were centrifuged and the pellet was resuspended in a 600μl solution of 5μg/ml mouse anti-human YB5.B8 antibody (donated by Dr. L. Ashman, Melbourne, Australia) which targets the stem cell factor receptor (c-kit) on the mast cell surface. The cells were incubated with this antibody as before for 75min, made up to 30ml with HBSS/FBS and filtered using a 100μm cell strainer, to remove cells which have clumped together to discourage further adhesion of cells.

The resulting cell pellet was resuspended with sheep anti-mouse coated Dyna beads (Dynal) in PBS blocking buffer for the selection of mast cells as identified by the

YB5.B8 antibody. The cell-bead mixture was incubated on ice at 4°c for 75min. The mast cells were selected by repeated exposure of the mixture to the magnet (Fig. 2.3). The initial beads selected by the magnet were washed and reapplied to the magnet to remove any contaminating cells. A high purity was confirmed by Kimura's staining (Kimura et al. 1973).

2.4.4 Culture of peripheral blood eosinophils

2.4.4.1. ***Inhibition of A23187 stimulated leukotriene C_4 production / release.***

Freshly isolated eosinophils were resuspended in prewarmed RPMI medium containing 2mM L-glutamine (Gibco) with the addition of 20mM L-serine (Sigma) and 0.1% BSA (Sigma) at a concentration of 200,000 cells per ml. Polypropylene tubes containing 100,000 cells in 0.5ml were preincubated in a shaking waterbath at 37°c for 10 minutes with varying concentrations of a selection of leukotriene modifier drugs or appropriate vehicles. Drugs used included the proposed inhibitor of multi-drug resistance associated protein (MRP), XR9173 (Xenova Limited, Slough, UK), the FLAP inhibitor MK886 (Alexis Corporation, Nottingham, UK) or MK-571 (Alexis Corporation) which has been reported to act as an MRP inhibitor in addition to its primary activity as a Cys-LT_1 receptor antagonist (Leier et al. 1994b).

Cells were then stimulated using the calcium ionophore A23187 1μM (Sigma) for a further 15 min after which reactions were halted by placing cells on ice. Cells were immediately centrifuged at 430 x g for 5 min at 4°c and supernatants removed into 1ml ice cold methanol for 30 min before freezing at -20°c. Cell pellets were resuspended in 0.5ml RPMI (2mM L-serine, 0.1% BSA) and 1ml ice cold methanol added for leukotriene extraction overnight before freezing at -20°c. Samples were centrifuged at 300g for 15 min at 4°c to remove cell debris and protein precipitates and the supernatants evaporated to dryness under vacuum using a Gyrovap rotary evaporator (Uniscience, Banbury UK). Samples were resuspended in appropriate volumes of PBS (Gibco) and frozen at -20°c before assaying for leukotrienes $C_4/D_4/E_4$ by EIA according to the manufacturers instructions (Amersham Pharmacia Biotech, Little Chalfont, UK).

2.4.4.2 Culture of eosinophils with GM-CSF and IL-5.

Eosinophils were cultured as previously reported (Cowburn et al. 1999). Purified eosinophils were cultured at 37°c under CO_2 in prewarmed RPMI 1640 medium containing 2mM L-glutamine (Gibco) with 10% FBS (Gibco), 100U/ml penicillin and

100μg/ml streptomycin (Gibco). Cells were cultured in a 24 well plate (Triple Red) at a concentration of 1 x10^6 cells per ml with the addition of 10ng/ml GM-CSF or 10ng/ml IL-5 (R&D systems, Abingdon, UK) for 0-24 hours. At relevant time points cells were removed from the plates, and centrifuged at 430 x g for 5 min at 4°c. Cell viability was determined by trypan blue exclusion and the cells were fixed with 4% paraformaldehyde for 2 hours rolling at 4°c. Fixed cells are required for intracellular immunostaining of 5-LO, FLAP, COX-1, COX-2 and EG2 for flow cytometric analysis as described in section 2.6 based on a method described by Kraft et al. (1998).

2.5 Processing of bronchial biopsies into GMA.

Biopsy specimens were immediately added to ice-cold acetone containing the protease inhibitors iodoacetamide (20mM) and phenylmethylsulphonylfluoride (PMSF) (2mM) (Sigma Chemical Co., Poole, UK) and fixed at -20°C overnight. Biopsies were transferred to fresh acetone and then to methyl benzoate for 15 minutes each at room temperature, before infiltration with glycol methacrylate (GMA) resin (Polysciences, Northampton, UK) as described (Britten et al. 1993). Polymerisation of the GMA resin took place in airtight capsules (Taab, UK) with methyl benzoate at 4°C overnight. The polymerised resin block was stored desiccated at -20°C.

The addition of protease inhibitors to acetone during the fixation of the biopsies and the use of methyl benzoate before embedding in GMA and during polymerisation improves the demonstration of antigens to a standard equivalent to that obtained with frozen sections (Britten et al. 1993).

2.5.1 Benefits of embedding biopsies in GMA.

Embedding of biopsies into GMA preserves optimal morphology of the tissue such that the antigen can be identified and localised to specific cells, while frozen sections, give relatively poor morphology (Britten et al. 1993). GMA resin is water permeable, which preserves antigens better than paraffin-fixed sections. GMA resin embedded tissue can be cut much thinner (1-2μm) than either frozen tissue (8μm) or paraffin embedded tissue (4μm). Thin sectioning allows an increased number of sections to be taken from one small biopsy and hence, a large number of different antigens can be investigated (Britten et al. 1993). Serial 2μm sections can also display the same cell (approx. 10μm), allowing localisation of multiple antigens to a particular cell (Britten et al. 1993). GMA is a hydrophilic resin and therefore, unlike paraffin, the resin does not need to be

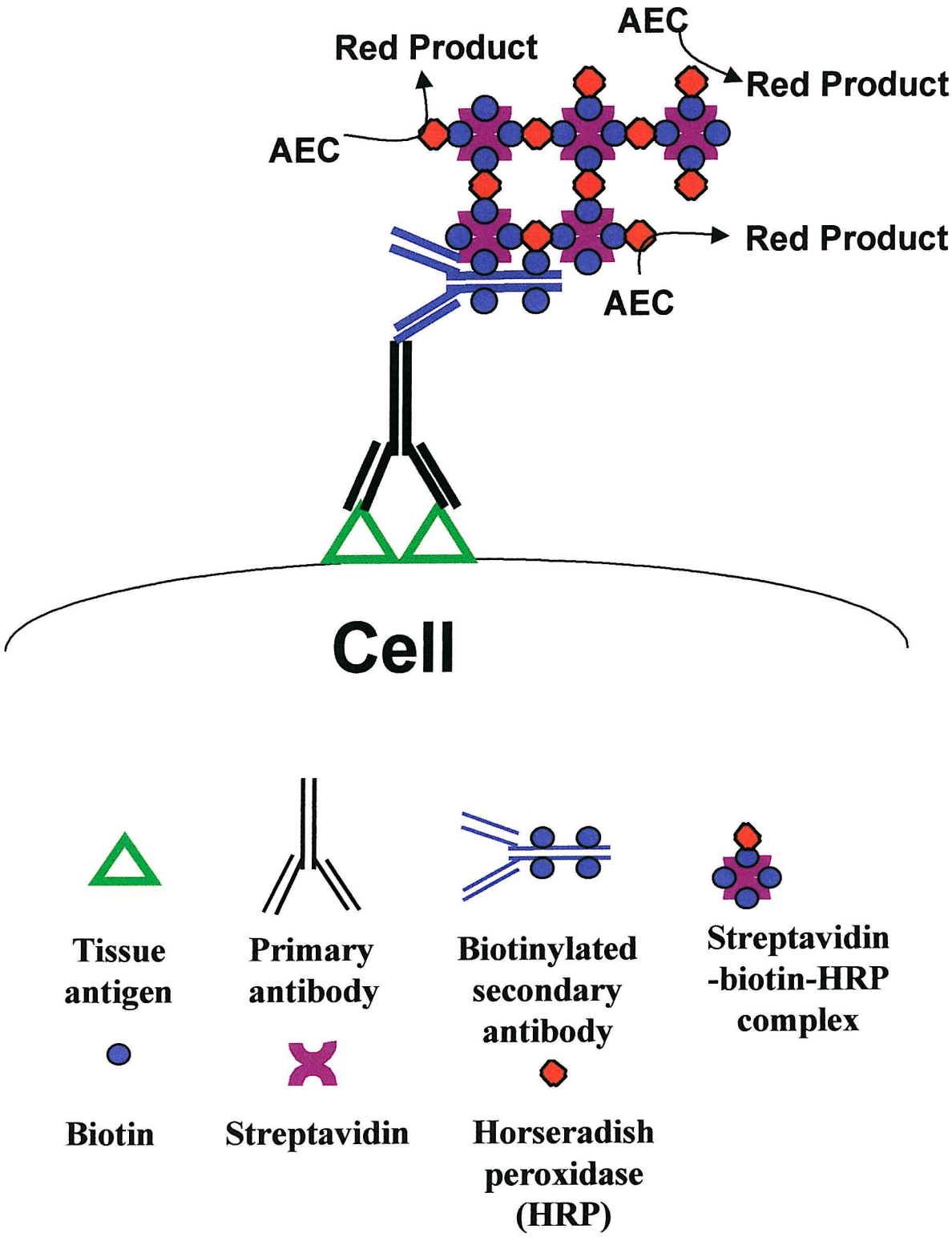
removed from the section before immunostaining. Trypsinisation is also not required to demonstrate antigens. (Britten et al. 1993).

2.5.2 Immunohistochemistry on GMA embedded bronchial biopsies.

Immunohistochemical staining for leukocyte markers and for 5-LO and COX pathway enzymes on GMA embedded bronchial biopsies was performed as described previously (**Fig 2.4**) (Britten et al. 1993; Cowburn et al. 1998) (**Fig. 2.5.1-2.6.3**). Serial semi-thin sections (2 μm) were cut by ultramicrotome using a glass knife and floated onto 0.2% ammonia solution for 90s, before adherence to microscope slides coated with poly-L-lysine (Sigma). The use of 0.2% ammonia in the water improves the demonstration of antigens. Sections were pre-treated with sodium azide (0.1%) and hydrogen peroxide (0.3%) for 30 minutes to irreversibly inhibit endogenous peroxidases. Sections were washed 3 times for 5min with TBS (0.15M NaCl (Sigma), 50μM Tris (BioRad), pH 7.66) and treated with blocking medium (Dulbecco's modified Eagle's medium (Gibco) containing 20% foetal calf serum (Gibco) and 1% bovine serum albumin (Sigma)) for 30 minutes to block non-specific binding sites. Slides were drained (but the blocking medium was not washed away) before the application of primary antibodies each section for one hour (polyclonals) or overnight (monoclonals) at room temperature.

For monoclonal primary antibodies, biotinylated rabbit anti-mouse IgG secondary antibody (1:300) (Dako) was applied for 2 hours at room temperature, followed by streptavidin-biotin horseradish peroxidase (SAB-HRP) complex (1:200) (Dako) for 2 hours. For polyclonal primary antibodies, biotinylated swine anti-rabbit IgG (1:300) (Dako) was applied for 1 hour followed by SAB-HRP complex for 1 hour. Incubation times and temperatures for antibody application were as previously determined for GMA embedded tissue to give optimum antigen demonstration (Britten et al. 1993). SAB-HRP forms a red reaction product from the substrate aminoethylcarbazole (AEC; 0.03%) in acetate buffer, incubated at 37°C for 25-30 min. Sections were counterstained with Meyer's haematoxylin for 1-1.5 min. Slides were treated with Crystal Mount (Biomedia, CA, USA) to prevent the red colour produced by AEC becoming soluble in the DPX (BDH, Poole, UK) with which coverslips were mounted. Positively stained nucleated cells in the total mucosal area (excluding epithelium, mucus glands, blood vessels and areas of forceps damage) of at least two non-adjacent sections from each biopsy were counted by Zeiss light microscope at x400 magnification. Counting was performed blind on coded sections. Submucosal areas in each section were determined using an image

Fig. 2.4 Principle of immunohistochemistry



analysis system (ColorVision 164SR, Analytical Measurement Systems, Cambridge, UK). Cell counts are expressed as mean cells per mm^2 of mucosa. Control sections were routinely immunostained in the absence of primary antibody or in the presence of an unrelated isotype-matched mouse IgG (for monoclonal primary antibodies) or non-immune rabbit serum (for polyclonal antibodies). In all cases there was no colour development (**Fig. 2.5.5**).

To determine the repeatability of cell counts from two non-adjacent sections of the same size the Bland-Altman coefficient of repeatability was calculated using the equation:

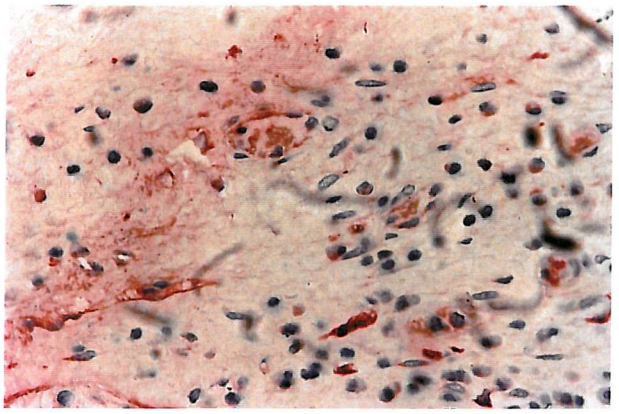
$$\left(\sqrt{\frac{\Sigma[(x-y)^2]}{n}} \right) * 2 = \text{Coefficient of Repeatability}$$

The repeatability coefficient for 5-LO (n=14) and FLAP (n=14) was 19.8 cells/mm^2 and 17.0 cell/mm^2 respectively. For COX-1 (n=14) it was 24.4 cells/mm^2, and for EG2 (n=14) and AA1 (n=14) was 24.1 cell/mm^2 and 20.0 cells/mm^2 respectively.

2.5.3 Titrations for primary antibodies.

Prior to use for immunohistochemistry, primary antibodies were titrated to determine the optimum dilution for use with bronchial biopsies embedded in GMA. Nasal polyp is an inflammatory tissue with high numbers of inflammatory leukocytes. As eicosanoid pathway enzymes are expressed primarily in these cells, nasal polyp was an appropriate tissue to titrate antibodies against eicosanoid pathway enzymes in preliminary experiments. The antibodies were titrated against biotinylated rabbit anti-mouse, or swine anti-rabbit secondary antibody at a standard dilution of 1:300, and the streptavidin biotin complex at a standard dilution of 1:200, which have previously been shown to be optimal for GMA sections. A range of dilutions of primary antibody between 1:50 and 1:1000 was applied to nasal polyp sections. The sections, stained as described in section 2.5.2, were examined under x400 light microscopy for the presence of a red coloration localised to cells. Dilutions of antibody which produced a red/pink wash and/or non-specific staining were disregarded, as were those which produced a very pale coloration. Photomicrographs illustrating a typical titration are shown in **Fig 2.5**. The titration was repeated for each new batch of antibody. Antibodies for cell markers are routinely titrated in the immunohistochemistry laboratory with Dr Susan Wilson in a similar manner (**Table 2.1, Fig.2.5.1 – 2.5.5**).

1:250



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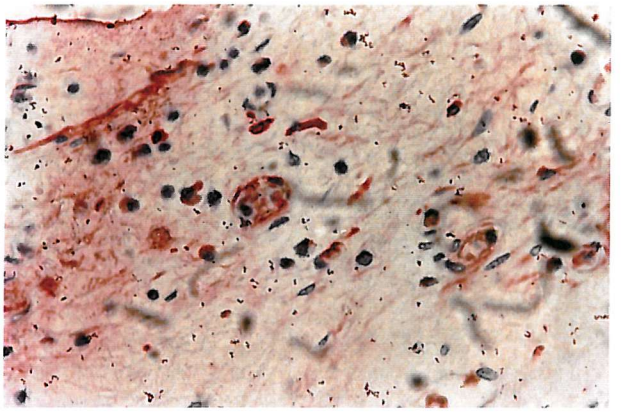

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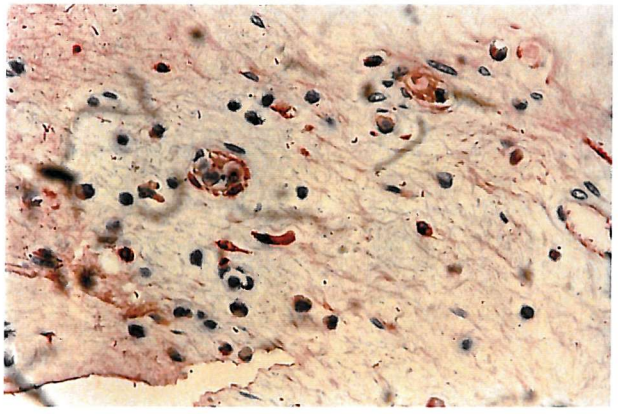

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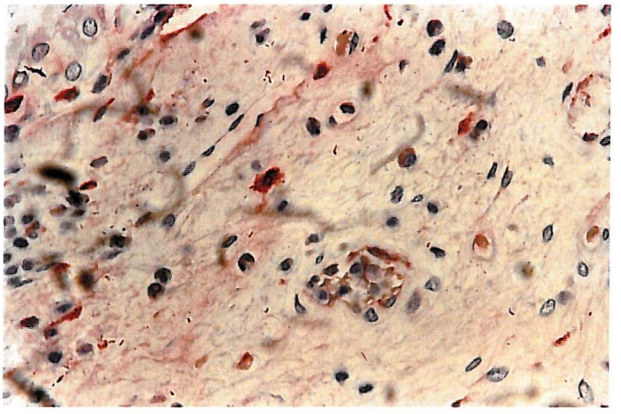

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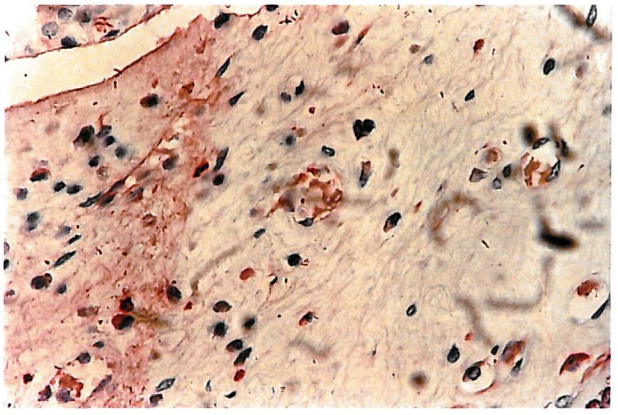

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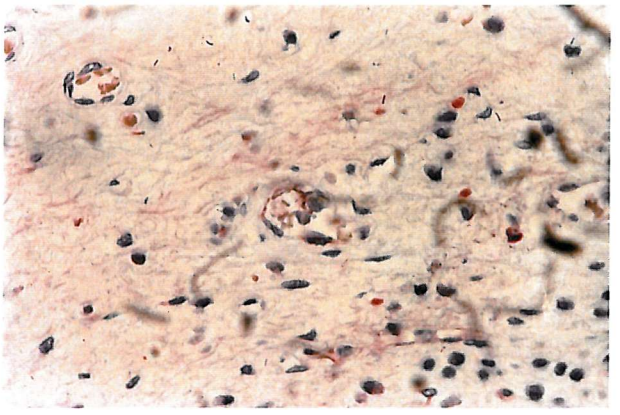


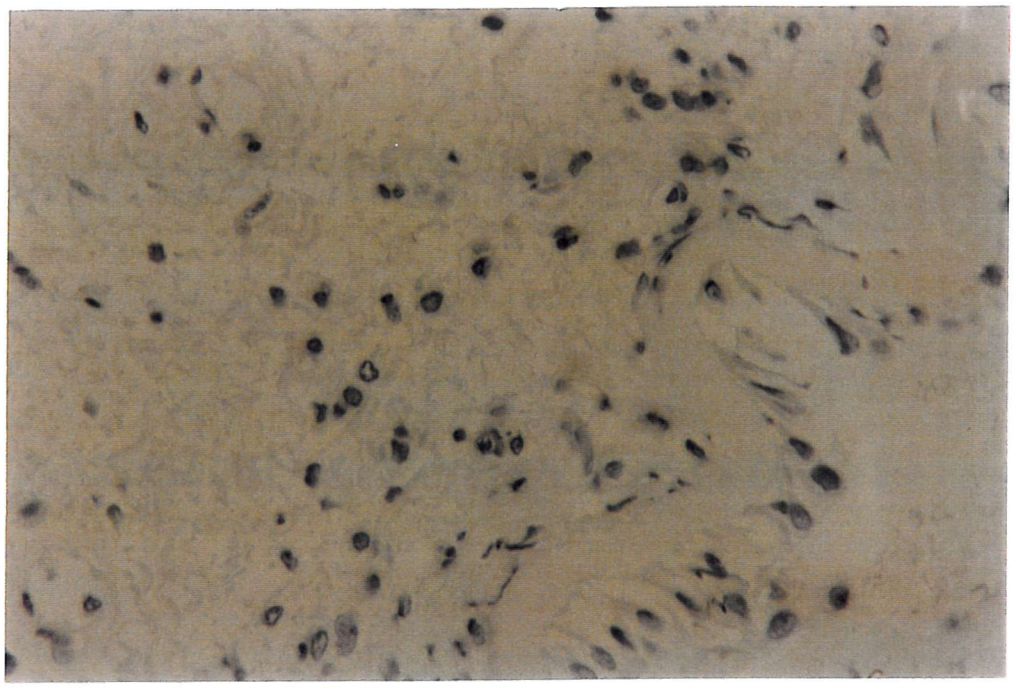
Fig.2.5 Photomicrographs of a representative titration of COX-1. Nasal polyp sections were immunostained using a monoclonal antibody against COX-1 (PGHS-1) at dilutions of 1in 250, 1in 500, 1in 1000, 1in 1500, 1in 2000 and 1in 3000. Optimal staining was achieved at 1in 1000. Positive AEC staining is red against the blue counterstain of Meyers haematoxylin.

Table 2.1 Mouse monoclonal antibodies to leukocyte markers.

Target antigen	Antibody	Typical dilution	Source	Reference
Mast cell tryptase	AA1 (IgG1, 18.2mg/ml)	1:1000	Dako, High Wycombe, UK	Walls et al. 1990
Eosinophil cationic protein (ECP)	EG2 (IgG_1 100μg/ml)	1:200	Pharmacia Biosystems Ltd., Milton Keynes, UK	Tai et al. 1984; Jahnsen et al. 1994
Macrophage-specific form of CD68	PG-M1 (IgG_3 360 μg/ml)	1:15	Dako	Falini et al. 1993
Monocyte endotoxin receptor (CD14)	TUK4 (IgG_{2A} 35μg/ml))	1:16	Dako	
$CD3^+$ pan-T-lymphocytes	UCHT1 (IgG1 300mg/ml)	1:100	Dako	
$CD4^+$ T-helper cells	IgG1 25mg/ml	1:10	Becton Dickinson Ltd., Oxford, UK	
$CD8^+$ cytotoxic/ suppressor T cells	DK25 (IgG1, 13.7mg/ml)	1:100	Dako	
CD25+ activated (IL-2 $receptor^+$) T-lymphocytes	ACT-1 (IgG1, 6mg/ml)	1:50	Dako	
Basophil	2D7 (IgG1 3mg/ml)	1:300	Gift from Dr L. Schwartz	Kepler et al. 1995
Basophil	BB1 (IgG_{2A} 0.5mg/ml)	1:10	Gift from Dr A.F. Walls. University of Southampton, UK	McEuen et al. 1999
Neutrophil Elastase	NP57 (IgG1 65μg/ml)	1:1000	Dako	

The mouse monoclonal antibody 2C7 (dilution 1:40) was used to identify cells which expressed activated NF-κB. (kindly donated by Pharmacia Upjohn, Kalamazoo, USA).

A



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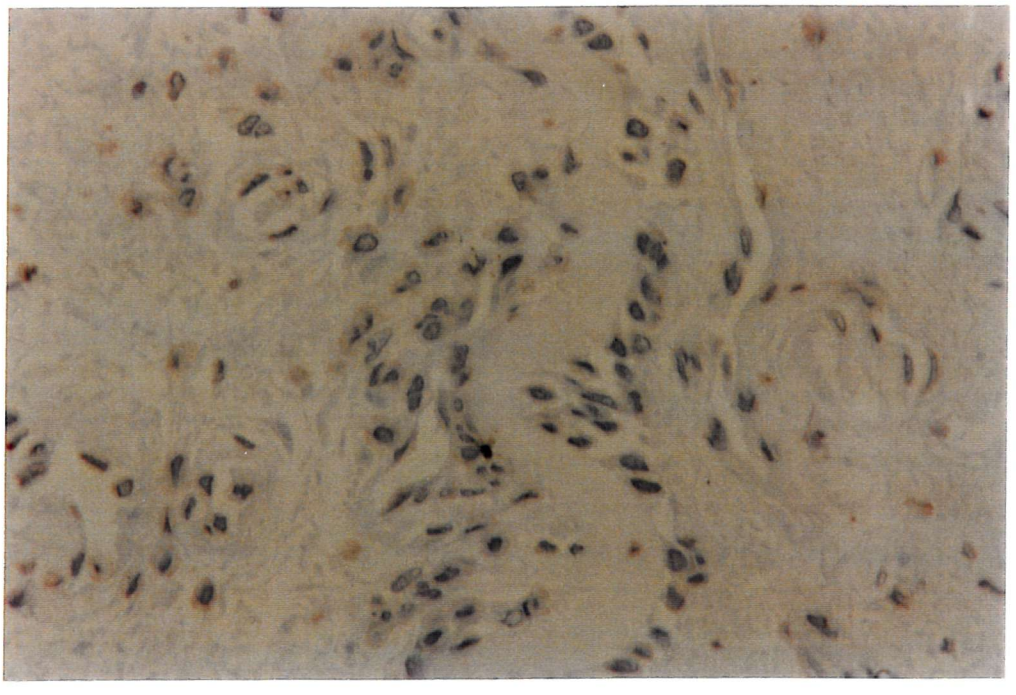
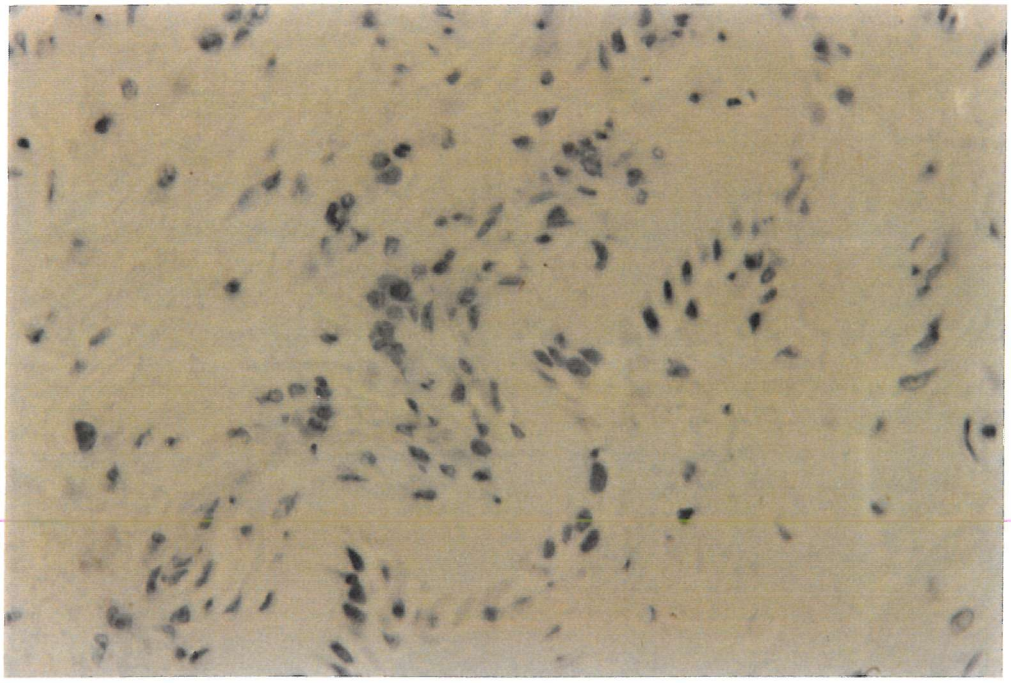


Fig 2.5.1 Photomicrographs of isotype control immunostaining in representative GMA embedded bronchial biopsies. Positive AEC immunostaining appears red against blue Meyers haematoxylin counterstain. ***Panel A.*** Immunostaining using IgG1 isotype control, this is the appropriate control for AA1, EG2, CD3, CD4, CD8, CD25, 2D7, COX-2, MRP-1 and MRP-2 antibodies. ***Panel B.*** Immunostaining using IgG2a isotype control, this is the appropriate control for CD14, BB1 and COX-1 antibodies.

A



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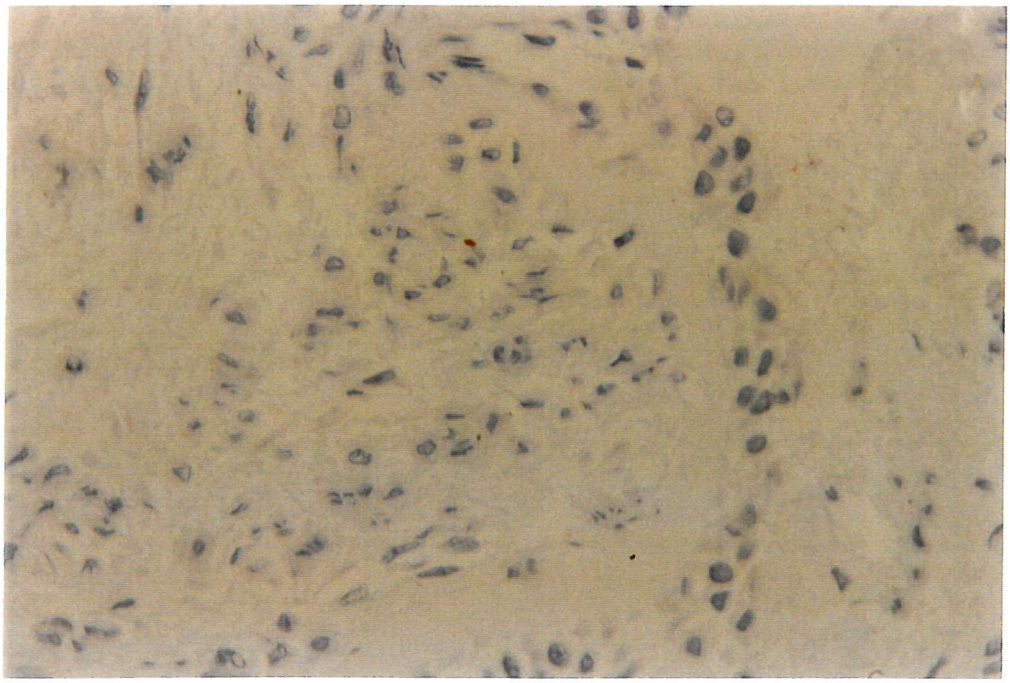
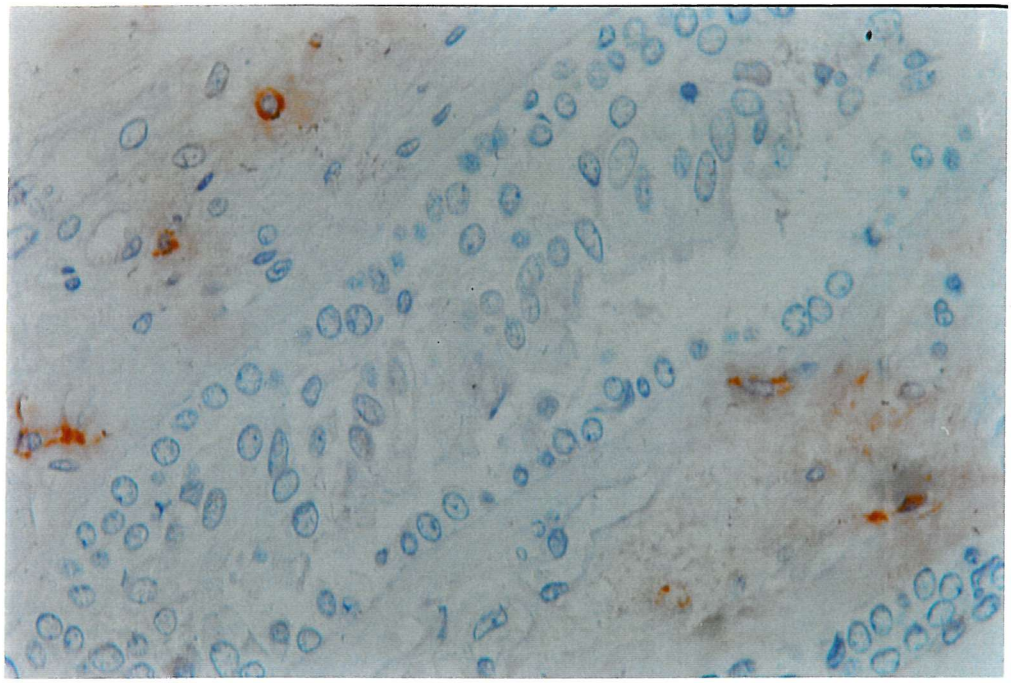


Fig 2.5.2 Photomicrographs of isotype control immunostaining in representative GMA embedded bronchial biopsies. Positive AEC immunostaining appears red against blue Meyers haematoxylin counterstain. ***Panel A.*** Immunostaining using IgG_{2B} isotype control, this is the appropriate control for the COX-1 antibody. ***Panel B.*** Immunostaining omitting the primary antibody, this is the appropriate control for the immunostaining procedure to ensure that there is no non-specific staining produced by secondary or tertiary antibodies.

A



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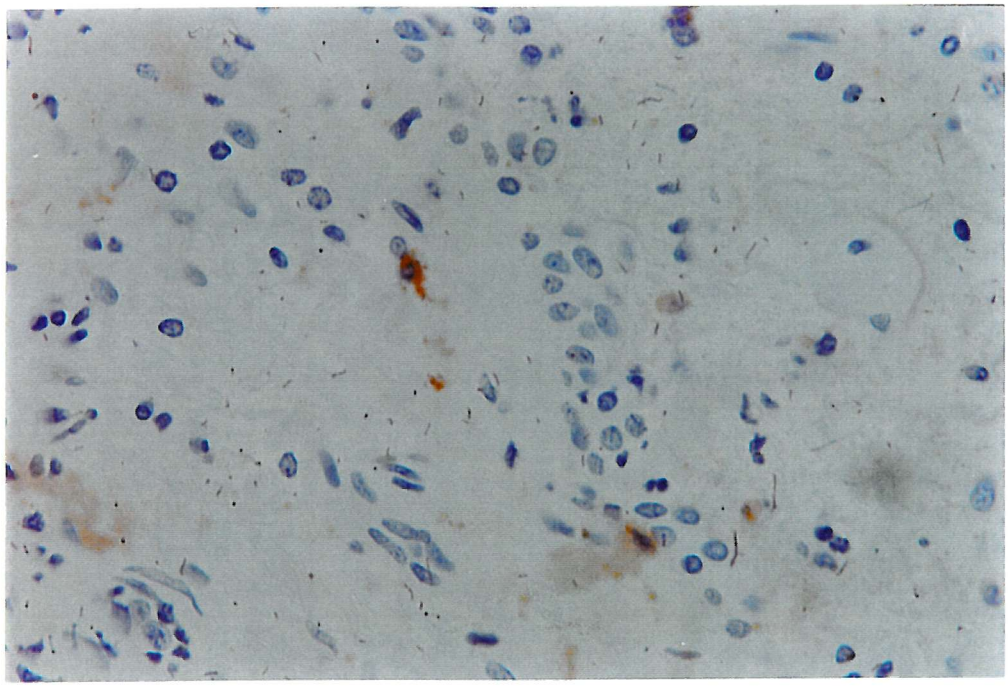
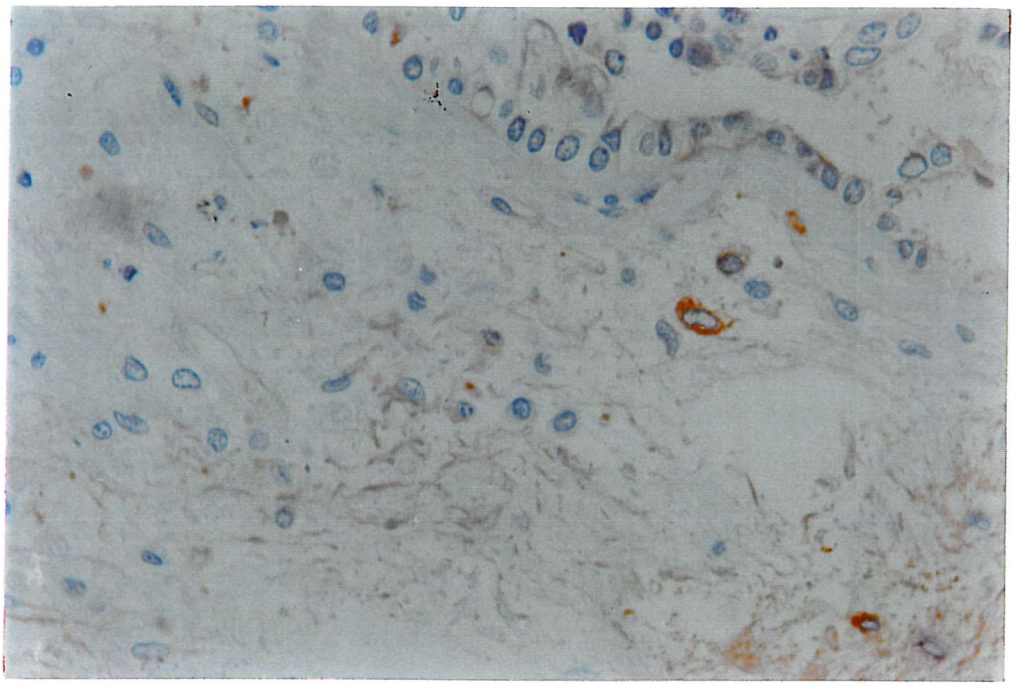


Fig. 2.5.3 Photomicrographs (x400) of immunostaining for mast cells and eosinophils in representative GMA embedded bronchial biopsies. Positive AEC immunostaining appears red against blue background of Meyers haematoxylin counterstain. ***Panel A.*** Immunostaining with the monoclonal antibody AA1 against mast cell tryptase. ***Panel B.*** Immunostaining with the monoclonal antibody EG2 against eosinophil cationic protein.

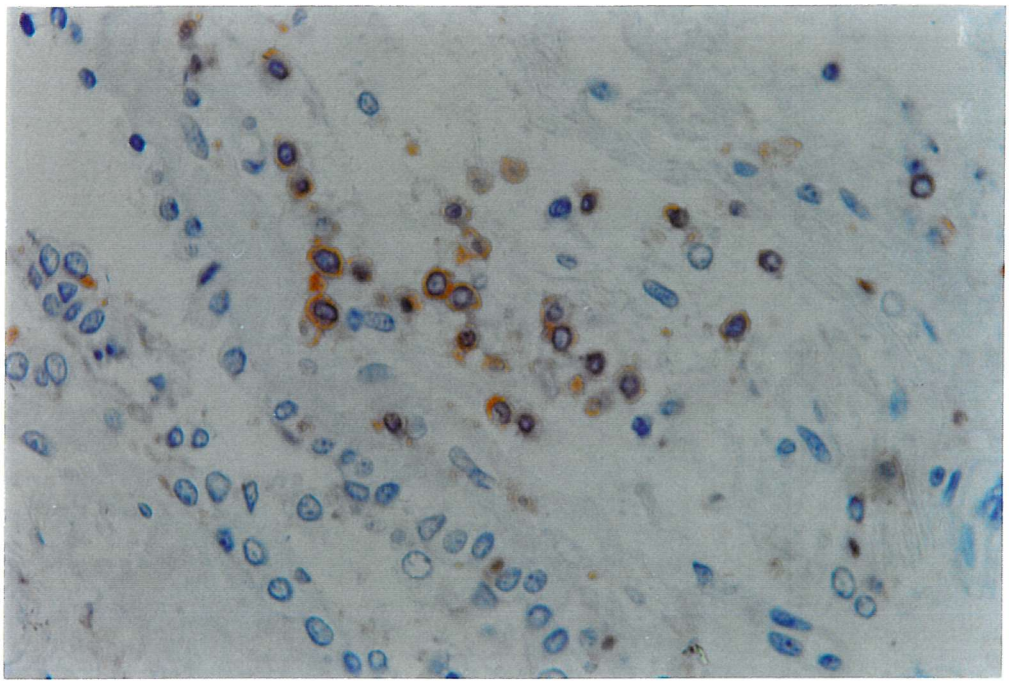
A



B

Fig. 2.5.4 Photomicrographs (x400) of immunostaining for macrophages and monocytes in representative GMA embedded bronchial biopsies. Positive AEC immunostaining appears red against blue background of Meyers haematoxylin counterstain. ***Panel A***. Immunostaining with the monoclonal antibody PG-M1 against the macrophage specific form of CD68. ***Panel B***. Immunostaining with the monoclonal antibody TUK4 against the monocyte endotoxin receptor CD14.

A



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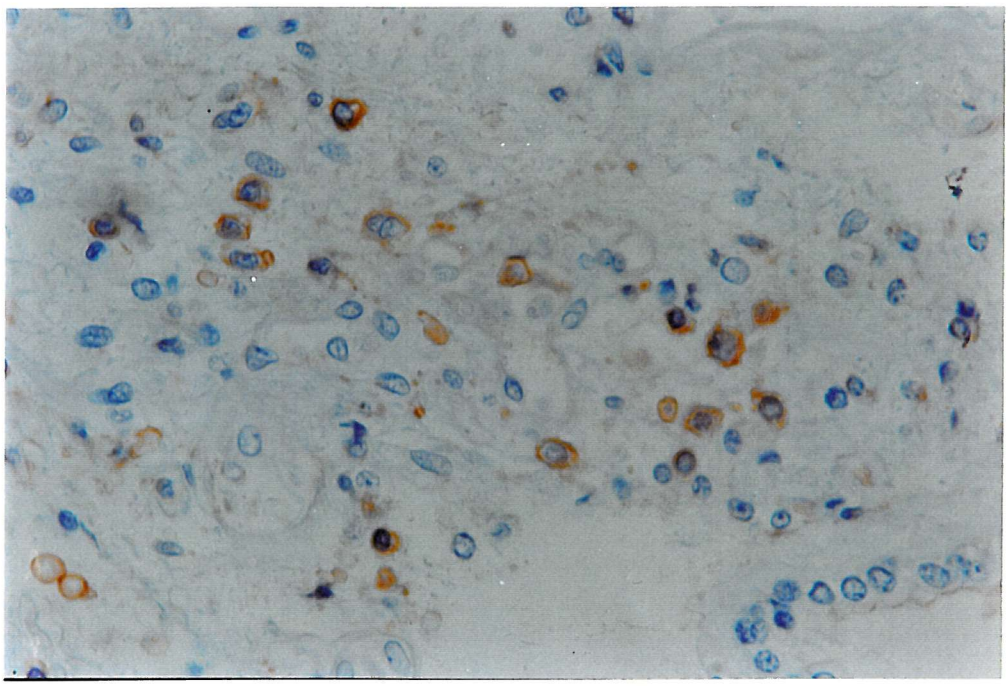
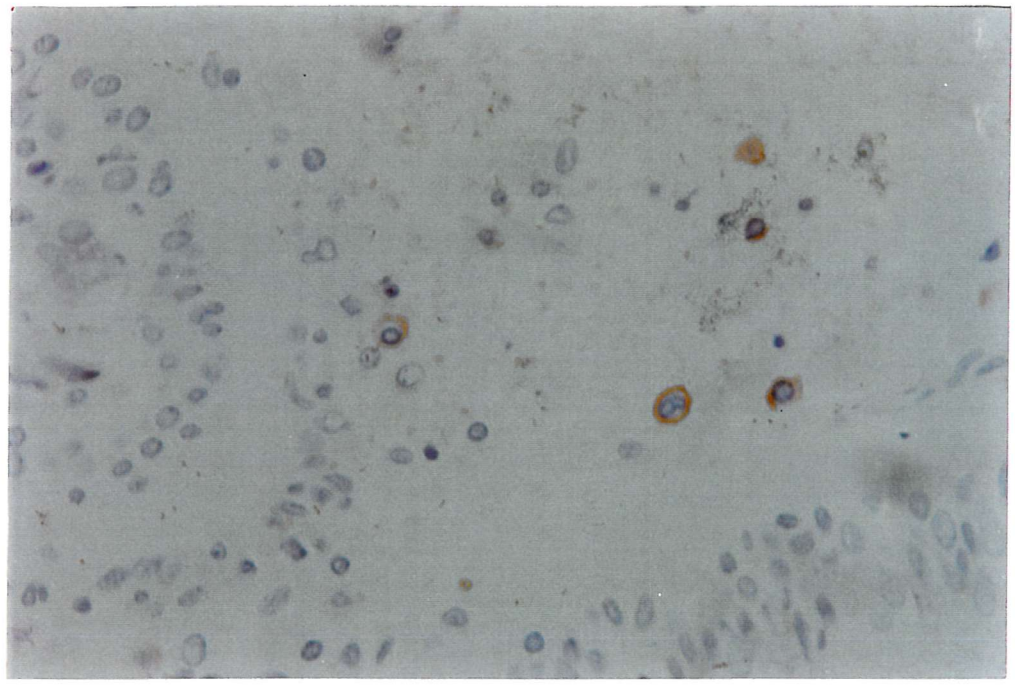


Fig. 2.5.5 Photomicrographs (x400) of immunostaining for T-cells in representative GMA embedded bronchial biopsies. Positive AEC immunostaining appears red against blue background of Meyers haematoxylin counterstain. ***Panel A***. Immunostaining using the monoclonal antibody UCTH1 for $CD3^+$ pan-T-cells. ***Panel B***. Immunostaining using the monoclonal antibody against $CD4^+$ helper T-cells.

A



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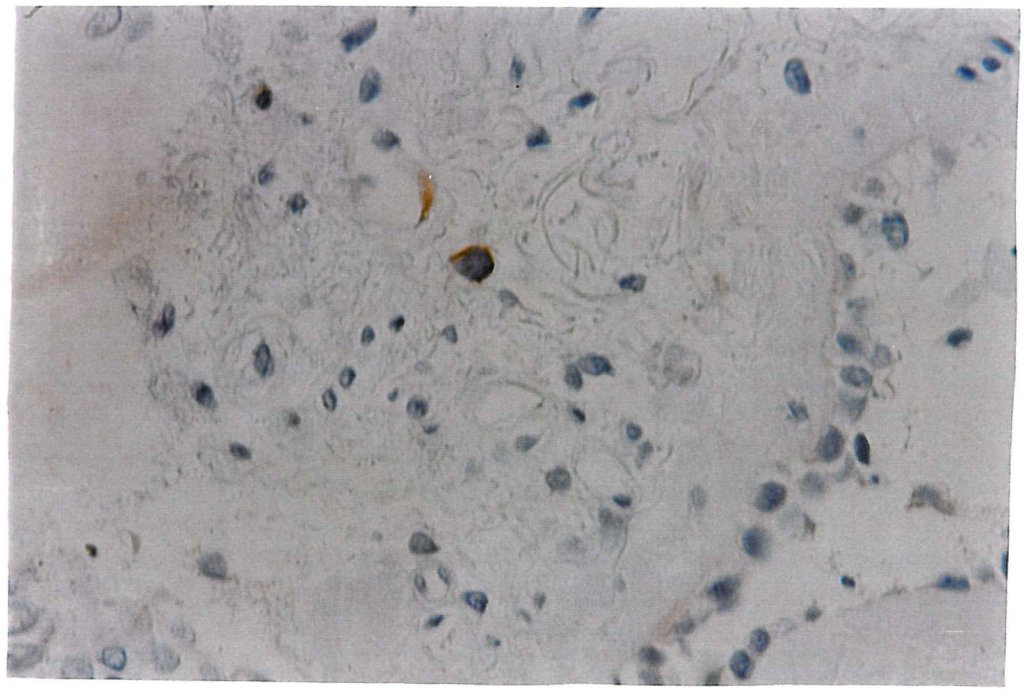
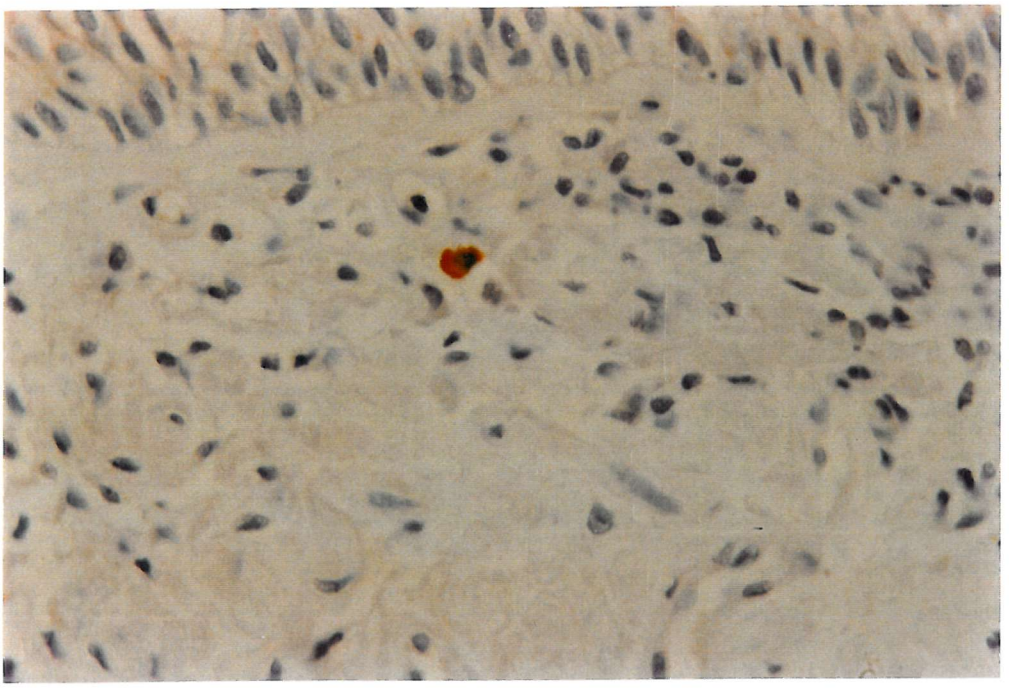


Fig. 2.5.6 Photomicrographs (x400) of immunostaining for T-cells in representative GMA embedded bronchial biopsies. Positive AEC immunostaining appears red against blue background of Meyers haematoxylin counterstain. ***Panel A***. Immunostaining using the monoclonal antibody DK25 against $CD8^+$ cytotoxic/suppressor T-cells. ***Panel B***. Immunostaining using the monoclonal antibody ACT-1 against $CD25\ (IL\text{-}2R)^+$ activated T-cells.

A



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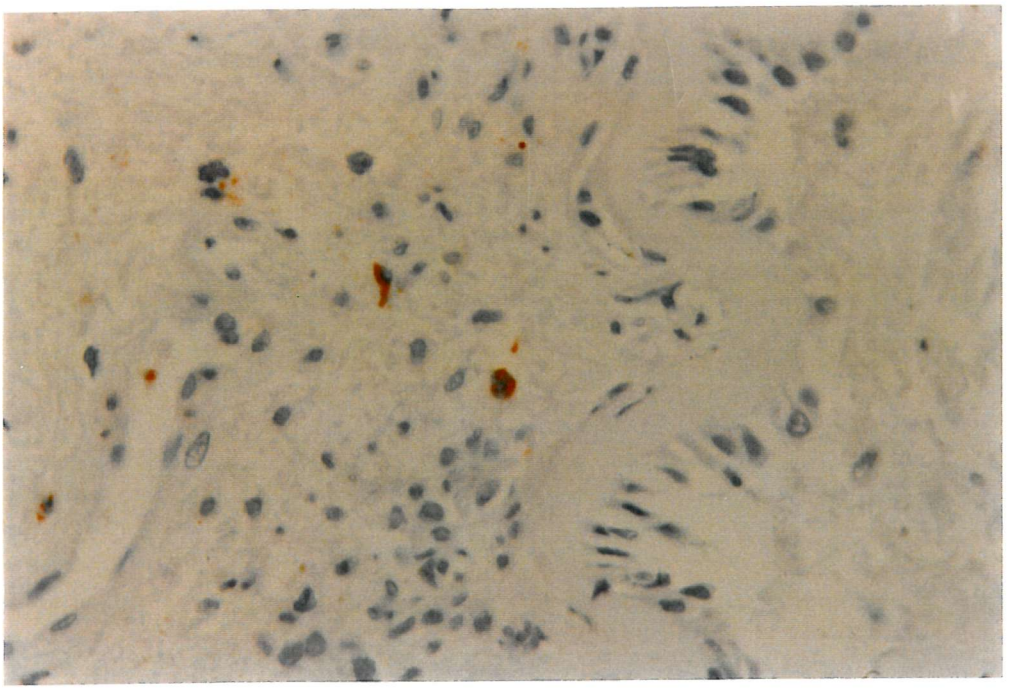
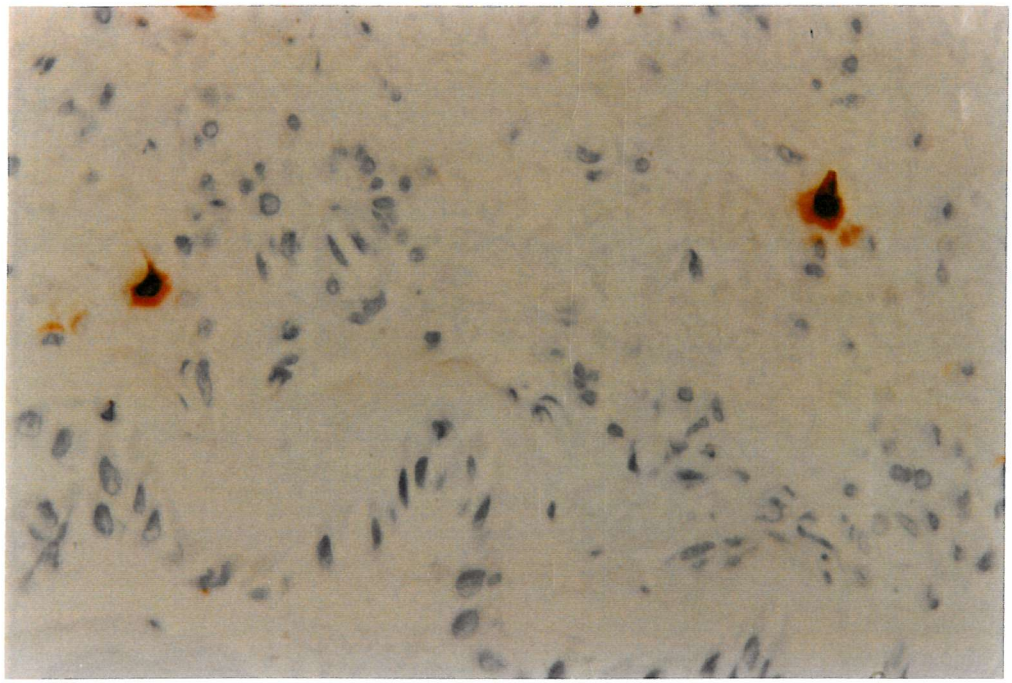


Fig. 2.5.7 Photomicrographs (x400) of immunostaining for basophils in representative GMA embedded bronchial biopsies. Positive AEC immunostaining appears red against blue background of Meyers haematoxylin counterstain. ***Panel A***. Immunostaining using the monoclonal antibody 2D7. ***Panel B***. Immunostaining using the monoclonal antibody BB1.

A



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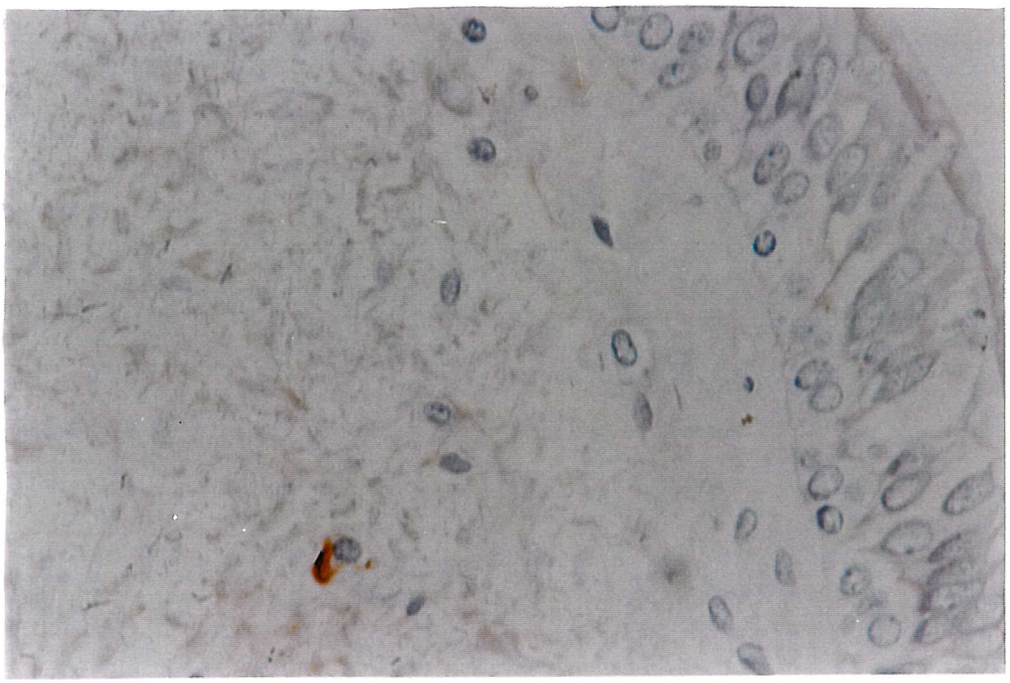


Fig. 2.5.8 Photomicrographs (x400) of immunostaining for neutrophils and activated NF-κB in representative GMA embedded bronchial biopsies. Positive AEC immunostaining appears red against blue background of Meyers haematoxylin counterstain. ***Panel A***. Immunostaining using the monoclonal antibody NP57 for neutrophil elastase ***(Panel B)***. Immunostaining using the monoclonal antibody 2C7 against activated NF-κB.

Table 2.2 Polyclonal and monoclonal antibodies against enzymes of the eicosanoid pathway.

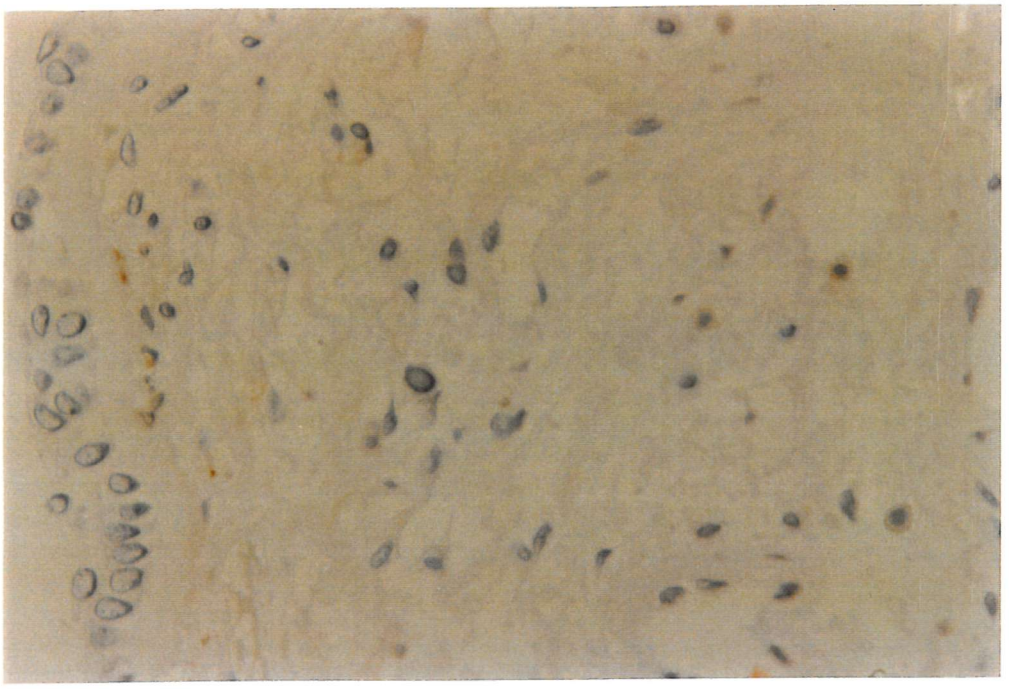
Target antigen	Antibody name	Monoclonal/ Polyclonal	Typical dilution	Source	Reference
Human 5-LO	LO-32	Rabbit polyclonal antiserum (stock diluted 15-fold)	1:1000	Gift from Dr Jilly Evans, Merck-Frosst, Quebec, Canada	Woods et al. 1993 Cowburn et al. 1998
Human FLAP	H4	Rabbit polyclonal antiserum (stock diluted 5-fold)	1:1000	Gift from Dr Jilly Evans, Merck-Frosst,	Woods et al. 1993 Cowburn et al. 1998
Human LTA_4 hydrolase		Rabbit polyclonal antiserum (stock diluted 5-fold)	1:1000	Gift from Dr Jilly Evans, Merck-Frosst,	Cowburn et al. 1998
Human LTC_4 synthase‡	HOMER	Rabbit polyclonal antibody affinity purified (147μg/ml)	1:100	Gift from K.F. Austen, Harvard Medical School, Boston, USA.	Penrose et al. 1995 Cowburn et al. 1998
Human MRP-1	MRPm6	Mouse monoclonal IgG1 500μg/ml	1:10	Alexis Corporation,	
Human MRP-2/ cMOAT	M_2 1-4	Mouse monoclonal IgG1 500μg/ml	1:100	Alexis Corporation,	Paulusma et al. 1996, Kool et al. 1997
Ovine COX-1*	PGHS-1	Mouse monoclonal IgG (500μg/ml)	1:400	Cayman Chemical Inc., Ann Arbor, MI, USA	Cowburn et al. 1998
Human COX-2†	PGHS-2	Mouse monoclonal IgG1 (50μg/ml)	1:400	Cayman Chemical Inc., Ann Arbor, MI, USA	Cowburn et al. 1998
Human PGD_2 synthase		Rabbit polyclonal antiserum (Stock diluted 10-fold)	1:1000	Gift from K.F. Austen,	Mahmud et al. 1997, Suzuki et al. 1997

*Antibody raised against purified sheep seminal vesicular COX-1 and shown to cross-react with the human enzyme.

†Antibody raised against a 19 amino acid synthetic peptide from the human COX-2 sequence.

‡Antibody raised from purified LTC_4 synthase from human lung.

A



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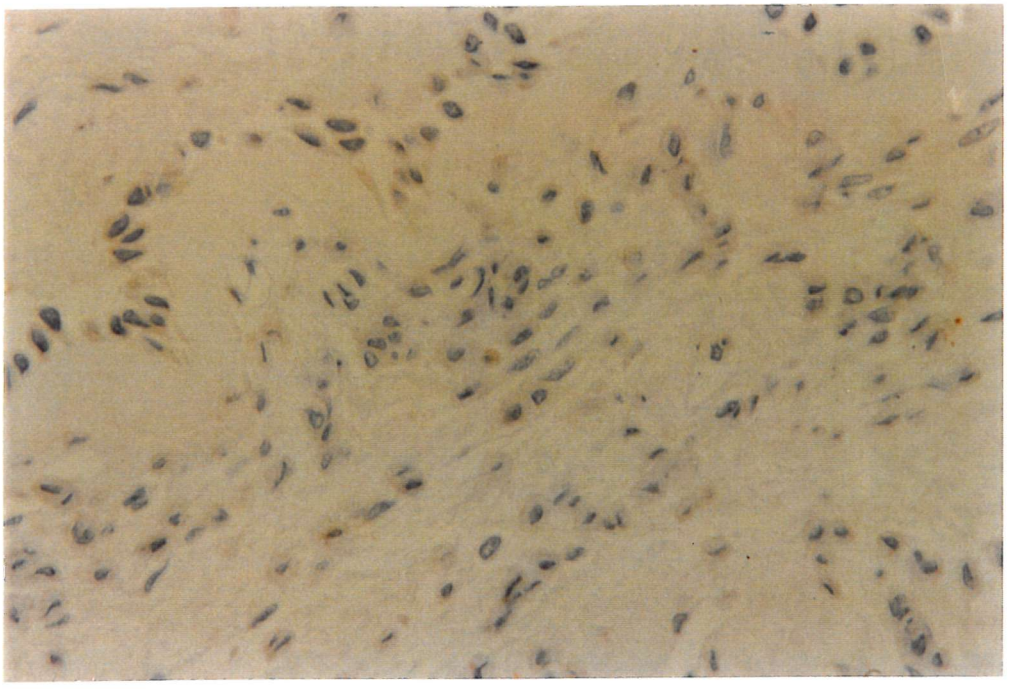
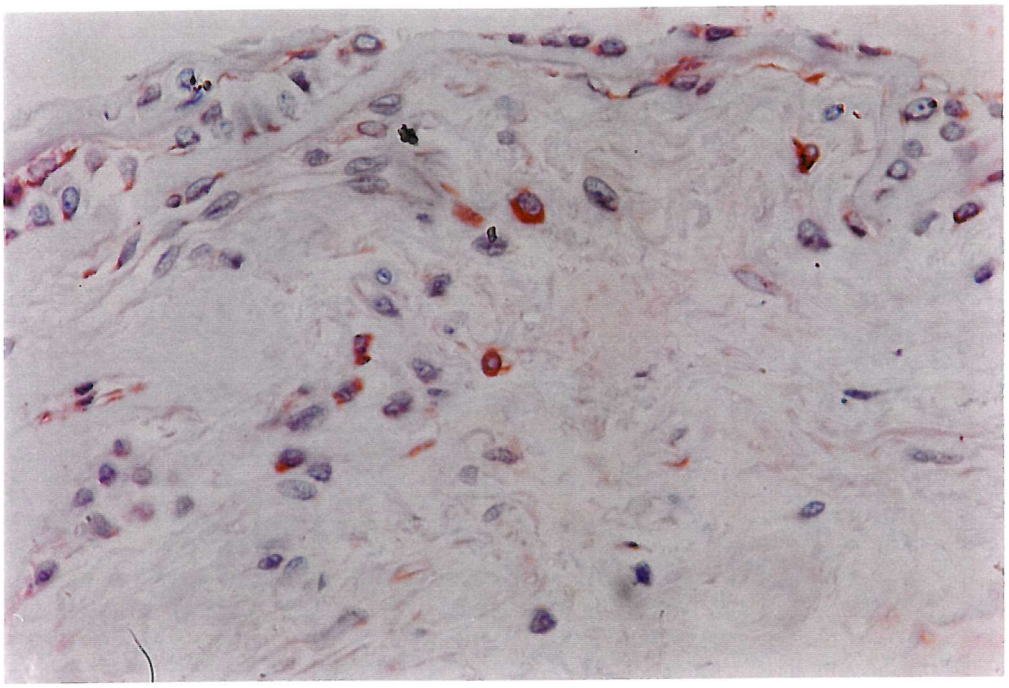


Fig 2.6.1 Photomicrographs of isotype control immunostaining in representative GMA embedded bronchial biopsies. Positive AEC immunostaining appears red against blue Meyers haematoxylin counterstain. ***Panel A.*** Immunostaining using normal rabbit serum isotype control, this is the appropriate control for 5-LO, FLAP, and PGD synthase antibodies. ***Panel B.*** Immunostaining using rabbit immunoglobulin isotype control, this is the appropriate control for LTC_4 synthase antibodies.

A



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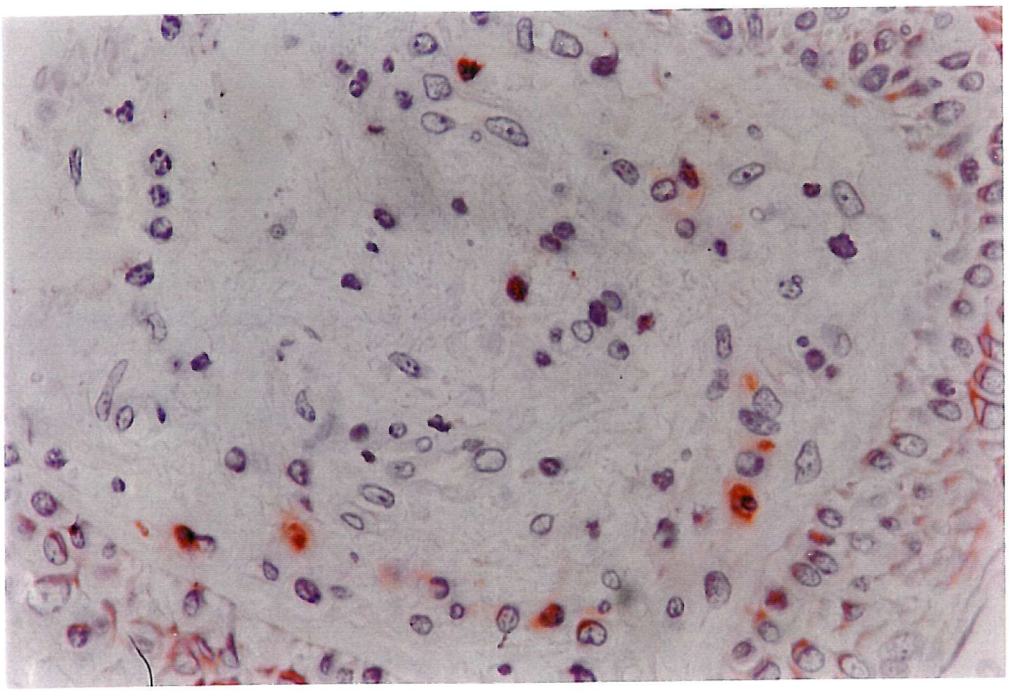
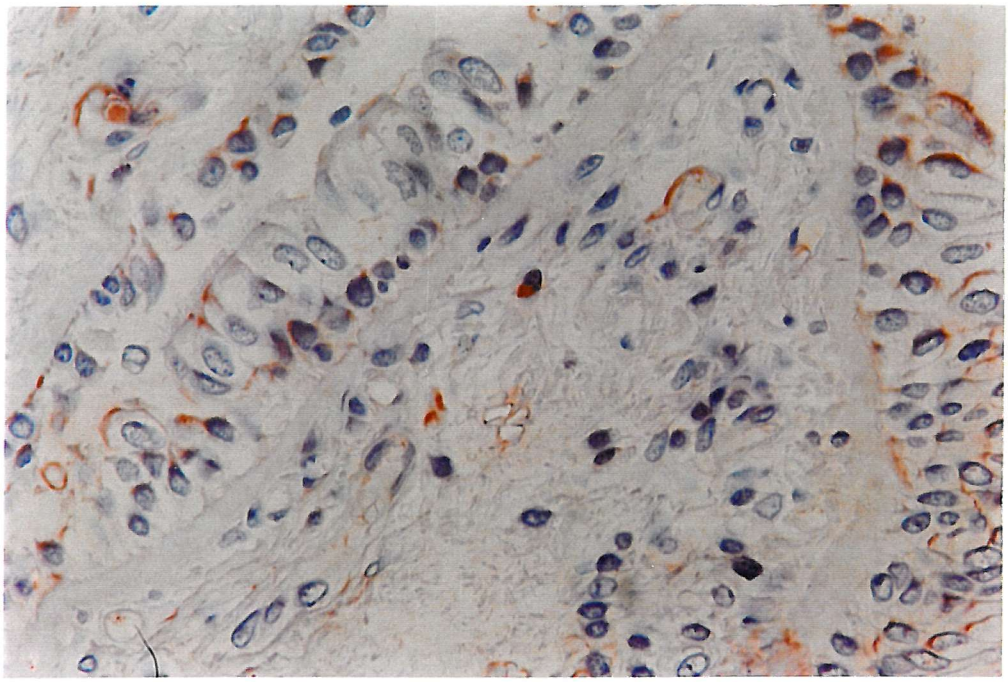


Fig. 2.6.2 Photomicrographs (x400) of immunostaining for 5-LO and FLAP in representative GMA embedded bronchial biopsies. Positive AEC immunostaining appears red against blue background of Meyers haematoxylin counterstain. ***Panel A***. Immunostaining using the polyclonal antibody LO-32 against 5-lipoxygenase (5-LO). ***Panel B***. Immunostaining polyclonal antibody H4 against 5-lipoxygenase activating protein (FLAP).

A



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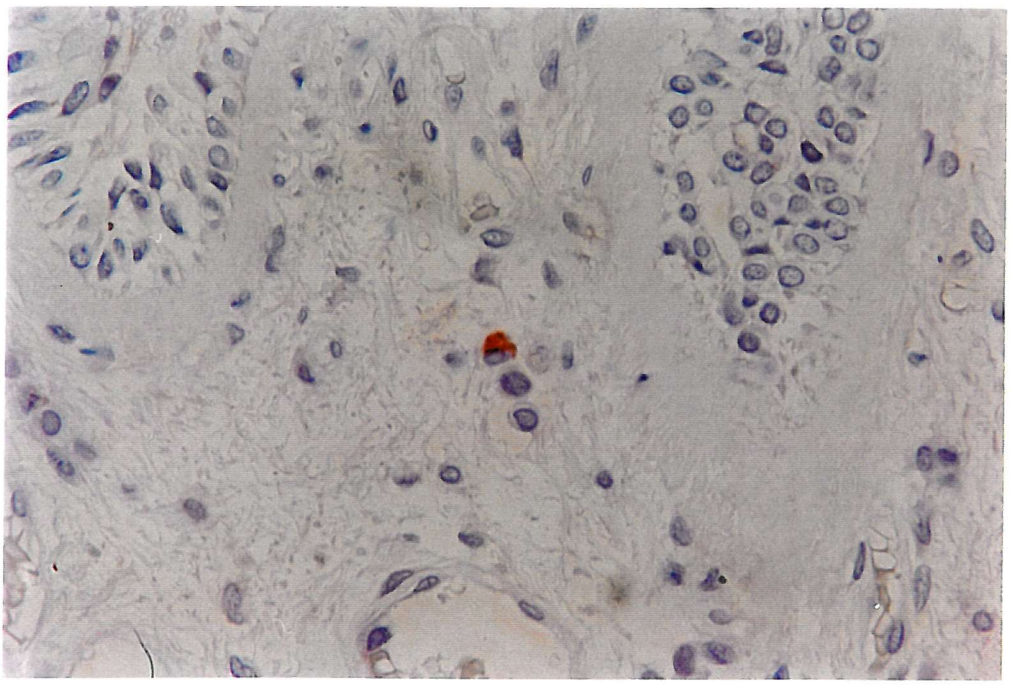
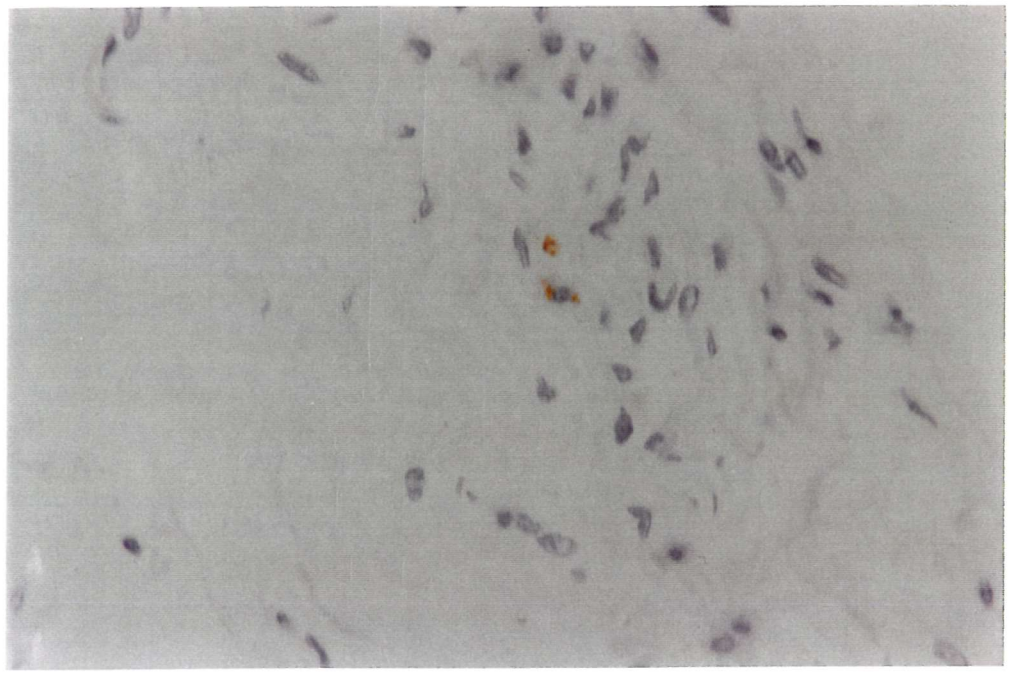


Fig. 2.6.3 Photomicrographs (x400) of immunostaining for LTA_4 hydrolase and LTC_4 synthase in representative GMA embedded bronchial biopsies. Positive AEC immunostaining appears red against blue background of Meyers haematoxylin counterstain. ***Panel A***. Immunostaining using the polyclonal antibody against LTA_4 hydrolase. ***Panel B***. Immunostaining using the polyclonal antibody THE against LTC_4 synthase.

A



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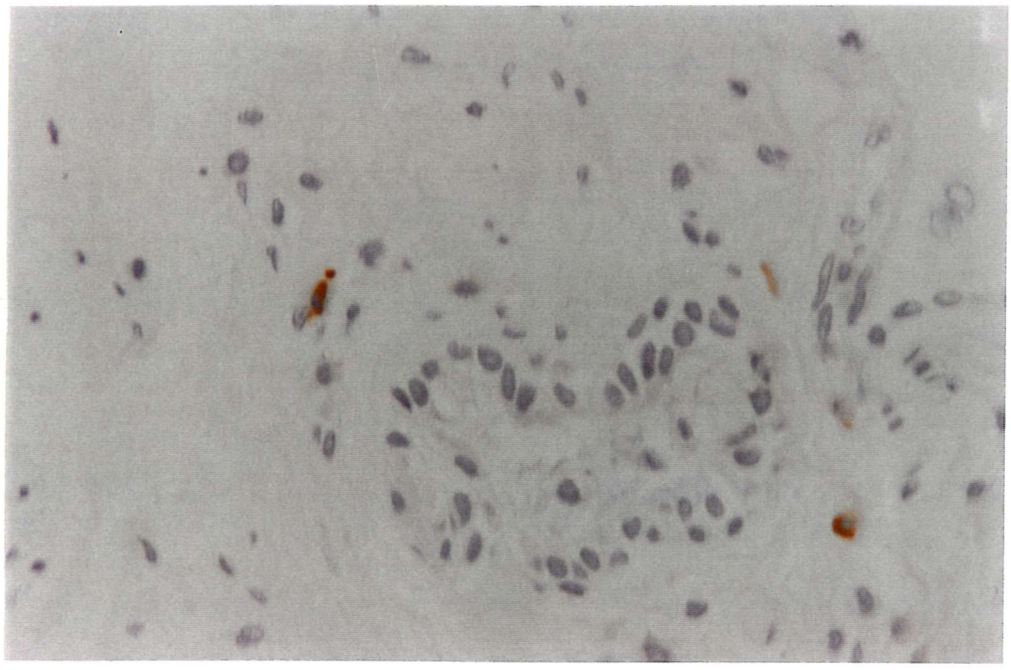
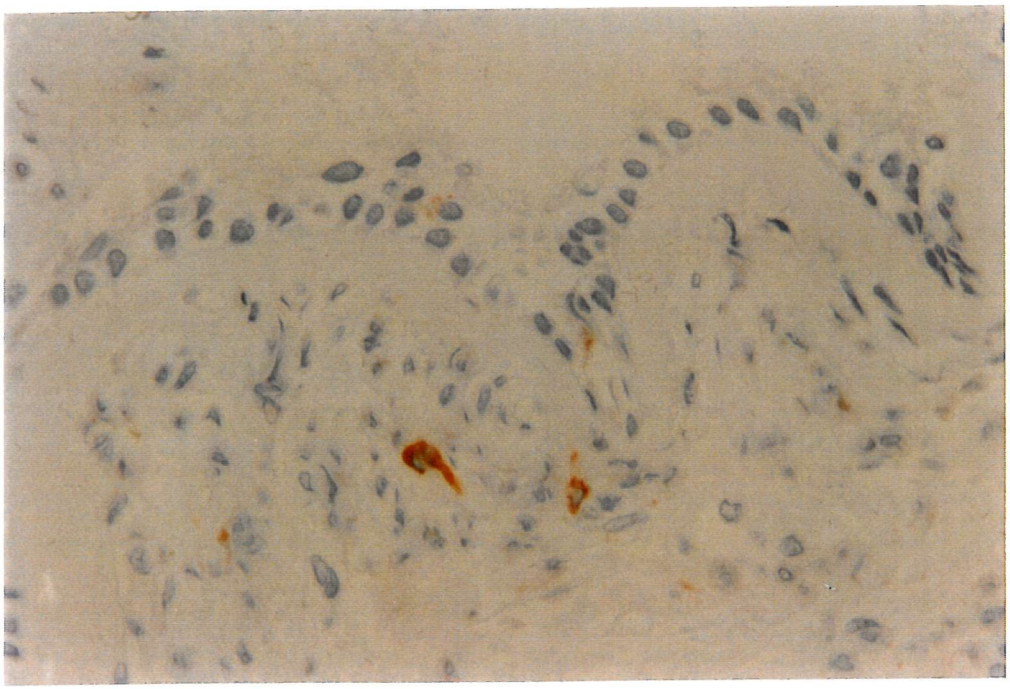


Fig. 2.6.4 Photomicrographs (x400) of immunostaining for MRP-1 and MRP-2 in representative GMA embedded bronchial biopsies. Positive AEC immunostaining appears red against blue background of Meyers haematoxylin counterstain. ***Panel A***. Immunostaining using the monoclonal antibody MRPm6 against MRP-1. ***Panel B***. Immunostaining using the monoclonal antibody M_2 1-4 against MRP-2 (cMOAT).

A



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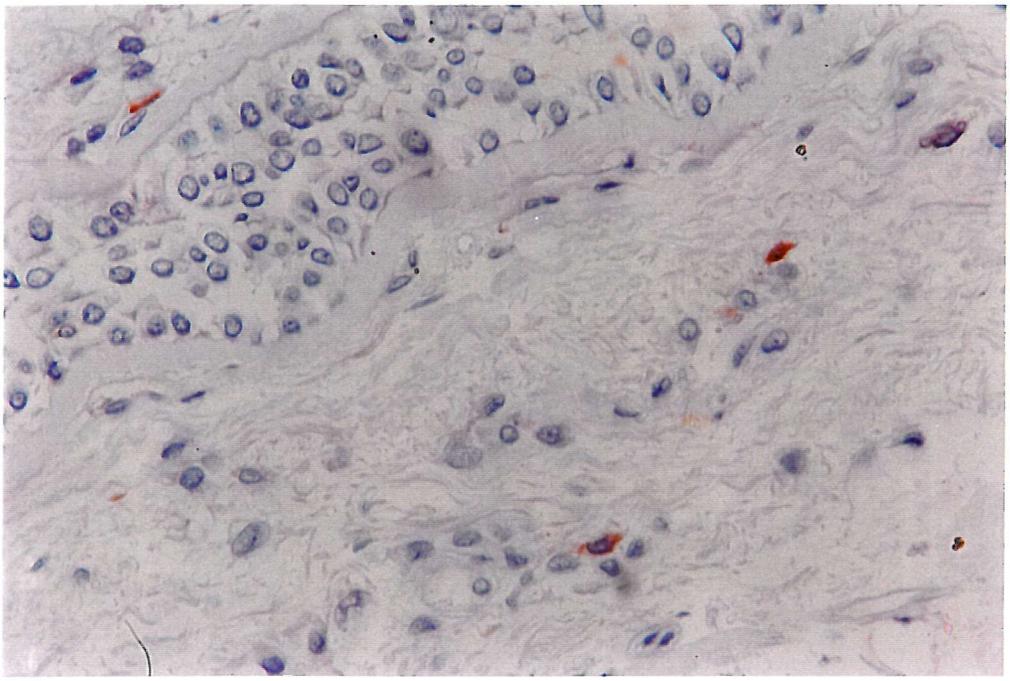
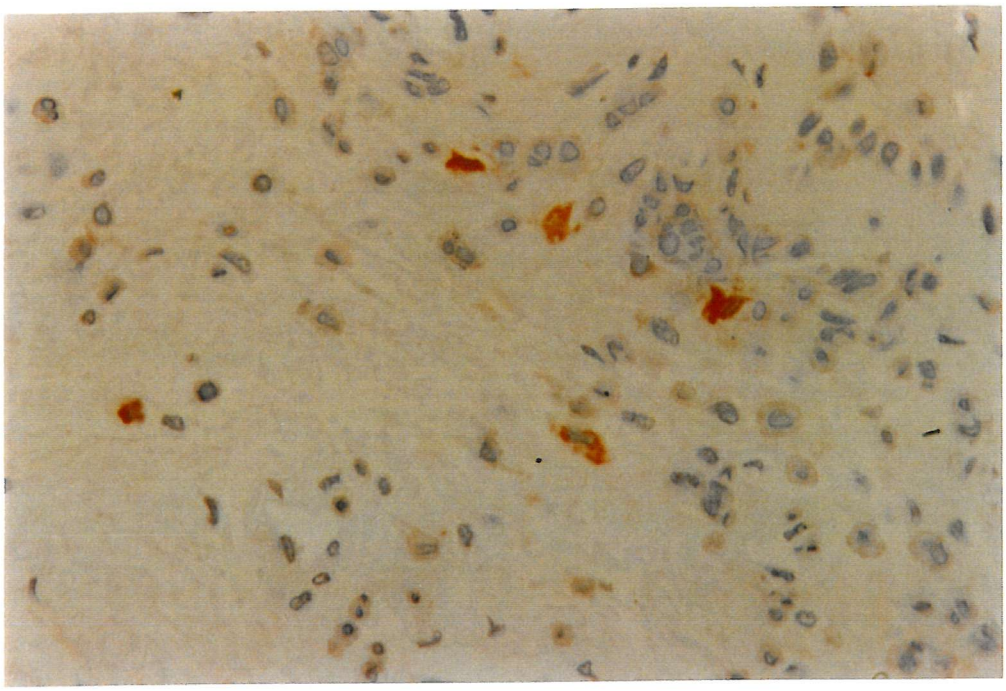


Fig. 2.6.5 Photomicrographs (x400) of immunostaining for COX isoenzymes in representative GMA embedded bronchial biopsies. Positive AEC immunostaining appears red against blue background of Meyers haematoxylin counterstain. ***Panel A***. Immunostaining using the monoclonal antibody against cyclooxygenase-1 (COX-1/PGHS-1). ***Panel B***. Immunostaining monoclonal antibody against cyclooxygenase-2 (COX-2/PGHS-2).

A



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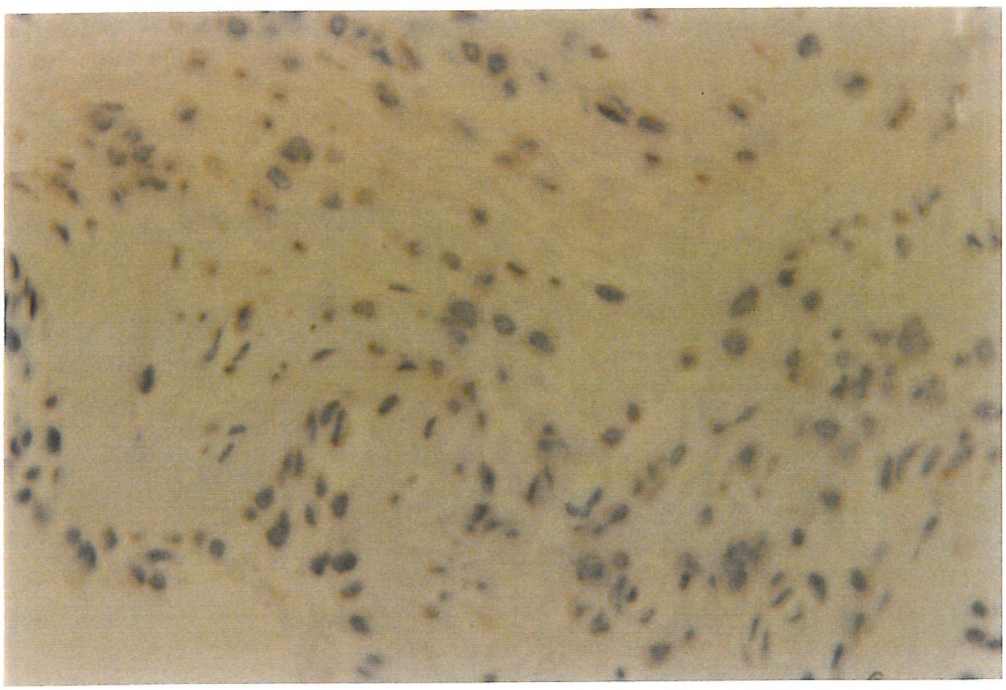
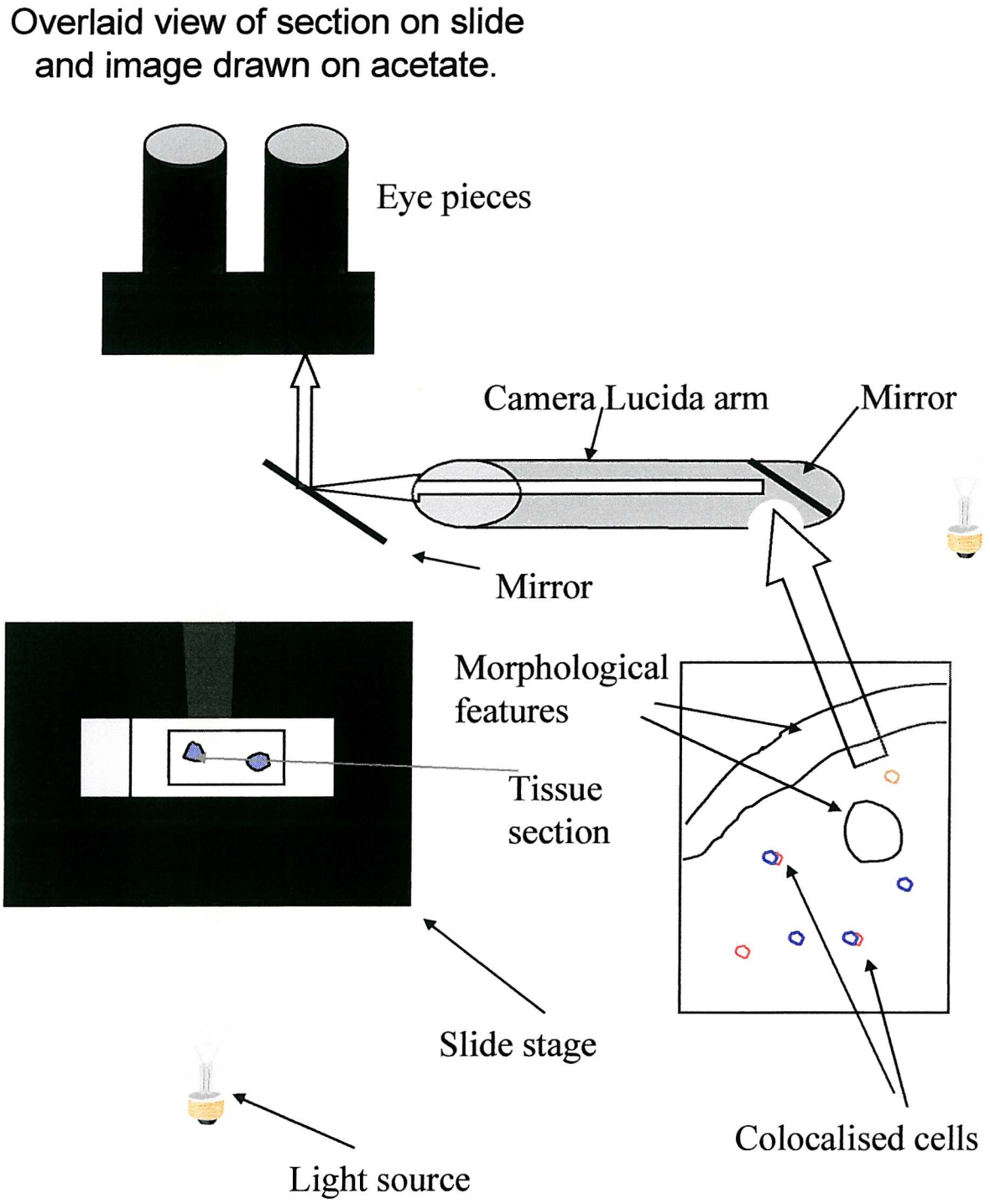


Fig. 2.6.6 Photomicrographs (x400) of immunostaining for PGD synthase in representative GMA embedded bronchial biopsies. Positive AEC immunostaining appears red against blue background of Meyers haematoxylin counterstain. ***Panel A***. Immunostaining using the polyclonal antibody against PGD synthase. ***Panel B***. Control immunostaining omitting the primary antibody, this is a control for the polyclonal staining procedure to confirm that there is no non-specific staining produced by secondary or tertiary antibodies.

Fig. 2.7 Principle of camera lucida technique



2.5.4 Colocalisation of eicosanoid pathway enzymes to cell markers.

To localise the expression of 5-LO and COX pathway enzymes to inflammatory cell types, adjacent sections were immunostained in parallel and optically superimposed using the camera lucida system (Leica UK Ltd., Milton Keynes, UK) as described (Bradding et al. 1992) (**Fig. 2.7**). Colocalisation allows the distribution of enzyme immunoreactivity in different cell types to be quantified e.g. that x% of COX-1$^+$ cells are mast cells, y% are eosinophils, etc. In addition, the proportion of a particular cell-type showing detectable immunoreactivity for a particular enzyme(s) can be calculated e.g. x% of eosinophils are 5-LO$^+$ but only y% are LTC_4 synthase$^+$. These analyses allow the relative contributions of cellular migration and intracellular induction of enzyme expression in resident cell populations to be dissected, and the likely cell sources of eicosanoids in the BAL fluid to be identified.

2.6 Intracellular staining of leukocytes for flow cytometry.

The location of eicosanoid pathway enzymes within the cell requires permeabilisation of the cell for access of the antibody to the antigen as previously described by Kraft et al. (1998). Cells were fixed in 4% paraformaldehyde washed in PBS/2% FBS and incubated in 0.1M glycine for 10 min on ice to prevent the non-specific binding of antibodies to paraformaldehyde cross links. Cells were then permeabilised using 0.5% Saponin (Sigma) in PBS containing 0.5% BSA for 30 min on ice, and this buffer was used for washing steps throughout the staining procedure as permeabilisation is reversible. For purified eosinophils 100,000 permeabilised cells were resuspended in 100µl saponin buffer with the addition of 10% human AB serum (HS) (Sigma) for blocking. For mixed leukocytes >1 x 10^6 cells were stained in 100µl saponin buffer/ 10% HS. Morphological regions for individual cell types were determined using specific cell markers, side scatter/forward scatter profile and natural autofluoresence. Morphological gating was not possible to distinguish between T and B lymphocyte cells and therefore were considered as one population for the purpose of this study. Primary antibodies or isotype-matched controls were added at appropriate dilutions as determined by titration (see section 2.6.1) and incubated on ice for 30 min before washing 3 times in 1ml cold saponin buffer. Cells were resuspended in 100µl saponin buffer / 10% HS and incubated with appropriate fluorescent labelled secondary antibodies for 30 min on ice. Cells were washed 3 times prior to resuspension in 250µl saponin buffer for flow cytometry using a

Hewlett Packard FACScan. Flow cytometry data was analysed using WinMDI 2.8 software package (Joseph Trotter) and expressed as % positive cells above control and geometric mean fluorescence intensity above control.

2.6.1 Titration of antibodies for flow cytometry.

Cells were fixed and permeabilised as previously described and primary antibodies at dilutions of 10:100, 5:100, 2.5:100, 1:100, 0.5:100 and 0.25:100 and equivalent concentrations (mg/ml) of isotype controls added for 30 min incubation on ice. Monoclonal antibodies were identified as IgG1, IgG2A etc. and the appropriate mouse IgG was applied, the polyclonal LTC_4 synthase antibody was affinity purified rabbit serum and hence rabbit immunoglobulins were used as isotype control. 5-LO, FLAP, LTA_4 hydrolase and PGD_2 synthase are diluted rabbit serum; a coomassie protein assay was performed to determine the relative albumin concentration of each antibody compared to normal rabbit serum, and rabbit serum was then used at the relevant dilution (approx. 1:10) as an isotype control. Fluorescent labelled secondary antibodies were applied at fixed dilutions for 30 min for flow cytometry. Secondary antibodies also required titration using a fixed concentration of primary antibody and applying varying concentrations of secondary antibody. Optimal concentrations were determined by plotting antibody concentration against median fluorescence intensity. The concentration at which these curves reached a plateau was the concentration selected for flow cytometry (**Fig 2.8**).

Table 2.3 Dilutions of antibodies against eicosanoid pathway enzymes used for staining mixed leukocytes and purified eosinophils for flow cytometry.

Antibody	Typical dilution	Stock concentration	Isotype control
5-LO	2.5:100	~1:10 rabbit serum	1:25 rabbit serum
FLAP	2.5:100	~1:10 rabbit serum	1:25 rabbit serum
LTA_4 hydrolase	2.5:100	~1:10 rabbit serum	1:25 rabbit serum
LTC_4 synthase	5:100	147μg/ml	Rabbit immunoglobulin
MRP-1	10:100	500μg/ml IgG1	Mouse IgG1
MRP-2	10:100	500μg/ml IgG1	Mouse IgG1
COX-1	1:100	2500 μg/ml IgG1	Mouse Ig
COX-2	1:100	250 μg/ml IgG1	Mouse IgG1
PGD_2 synthase	2.5:100	~1:10 rabbit serum	1:25 rabbit serum

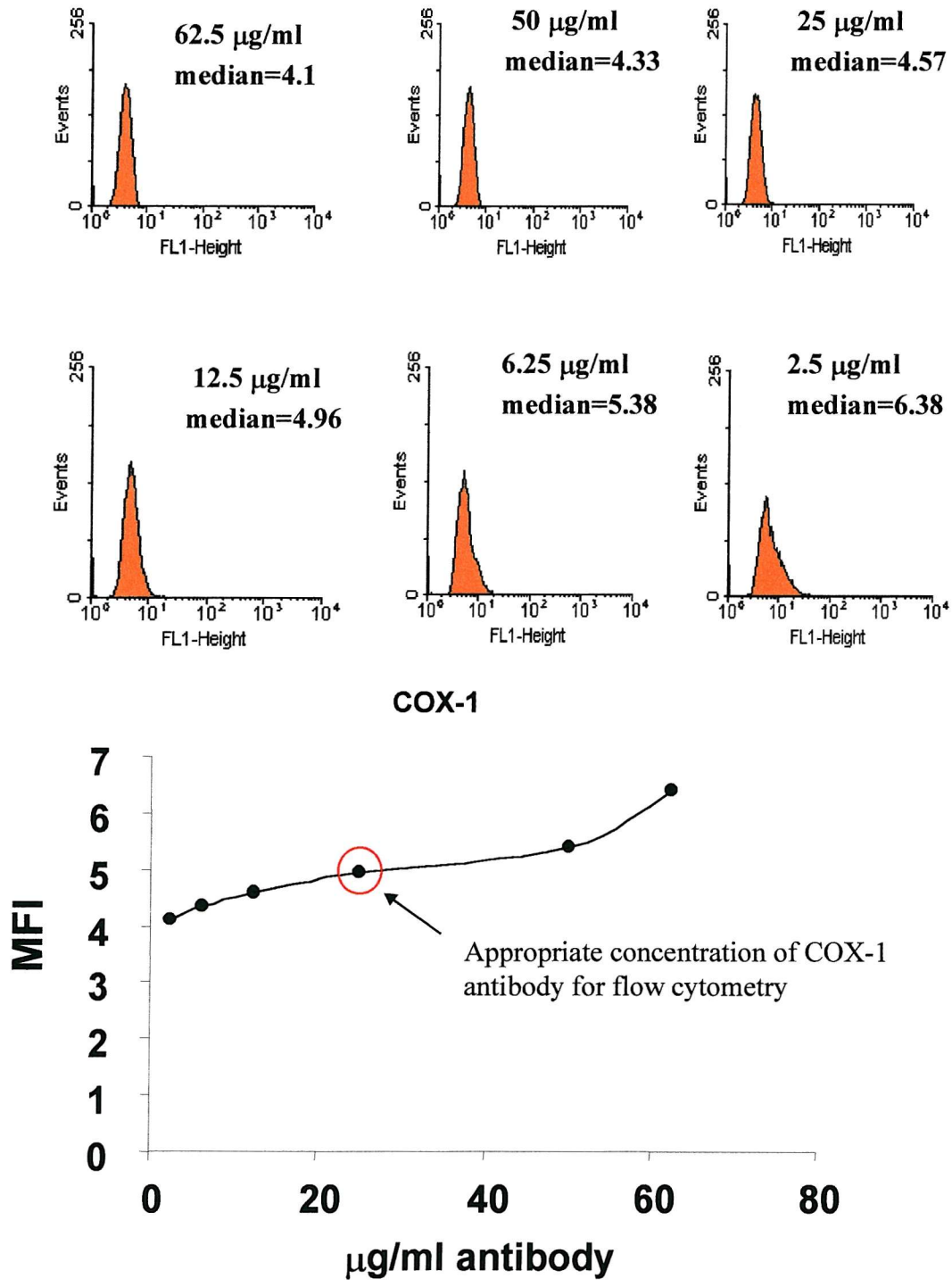


Fig 2.7 Titration of COX-1 monoclonal antibody between 2.5 and 62.5 µg/ml. The MFI reaches a plateau at 25 µg/ml. This is the appropriate concentration of COX-1 antibody to use for flow cytometry. At concentrations above 50 µg/ml the antibody is binding non-specifically.

Table 2.4 Dilutions of antibodies cell markers used for staining mixed leukocytes and purified eosinophils for flow cytometry.

EG2	1:100	100μg/ml IgG1	Mouse IgG1
NE	5:100	65μg/ml IgG1	Mouse IgG1
CD14	1:10	35 mg/ml IgG2A	Mouse IgG2A
CD3	2.5:100	300 mg/ml IgG1	Mouse IgG1
CD20	2.5:100	174 μg/ml IgG2A	Mouse IgG2A

2.6.1.1 *Coomassie Protein assay.*

In a 96 well microtitre plate a standard curve was constructed from bovine serum albumin in PBS in doubling dilutions from 62.5 mg/ml to 1.95 mg/ml in 10μl duplicates including plate blank. Ten fold dilutions of rabbit serum, or polyclonal antibodies against 5-LO, FLAP, LTA_4 hydrolase, or PGD synthase were also added to the plate in 10μl duplicate in dilutions ranging from 1:1000 to $1:1x10^6$. 90μl coomassie blue was added to each well and incubated for 10 min at room temperature. The blue colour that developed was read at 595nm. The relative dilutions of antibody compared to rabbit serum was calculated.

2.7 Immunocytochemistry of mast cell cytospins.

Cytocentrifuge preparations of human lung mast cells were fixed in anhydrous acetone for 15 min. prior to storage no more than 2 weeks at -20°C. Endogenous peroxidases were irreversibly blocked (as described in section 2.5.2) using 0.3% hydrogen peroxide containing 0.1% sodium azide for 30min. Slides were washed in TBS (0.005M Tris and 0.15M NaCl). The subsequent immunostaining procedure was as described in section 2.5.2, differing only in the use of an AEC chromagen kit (Biogentix, CA, USA) which was applied following the manufacturer's instructions. The kit was preferred for reasons of safety and for the production of cleaner immunostaining. The time taken for the development of a red reaction product by AEC at room temperature was 5 min. Cells were examined under x400 or x1000 light microscopy and a minimum of 400 cells was counted. Results are expressed as the percentage of positive cells. The purity of the cell preparation was confirmed by staining with AA1 (Pharmacia), a marker for mast cell tryptase (Walls et al. 1990). Antibodies against CD68 and CD3 were used to determine proportion of major contaminating cells (macrophages and T-lymphocytes respectively). To confirm the absence of non-specific staining, cytospins were also stained using the

eosinophil marker EG2 as an irrelevant antibody, and with the primary antibody omitted. When titrated on mast cell preparations optimal primary antibody concentrations were lower than when staining GMA sections, probably due to better access to the antigens in isolated cells than in GMA resin-embedded tissue.

Table 2.5 Dilutions of antibodies against eicosanoid pathway enzymes used for staining mast cell cytospin preparations.

Antibody	Typical dilution
5-LO	1:2000
FLAP	1:800
LTC_4 synthase	1:50
COX-1	1:2000
COX-2	1:2000
PGD_2 synthase	1:1000

2.8 Measurement of eicosanoids in BAL fluid.

BAL fluid was immediately frozen at -20°C for later analysis. One ml of the second (30ml) wash of BAL fluid from each patient was deproteinised with 2ml of ice-cold methanol for 30 min and centrifuged at 1000g to pellet cell debris and precipitated protein. Eicosanoids in the supernatants were concentrated five-fold by evaporation of 1ml BAL fluid to dryness *in vacuo* and resuspension in 0.2ml of phosphate-buffered saline containing 0.1% fatty acid-free bovine serum albumin (BSA).

2.9 Enzyme Immunoassay of Leukotrienes $C_4/D_4/E_4$.

Aliquots (50 µl) were assayed in duplicate for total cys-LT levels using the Biotrak leukotriene $C_4/D_4/E_4$ enzyme immunoassay (EIA) kit following the manufacturer's instructions (Amersham International Ltd., Little Chalfont, UK). The polyclonal antiserum cross-reacts with LTC_4 (100%), LTD_4 (100%), LTE_4 (70%) and their 11-trans isomers, but negligibly with LTB_4 (0.3%), prostaglandin $F_{2\alpha}$, prostaglandin D_2, prostaglandin E_2, 6-keto-prostaglandin $F_{1\alpha}$, thromboxane B_2 and glutathione (<0.006%). The standard curve of the EIA equates to original concentrations of cys-LTs in BAL fluid between 3 pg/ml and 200 pg/ml.

2.10 Enzyme Immunoassay of Prostaglandin D_2.

BAL fluid aliquots (50 µl) were assayed for PGD_2 by competitive EIA according to the

manufacturer's instructions (Cayman Chemical), after conversion of PGD_2 to its methoxime derivative (PGD_2-MOX) with methoxamine hydrochloride. The polyclonal antiserum recognises PGD_2-MOX (100%), but not its parent compound PGD_2 (0.2%), PGE_2, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, or thromboxane B_2, or their methoxime derivatives. The EIA standard curve equates to original concentrations of PGD_2 in BAL fluid between 5 pg/ml and 1000 pg/ml.

2.11 Statistical analysis.

Positive cell-counts, BAL fluid cys-LT concentrations, and lung function data (FEV_1 etc.) are expressed as mean ± SEM. Methacholine PC_{20} FEV_1 values are given as median and 95% confidence interval. Comparisons between pre-season and in-season group values (Chapter 3), baseline and infection group values (Chapter 4) and filtered air and ozone (Chapter 5) were performed by the Wilcoxon signed rank test for paired non-parametric data. Correlations between data sets were performed using Pearsons test of correlation, a correlation coefficient of >0.6 with $p<0.05$ was considered significant. Comparisons between unrelated subject groups i.e. normal and asthmatic group values were performed by the Mann Whitney test for unpaired non-parametric data. Enzyme expression in eosinophils incubated with cytokine was compared to untreated samples over time using two-way ANOVA. On achieving a significant ANOVA, each time point was tested individually by paired Students T-test. Paired T-test was also used to compare the effect of increasing doses of cytokine on enzyme expression in eosinophils. Protein expression in mixed leukocytes from normal and asthmatic subjects was compared by the Mann Whitney U-test for unpaired non-parametric data, and by Two-sample T-test. All statistical tests were performed using Minitab Statistical software (Minitab Inc., State Co., PA, USA). In all cases $P\leq0.05$ was considered significant.

CHAPTER 3

Effect of seasonal allergen exposure on leukotriene pathway enzyme expression in inflammatory cells

Hypothesis: *Allergic asthmatics experience increased clinical symptoms of asthma due to eosinophil influx, and an increased expression of leukotriene and/or prostanoid pathway enzymes in inflammatory cells producing more cys-LTs and/or PGD_2 with seasonal allergen exposure.*

RESULTS.

3.1 Pollen Counts

Airborne birch pollen counts in Gothenburg, Sweden were monitored daily during 1997 with a Burkard volumetric trap. (**Figure 3.1**). The birch pollen season occurred between April 24th and June 9th reaching its peak on May 16th with pollen counts of 354 grains/m^2/day. The birch pollen season was preceded by the alder and hazel seasons that produced much lower pollen counts in comparison to birch. Alder pollen grains were detected between March 1st and April 11th with a peak of 30 grains/m^2/day on 20th March, and hazel detected at very low levels between March 19th and March 30th with a peak pollen count of 4 grains/m^2/day on 29th March. The grass pollen season began on the 30th May but did not reach its peak of 42 grains/m^2/day until17th June.

Patients were recruited for the study between 24th February and 20th March for pre-season samples and between 20th May and 18th June for in-season samples. Pre-season samples were, therefore, taken before the peak of the alder pollen season when pollen counts were below 20 grains/m^2/day. The in-season samples were taken shortly after the peak of the birch pollen season and at the beginning of the grass pollen season ensuring that patients had been exposed to maximal levels of the pollens to which they were sensitive.

3.2 Effect of seasonal allergen exposure on clinical measures of asthma severity.

Compared with pre-season measurements, patients experienced a significant deterioration in lung function during the pollen season indicated by falls in morning and evening peak flow. Mean morning PEF values of 590 ± 30 l/min (pre-season) fell to 569 ± 27 l/min in-season (P=0.023), and mean evening PEF of 600 ± 29 l/min (pre-season) fell to 572 ± 27 l/min in-season (p=0.014). (**Figure 3.2B**). This was accompanied by increasing bronchial responsiveness to methacholine, with median (95% confidence interval) methacholine $PC_{20}FEV_1$ values falling from 14.0 mg/ml (9.0-23.8) pre-season to 2.7 mg/ml (1.4-6.5) in-season (P=0.001, n=12, Wilcoxon) (**Figure 3.2A**). Symptom scores as assessed by the patient showed a worsening in both asthma and rhinitis symptoms. Asthma symptom scores rose significantly from 0.18 ± 0.09 pre-season to 0.68 ± 0.10 in-season (p=0.004) indicating the induction of mild asthmatic symptoms

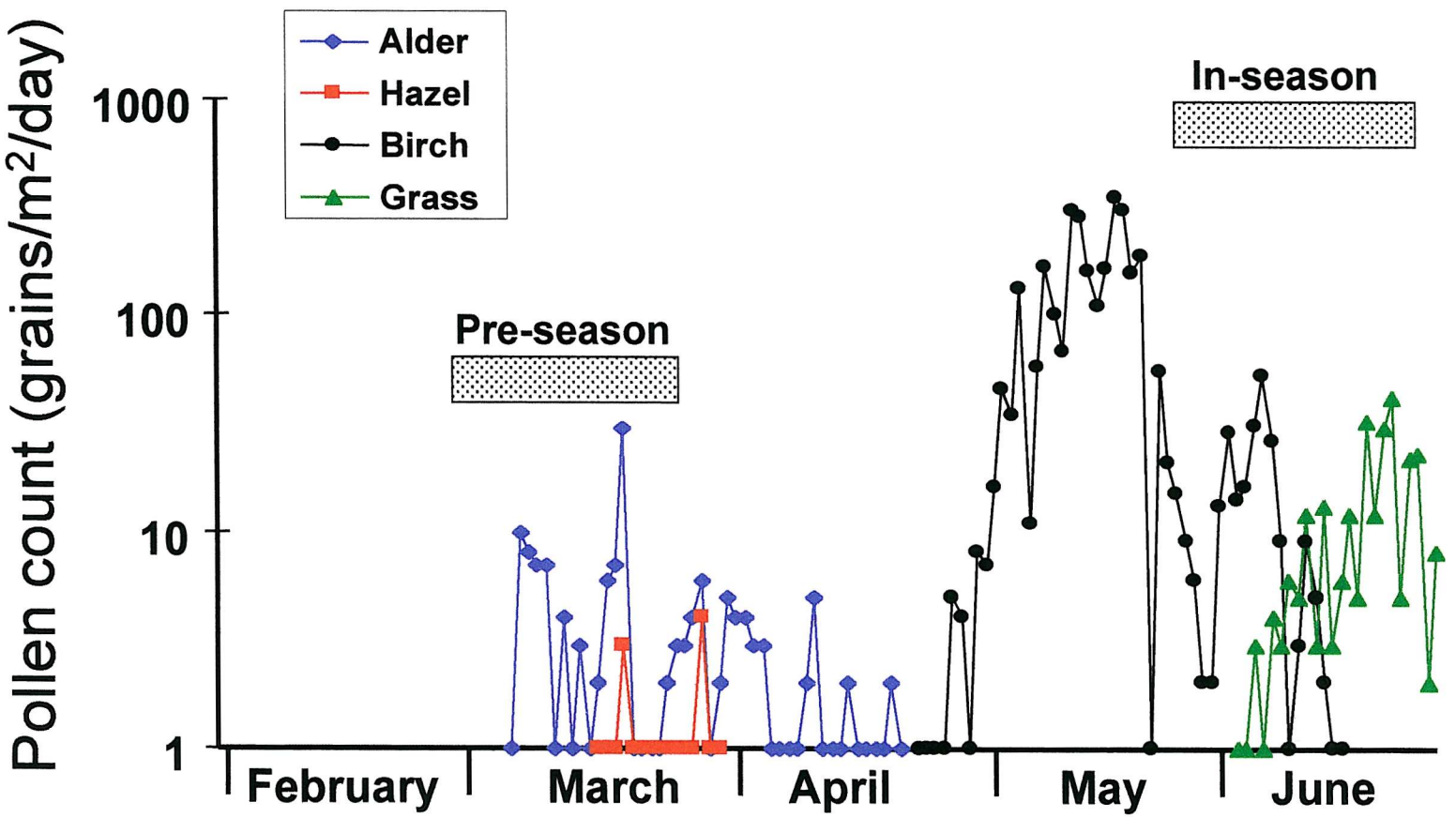


Fig 3.1. Airborne counts (grains per m^2 per day) of alder, hazel, birch and grass pollens between February and June 1997 in Gothenburg, Sweden. Bronchoscopies were carried out between 24th February and 20th March (pre-season) and between 20th May and 18th June (in-season).

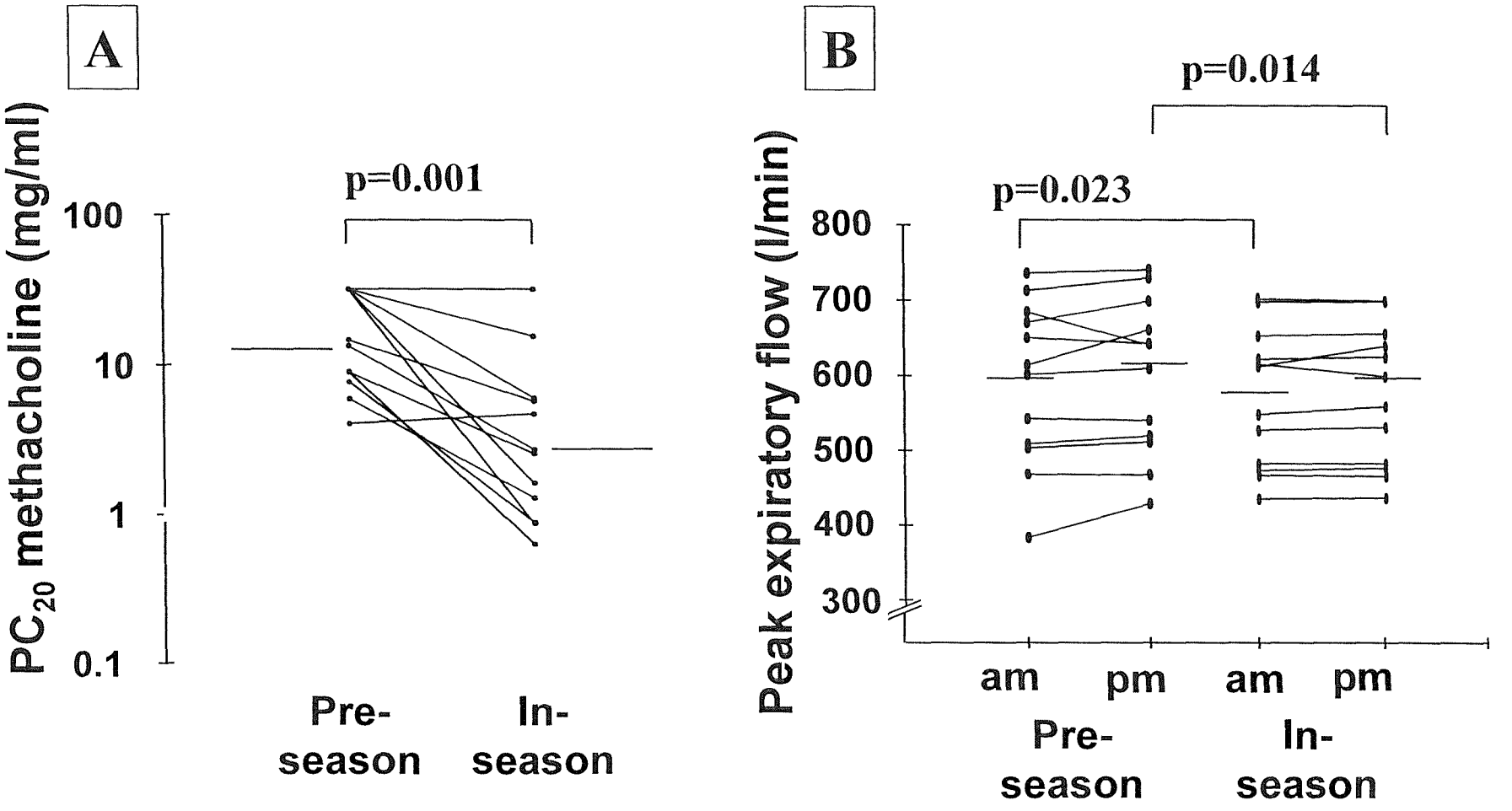


Fig. 3.2 Clinical measures of asthma severity before and during the birch pollen season. **Panel A:** The concentration of methacholine required to reduce FEV_1 by 20% (PC_{20}) is lower in birch pollen-allergic asthmatics in-season than pre-season (p=0.001). **Panel B:** Morning (am) and evening (pm) peak expiratory flow (l/min) in birch pollen-allergic asthmatics is decreased in-season (p=0.023, p=0.014 respectively).

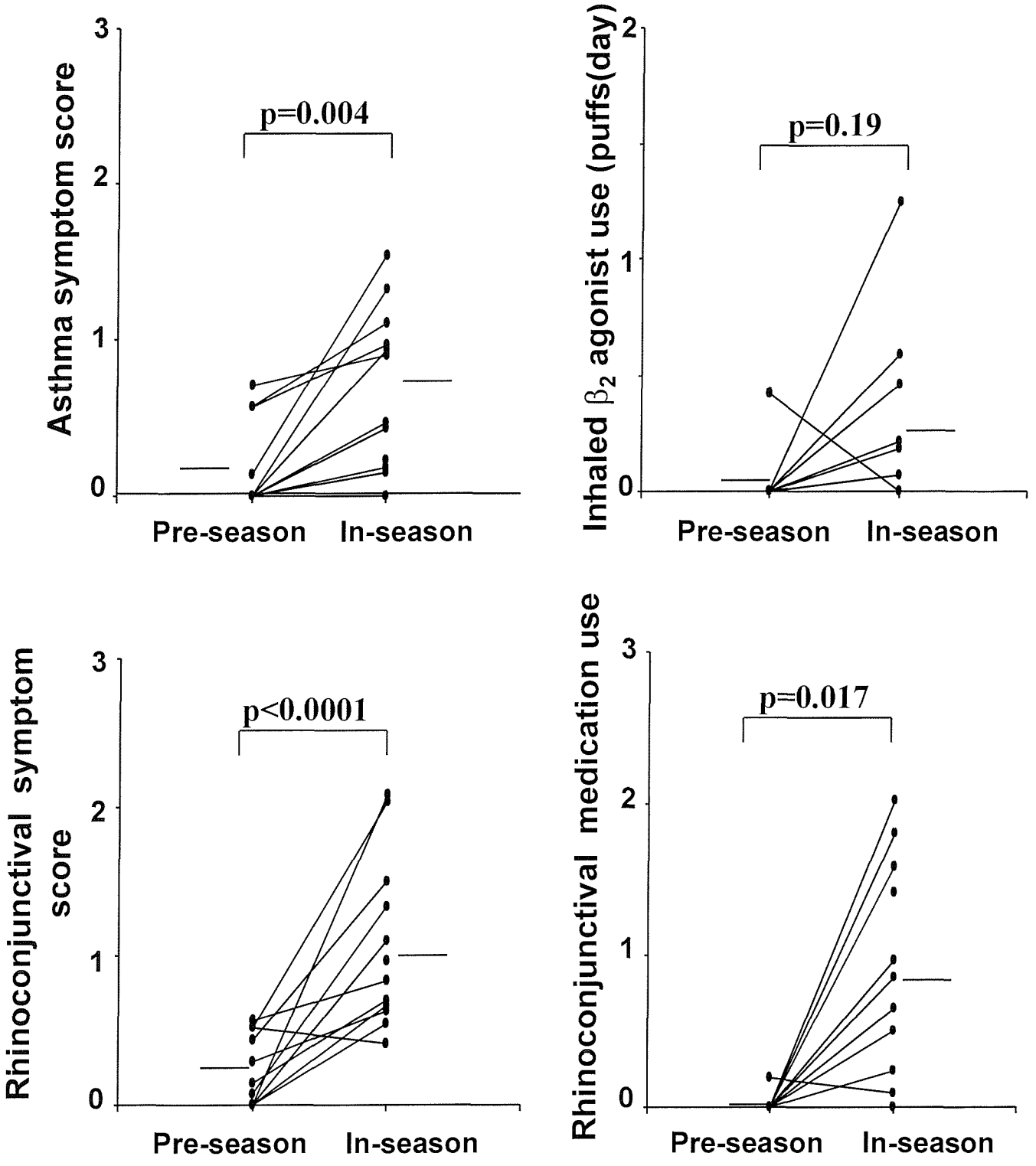


Fig 3.3 Asthma and rhinoconjunctival symptom scores, and use of related medication in 12 allergic asthmatics before and during the birch pollen season. Symptom scores are 0-3 with 3 being most severe. Asthma symptom scores (***top left***) and rhinoconjunctival symptom scores (***bottom left***) increase significantly with seasonal allergen exposure (p=0.004, p<0.0001 respectively). Inhaled β_2 agonist use (***top right***) is measured as puffs of inhaled β_2 agonist per day and shows a non-significant increase with allergen exposure (p=0.19). Rhinoconjunctival medication use (antihistamines) (***bottom right***) increased significantly with allergen exposure. Horizontal lines show mean values.

with seasonal allergen (**Figure 3.3**). This was accompanied by a trend to increased use of inhaled β_2 agonists, rising from 0.04 ± 0.04 puffs/day pre-season to 0.23 ± 0.07 puffs/week in-season (P=0.19) (**Figure 3.3**). Seasonal allergen exposure also significantly increased rhinoconjunctival symptom scores from 0.23 ± 0.07 pre-season to 1.07 ± 0.10 in-season (P<0.0001) (**Figure 3.3**), accompanied by a significant rise in the use of oral and topical anti-histamines from 0.02 ± 0.02 pre-season to 0.84 ± 0.14 in-season (P=0.017) (**Figure 3.3**).

3.3 Immunohistochemical analysis of leukocyte markers in bronchial biopsies.

Biopsies were immunostained using monoclonal antibodies against leukocyte markers and counts of cells per mm^2/mucosa were determined by light microscopy. Statistical comparisons were made between cell counts from pre-season biopsies and from matching biopsies taken during the birch pollen season for 10-12 subjects.

Mean counts of eosinophils identified using monoclonal antibody EG2 to eosinophil cationic protein (ECP) (Tai et al.1984; Jahnsen et al.1994) more than doubled from 15.5 ± 3.3 cells/mm^2 pre-season to 35.0 ± 10.4 cells/mm^2 in-season (p=0.035 Wilcoxon) (**Figure 3.4**). Macrophages were identified using the monoclonal antibody PG-M1 directed against the macrophage-specific form of CD68 (Falini et al.1993). Mean $CD68^+$ macrophage counts rose significantly from 8.3 ± 1.8 cells/mm^2 pre-season to 14.9 ± 2.6 cells/mm^2 in-season (p=0.019) (**Figure 3.5**). Staining was strong and appeared cytoplasmic. There was a weak trend towards an increase in the $CD14^+$ (lipopolysaccharide $receptor^+$) monocyte population from 5.7 ± 2.6 cells/mm^2 pre-season to 11.3 ± 2.8 cells/mm^2 in-season, but this did not reach statistical significance (p=0.17) (**Figure 3.5**). Pre-season counts of $tryptase^+$ mast cell counts as identified by the monoclonal antibody AA1 were 44.3 ± 3.8 cells/mm^2 mucosa and remained stable at 46.5 ± 4.8 cells/mm^2 mucosa with seasonal allergen exposure (**Figure 3.4**). Neutrophils were identified using the monoclonal antibody NP57 against neutrophil elastase (NE) (**Fig 2.4.8**), there was a strong trend towards a doubling of counts of neutrophils with seasonal allergen exposure (41.6 ± 11.9 cells/mm^2 pre-season; 79.1 ± 28.2 cells/mm^2 in-season, p=0.083, n=10) (**Fig 3.6**). Two antibodies were used for the identification of basophils, 2D7 and BB1. The antibody 2D7 immunostained 1.9 ± 0.4 cells/mm^2 pre-season, and this more than doubled with seasonal allergen exposure to 4.8 ± 0.9 cells/mm^2 (p=0.019, n=10) (**Fig 3.7**) staining with 2D7 appeared to be located in the granules (**Fig 2.5.7**). However, using the newer, less well-characterised BB1 antibody, fewer cells were immunostained. Pre-season 0.3 ± 0.1 cells/mm^2 were $BB1^+$, this rose to 1.3 ± 0.5 in-season but was not statistically significant (p=0.359, n=10) (**Fig 3.7**) staining

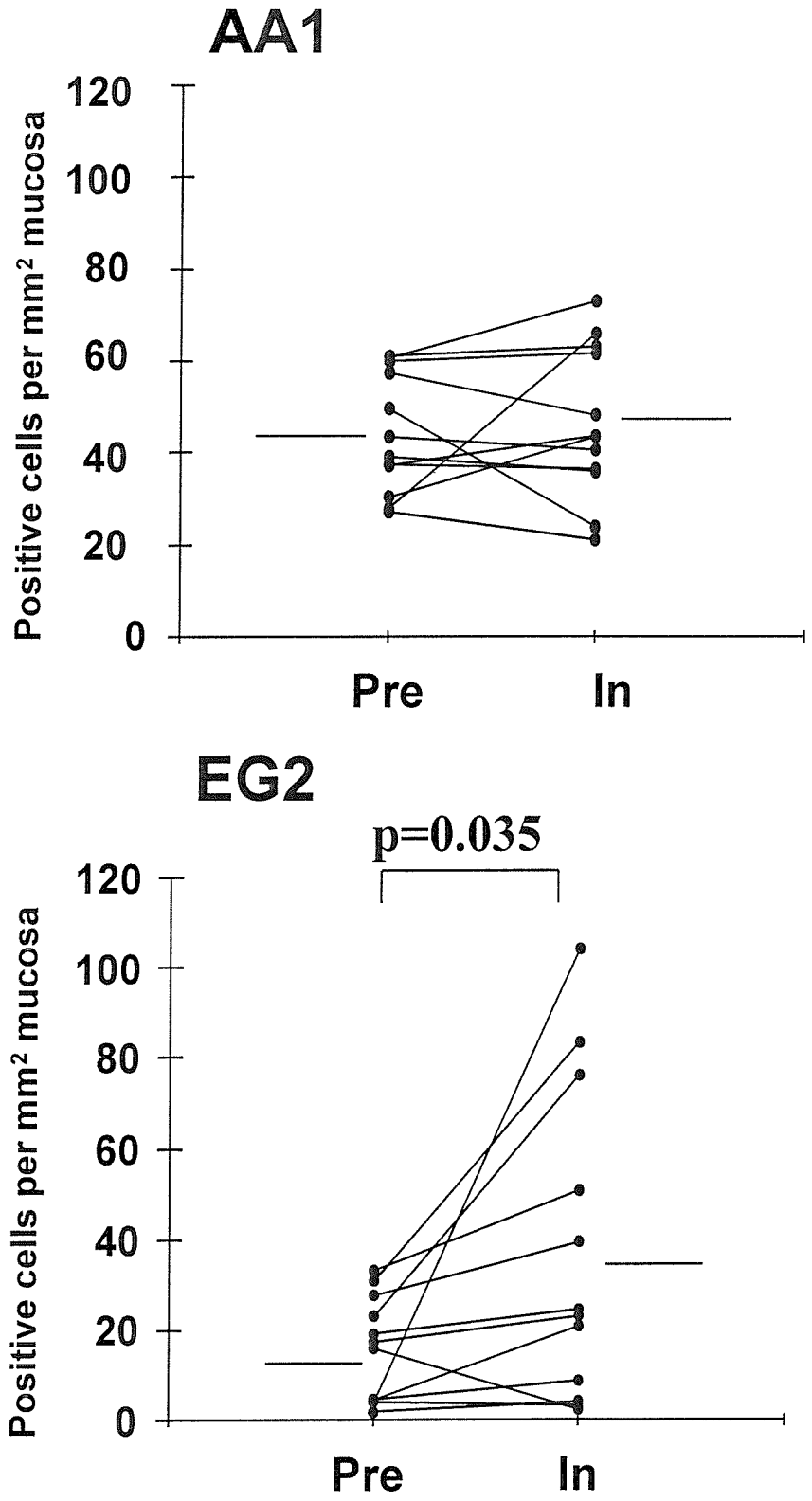


Fig. 3.4 Counts of mast cells and eosinophils per mm^2 bronchial mucosa from 12 allergic asthmatics before (pre) and during (in) seasonal allergen exposure. Counts of tryptase positive (AA1) mast cells (***top***) are unchanged with seasonal allergen exposure ($p>0.5$). Counts of $EG2^+$ eosinophils (***bottom***) are significantly increased in-season ($p=0.035$). Horizontal lines represent mean values.

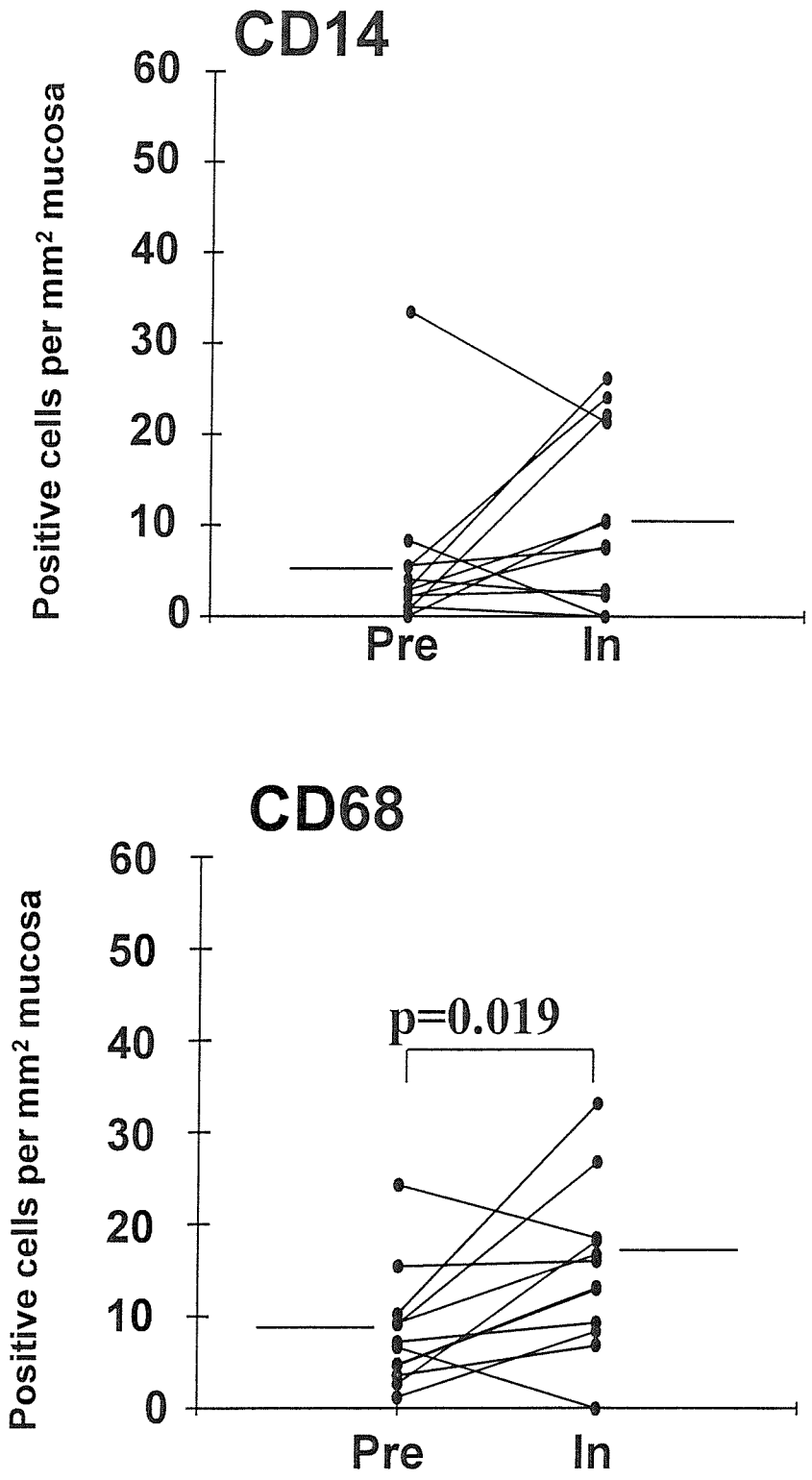


Fig. 3.5 Counts of positively stained cells of monocyte lineage per mm^2 bronchial mucosa from 12 allergic asthmatics before (pre) and during (in) seasonal allergen exposure. Counts of $CD14^+$ monocyte/macrophages (***top***) are increased with allergen exposure but the increase does not reach significance (p=0.17). Counts of CD68+ macrophages (***bottom***) were significantly increased in-season (p=0.019). Horizontal lines represent mean values.

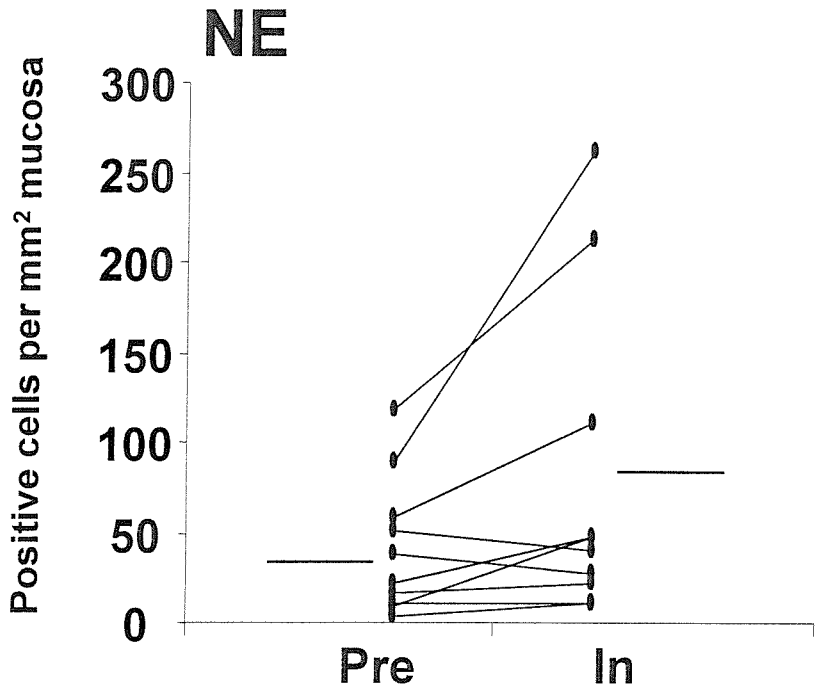


Fig. 3.6 Counts of positively stained ($NP57^+$) neutrophils per mm^2 bronchial mucosa from 10 allergic asthmatics before (pre) and during (in) seasonal allergen exposure. Counts of neutrophils are increased with allergen exposure but the increase does not reach significance ($p=0.083$). Horizontal lines represent mean values.

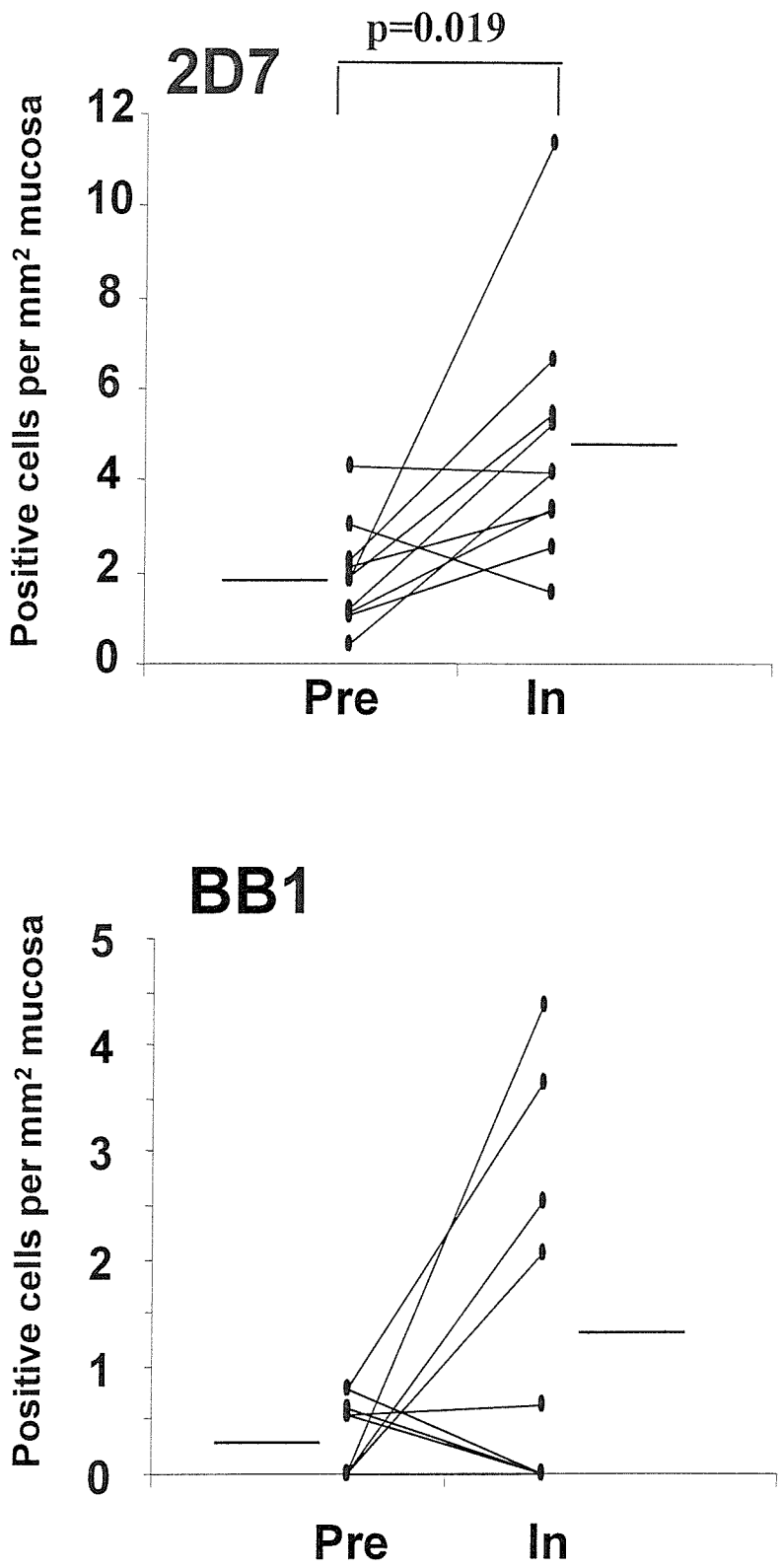


Fig. 3.7 Counts of positively stained cells of basophils per mm^2 bronchial mucosa from 10 allergic asthmatics before (pre) and during (in) seasonal allergen exposure. Counts of basophils using the 2D7 antibody (***top***) increased with allergen exposure (p=0.019). Counts of basophils using the BB1 antibody (***bottom***) did not significantly increase in-season (p=0.359). Horizontal lines represent mean values.

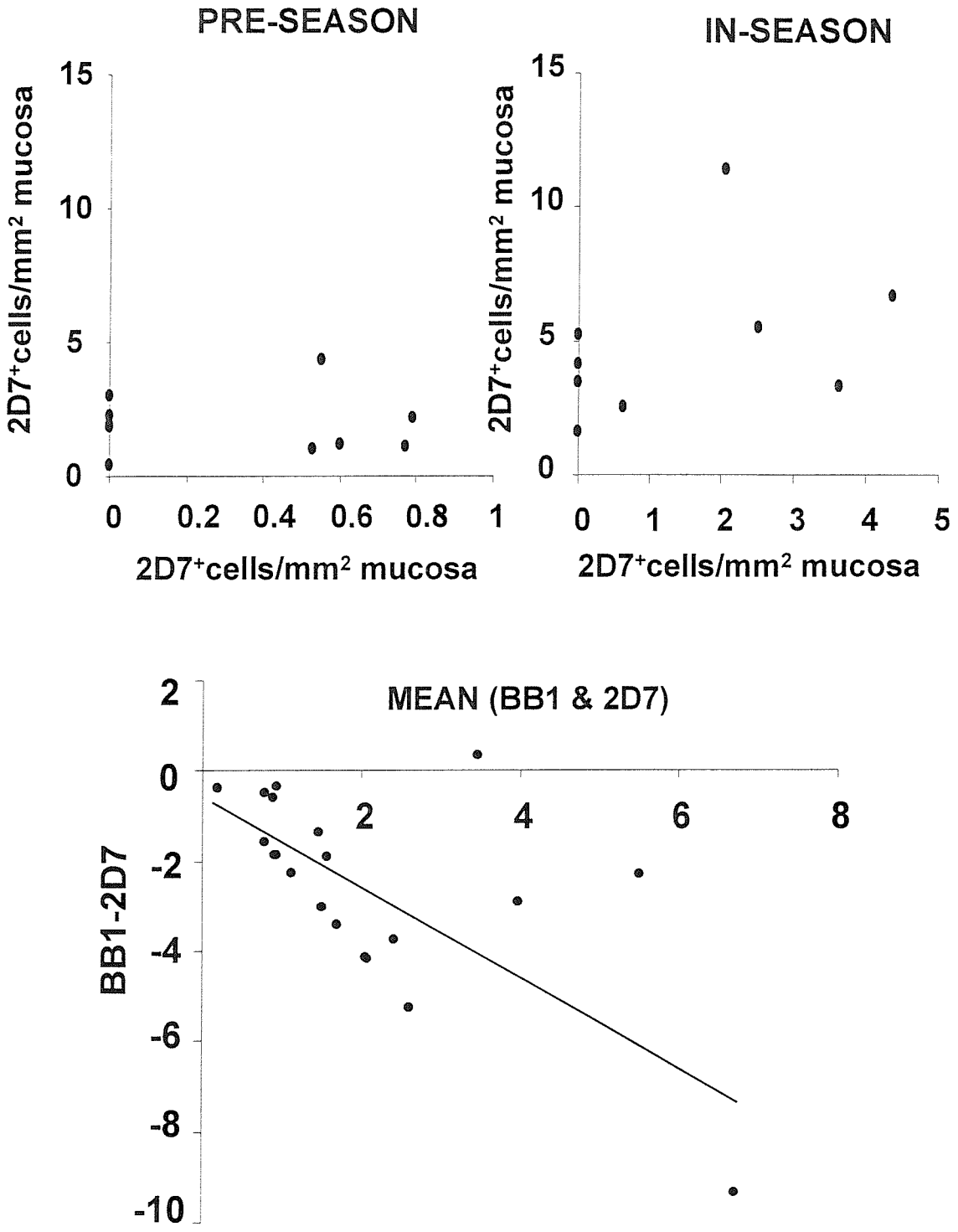


Fig. 3.8 Comparison of counts of positively stained cells of basophils per mm^2 bronchial mucosa using the 2D7 antibody (***top***) and the BB1 antibody. ***Top***: Counts of $BB1^+$ cells and $2D7^+$ cells were not significantly correlated either pre-season (r=-0.012, p=0.975) or in-season (r=0.395, p=0.258). ***Bottom***: Bland-Altman analysis of same-parameter measurement.

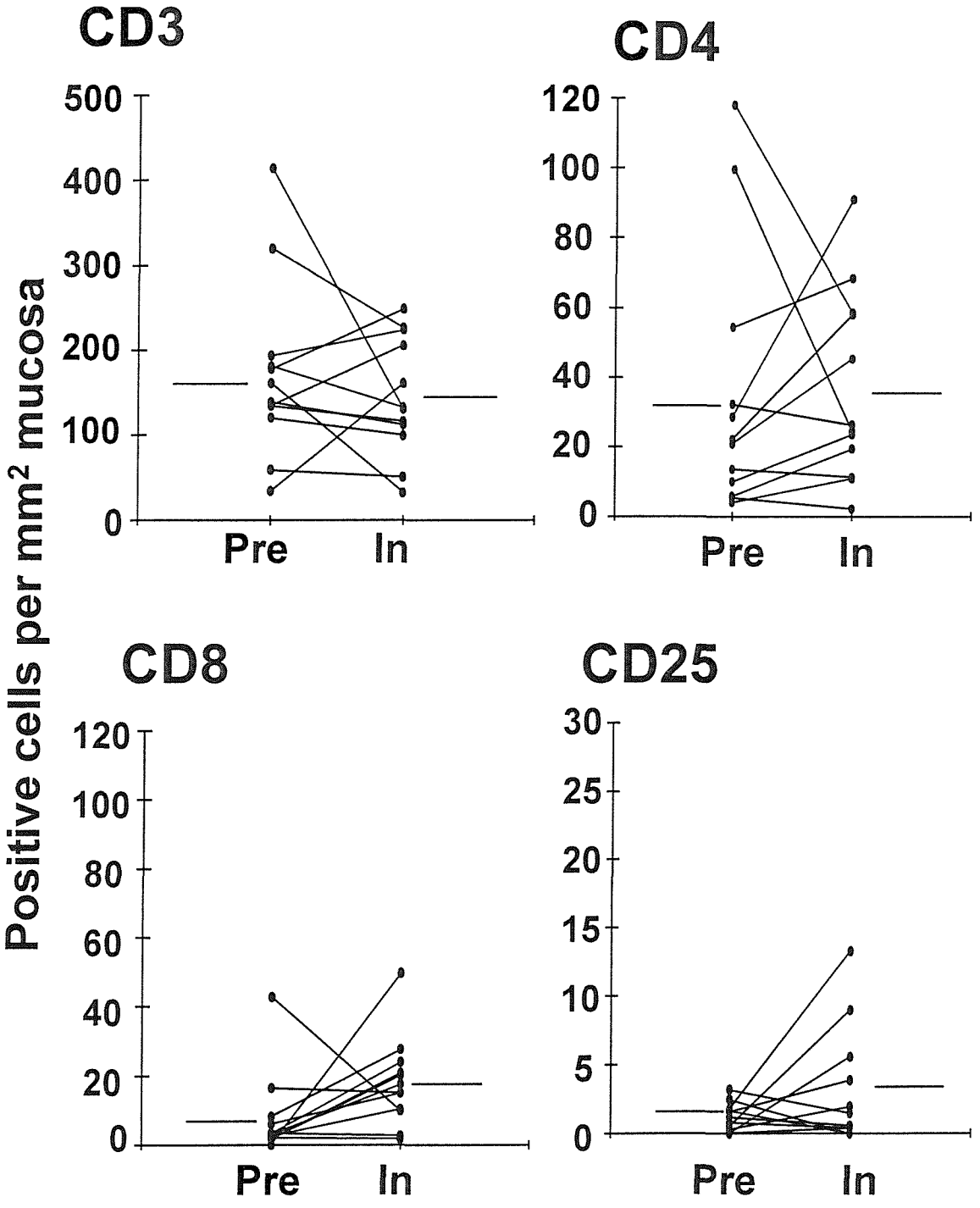


Fig. 3.9 Positive cell counts for T-lymphocyte cell markers: CD3 (pan T-cells), CD4 (T-helper cells), CD8 (cytotoxic T-cells), and CD25 (activated (IL-2 receptor positive) T-cells) per mm^2 of bronchial mucosa of allergic asthmatics before and during the birch pollen season. Counts of $CD3^+$ T-cells (***top left***) were unchanged with seasonal allergen exposure (p=0.49), as were $CD4^+$ cell counts (p=0.4) (***top right***). The trend to increased CD8+ T-cells (***bottom left***) did not reach significance (p=0.08). Counts of CD25+ cells (***bottom right***) were unchanged (p=0.4) and remained unchanged when expressed as a percentage of total ($CD3^+$) T-cells (p>0.5). Horizontal lines represent mean values.

using BB1 appeared granular (**Fig 2.5.7**). Bland-Altman analysis of cell counts for BB1 and 2D7 suggests that neither antibody is staining non-specifically because the curve crosses zero at low mean values (**Fig 3.8**). The curve has a downward slope, which suggests that BB1 is only identifying a fixed proportion of the cells that are detected by 2D7 (**Fig 3.8**). However, $BB1^+$ cell counts and $2D7^+$ cell counts did not correlate significantly with each other either pre-season (r=-0.012 p=0.975) or in-season (r=0.395, p=0.258) (**Fig 3.8**). BB1, therefore, may be a less sensitive antibody for the detection of basophils, but the fact should not be ignored that this analysis may also suggest that 2D7 is less selective than BB1 and may be detecting another cell type e.g. Eosinophils or mast cells. However, 2D7 is the better characterised of the two antibodies and therefore for the purpose of this study 2D7 will be considered to selectively detect the basophil population.

There was a trend towards an increase in the cytotoxic ($CD8^+$) T-lymphocyte subpopulation with seasonal allergen exposure (**Figure 3.9**). Cell counts rose from 7.7 ± 3.4 cells/mm^2 pre-season to 18.0 ± 3.7 cells/mm^2 during the birch pollen season (P=0.085). There were no associated changes in $CD3^+$ pan-T-lymphocytes with pre-season and in-season cell counts of 163.7 ± 32.3 cells/mm^2 and 145.2 ± 20.1 cells/mm^2 respectively, (P>0.4). There was also no change in the helper ($CD4^+$) T-lymphocyte subpopulation with pre-season cell counts of 34.6 ± 10.9 cells/mm^2 pre-season remaining stable at 36.6 ± 7.9 cells/mm^2 in-season (P>0.4) **Figure 3.9**. Numbers of the activated subset of T-lymphocytes expressing the IL-2 receptor ($CD25^+$) showed no change with allergen exposure when expressed as cell counts per mm^2 mucosa (1.1 ± 0.3 cells/mm^2 pre-season; 3.1 ± 1.2 cells/mm^2 in-season, P>0.4) **Figure 3.9,** or when expressed as a percentage of total T-lymphocytes (0.9 ± 0.4% pre-season; 2.2 ± 1.0% in-season, P>0.6).

3.3.1 Relationship of bronchial biopsy leukocyte counts to clinical measures of asthma severity.

During the birch pollen season, asthmatic patients exhibited increased bronchial responsiveness to inhaled methacholine, lower morning and evening peak expiratory flow (PEFam and PEFpm)(**Figure 3.2**) and higher asthma symptom scores, compared to pre-season values. Of these clinical measures, only PEF values showed meaningful relationships to counts of inflammatory cells in bronchial biopsies.

Pre-season PEFam and PEFpm values showed significant inverse correlations with counts of $CD3^+$ pan-T-lymphocytes (ρ=-0.67, p=0.018, and ρ=-0.66, p=0.019, respectively; n=12). Pre-season PEFam and PEFpm values also showed non-significant

inverse relationships to $CD68^+$ macrophage counts (ρ=-0.49, p=0.1, and ρ=-0.53, p=0.08 respectively, n=12), but not to counts of $CD4^+$, $CD8^+$ or $CD25^+$ T-cells, $AA1^+$ mast cells, $EG2^+$ eosinophils, NE^+ neutrophils, $2D7^+$ or $BB1^+$ basophils, or $CD14^+$ monocytes (all p>0.1).

In-season PEF values correlated inversely with counts of $CD68^+$ macrophages (PEFam: ρ=-0.78, p=0.003; PEFpm: ρ=-0.76, p=0.004; n=12, but not with counts of eosinophils, mast cells, monocytes, neutrophils, basophils or T-lymphocytes. Significantly poorer lung function during the pollen season was thus uniquely associated with higher macrophage counts.

3.4 Immunohistochemical analysis of eicosanoid pathway protein expression in bronchial biopsies.

Immunohistochemistry using polyclonal antibodies directed against the enzymes of the leukotriene pathway 5-LO, FLAP, LTA_4 hydrolase (Woods et al. 1993) and LTC_4 synthase (Penrose et al. 1995) showed that immunoreactivity of all four enzymes was increased with seasonal allergen exposure. Counts of cells staining positive for 5-LO almost doubled from 31.7 ± 7.9 cells/mm^2 pre-season to 58.2 ± 8.2 cells/mm^2 in-season (p=0.02, n=12, Wilcoxon) (**Figure 3.10**). Similarly there was a doubling of $FLAP^+$ cell counts from 8.7 ± 2.9 cells/mm^2 pre-season to 18.1 ± 3.7 cells/mm^2 in-season (P=0.04) (**Figure 3.10**). The number of cells expressing LTA_4 hydrolase, the terminal enzyme for LTB_4 synthesis, showed a smaller but still significant increase from pre-season counts of 9.5 ± 2.8 cells/mm^2 to 16.4 ± 4.1 cells/mm^2 in–season (P=0.05) (**Figure 3.11**). There was a near four fold increase in cells staining positively for LTC_4 synthase, the terminal enzyme for the synthesis of the cysteinyl leukotrienes, with pre-season cell counts of 1.06 ± 0.25 cells/mm^2 rising to 3.87 ± 1.03 cells/mm^2 in-season (P=0.021) (**Figure 3.11**).

Counts of cells immunopositive for MRP 1 were low at baseline (0.5 ± 0.4 cells/mm^2) and were not significantly different in-season (0.4 ± 0.2 cells/mm^2) (p=0.415) (**Fig 3.12**). Counts of cells staining positive for MRP-2 were higher than MRP-1 both at baseline (1.0 ± 0.4 cells/mm^2) and with seasonal allergen exposure (1.7 ± 0.6 cells/mm^2), counts of MRP-2 positive cells were not different in-season compared to pre-season values (p=0.61) (**Fig 3.12**). MRP-2 cell counts correlated significantly with eosinophil counts in-season (r=0.679, p=0.031) and the change in MRP-2 cell counts also correlated significantly with the change in eosinophil counts with seasonal allergen exposure (r=0.650, p=0.042) (**Fig 3.13**). This would suggest that eosinophils express MRP-2 and that those patients who have raised eosinophil counts in-season also have higher $MRP\text{-}2^+$ cell counts. MRP-1 and MRP-2 did not show any relationship with LTC_4 $synthase^+$

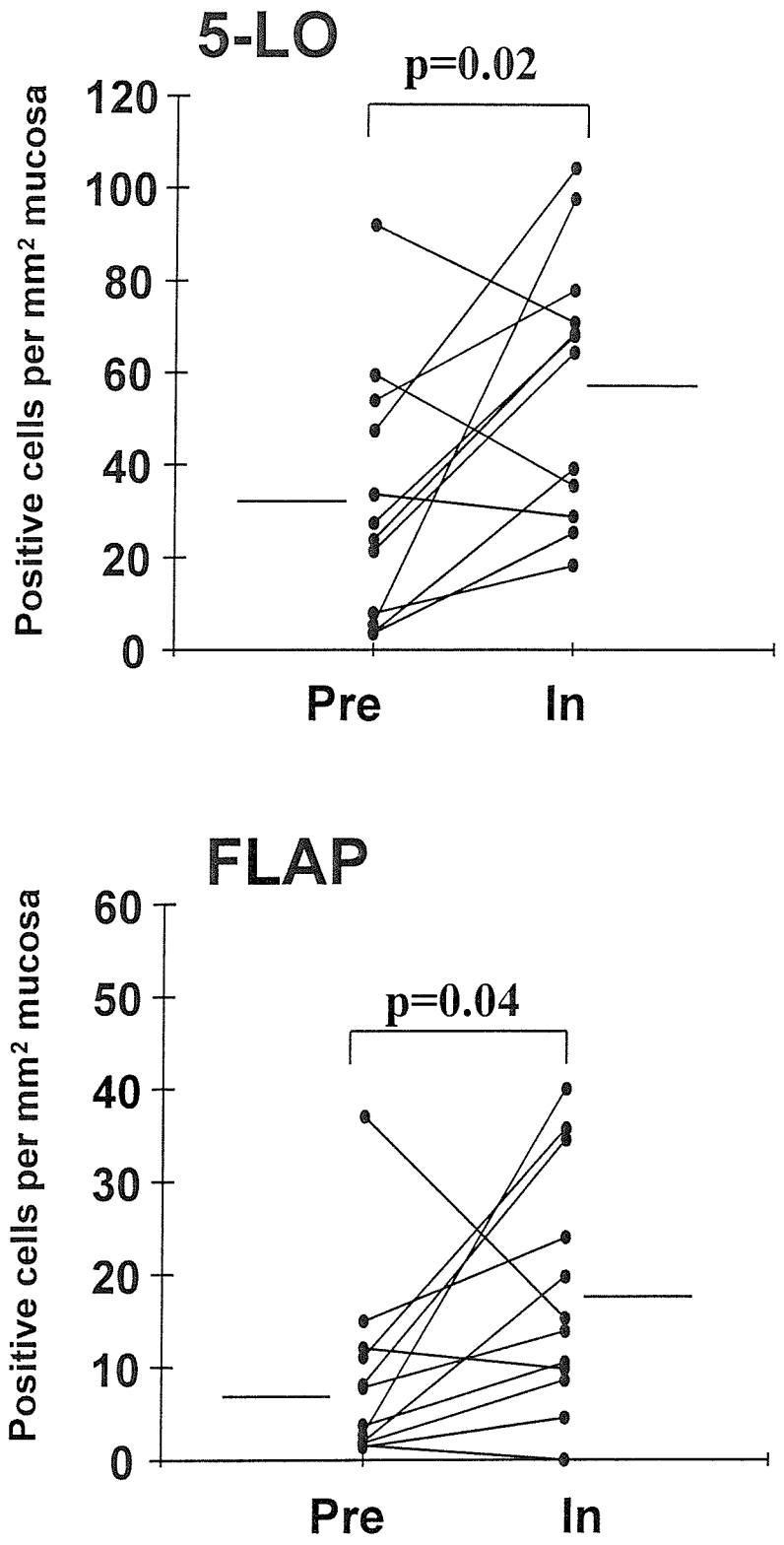


Fig. 3.10 Counts of cells immunostaining for 5-lipoxygenase (5-LO) (***top***) and 5-LO activating protein (FLAP) (***bottom***) per mm^2 mucosa in bronchial biopsies from 12 allergic asthmatics before (pre) and during (in) the birch pollen season. There were significant increases in 5-LO^+ (p=0.02) and $FLAP^+$ (p=0.04) cell counts in season. Horizontal lines represent mean cell counts.

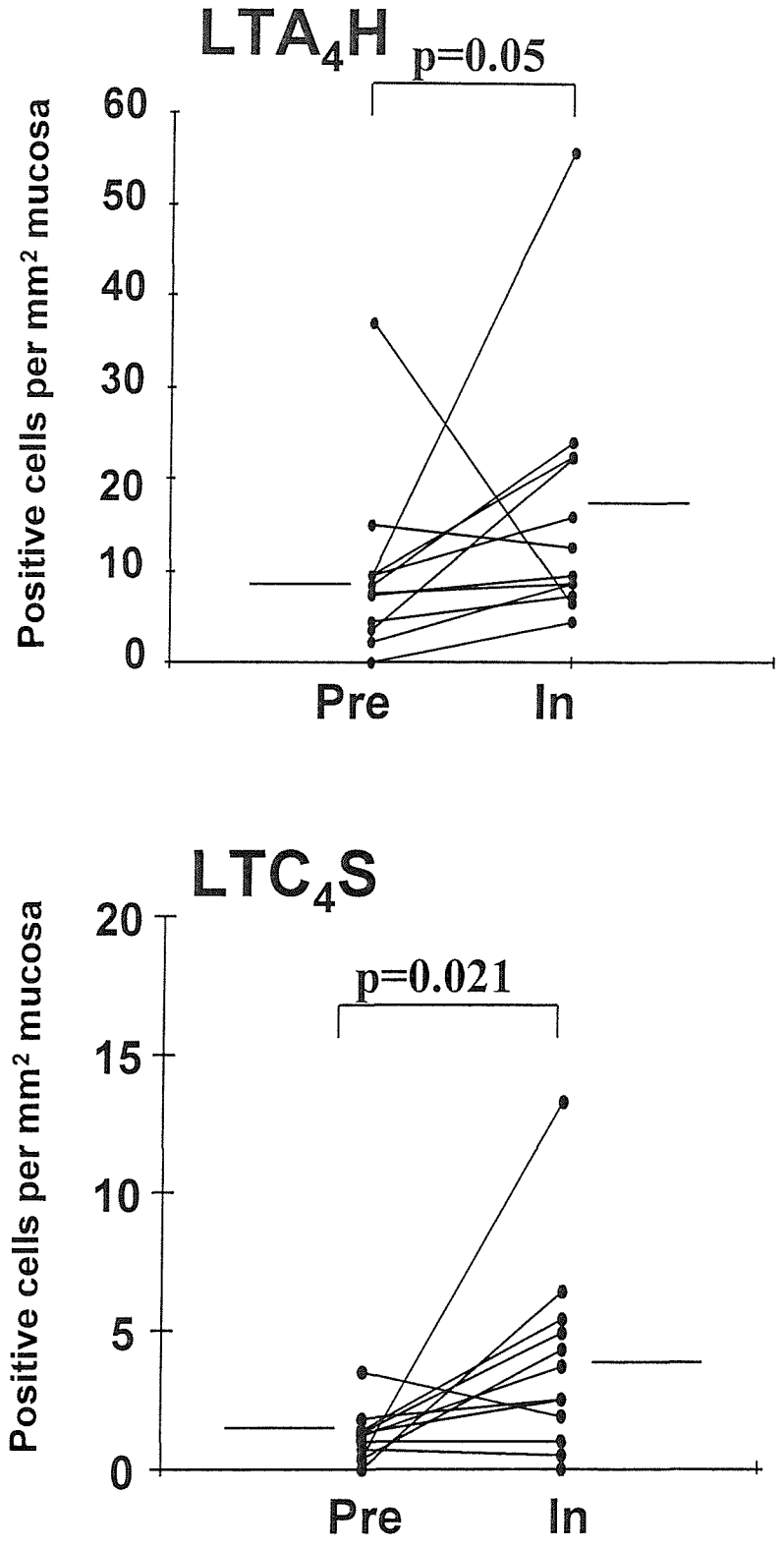


Fig. 3.11 Counts of cells immunostaining for leukotriene A_4 hydrolase (LTA_4H) (***top***) and leukotriene C_4 synthase (LTC_4S) (***bottom***) per mm^2 mucosa in bronchial biopsies from 12 allergic asthmatics before (pre) and during (in) the birch pollen season. There were significant increases in LTA_4 hydrolase$^+$ (p=0.05) and LTC_4 synthase$^+$ (p=0.021) cell counts with seasonal allergen exposure. Horizontal lines represent mean cell counts.

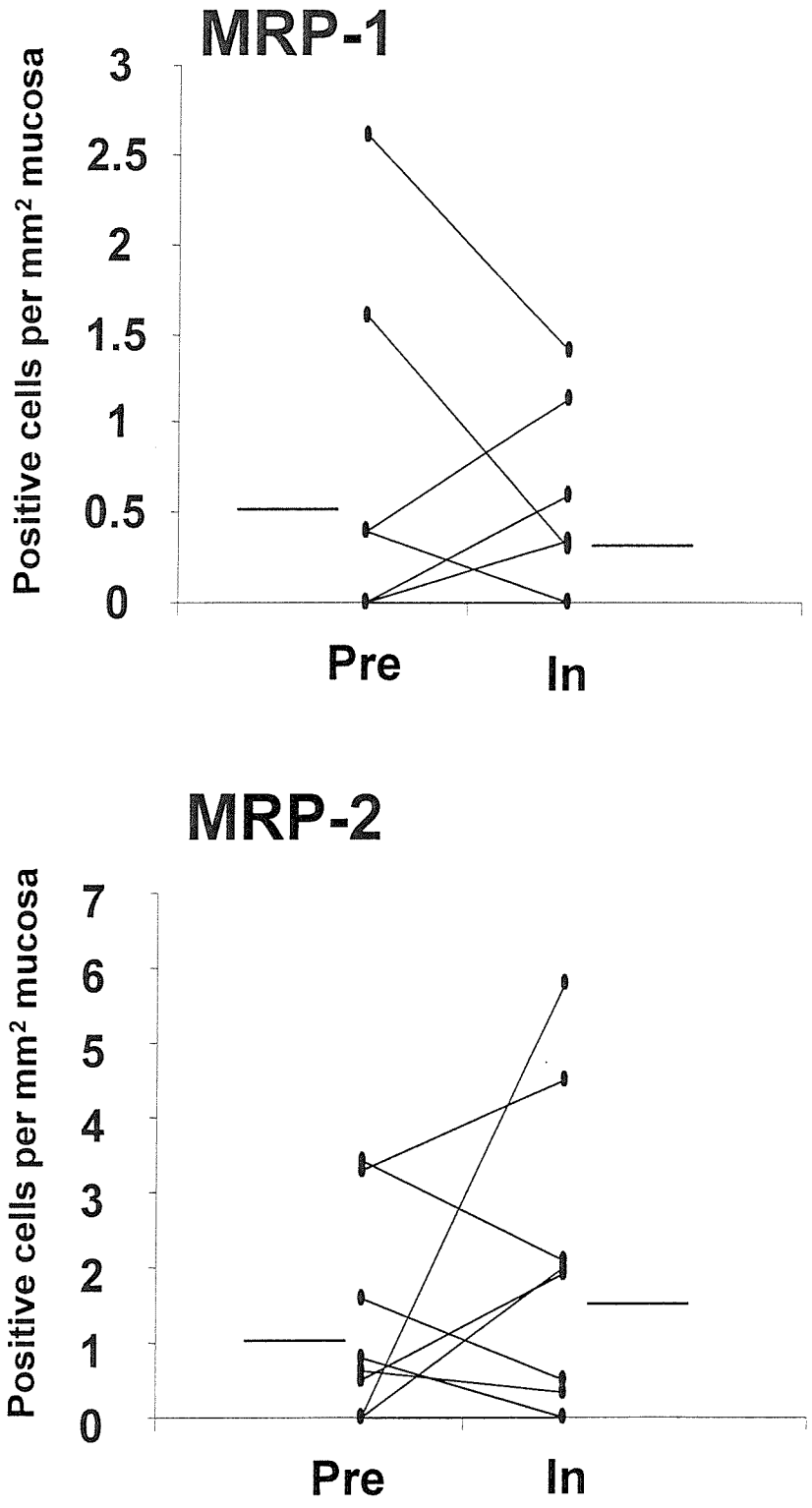


Fig. 3.12 Counts of cells immunostaining for MRP-1 (***top***) and MRP-2(***bottom***) per mm^2 mucosa in bronchial biopsies from 10 allergic asthmatics before (pre) and during (in) the birch pollen season. There were no changes in counts of cells immunostaining for MRP-1 (p=0.415) a or MRP-2 (p=0.61) with seasonal allergen exposure. Horizontal lines represent mean cell counts.

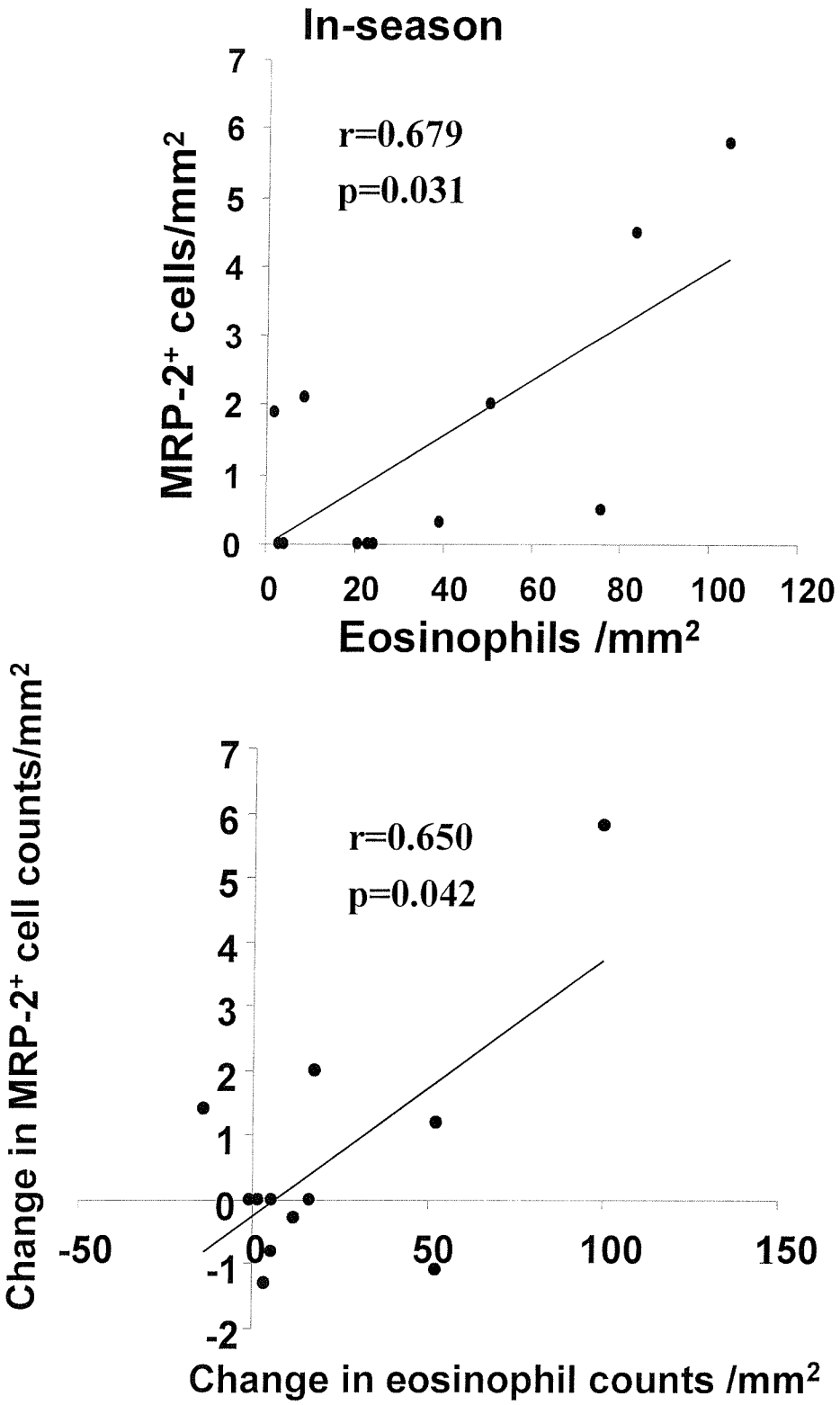


Fig. 3.13 Correlations between counts of cells expressing MRP-2 and eosinophil counts from 10 allergic asthmatics during the birch pollen season, and the change with seasonal allergen exposure. ***Top***: There is a significant correlation between MRP-2+ cell counts and eosinophil counts in-season (ρ=0.679, p=0.031). ***Bottom***: There is a relationship between the change in MRP-2+ cell counts and the change in eosinophil counts with seasonal allergen (ρ=0.650, p=0.042).

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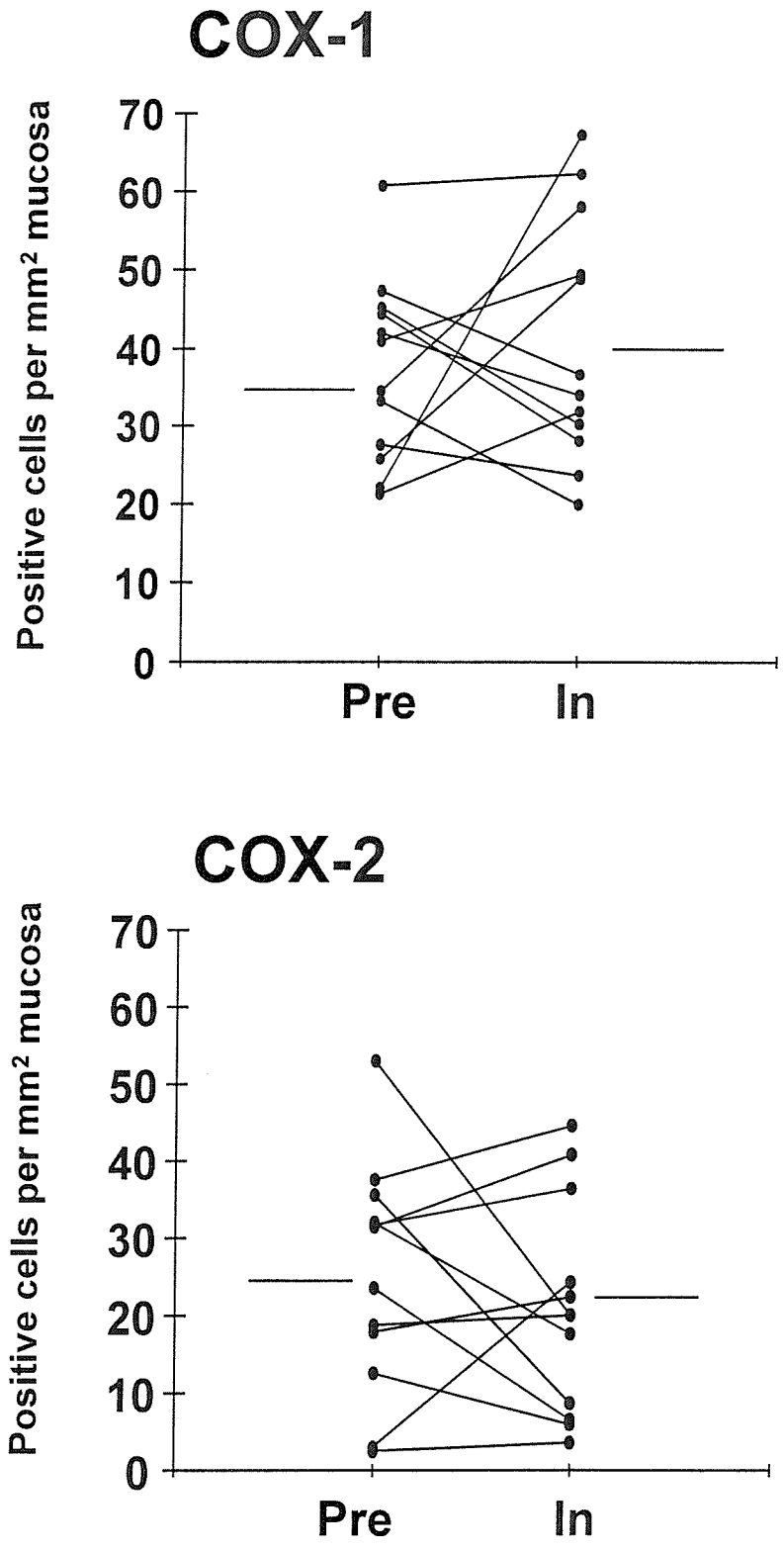


Fig. 3.14 Counts of cells staining positive for cyclooxygenases (COX) 1(***top***) and 2 (***bottom***) per mm^2 of bronchial mucosa from allergic asthmatics before (pre) and during (in) the birch pollen season. There were no significant changes in $COX\text{-}1^+$ or $COX\text{-}2^+$ cell counts ($p>0.5$ for both). Horizontal lines represent mean cell counts.

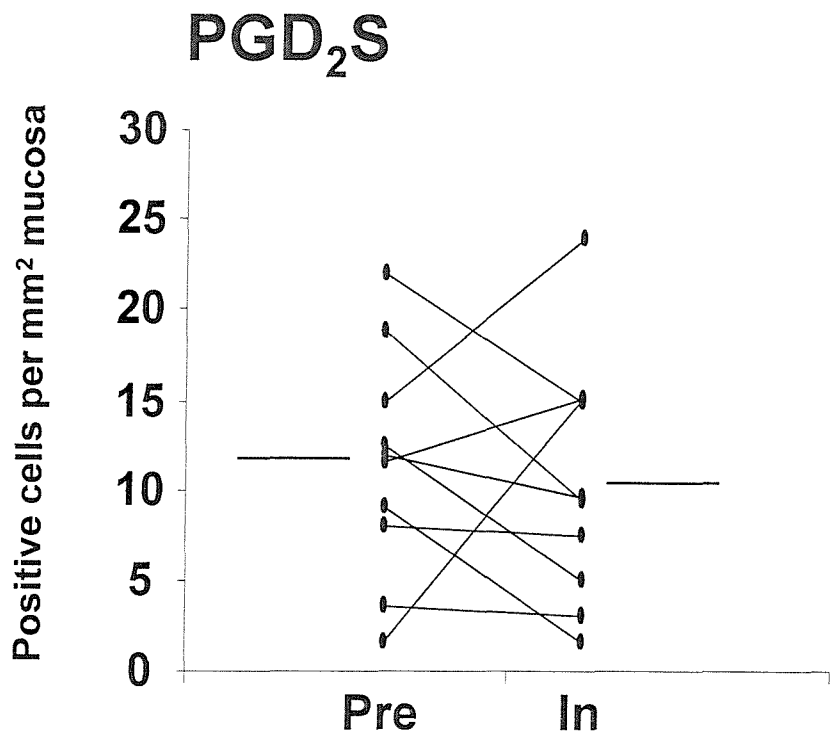


Fig. 3.15 Counts of cells staining positive for PGD_2 synthase per mm^2 of bronchial mucosa from 10 allergic asthmatics before (pre) and during (in) the birch pollen season. There were no significant changes in PGD_2 synthase cell counts ($p>0.61$). Horizontal lines represent mean cell counts.

cell counts.

In contrast to the leukotriene pathway enzymes, immunostaining using monoclonal antibodies for the enzymes of the prostanoid pathway COX-1 (PGHS-1) and COX-2 (PGHS-2) showed no changes in cells expressing the enzymes with seasonal allergen exposure. Mean COX-1^+ cell counts remained stable (pre-season: 37.1 ± 3.4 cells/mm^2; in-season: 41.0 ± 4.6 cells/mm^2; $p>0.5$ Wilcoxon) as did COX-2^+ cell counts (pre-season: 25.0 ± 4.3 cells/mm^2; in season: 21.0 ± 4.0 cells/mm^2; $p>0.5$) (**Figure 3.14**). 11.42 ± 2.0 cells/mm^2 were immunopositive for PGD_2 synthase at baseline and were not significantly different in-season (10.52 ± 2.16 cells/mm^2, $p=0.61$) (**Fig 3.15**).

3.4.1 Relationship of eicosanoid enzyme expression to clinical measures of asthma severity.

Bronchial responsiveness, asthma symptom scores, and use of asthma medication showed no significant relationships to counts of cells immunostaining for eicosanoid pathway enzymes either before or during the pollen season.

However, significant relationships were observed between the counts of cells immunostaining for eicosanoid pathway enzymes and lung function (PEF). With respect to 5-LO pathway enzymes, pre-season PEFam and PEFpm values showed significant inverse correlations with pre-season biopsy counts of cells expressing 5-LO (ρ= -0.59, $p=0.04$, and $\rho=-0.61$, $p=0.036$, respectively; $n=12$)(**Figure 3.16**). The correlations depended particularly on the numbers of 5-LO^+ eosinophils ($\rho=-0.80$, $p=0.017$, and $\rho=-0.78$, $p=0.023$, respectively, $n=8$), with non-significant correlations with counts of 5-LO^+ mast cells ($\rho=-0.69$; $p=0.06$, and $\rho=-0.68$, $p=0.06$, respectively, $n=8$) and 5-LO^+ macrophages ($\rho=-0.57$,$p=0.14$, and $\rho=-0.59$, $p=0.13$, respectively, $n=8$). Pre-season PEFam and PEFpm values also correlated with counts of cells immunostaining for FLAP ($\rho=-0.62$, $p=0.032$, and $\rho=-0.64$, $p=0.026$; $n=12$) and LTA_4 hydrolase ($\rho=-0.70$, $p=0.012$, and $\rho=-0.68$, $p=0.015$; $n=12$), but not significantly with counts of cells expressing LTC_4 synthase ($\rho=-0.40$, $p=0.2$, and $\rho=-0.43$, $p=0.16$; $n=12$). LTC_4 $synthase^+$ macrophage counts did correlate with the pre-season diurnal variability in PEF values (i.e. difference between morning and evening PEF) ($\rho=-0.8$, $p=0.057$, $n=6$).

During the pollen season, PEFam and PEFpm values again correlated significantly with counts of 5-LO^+ cells ($\rho=-0.65$, $p=0.023$, and $\rho=-0.66$, $p=0.021$, respectively, $n=12$) (**Figure 3.16**), with the strongest influence being the counts of 5-LO^+ macrophages ($\rho=-0.64$, $p=0.09$, and $\rho=-0.62$, $p=0.10$), with no correlations with 5-LO^+ eosinophils or 5-LO^+ mast cells ($\rho<0.4$, $p>0.2$). In-season PEFam and PEFpm values did

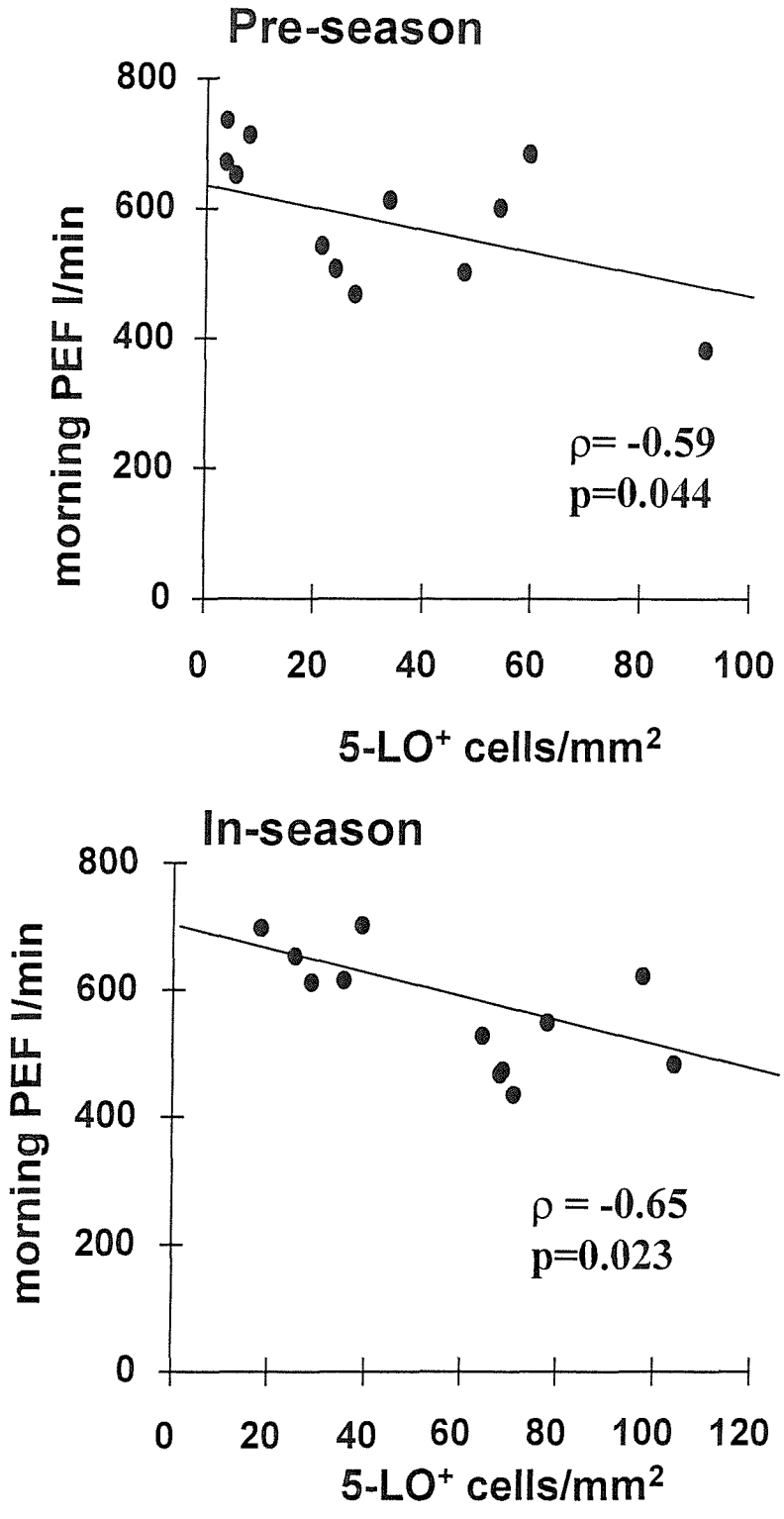


Fig. 3.16 Inverse correlations between morning peak expiratory flow (l/min) and 5-LO positive cell counts per mm^2 mucosa from 12 allergic asthmatics both pre-season (***top***) (ρ=-0.59, p=0.044) and in-season (***bottom***) (ρ=-0.65, p=0.023).

not correlate with counts of cells expressing FLAP, LTA_4 hydrolase, or LTC_4 synthase (all $\rho<0.4$, $p>0.2$), or with the sub-populations of leukocytes expressing these enzymes.

With respect to cyclooxygenase pathway enzymes, pre-season PEFam and PEFpm values showed no relationship to counts of cells expressing COX-1 (both $\rho<0.40$, $p> 0.2$), but showed a significant inverse correlation with counts of COX-2-positive cells ($\rho=-0.69$, $p=0.019$, and $\rho=-0.68$, $p=0.009$ respectively, $n=12$). In-season PEFam and PEFpm values showed only a non-significant trend to an inverse correlation with COX-1-positive cell counts ($\rho=-0.54$, $p=0.07$, and $\rho=-0.53$, $p=0.08$, respectively, $n=12$) and no relationship to COX-2-positive cell counts ($\rho<0.3$, $p>0.4$). PGD_2 synthase$^+$ cell counts did not show any significant relationships with measures of asthma severity.

3.4.2 Cellular localisation of eicosanoid pathway enzymes.

A change in the expression of eicosanoid pathway enzymes was shown to be associated with changes in leukocyte populations. The *camera lucida* technique (Bradding et al. 1992) allows identification of the cells staining positively for an enzyme. Adjacent sections from a representative subgroup of 6-8 of 12 patients were immunostained for 5-LO, FLAP, LTC_4 synthase, COX-1 and COX-2 and colocalised to eosinophils, mast cells and macrophages pre-season and in-season.

3.4.2.1 Colocalisation of 5-lipoxygenase.

5-lipoxygenase was localised to EG2$^+$ eosinophils, AA1$^+$ mast cells and CD68$^+$ macrophages in 8 representative patients (**Figure 3.17**). At baseline there were 39.2 ± 10.5 cells/mm^2 mucosa immunostaining for 5-LO, of which 9.1 ± 3.2 were eosinophils, 13.3 ± 4.1 were mast cells and 6.6 ± 1.6 were macrophages.

Seasonal allergen exposure increased the number of 5-LO$^+$ cells identified as eosinophils from 9.1 ± 3.2 cells/mm^2 pre-season to 17.9 ± 4.7 cells /mm^2 in-season ($P=0.18$). There was no change in the proportion of eosinophils staining positive for 5-LO with a pre-season value of 38.4 ± 10.5% and an in-season value of 32.7 ± 3.9% ($P>0.5$) (**Figure 3.17**). This suggests that eosinophil influx contributed to the increase in 5-LO cell counts.

There was no change in the numbers of 5-LO$^+$ cells identified as mast cells (pre-season: 13.3 ± 4.10 cells/mm^2; in-season: 16.6 ± 2.2 cells/mm^2; $P>0.4$) or in the percentage of mast cells expressing 5-LO (pre-season: 35.5 ± 4.3%; in-season: 34.7 ± 7.2%; $P>0.6$). The mast cell is therefore unlikely to directly contribute to the increased

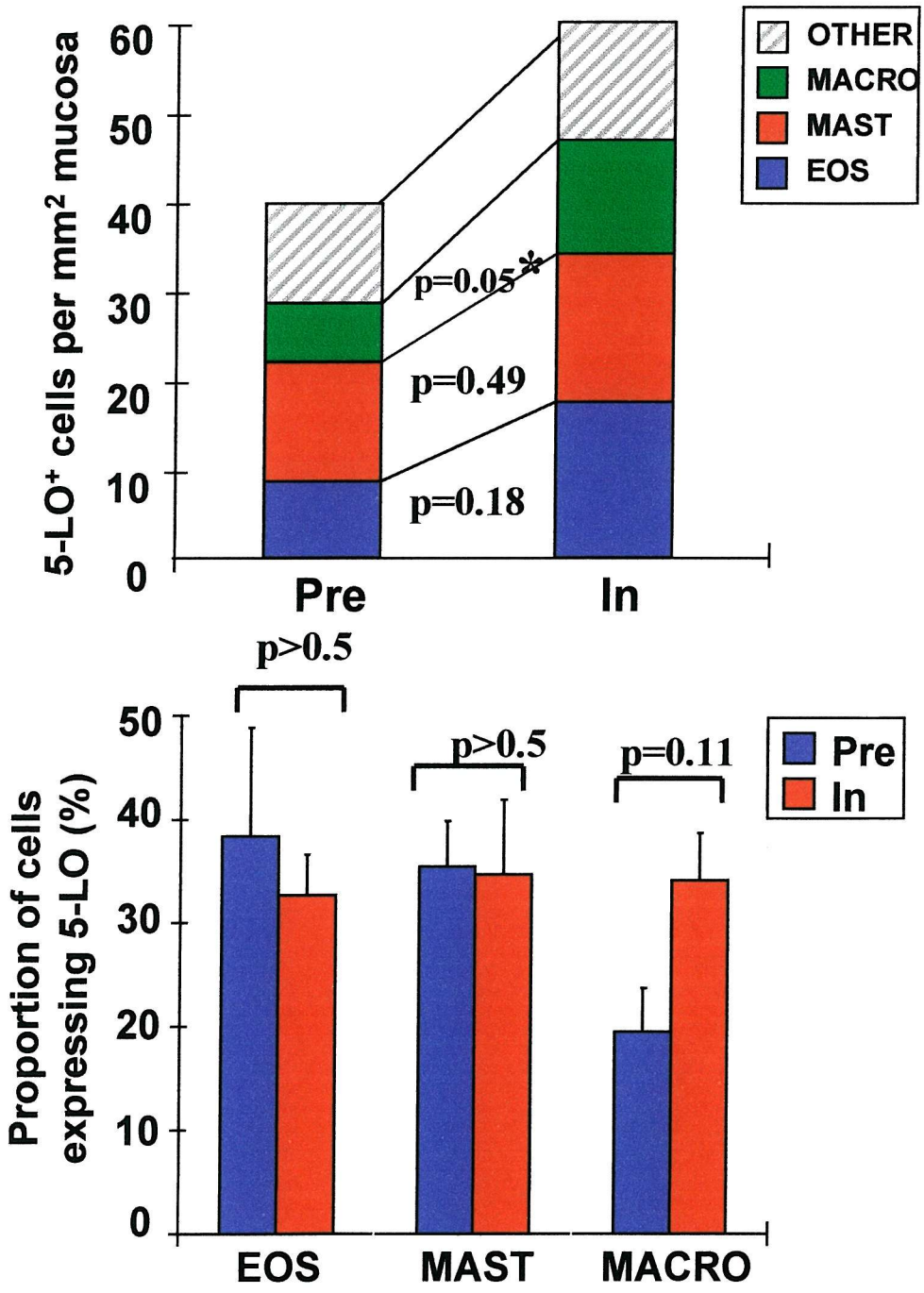


Fig. 3.17 Colocalisation of 5-LO positive cells to eosinophils, mast cells and macrophages in the bronchial mucosa of 8 representative allergic asthmatics before (pre) and during (in) the birch pollen season. ***Top:*** Numbers of 5-LO$^+$ cells which are identified as eosinophils, mast cells or macrophages. There was a significant increase in the number of 5-LO$^+$ macrophages (p=0.05), and a weak trend towards increased 5-LO$^+$ eosinophil counts (p=0.18) with seasonal allergen exposure. There was no change in 5-LO$^+$ mast cell numbers (p=0.49). ***Bottom:*** Graph shows the proportion of each cell population that expressed 5-LO. There was a trend towards an increased percentage of macrophages expressing 5-LO (p=0.11). The proportions of eosinophils and mast cells expressing 5-LO did not change (p>0.5 for both).

expression of 5-LO in the bronchial mucosa with seasonal allergen exposure. There was a two-fold increase in the number of 5-LO$^+$ cells identified as macrophages from 6.6 ± 1.6 cells/mm^2 pre-season to 12.7 ± 2.8 cells/mm^2 in-season (P=0.05). This was also associated with an increase in the proportion of macrophages expressing 5-LO (19.5 ± 4.2% pre-season; 34.1 ± 4.6% in-season; P=0.11) suggesting that there is an upregulation of expression of 5-LO in the macrophage combined with macrophage influx. In conclusion, influx of eosinophils and macrophages expressing 5-LO and an increased expression of 5-LO in resident macrophages contributed to the increase in 5-LO$^+$ cells in the bronchial mucosa of allergic asthmatics during the birch pollen season.

3.4.2.2 Colocalisation of FLAP.

FLAP was localised to EG2$^+$ eosinophils, AA1$^+$ mast cells and CD68$^+$ macrophages in 8 representative patients (**Figure 3.18**). From a total of 10.9 ± 4.1 FLAP$^+$ cells/mm^2 mucosa, 1.4 ± 0.8 were eosinophils, 1.5 ± 0.4 were mast cells and 1.0 ± 0.6 were macrophages at baseline, the remaining cells are likely to be neutrophils, basophils and T-cells .

There were 7-fold increases in the numbers of FLAP$^+$ cells identified as eosinophils (pre-season: 1.4 ± 0.8 cells/mm^2: in-season: 7.8 ± 2.9 cells/mm^2; P=0.089) and mast cells (pre-season: 1.5 ± 0.4 cells/mm^2; in-season: 7.7 ± 2.6 cells/mm^2; P=0.03) with seasonal allergen exposure. This was associated with 2-fold increases in the percentage of mast cells expressing FLAP from 8.4 ± 1.7% pre-season to 17.4 ± 4.4% in-season (P=0.06) and in the proportion of eosinophils staining positive for FLAP (pre-season: 7.9 ± 3.1%; in-season: 14.2 ± 3.9%; P=0.1). In contrast to 5-LO there was no change in the expression of FLAP in macrophages. The number of FLAP$^+$ cells which were macrophages remained low with seasonal allergen (pre-season: 1.0 ± 0.6 cells/mm^2; in-season: 3.7 ± 2.1 cells/mm^2; P=0.3) and similarly the proportion of macrophages expressing FLAP was low and did not change (pre-season: 5.6 ± 2.4%; in-season: 8.1 ± 3.4%; P>0.9).

These results suggest that the increase observed in FLAP$^+$ cell counts during the birch pollen season can be attributed to an increase in expression of FLAP in resident mast cells and eosinophils with a further contribution from eosinophil influx. The macrophage did not appear to have the same influence on FLAP expression as it does on 5-LO expression.

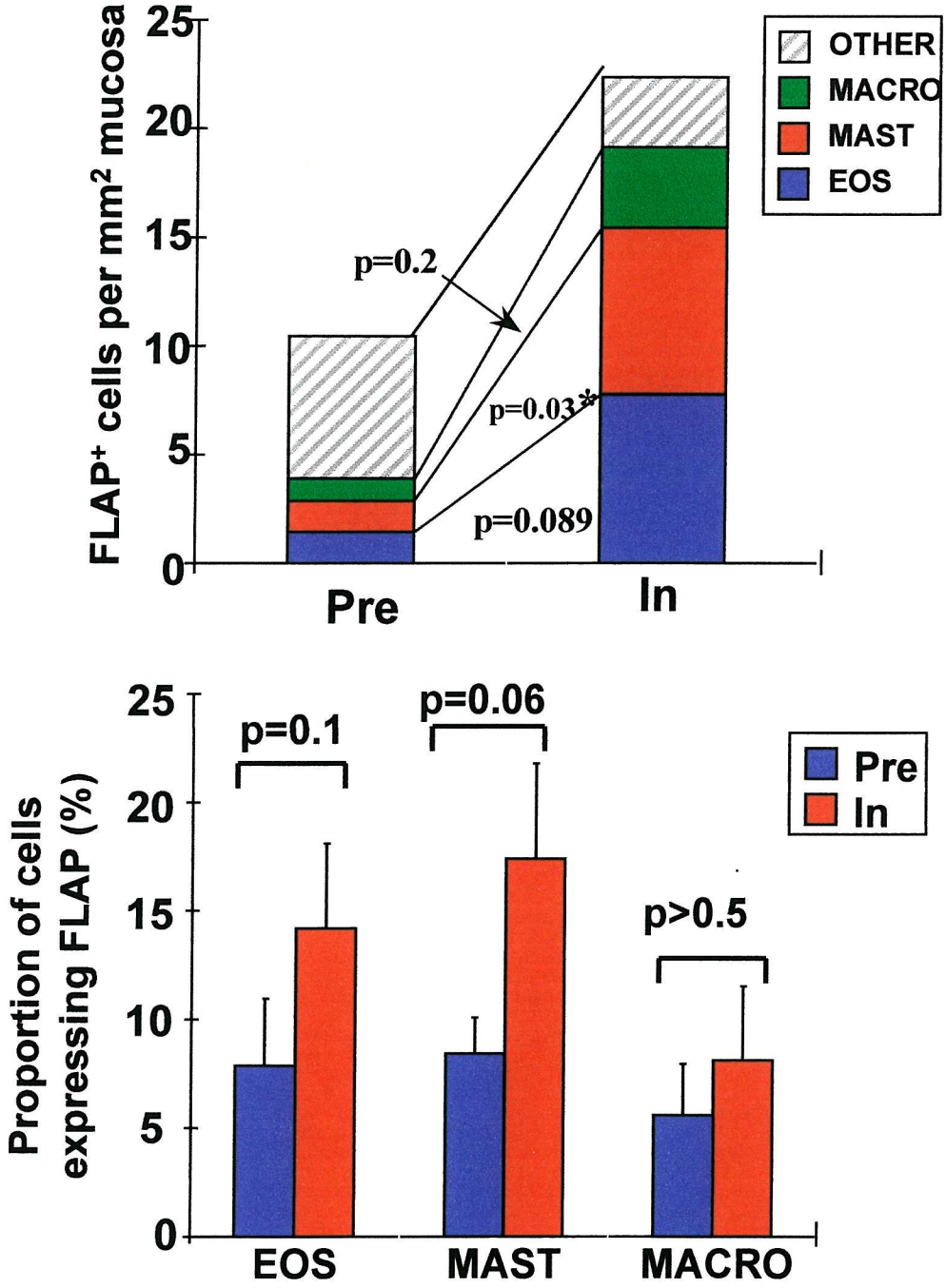


Fig. 3.18 Colocalisation of FLAP positive cells to eosinophils, mast cells and macrophages in the bronchial mucosa of 8 representative allergic asthmatics before (pre) and during (in) the birch pollen season. ***Top:*** Graph shows numbers of $FLAP^+$ cells which were identified as eosinophils, mast cells or macrophages. There was a significant increase in the number of $FLAP^+$ mast cells ($p=0.03$), and a trend towards increased $FLAP^+$ eosinophil counts ($p=0.089$) with seasonal allergen exposure. There was no significant change in $FLAP^+$ macrophage numbers ($p=0.2$). ***Bottom:*** Graph shows the proportion of a cell population expressing FLAP. There were trends towards an increased percentage of eosinophils and mast cells expressing FLAP ($p=0.1$, $p=0.06$ respectively). The proportion of macrophages expressing FLAP did not change ($p>0.5$).

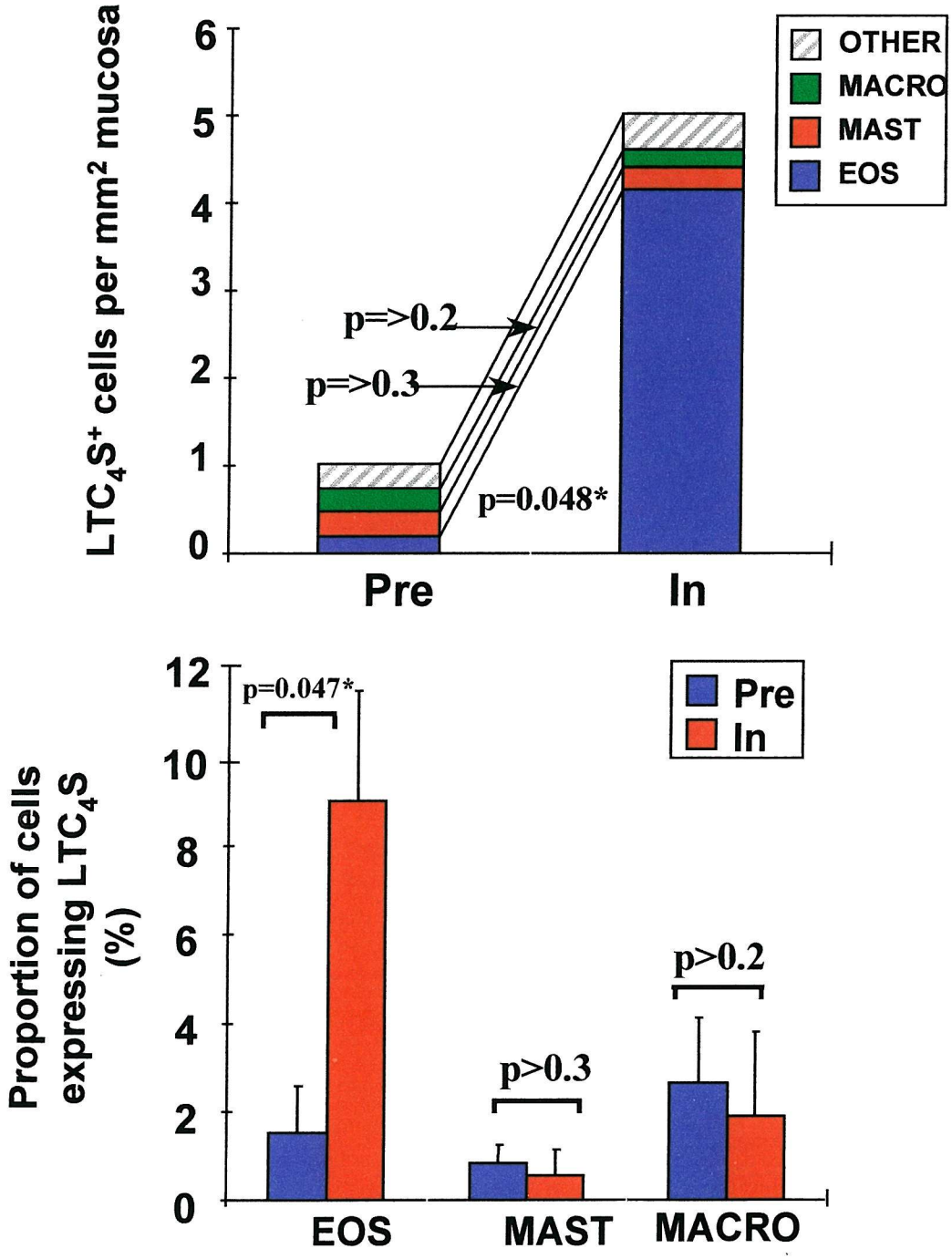


Fig. 3.19 Colocalisation of LTC_4 synthase positive cells to eosinophils, mast cells and macrophages in the bronchial mucosa of 6 representative allergic asthmatics before (pre) and during (in) the birch pollen season. ***Top:*** Graph shows numbers of LTC_4S^+ cells identified as eosinophils, mast cells or macrophages. Uniquely, there was a significant increase in the number of LTC_4S^+ eosinophils (p=0.048) with seasonal allergen exposure, with no changes in LTC_4S^+ mast cells or macrophages (p>0.3, p>0.2 respectively). ***Bottom:*** Graph shows the proportion of a cell population expressing LTC_4S. There was an increased percentage of eosinophils expressing LTC_4S (p=0.047). In season the proportion of mast cells and macrophages expressing LTC_4S did not change (p>0.3, p>0.2 respectively).

3.4.2.3 ***Colocalisation of leukotriene C_4 synthase.***

Leukotriene C_4 synthase was colocalised to $EG2^+$ eosinophils, $AA1^+$ mast cells and $CD68^+$ mast cells in 6 representative subjects (**Figure 3.19**). At baseline, expression of LTC_4 synthase was evenly distributed between eosinophils, mast cells and macrophages. Of a total of 1.03 ± 0.23 LTC_4 synthase$^+$ cells/mm^2 mucosa, 0.2 ± 0.11 were eosinophils, 0.28 ± 0.13 were mast cells, and 0.26 ± 0.16 were macrophages.

Seasonal allergen exposure induced a substantial 20-fold increase in the number of LTC_4 synthase$^+$ cells which are eosinophils rising from 0.2 ± 0.11 cells/mm^2 pre-season to 4.2 ± 2.0 cells/mm^2 in-season (P=0.048). This was associated with a 6-fold increase in the proportion of eosinophils expressing LTC_4 synthase from 1.5 ± 1.1% pre-season to 9.0 ± 2.6% in-season (P=0.047). The combination of an influx of eosinophils expressing LTC_4 synthase and an increase in the expression of LTC_4 synthase in resident eosinophils thus accounts for the total increase in counts of cells expressing the enzyme. The eosinophil, therefore, is the primary cell responsible for this increase as expression in mast cells and macrophages is unchanged. The number of LTC_4 synthase$^+$ cells which were mast cells remained stable at 0.28 ± 0.13 cells/mm^2 pre-season, and 0.27 ± 0.27 cells/mm^2 in-season and the proportion of mast cells expressing LTC_4 synthase was low at 0.83 ± 0.42% pre-season and remained low at 0.6 ± 0.6% in-season. A similar situation was apparent in macrophages. The number of LTC_4 synthase$^+$ macrophages is unchanged at 0.26 ± 0.16 cells/mm^2 pre-season and 0.18 ± 0.18 cells/mm^2 in-season, and the proportion of macrophages expressing the enzyme is 2.7 ± 1.5% pre-season and 1.9 ± 1.9% in-season.

3.4.2.4 Colocalisation of Cyclooxygenase-1.

Cyclooxygenase-1 was colocalised to $EG2^+$ eosinophils and $AA1^+$ mast cells in 6 representative patients (**Figure 3.20**). There was no change in the numbers of COX-1^+ cells which are eosinophils (pre-season: 4.7 ± 1.9 cells/mm^2; in-season 7.5 ± 1.5 cells/mm^2; P=0.35) or in the proportion of eosinophils expressing COX-1 (pre-season: 20.3 ± 5.9%; in-season: 18.8 ± 3.3%; P=0.47). There was no change in the number of COX-1^+ cells identified as mast cells (pre-season: 21.5 ± 4.0 cells/mm^2; in-season 21.7 ± 3.5 cells/mm^2; P>0.9). However, there was a significant decrease in the proportion of mast cells expressing COX-1 from 71.6 ± 4.6% pre-season to 46.1 ± 7.1% in-season (P<0.0001).

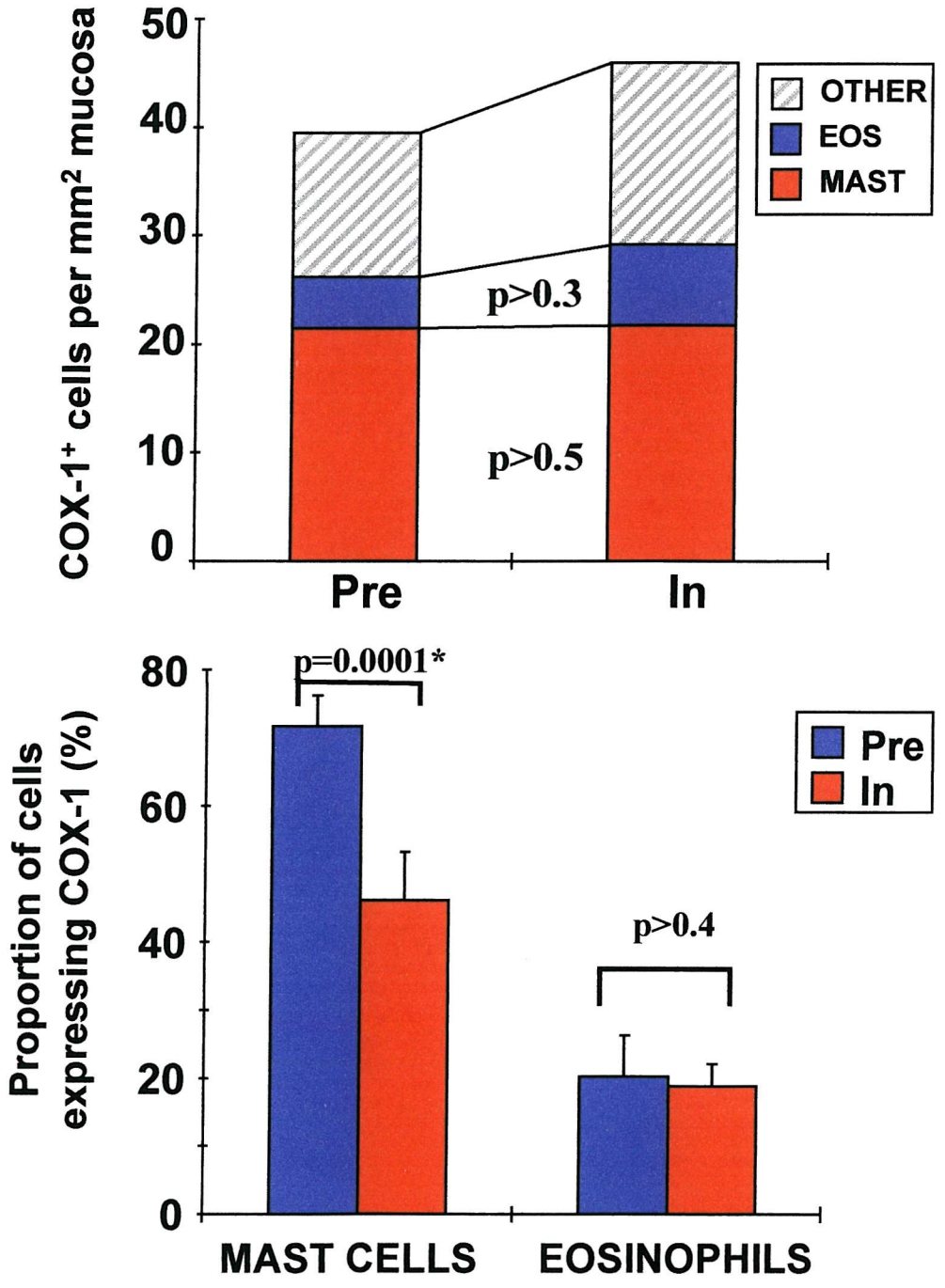


Fig. 3.20 Localisation of COX-1 to eosinophils and mast cells in 6 representative allergic asthmatics before (pre) and during (in) the birch pollen season. The distribution of COX-1$^+$ cells between mast cells and eosinophils (***top***) does not change with seasonal allergen exposure ($p>0.5$, $p>0.3$ respectively). The proportion of mast cells which express COX-1 (***bottom***) decreases with seasonal allergen exposure ($p=0.0001$), with no change in eosinophil COX-1 expression ($p>0.4$).

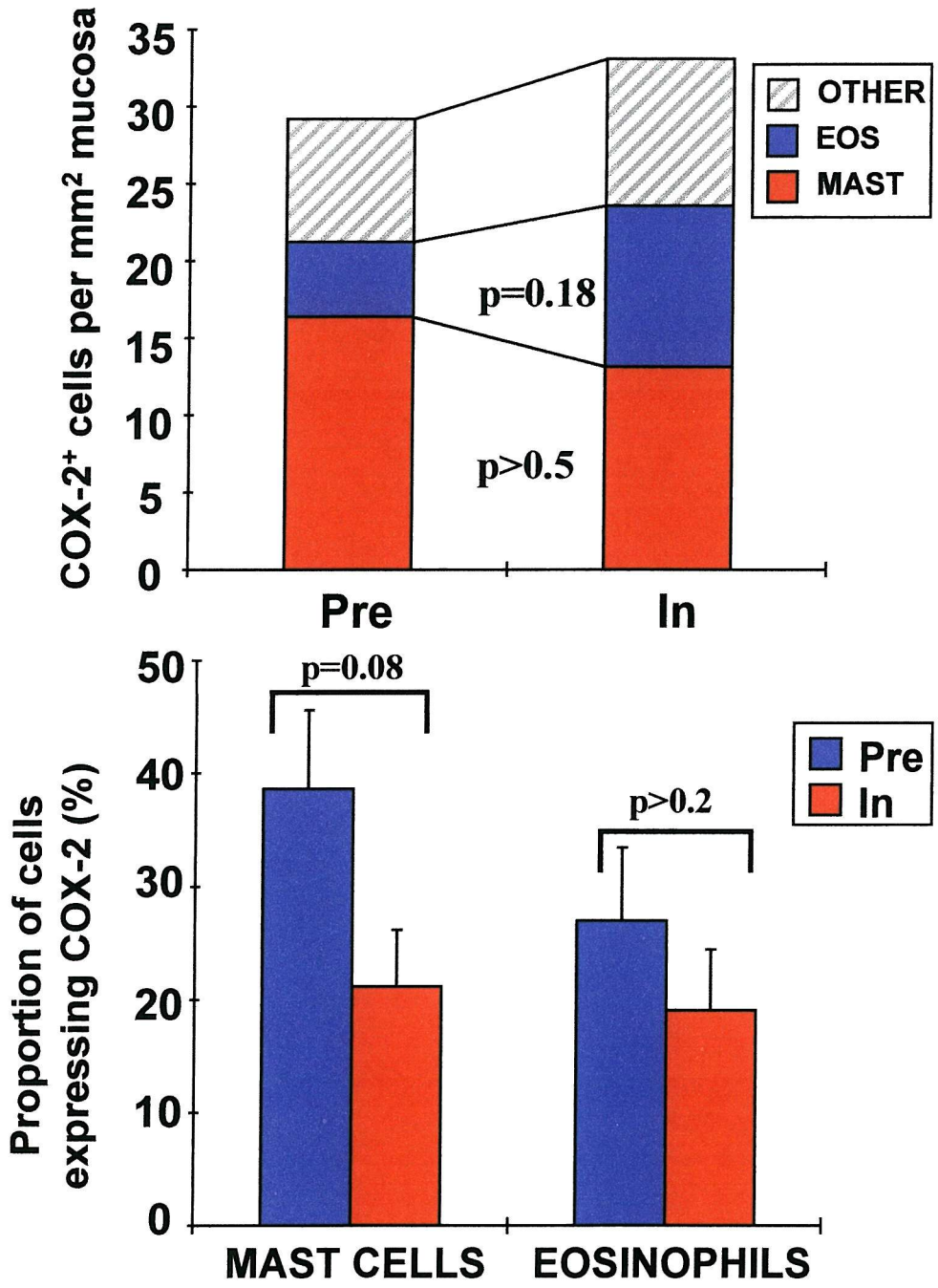


Fig. 3.21 Localisation of COX-2 to eosinophils and mast cells in 6 representative allergic asthmatics before (pre) and during (in) the birch pollen season. The distribution of COX-1^+ mast cells (***top***) did not change with seasonal allergen exposure ($p>0.5$). There was a very weak trend towards an increase in COX-2^+ eosinophils in season ($p=0.18$). The proportion of mast cells which express COX-2 (***bottom***) decreased with seasonal allergen exposure but the decrease did not reach significance ($p=0.08$). There was no change in eosinophil COX-1 expression ($p>0.2$).

3.4.2.5 Colocalisation of Cyclooxygenase-2

Cyclooxygenase-2 was colocalised to $EG2^+$ eosinophils and $AA1^+$ mast cells in 6 representative patients (**Figure 3.21**). There was a trend towards an increase in the numbers of $COX\text{-}2^+$ eosinophils with seasonal allergen exposure (pre-season: 4.8 ± 1.4 cells/mm^2; in-season: 10.5 ± 3.2 cells/mm^2; P=0.18) but no change in the proportion of eosinophils expressing COX-2 (pre-season: 27.0 ± 6.5%; in-season 19.1 ± 5.3%; P=0.25). Similarly to COX-1, there was no change in the numbers of $COX\text{-}2^+$ mast cells (pre-season: 16.4 ± 5.1 cells/mm^2; in-season: 13.1 ± 3.2 cells/mm^2; P=0.61) but there was a trend towards a decrease in the proportion of mast cells expressing COX-2 from 38.7 ± 7.0% pre-season to 21.2 ± 5.0% in-season (P=0.18).

3.5 Cysteinyl leukotriene and Prostaglandin D_2 levels in BAL fluid.

Bronchoalveolar lavage fluid taken during the same bronchoscopies as the pre-season and in-season biopsies was assayed for cys-LT and PGD_2 levels using enzyme-immunoassays as described in section 2.3.

Despite significant rises in 5-LO pathway enzyme expression in bronchial biopsies in-season, the mean total concentration of the three cys-LTs (LTC_4, LTD_4, and LTE_4) in BAL fluid did not change in-season (19.8 ± 1.3 pg/ml) compared with pre-season values (21.3 ± 1.4 pg/ml; p=0.4, n=12, Wilcoxon) (**Figure 3.22**). Surprisingly, PGD_2 levels in BAL fluid fell significantly during the pollen season from 12.1 ± 2.4 pg/ml to 8.5 ± 1.2 pg/ml (P<0.005) (**Figure 3.22**).

3.5.1 Relationships between BAL fluid cysteinyl leukotriene levels and inflammatory cell types.

Both pre-season and in-season there were few meaningful relationships between cys-LT levels in BAL fluid and counts of cells immunostaining for the 5-LO pathway enzymes (5-LO, FLAP or LTC_4 synthase) or between inflammatory cell types (eosinophils, mast cells and macrophages) in bronchial biopsies. BAL cys-LT levels did show weak correlations with bronchial mucosal counts of eosinophils (ρ=0.48, p=0.12, n=12) and with LTC_4 $synthase^+$ cell counts (ρ=0.43, p=0.16, n=12). Similar, significant relationships exist between the change in eosinophil counts with seasonal allergen exposure and the change in BAL fluid cys-LT levels (ρ=0.782, p=0.003) (**Figure 3.23**) such that those patients who exhibited an increase in cys-LT levels had the largest increases in eosinophil counts with allergen exposure. No single cell type is solely responsible for the cys-LT production and only a small subpopulation of each cell type

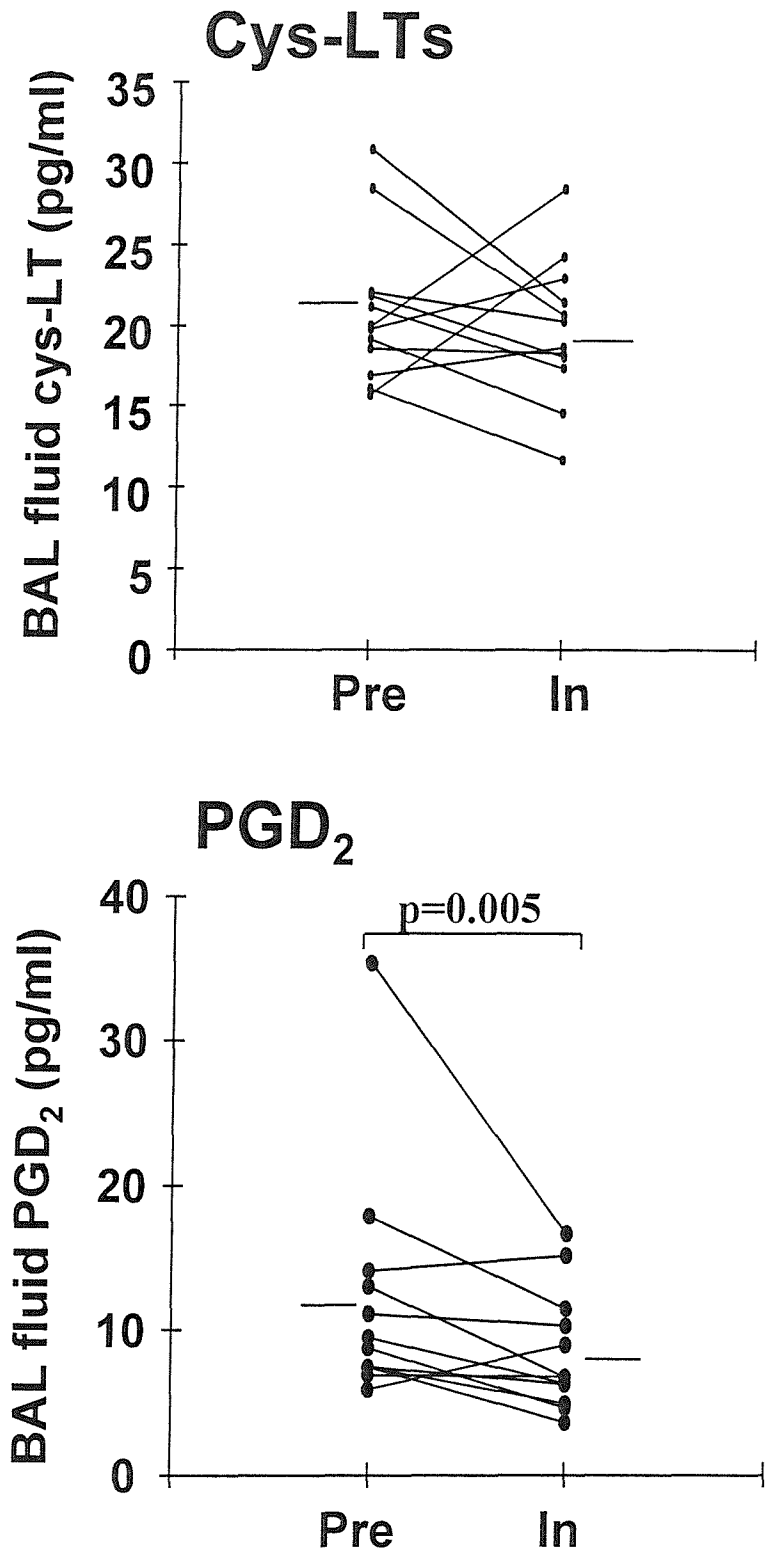


Fig. 3.22 Concentration of eicosanoid mediators in BAL fluid (pg/ml) from 12 allergic asthmatics before (pre) and during (in) the birch pollen season. Cysteinyl leukotriene levels in BAL fluid (***top***) were unchanged with seasonal allergen exposure (p=0.4). There was a significant decrease in BAL fluid PGD_2 levels (***bottom***) (p=0.005).

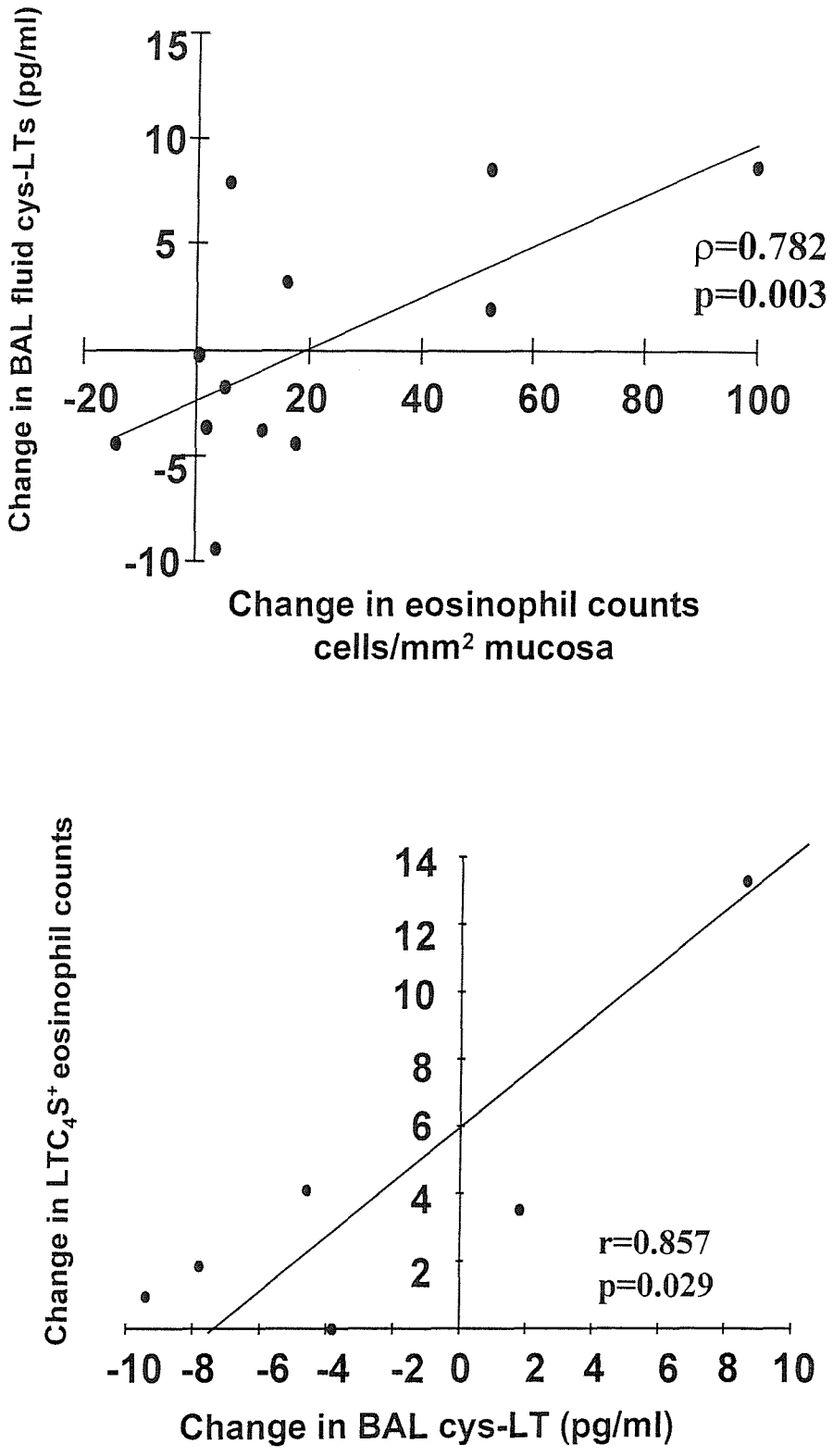


Fig. 3.23 Correlation (ρ=0.782, p=0.003) between the change in bronchoalveolar lavage fluid cys-LT levels with seasonal allergen exposure and the change in $EG2^+$ cell counts per mm^2 mucosa of bronchial biopsies from 12 allergic asthmatics before and during the birch pollen season. With seasonal allergen exposure, the change in BALfluid cys-LT levels correlates significantly with the change in LTC_4 $synthase^+$ eosinophil counts (ρ=0.857, p=0.029) (***bottom***).

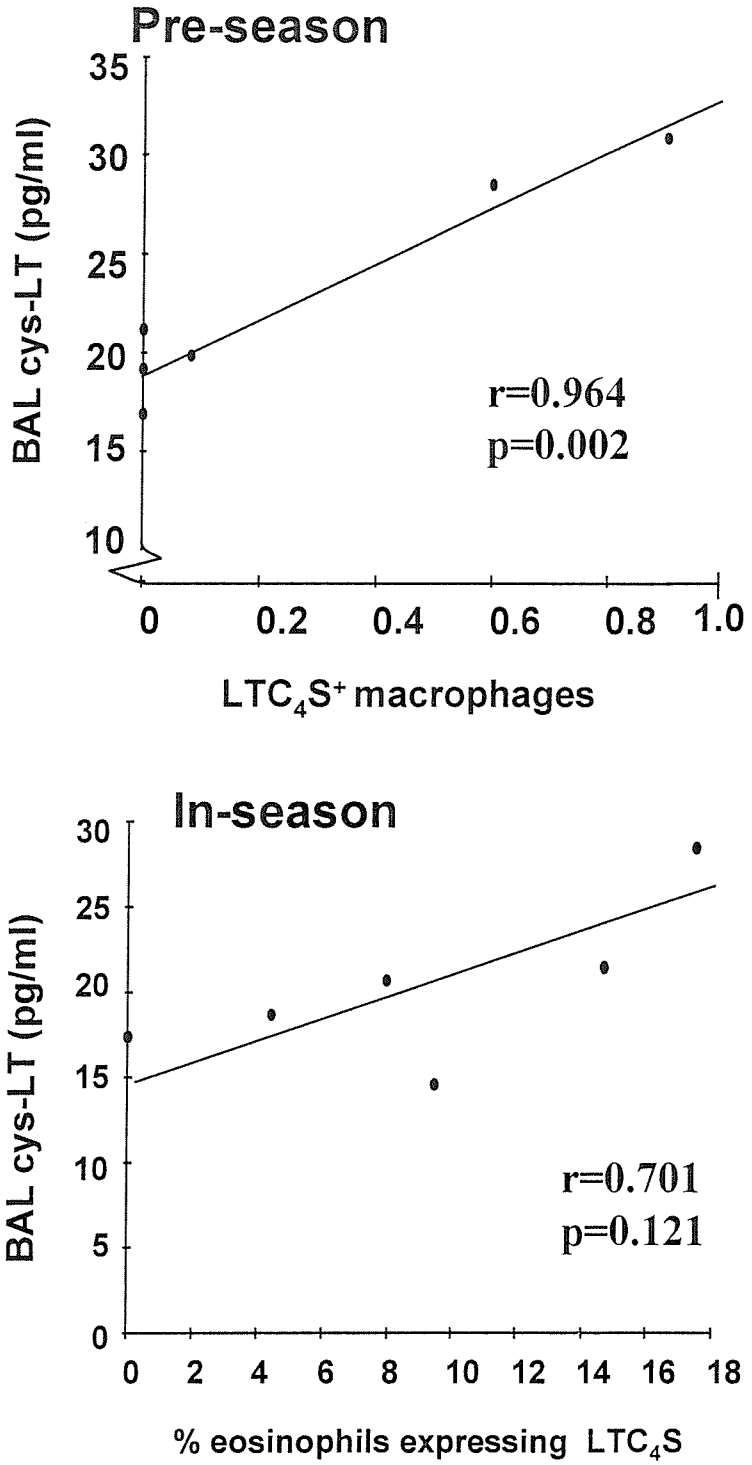


Fig. 3.24 Correlations between BAL cys-LT levels (pg/ml) and cell populations expressing LTC_4 synthase from 6 representative allergic asthmatics before and during the birch pollen season, and the change with seasonal allergen exposure. There is a significant correlation between BAL fluid cys-LTs and LTC_4 synthase$^+$ macrophages pre-season (ρ=0.964, p=0.002) (***top***). In-season there is a relationship between BAL fluid cys-LTs and the proportion of eosinophils expressing LTC_4 synthase (ρ=0.701, p=0.121) (***bottom***).

may express the relevant enzymes. BAL fluid cys-LT levels were therefore related to the subpopulations of eosinophils, macrophages and mast cells which were found to express the leukotriene pathway enzymes as determined by the *camera lucida* technique.

BAL fluid cys-LT levels did not show a correlation with the 5-LO$^+$ or FLAP$^+$ subpopulations of any cell type either pre-season or in-season. There was, however, a strong significant relationship with the subpopulation of LTC_4 synthase$^+$ macrophages pre-season (ρ=0.964, p=0.002, n=6) (**Figure 3.24**). No such relationship existed with LTC_4 synthase$^+$ eosinophils (ρ=-0.15, p=0.8, n=6) or mast cells (ρ=0.04, p>0.9, n=6). In-season, consistent with the weak relationship observed between cys-LT levels and eosinophils, and between cys-LT levels and LTC_4 synthase$^+$ cells there were also trends towards correlations between BAL fluid cys-LT levels and the proportion of eosinophils expressing LTC_4 synthase (ρ=0.70, p=0.121, n=6) (**Figure 3.24**). The relationship between LTC_4 synthase$^+$ macrophages and BAL fluid cys-LTs was no longer apparent (ρ=0.05, p>0.9, n=6) and there was no relationship between cys-LTs and LTC_4 synthase$^+$ mast cells (ρ=0.05, p>0.9, n=6).

Importantly, there was a significant relationship between the changes in BAL fluid cys LTs from pre-season to in-season and the change in the counts of eosinophils (ρ=0.78, p=0.003, n=12) (**Figure 3.23**) and particularly with changes in the counts of the subpopulation of LTC_4 synthase$^+$ eosinophils (ρ=0.857, p=0.029, n=6) (**Figure 3.23**). Such relationships were not apparent between cys-LT levels and other enzymes or cell types.

The strong relationship between LTC_4 synthase$^+$ macrophages and BAL fluid cys-LTs pre-season suggests that the macrophage may be responsible for the baseline levels of cys-LT production in the asthmatic lung. The substantial increase in eosinophil numbers and expression of LTC_4 synthase suggests eosinophil influx and activation may, therefore, determine variability in BAL fluid cys-LT levels during seasonal allergen exposure.

3.5.2 Relationships between BAL fluid Prostaglandin D_2 levels and cyclooxygenase pathway enzymes in inflammatory cell types.

There was a decrease in BAL fluid PGD_2 with seasonal allergen exposure (P=0.005) but there was no change in COX-1, COX-2, or PGD_2 synthase positive cell counts (P>0.5). PGD_2 is predominantly a mast cell product and it is frequently measured as a marker of mast cell activation. There was a decrease in the proportion of mast cells expressing COX-1 (P=0.0001) and COX-2 (P=0.08) in-season associated with the decrease in BAL fluid PGD_2 levels. A decrease in COX expression in mast cells may therefore contribute

to the fall in PGD_2 with seasonal allergen, although there are no direct relationships between the number of COX-1^+ or COX-2^+ mast cells and the concentration of PGD_2 in BAL fluid. There was no significant correlation between PGD_2 synthase positive cell counts and BAL fluid PGD_2, PGD_2 synthase expression in an individual cell type may be related to BAL fluid PGD_2. Other cell types may contribute to PGD_2 production.

3.6 Summary

Exposure of birch pollen allergic asthmatics to a natural birch pollen season significantly worsened peak flow and increased bronchial responsiveness, symptom scores for asthma and rhinitis were elevated associated with increased use of related medication compared to pre-season measurements. Whilst there was no increase in BAL fluid cys-LTs, counts of cells immunostaining for 5-LO, FLAP, LTC_4 synthase and LTA_4 hydrolase in bronchial biopsies were increased in-season. However, counts of cells immunostaining for MRP-1 and MRP-2 were low and unchanged with seasonal allergen exposure. These increases in leukotriene pathway enzymes were associated with increased counts of eosinophils, macrophages, and $2D7^+$ basophils and a trend towards more neutrophils. Localisation of 5-LO, FLAP and LTC_4 synthase to mast cells, eosinophils and macrophages, has shown that influx of eosinophils and macrophages contributes significantly to the increased representation of these enzymes in the lung.

There were no changes in COX-1, COX-2 or PGD_2 synthase positive cell counts, but BAL fluid PGD_2 was reduced in-season. Localisation of COX isoenzymes to mast cells and eosinophils showed a decrease in the percentage of mast cells expressing these enzymes, which might be related to the decrease in BAL fluid PGD_2.

3.7 Discussion

Allergic asthmatic subjects experience a decrease in peak flow, increased bronchial responsiveness asthma and rhinoconjunctival symptom scores and medication use during seasonal allergen exposure to birch pollen.

This study has shown for the first time that deterioration in lung function is associated with an increase in bronchial mucosal eosinophils with seasonal allergen exposure, only previously seen in the bronchial mucosa after allergen challenge (Rossi et al. 1991) and in BAL with exposure to high pollen counts (Rak et al. 1991). Djukanovic and colleagues (1996) did not observe increased eosinophils during the grass pollen season as a large proportion of their subjects were sensitive to perennial allergens, especially house dust mite, which may have produced airway inflammation out of the

grass pollen season. The present study showed an increased mucosal count of macrophages, confirming previous findings with allergen challenge (Poston et al. 1992). Increased mucosal counts of 2D7 positive basophils were seen during the birch pollen season, this is in agreement with the finding that $2D7^+$ basophils were elevated in skin 6-24 hours after allergen challenge (Irani et al. 1998). Basophil counts have also been shown to be elevated prior to an asthma attack but not during or after the attack (Kimura et al. 1973). There was a trend towards an increase in neutrophils in the bronchial mucosa, associated with increased counts of LTA_4 hydrolase positive cells with allergen exposure. The increased neutrophil count may be a consequence and/or a source of elevated LTB_4 produced by increased numbers of LTA_4 hydrolase$^+$ cells. Neutrophils are elevated in sputum from severe asthmatics (Fahy et al. 1995), and in BAL from patients with nocturnal asthma (Martin et al. 1991) and is considered to be associated with late phase asthmatic reactions (Metzger et al. 1986, Diaz et al. 1986).

In contrast to the study performed by Djukanovic and colleagues, (1996) there was no change in mucosal counts of $CD4^+$ T-lymphocytes with seasonal allergen exposure, Djukanovic and colleagues also observed an increase in $CD3^+$ T-lymphocytes expressing the IL-2R ($CD25^+$) in BAL. Allergen challenge may also increase counts of mast cells, $CD3^+$, $CD4^+$ and $CD25^+$ T-cells in bronchial biopsies (Virchow et al. 1995, Montefort et al. 1994, Diaz et al. 1989). No increases in counts of these cells were seen in the present study, perhaps by virtue of the difference between a single high dose of allergen and longer, lower levels of allergen exposure in the natural pollen season.

The cys-LTs are the only mediators proven to be chemotactic for eosinophils *in vivo*. Inhalation of LTE_4 by asthmatic subjects causes an increase in numbers of eosinophils in the lamina propria 4 hours after challenge (Laitinen et al. 1993), LTD_4 inhalation has been shown to increase eosinophil counts in sputum from asthmatic subjects (Diamant et al. 1997). The cys-LT receptor antagonist pranlukast has reduced counts of $EG2^+$ eosinophils in the bronchial mucosa of allergic asthmatics compared to placebo (Nakamura et al. 1998). Zileuton also inhibited an allergen-induced increase in BAL eosinophilia in allergic asthmatics (Kane et al. 1996, Wenzel et al. 1995), and sputum eosinophils were reduced by a four week treatment with montelukast in asthmatics (Leff et al. 1997).

The present study also demonstrated for the first time an increase in immunostaining of bronchial mucosal cells for the four major enzymes of the leukotriene pathway 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase during the birch pollen season. 5-LO^+ cell counts were inversely related to morning and evening peak flow both pre- and in-season, suggesting that leukotrienes adversely influence lung function.

The eosinophil is entirely responsible for the 3-fold rise in counts of LTC_4 synthase$^+$ cells in season, both by influx and an increased expression of LTC_4 synthase. Influx of macrophages and eosinophils, and an increase in 5-LO expression in macrophages can account for allergen induced increases in 5-LO$^+$ cell counts. FLAP expression increased in eosinophils and mast cells, and FLAP$^+$ cell counts may also be increased by influx of macrophages.

In an *in vitro* study by Boyce and colleagues (1996) expression of LTC_4 synthase in eosinophils was examined during maturation in the presence of IL-3 and IL-5. In the first week of culture the eosinophils had $cPLA_2$, 5-LO and FLAP but did not express LTC_4 synthase or produce cys-LTs when stimulated with calcium ionophore. During weeks 2-4 the eosinophil matured and showed increasing LTC_4 synthase expression, cys-LT production, and increased expression of 5-LO and FLAP (Boyce et al. 1996). $cPLA_2$, 5-LO, FLAP and LTC_4 synthase are thus expressed sequentially in eosinophils. Expression of cytokines, chemokines and leukotriene C_4 which promote adhesion and migration, and reduce apoptosis of eosinophils *in vitro* are increased in BAL and the bronchial mucosa of symptomatic asthmatics, including IL-3, IL-4, IL-5, GM-CSF, eotaxin and RANTES (Robinson et al. 1993; Venge et al. 1996; Jose et al. 1994; Humbert et al. 1997). Maturation of eosinophils by increased expression of eosinophilopoietic cytokines by T-cells, mast cells and eosinophils in the asthmatic airway (Robinson et al. 1993; Bradding et al. 1994; Venge et al. 1996; Humbert et al. 1997; Virchow et al. 1995) could be contributing to increased expression of LTC_4 synthase, 5-LO and FLAP and increases cys-LT synthesis in asthmatics in-season. IL-5 also enhances the expression of FLAP in human peripheral blood eosinophils (Cowburn et al. 1999).

Baseline BAL fluid cys-LT levels correlated with the subset of macrophages expressing LTC_4 synthase indicating that basal levels of cys-LT production in the lung are macrophage derived. However, in-season, eosinophil expression of LTC_4 synthase was related to BAL cys-LTs. The high number of eosinophils in-season may therefore be responsible for a large proportion of cys-LT production in-season. Despite the raised level of leukotriene pathway enzymes in the tissue, there was no increase in BAL fluid cys-LTs with seasonal allergen exposure in the present study. Inhaled allergen challenge increases levels of LTC_4 in BAL fluid within 5 minutes (Wenzel et al. 1990). The lack of change in BAL fluid cys-LT following seasonal allergen exposure again may be indicative of the difference between the response to allergen challenge in the laboratory and to sustained exposure to low levels of allergen in clinical asthma. For example, the present study did not show the increased bronchial counts of mast cells, $CD3^+$, $CD4^+$ and

$CD25^+$ cells in-season which were observed after allergen challenge by Virchow and colleagues (1995), Montefort and colleagues (1994) and Diaz and colleagues (1989). In addition, activated eosinophils contribute to a respiratory burst, which produce oxygen radicals known to participate in the local peroxidative degradation of the cys-LTs (Lee et al. 1983a). This may be such that total BAL fluid cys-LT is not measurable in-season. MRP-1^+ and MRP-2^+ cell counts did not change with seasonal allergen exposure, this may indicate that while cells are synthesising more LTC_4 it cannot be exported any more efficiently. However, MRP-2^+ cell counts correlated with eosinophil counts in-season, this would suggest that patients with raised eosinophil counts have proportionally more MRP-2 and hence may release more LTC_4. Expression of MRP-1 and MRP-2 has never previously been studied in relation to asthma or allergy.

There was no change in counts of bronchial mucosal cells immunostaining for COX-1 or COX-2 with seasonal allergen exposure. Pre-season COX-1 and COX-2 expression was predominantly localised to mast cells. In-season, the proportion of mast cells immunostaining for COX-1 and COX-2 decreased by approx. 30%, ($p<0.0001$) and 20% ($p<0.08$) respectively. This was associated with a decrease in PGD_2 in BAL fluid in season. These results contrast with an increase in BAL PGD_2 after allergen challenge (Wenzel et al. 1992), but again highlight the difference between a single high provocation dose of allergen and continuous low level exposure to seasonal allergen. There were no relationships between COX and PGD_2 synthase immunostaining, BAL fluid PGD_2 levels, and clinical measures of asthma severity, suggesting that prostanoids do not contribute to decreased PEF, increased bronchial responsiveness, and higher symptom scores in-season.

NSAIDs have been reported to inhibit the early response to allergen by 30% (Manning et al. 1991), and blocking the TP receptor can also block this response in some patients (Beasely et al. 1989), suggesting a role for prostanoids in the pathophysiology of asthma. However, studies with indomethacin directly contradict this, this NSAID had no effect on allergen-induced bronchoconstriction in atopic asthmatics (Skoner et al. 1988). The results from the present study suggest that the bronchoconstrictor response to allergen in allergic asthmatics is more dependent on cys-LT production than on the production of prostanoids. It would have been useful to take a third biopsy from these patients after a period of treatment with a 5-LO inhibitor or COX inhibitor to determine the relative contributions of the leukotrienes or prostanoids respectively to clinical symptoms and bronchial eosinophilia in seasonal asthma.

CHAPTER 4

Effect of infection with human rhinovirus on eicosanoid enzyme expression in bronchial biopsies from normal and asthmatic subjects

Hypothesis: *Increased symptoms associated with rhinovirus infection in asthmatics, and the respiratory symptoms of a rhinovirus cold in normal subjects is caused by eicosanoid production. This increase in eicosanoid production is the result of an influx of inflammatory cells expressing the eicosanoid pathway enzymes and/or an increase in expression of eicosanoid pathway enzymes in resident inflammatory cells.*

RESULTS

4.1 Confirmation of viral infection.

All subjects were successfully infected with HRV as shown by positive viral cultures from nasal lavage taken 3-5 days after inoculation. Four of the 9 normal subjects, and 4 of the 6 asthmatic subjects also demonstrated seroconversion, with at least four-fold rises in their antibody titres (**Table 4.1**). Four of the 9 normal subjects and all 6 of the asthmatic subjects satisfied the modified Jackson's criteria for a symptomatic cold with a symptom score of 14 or more. There were no significant differences in viral infection between the normal (n=9) and asthmatic (n=6) subjects as shown by median culture scores (normal: 12, range 4 - 12; asthmatic: 11.5, range 6-12; p=0.86) and median titre rises (normal: 8, range 0-64, n=7); (asthmatic: 4, range 0-16, n=5; p=0.46) (**Table 4.1**). The response to the infection was also comparable between normals and asthmatics as assessed by median cold score, (normals: 13, range 5-29; asthmatics: 21, range 14-24; p=0.3) (**Table 4.1**).

4.2 Effect of HRV 16 infection on clinical measures of lung function.

Before infection, asthmatic subjects (n=6) had significantly lower FEV_1 (90.3 ±2.9% of predicted) than the normal subjects (99.8 ± 2.8% predicted, n=9, p=0.04). During HRV infection, FEV_1 did not change significantly compared to baseline in the asthmatic (89.2 ±3.7% n=6, p=0.7 Wilcoxon) or normal subjects (101.9 ± 2.8%, p=0.15) (**Figure 4.1**).
Before HRV infection, asthmatic subjects had significantly lower PC_{20} FEV_1 to inhaled histamine (geometric mean: 3.4mg/ml, 95% CI:0.14-7.03, n=5) than the normal subjects (geometric mean: 29.2mg/ml, 11.78-38.6, n=8; p=0.023) (**Figure 4.1**). In the asthmatic patients, HRV infection significantly increased bronchial responsiveness to histamine, with geometric mean PC_{20} values falling to 1.6mg/ml (95% CI: 0.24-23.2, P=0.001) (**Figure 4.1**). There was no significant change in PC_{20} in the normal subjects (geometric mean 18.0 mg/ml, 95% CI: 8.3-28.6, p=0.24) (**Figure 4.1**).

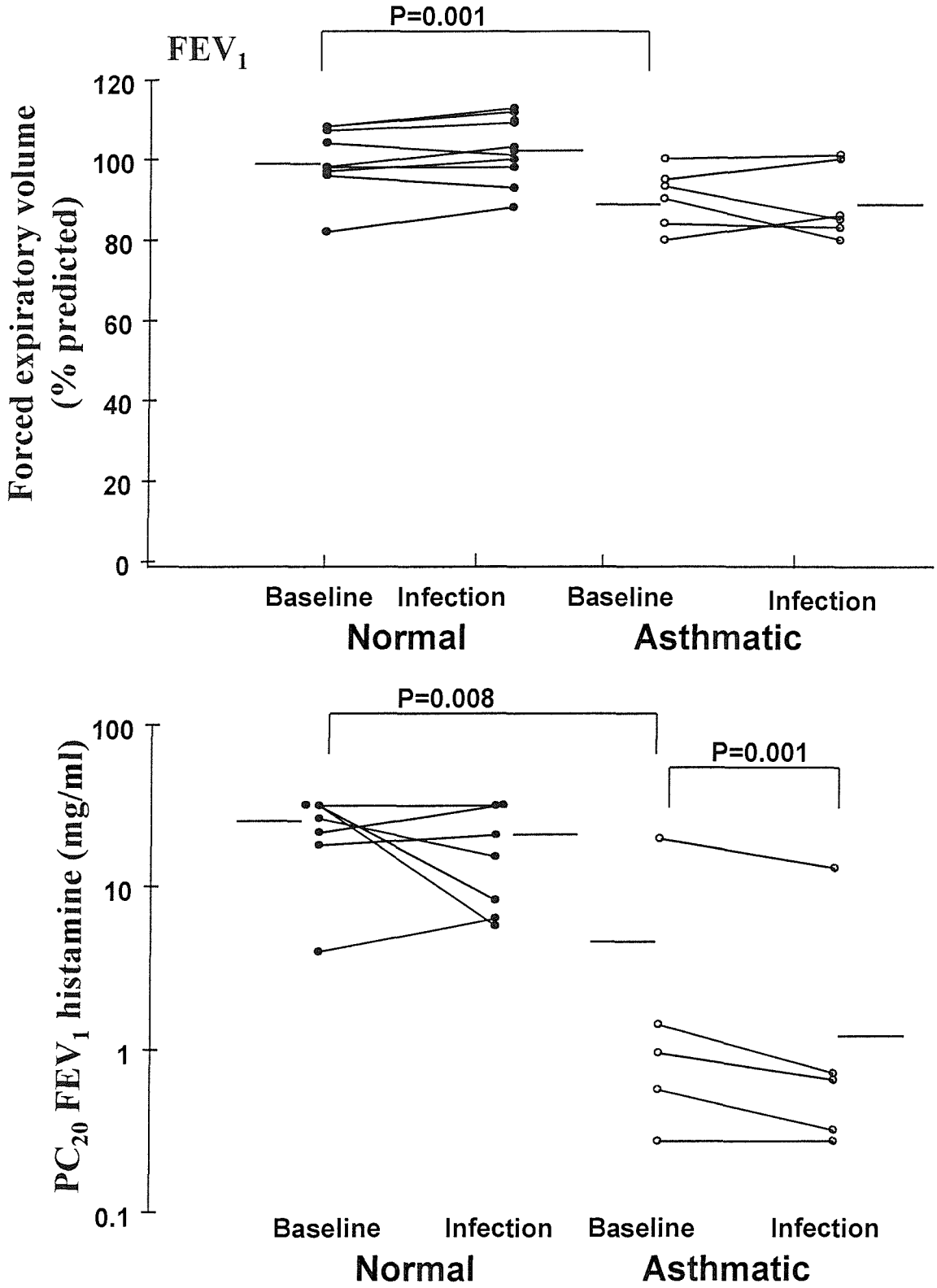


Fig.4.1 Clinical measures of lung function in 9 normal subjects (n=8 for PC_{20}) (solid circles) and 6 asthmatic subjects (n=5 for PC_{20}) (open circles) before and during HRV infection. Forced expiratory volume in 1s (FEV_1) (*top*) is unchanged in both normal (P=0.15) and asthmatic subjects (P=0.7). The asthmatic subjects had significantly lower FEV_1 than the normal subjects at baseline (P=0.04). During infection there was no change in bronchial responsiveness to histamine (PC_{20}) (*bottom*) in normal subjects (P=0.24) but there was a significant decrease in PC_{20} in the asthmatic subjects (P=0.001).

Table 4.1 Culture scores, cold scores and viral titre measurements in 9 normal subjects and 6 asthmatic subjects for the confirmation of viral infection.

Subject	Cold Score	Culture Score	Titre rise (x)
NORMALS			
GW	5	12	16
MC	6	12	2
CF	24	12	
SP	13	10	32
CS	10	4	1
CB	28	12	8
AM	29	10	64
JP	15	12	4
DB	11	11	
median	**13**	**12**	**8**
ASTHMATICS			
PB	18	6	1
MB	24	12	4
KM	24	6	4
PP	24	12	4
GM	18	12	16
JS	14	11	
median	**21**	**11.5**	**4**

4.3 Immunohistochemical analysis of leukotriene pathway enzyme expression in bronchial biopsies.

Immunohistochemical analysis of bronchial biopsies from normal subjects showed a two-fold increase in the number of cells staining positively for 5-LO from 2.1 ± 0.9 cells/mm^2 pre-infection to 5.0 ± 0.9 cells/mm^2 during infection (p=0.05, n=9) (**Figure 4.2**). This rise is accompanied by a strong trend towards a 2.5 fold increase in the counts of cells staining positively for FLAP from 3.76 ± 1.6 cells/mm^2 to 10.1 ± 2.6 cells/mm^2 (p=0.07, n=9) (**Figure 4.2**). There were no changes in counts of cells staining positive for LTA_4 hydrolase (2.8 ±1.2 cells/mm^2 pre-infection, 2.2 ± 0.6 cells/mm^2 during

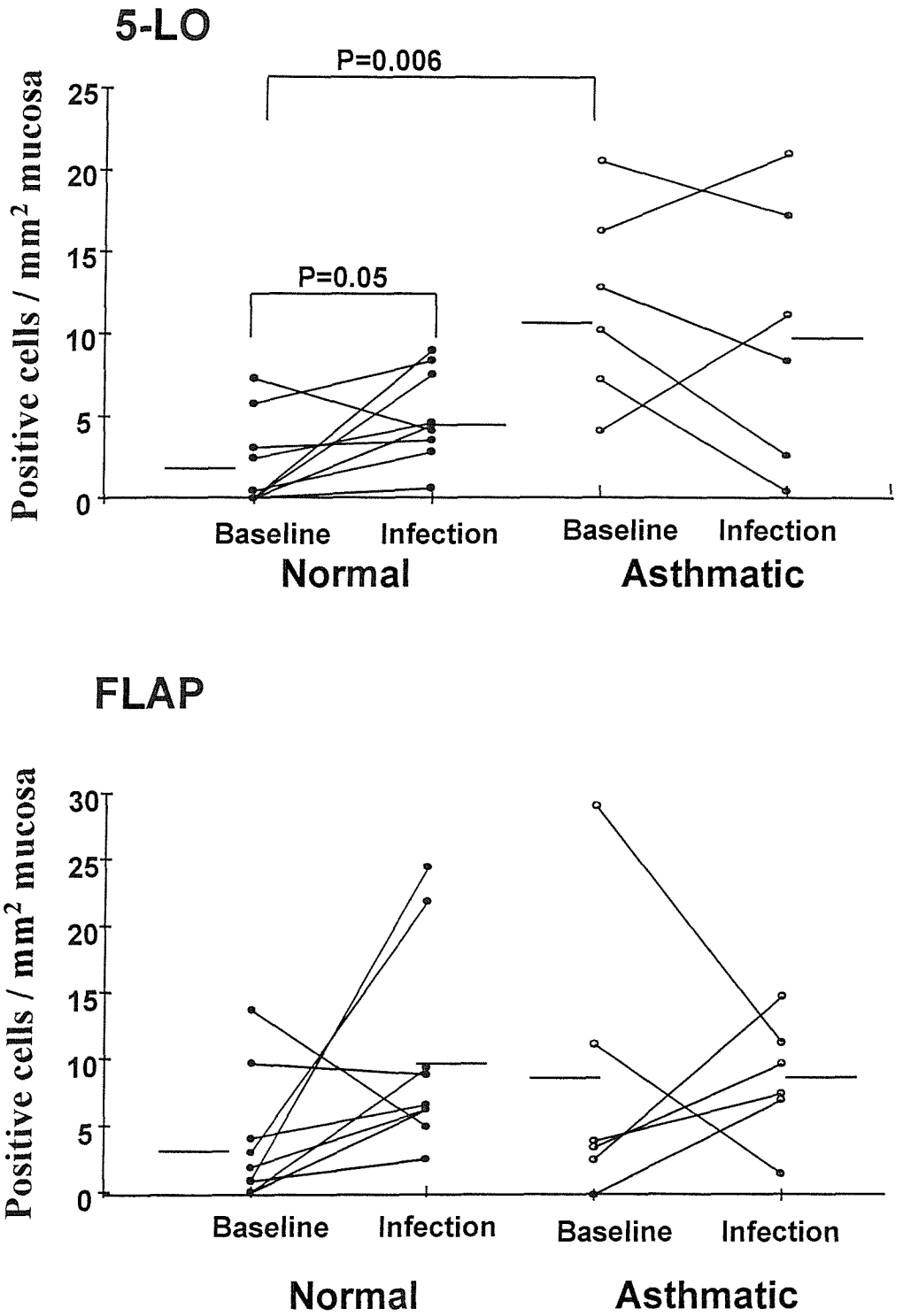


Fig. 4.2 Counts of cells immunostaining for 5-LO (*top*) and FLAP (*bottom*) in the bronchial mucosa of 9 normal and 6 asthmatic subjects before and during HRV infection. There was a 2-fold increase in 5-LO$^+$ cells in normals with HRV ($p=0.05$). Counts were unchanged in asthmatics ($p>0.6$) but at baseline the asthmatics had higher counts than normals ($p=0.006$). FLAP$^+$ cell counts trended towards an increase in normal subjects ($p=0.07$). Counts were unchanged in asthmatics ($p>0.9$) and there was no difference between the normal and asthmatic groups at baseline ($p>0.3$).

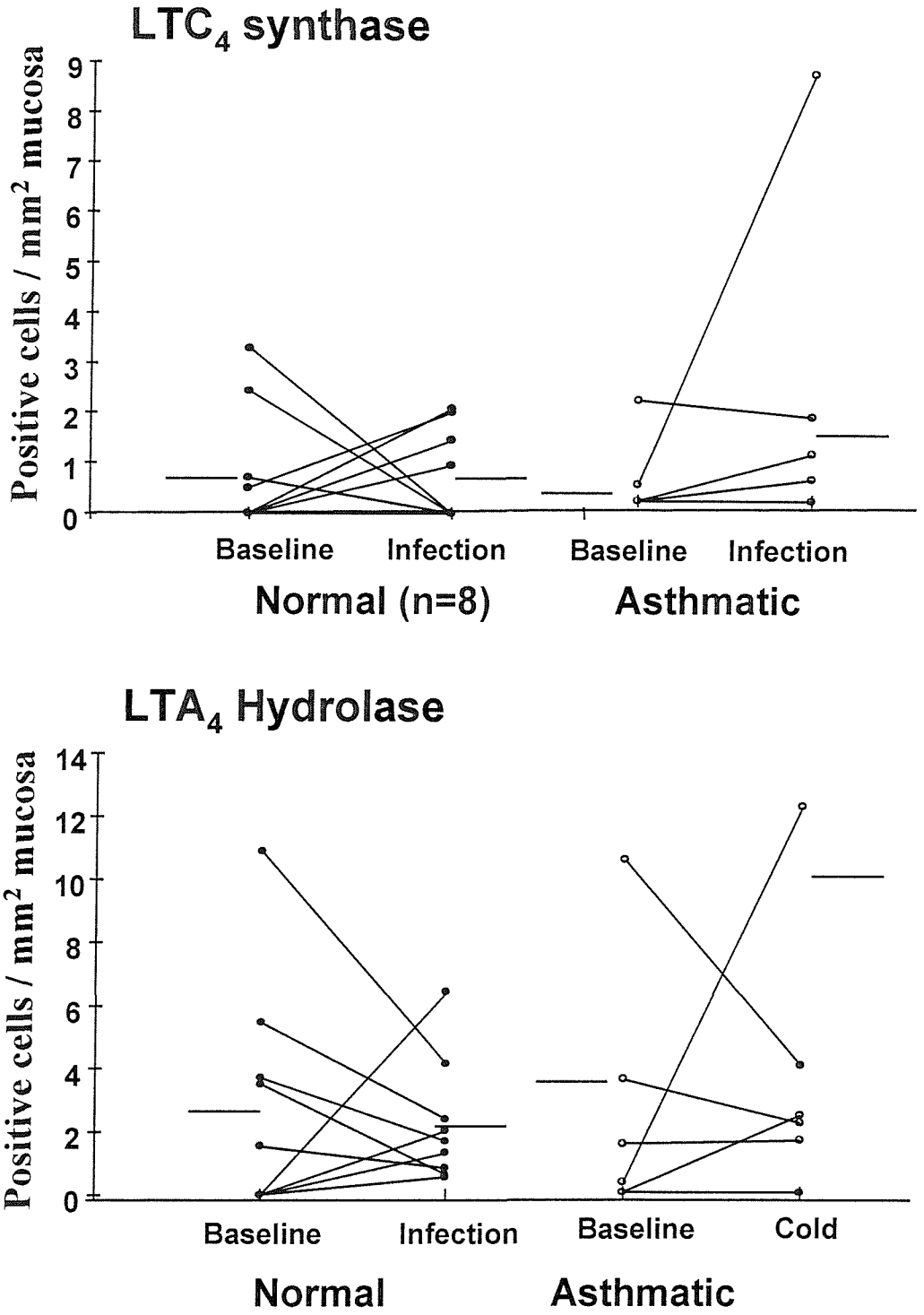


Fig. 4.3 Counts of cells immunostaining for LTC_4 synthase (*top*) and LTA_4 hydrolase (*bottom*) in the bronchial mucosa of 9 normal (n=8 for LTC_4 synthase) and 6 asthmatic subjects before and during HRV infection. There were no changes in LTC_4 synthase$^+$ or LTA_4 hydrolase$^+$ cell counts in either normal ($P>0.5$) or asthmatic subjects ($P>0.15$) with HRV infection. Counts of cells at baseline were not different between normal and asthmatic subjects ($p>0.4$).

infection; p>0.5, n=9) or LTC_4 synthase (0.9 ± 0.4 cells/mm^2 pre-infection, 0.8 ± 0.3 cells/mm^2 during infection; p>0.5, n=8) (**Figure 4.3**).

At baseline, the asthmatic group had higher counts of 5-LO$^+$ cells than the normal group (p=0.006, n=9 v n=6) but FLAP$^+$ cell counts were not significantly different between the groups (p=0.32) (**Figure 4.2**). There was no difference between LTC_4 synthase$^+$ cell counts between the asthmatic and normal groups at baseline (p>0.4, n=8 v n=6) (**Figure 4.3**). There was no difference in counts of cells staining for LTA_4 hydrolase when comparing the normals and asthmatics at baseline (p>0.9, n=9 v n=6) (**Figure 4.3**).

In contrast to the normal subjects, in the asthmatic group, there is no change in the counts of cells staining positively for 5-LO (11.7 ± 2.5 cells/mm^2 pre-infection, 9.95 ± 3.4 cells/mm^2 during infection, p>0.6, n=6) or FLAP (9.1 ± 4.8 cells/mm^2 pre-infection, 9.41 ±2.0 cells/mm^2 during infection, p>0.9, n=6) (**Figure 4.2**). Counts of LTC_4 synthase$^+$ cells were unchanged with HRV infection (0.4 ± 0.33 cells/mm^2 pre-infection, 2.0 ± 1.4 cells/mm^2 during infection, p>0.3, n=6). There was no change in LTA_4 hydrolase$^+$ cell counts (4.12 ± 1.7 cells/mm^2 pre-infection, 9.80 ± 6.3 cells/mm^2 during infection, p=0.18, n=6) (**Figure 4.3**).

There are consistent strong positive correlations between all the enzymes involved in leukotriene synthesis (5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase) in normal subjects at baseline (ρ>0.89, p<0.001 for all combinations). During infection these correlations are lost (ρ<0.4, p>0.2).

4.4 Immunohistochemical analysis of prostanoid pathway enzyme expression in bronchial biopsies.

In normal subjects, there were significant three-fold increases in the counts of cells staining positive for COX-2 from 1.4 ± 0.9 cells/mm^2 pre-infection to 4.6 ± 1.7 cells/mm^2 during infection (p=0.01, n=9) (**Figure 4.4**). This was associated with a trend towards an increase in COX-1$^+$ cells from 9.8 ± 5.5 cells/mm^2 pre-infection to 11.6 ± 2.2 cells/mm^2 during infection (p=0.09, n=9) (**Figure 4.4**).

The asthmatic group had significantly more COX-2$^+$ cells at baseline than the normal subjects (p=0.02, n=9 v n=6), this was not seen with COX-1 (p>0.26 n=9 v n=6) (**Figure 4.4**).

The asthmatic group showed a trend towards increases in positive cell counts for COX-1 (21.9 ± 12.0 cells/mm^2 pre-infection, 37.8 ± 20.0 cells/mm^2 during infection, p=0.08, n=6). There was no change in COX-2$^+$ cell counts with infection (14.6 ± 6.9

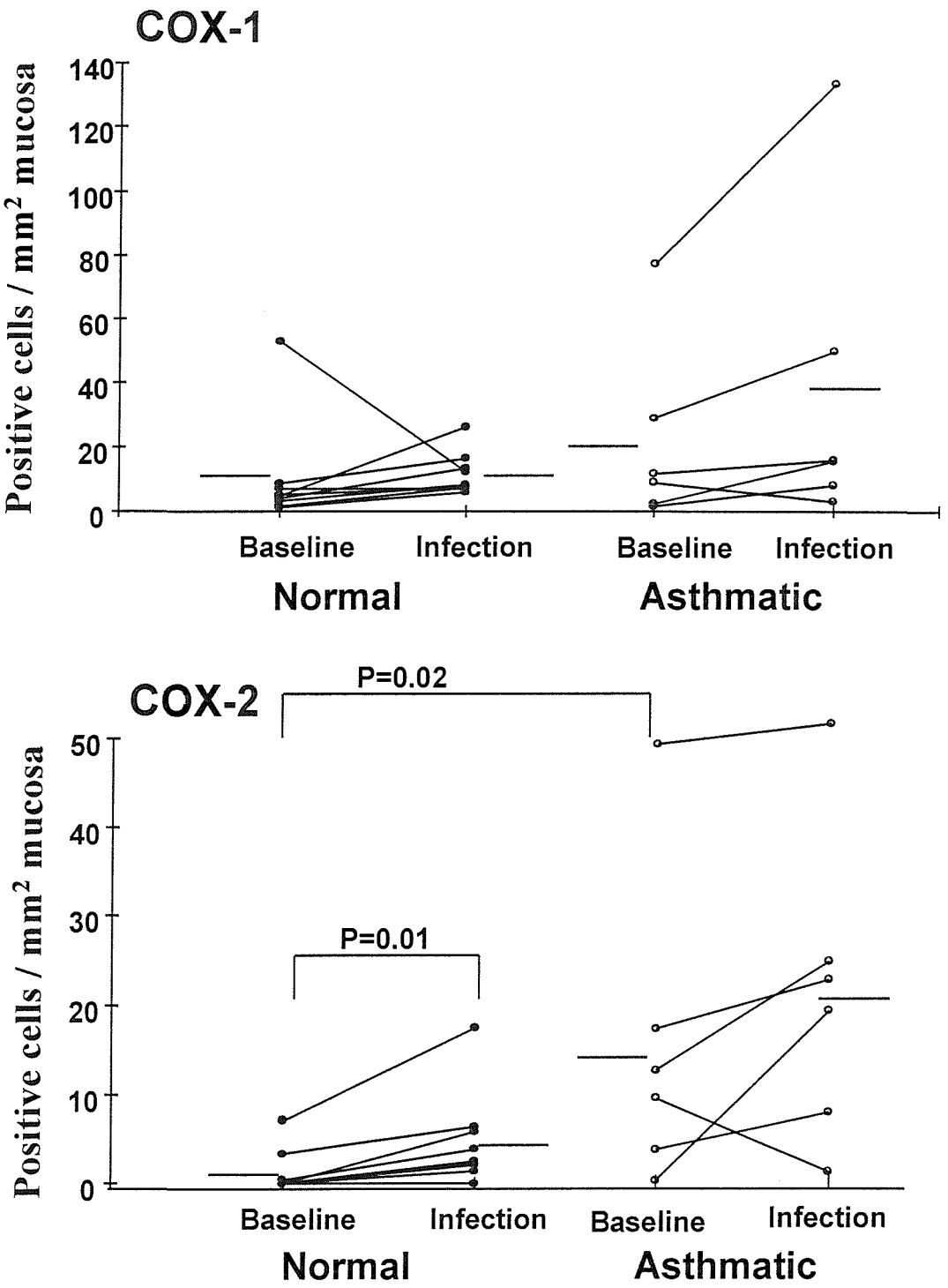


Fig.4.4 Counts of cells immunostaining for COX-1 (*top*) and COX-2 (*bottom*) in the bronchial mucosa of 9 normal and 6 asthmatic subjects before and during HRV infection. COX-1$^+$ cell counts trended towards an increase in asthmatic subjects with HRV ($P<0.09$). There was no difference between normal and asthmatic subjects at baseline ($P>0.25$). There was a 3-fold increase in COX-2$^+$ cell counts in normal subjects with infection ($p=0.01$), but there was no change in counts in asthmatics ($p=0.18$). COX-2$^+$ cell counts were higher in asthmatics than in normals at baseline ($p=0.02$).

cells/mm^2 pre-infection, 20.1 ± 6.8 cells/mm^2 during infection, p=0.18, n=6) (**Figure 4.4**).

4.5 Immunohistochemical analysis of leukocyte cell markers in the bronchial mucosa.

Immunohistochemical analysis of bronchial biopsies from normal subjects showed that counts of CD68$^+$ macrophages rose significantly from 4.8 ± 2.4 cells/mm^2 pre-infection to 10.6 ±1.9 cells/mm^2 during infection (p=0.02 Wilcoxon, n=8). (**Figure 4.6**). There were also strong trends to rises in counts of tryptase positive (AA1$^+$) mast cells from 9.71 ± 3.6 cells/mm^2 to 19.9 ± 3.5 cells/mm^2 (p=0.09 Wilcoxon n=9) (**Figure 4.5**). CD4$^+$ T-helper cell counts also tended to increase from 15.7 ± 7.9 cells/mm^2 to 52.0 ± 31.8 cells/mm^2 (p=0.086 Wilcoxon, n=9) (**Figure 4.7**). There were no changes in counts of eosinophils (8.3 ± 4.3 cells/mm^2 pre-infection; 11.5 ± 4.6 cells/mm^2 during infection, p=0.27, n=9) (**Figure 4.5**), CD3$^+$ T-cells (26.5 ± 13.2 cells/mm^2 pre-infection; 82.5 ± 36.9 cells/mm^2 during infection, p=0.13, n=8) (**Figure 4.6**) or CD8$^+$ T-cells (4.69 ± 2.7 cells/mm^2 pre-infection; 22.3 ± 12.1 cells/mm^2 during infection, p=0.18, n=9) (**Figure 4.7**).

Baseline mast cell counts were significantly higher in the asthmatic subjects than in the normal subjects (p=0.039, n=9 v n=6) (**Figure 4.5**). There was no difference in eosinophil counts between the two groups at baseline (p=0.18, n=9 v n=6) (**Figure 4.5**). or in counts of CD3$^+$ cells (p=0.11, n=8 v n=6) (**Figure 4.6**). Counts of CD4$^+$ T-cells were not significantly different between the groups at baseline (p=0.18, n=9 v n=6) (**Figure 4.7**). but the asthmatic subjects had significantly more CD8$^+$ cells than normals (p=0.05, n=9 v n=6) (**Figure 4.7**).

Analysis of bronchial biopsies from asthmatics showed no rise in macrophage counts with HRV infection (14.1 ± 5.6 cells/mm^2 pre-infection, 11.86 ± 2.9 cells/mm^2 during infection, p=0.13, n=6), but there was a strong trend towards higher baseline macrophage counts in asthmatics compared to normal subjects (p=0.08 Mann Whitney U test n=8 v n=6) (**Figure 4.6**). There was no change in mast cell counts in the asthmatic subjects (37.3 ± 12.0 pre-infection, 46.3 ± 14.0 during infection, p=0.22, n=6) (**Figure 4.5**). There was no increase in mucosal eosinophil counts in the asthmatic group (11.9 ± 1.7 cells/mm^2 pre-infection, 14.5 ± 4.6 cells/mm^2 during infection, p=0.61, n=6). CD3$^+$ T-cell counts in the asthmatic subjects were very weakly trending towards an increase from 48.1 ± 13.0 cells/mm^2 pre-infection to 81.3 ± 27.0 cells/mm^2 during infection, (p=0.25, n=6) (**Figure 4.6**). There was no increase in CD4$^+$ T-cell counts in the asthmatic group (22.3 ± 4.9 cells/mm^2 pre-infection, 27.0 ± 7.5 cells/mm^2 during

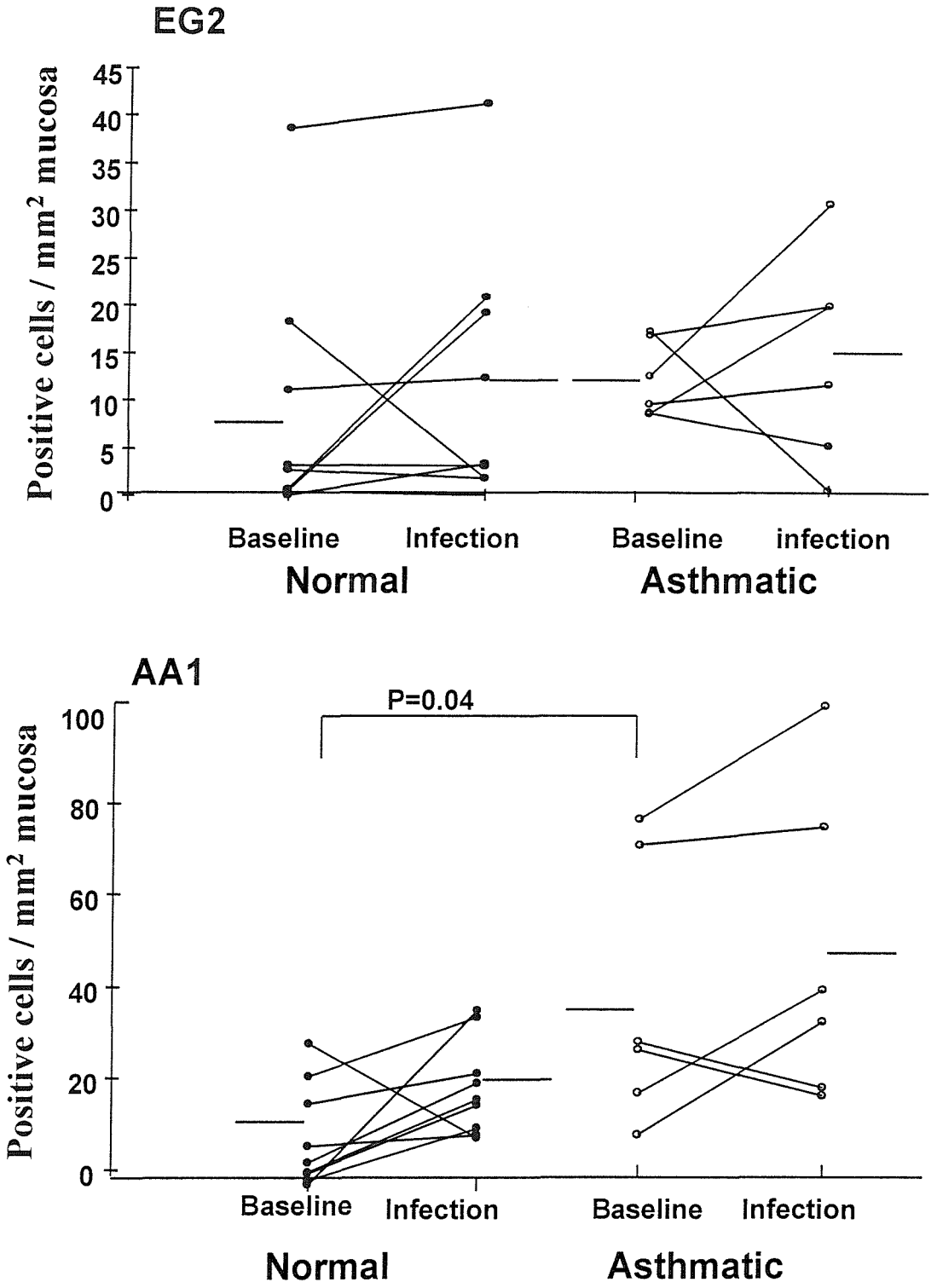


Fig. 4.5 Counts of eosinophils (EG2) (*top*) and mast cells (AA1) (*bottom*) in the bronchial mucosa of 9 normal and 6 asthmatic subjects before and during HRV infection. HRV caused no changes in eosinophil counts in either group ($p>0.25$). There was no difference between the normals and asthmatics at baseline ($p=0.18$). There was a strong trend towards higher mast cell counts in normal subjects with infection ($p=0.09$) but counts were unchanged in asthmatics ($p=0.22$). Baseline mast cell counts were significantly higher in asthmatics compared to normals ($p=0.04$).

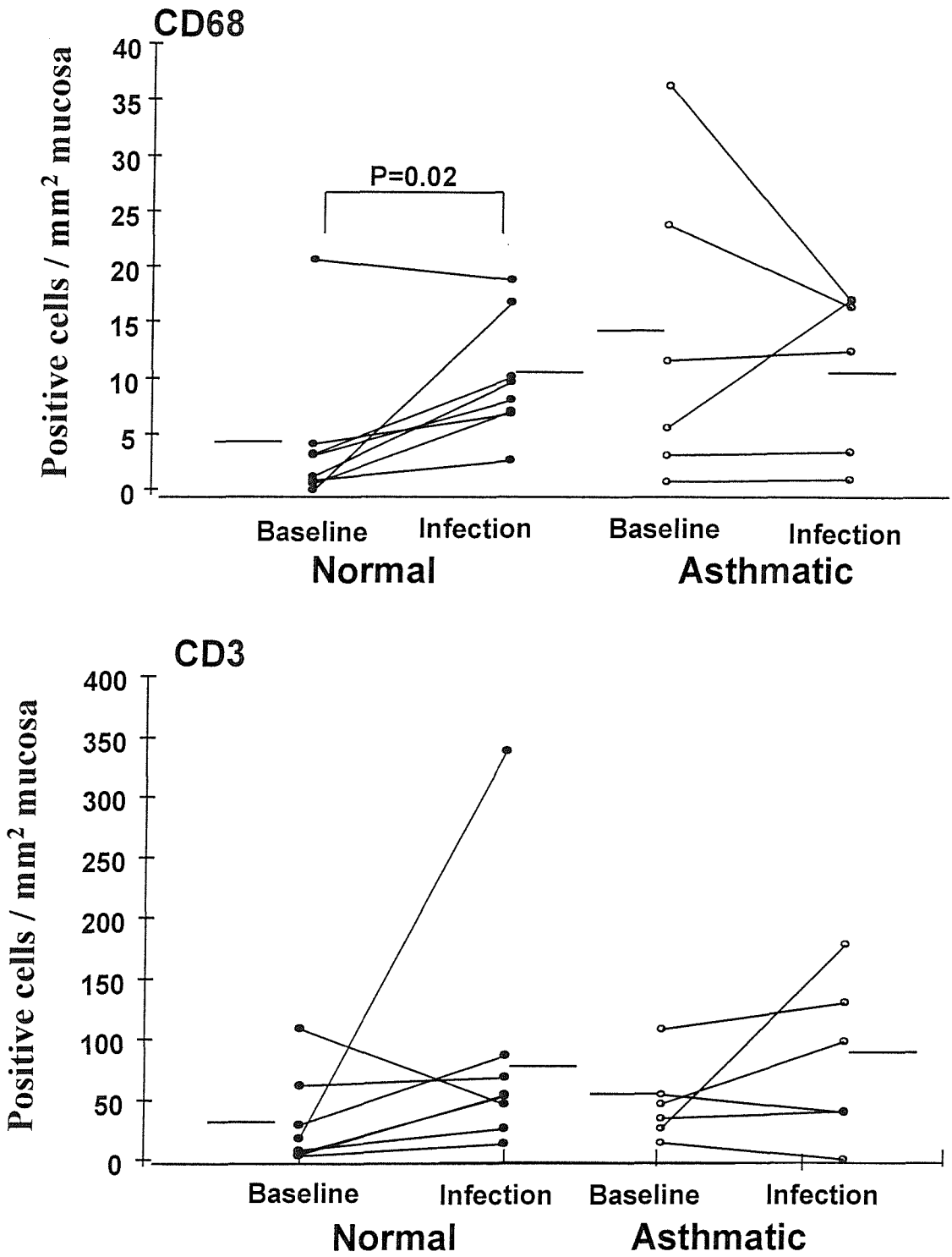


Fig.4.6 Counts of macrophages (CD68) (*top*) and total T-cells (CD3) (*bottom*) in the bronchial mucosa of 8 normal and 6 asthmatic subjects before and during HRV infection. The asthmatic subjects had a trend towards higher macrophage counts than normals at baseline (P=0.08). There was no difference in $CD3^+$ cell counts between the two groups at baseline (P=0.11). Counts of $CD68^+$ macrophages increased in normal subjects with infection (P=0.02), there was no change in counts in asthmatic subjects with infection (P=0.13) There was no increase in $CD3^+$ T-cell counts in either group with infection (normals P=0.13, asthmatics P=0.25).

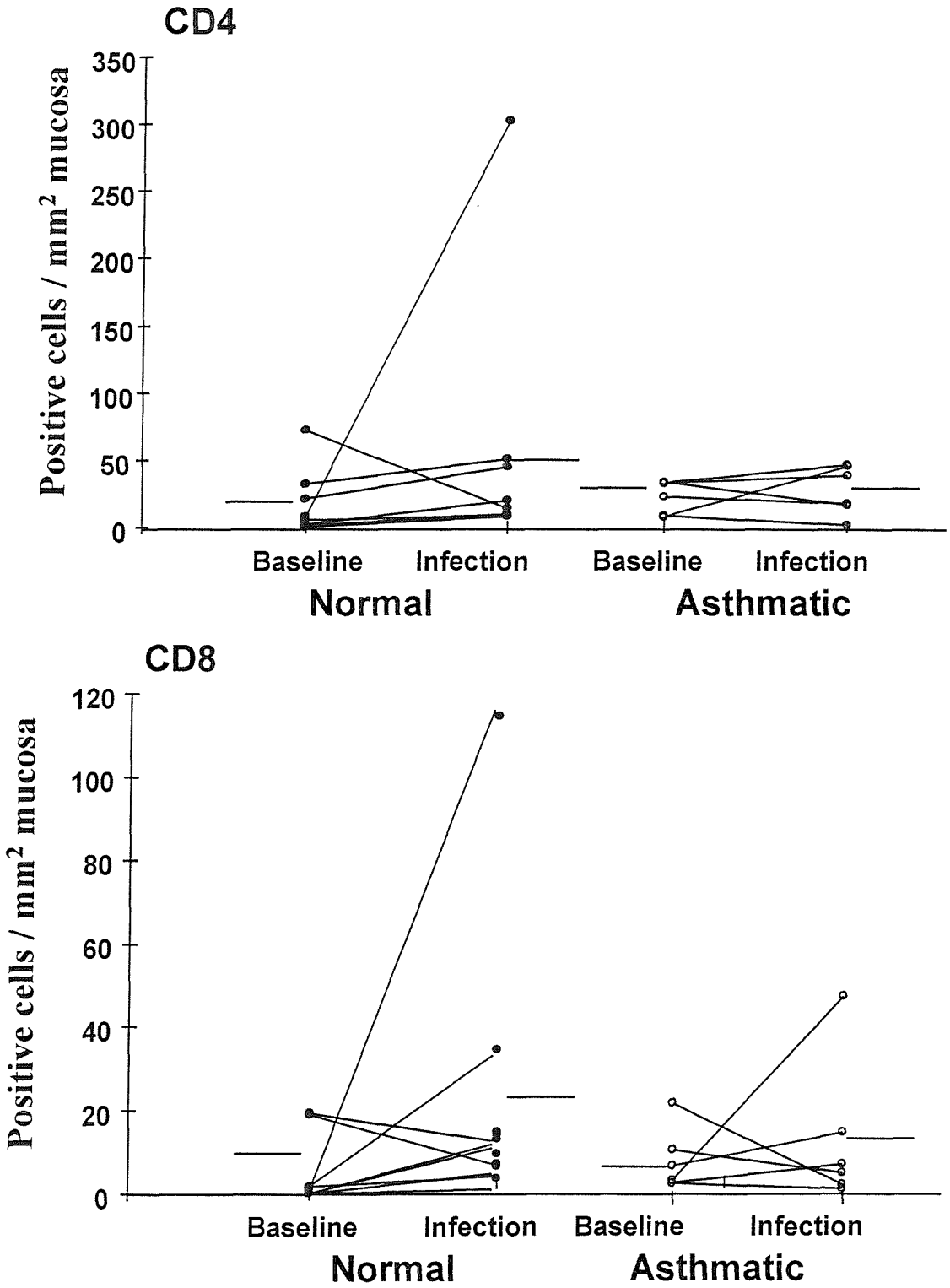


Fig.4.7 Counts of $CD4^+$ T-helper cells (*top*) and $CD8^+$ cytotoxic T-cells (*bottom*) in the bronchial mucosa of 9 normal and 6 asthmatic subjects before and during HRV infection. There was also no difference in CD4+ cell counts at baseline (P=0.18) but the asthmatic subjects had significantly more $CD8^+$ T-cells than normals (p=0.05). $CD4^+$ T-cell counts trended towards an increase in the normal group with infection (P=0.086), but there was no change in asthmatics (P=0.8). $CD8^+$ T-cell counts were unchanged in both the normal (P=0.18) and asthmatic (P=0.8) groups.

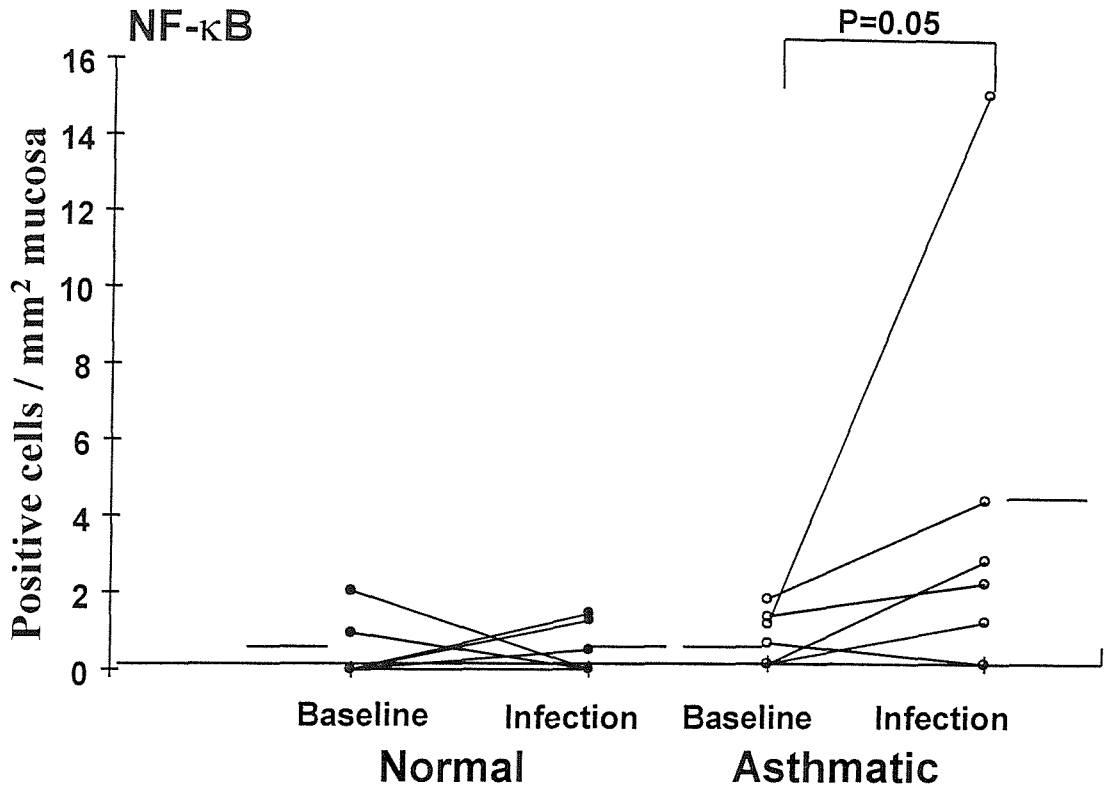


Fig.4.8 Counts of cells immunostaining for activated NF-κB (2C7) in the bronchial mucosa of 6 normal (closed circles) and 6 asthmatic (open circles) subjects before and during HRV infection. There was no difference in counts of NF-κB$^+$ cells between the 2 groups at baseline (P=0.078). There was no change in 2C7$^+$ cell counts in the normal subjects with infection (P>0.9), but counts were significantly increased in the asthmatic subjects (P=0.05).

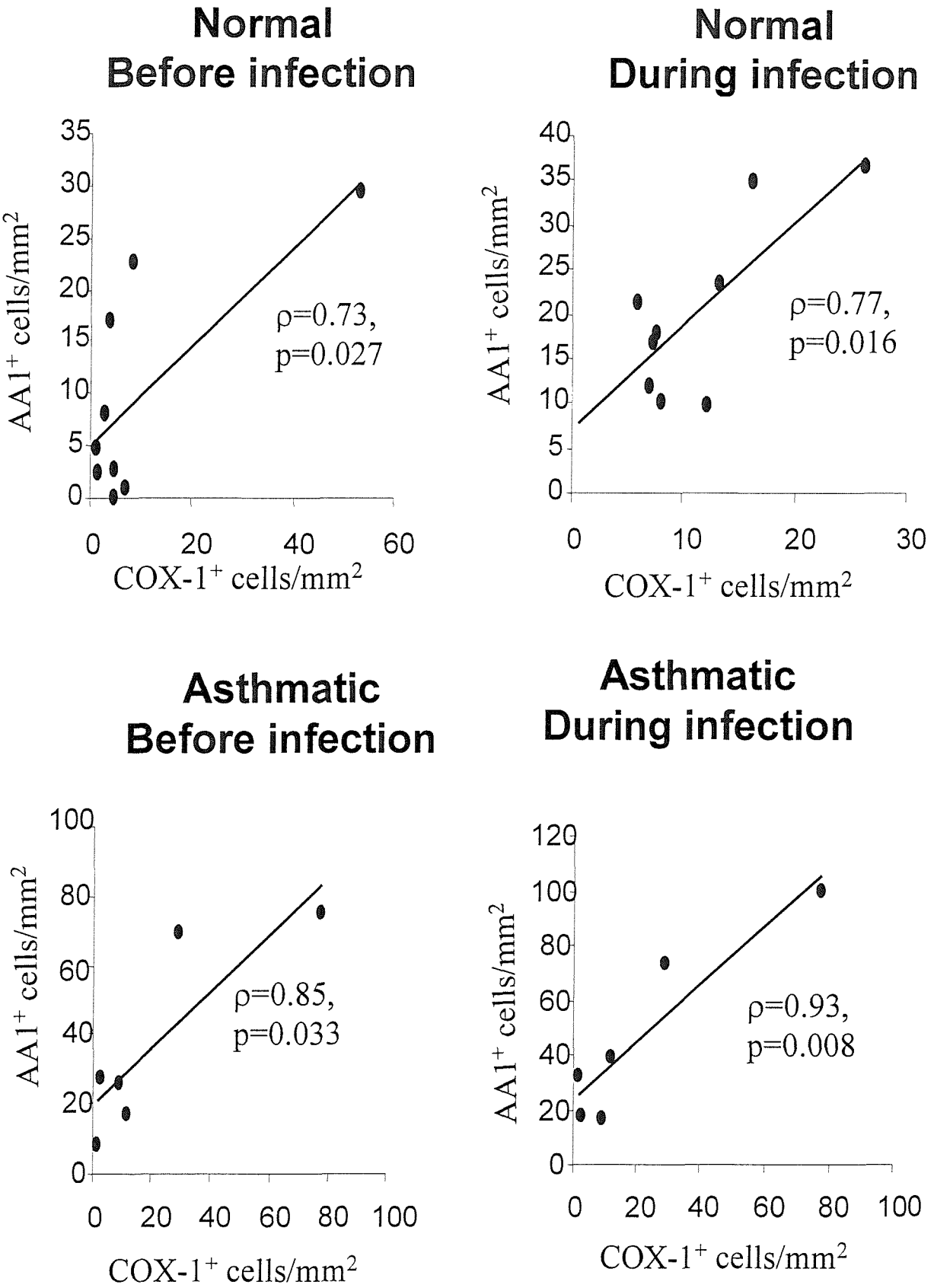


Fig. 4.9 Correlation between counts of mast cells and COX-1$^+$ cells in normal and asthmatic subjects before and during HRV infection

infection, p=0.8, n=6), $CD8^+$ cell counts were also unchanged with HRV (8.69 ± 3.0 cells/mm^2 pre-infection, 13.8 ± 7.1 cells/mm^2 during infection, p=0.80, n=6).

In the normal subjects during infection there were unique correlations between titre rise and all three T-lymphocyte populations ($\rho>0.83$, $p<0.04$). These relationships were not apparent in the normal subjects before infection, or in the asthmatic subjects.

4.6 Immunohistochemical analysis of activated NF-κB in bronchial biopsies.

The antibody 2C7 recognises the nuclear localisation sequence (NLS) of the activated form of nuclear factor κB (NF-κB) which is masked by I-κB binding. At baseline, there was no difference in counts of NF-$κB^+$ cells between the normal and asthmatic groups ($p>0.4$, n=6 v n=6) (**Figure 4.8**). There was no change in immunostaining for NF-κB in the normal group where positive cell counts remained low (0.50 ± 0.34 cells/mm^2 pre-infection, 0.54 ± 0.27 cells/mm^2 during infection, $p>0.9$, n=6) (**Figure 4.8**).

However, in the asthmatic group there was a significant 5-fold increase in counts of cells staining positively for NF-κB from 0.76 ± 0.28 cells/mm^2 pre-infection to 4.2 ± 2.24 cells/mm^2 during infection (p=0.05, n=6) (**Figure 4.8**).

4.7 Relationship between cyclooxygenase and mast cells.

There were consistent correlations between COX-1^+ cell counts and counts of $AA1^+$ mast cells both in normals (n=9) before ($\rho=0.73$, p=0.027) and during ($\rho=0.77$, p=0.016) HRV infection, and in asthmatics (n=6) before ($\rho=0.85$, p=0.033) and during ($\rho=0.93$, p=0.008) HRV infection (**Fig. 4.9**). In normals, the HRV induced change in COX-1^+ cell counts correlated strongly with the change in mast cell counts ($\rho=0.88$, $p<0.001$; n=9) suggesting that COX-1 is constitutively expressed in the mast cell. There was a strong trend towards a significant correlation between COX-2^+ cells and mast cell counts ($\rho=0.62$, p=0.076) in normals during infection.

To further examine the effect of HRV infection on the expression of COX-2 in inflammatory cells, COX-2 was localised using the *camera lucida* technique to mast cells, macrophages and eosinophils in 6 representative normal subjects both before and during HRV infection. Before infection, counts of COX-2^+ cells in normal subjects at baseline were very low 0.63 cells/mm^2, and only 5% of mast cells immunostained for COX-2. COX-2 was not detectable in macrophages or eosinophils at baseline (**Fig 4.10**). During infection 8% of mast cells, 5% of macrophages, and 3% of eosinophils immunostained for COX-2 (**Fig. 4.10**). An increase in expression of COX-2 in mast cells, macrophages and eosinophils could entirely account for the increase in COX-2 in the bronchial mucosa of normal subjects with HRV infection. Of a total count of COX-2^+

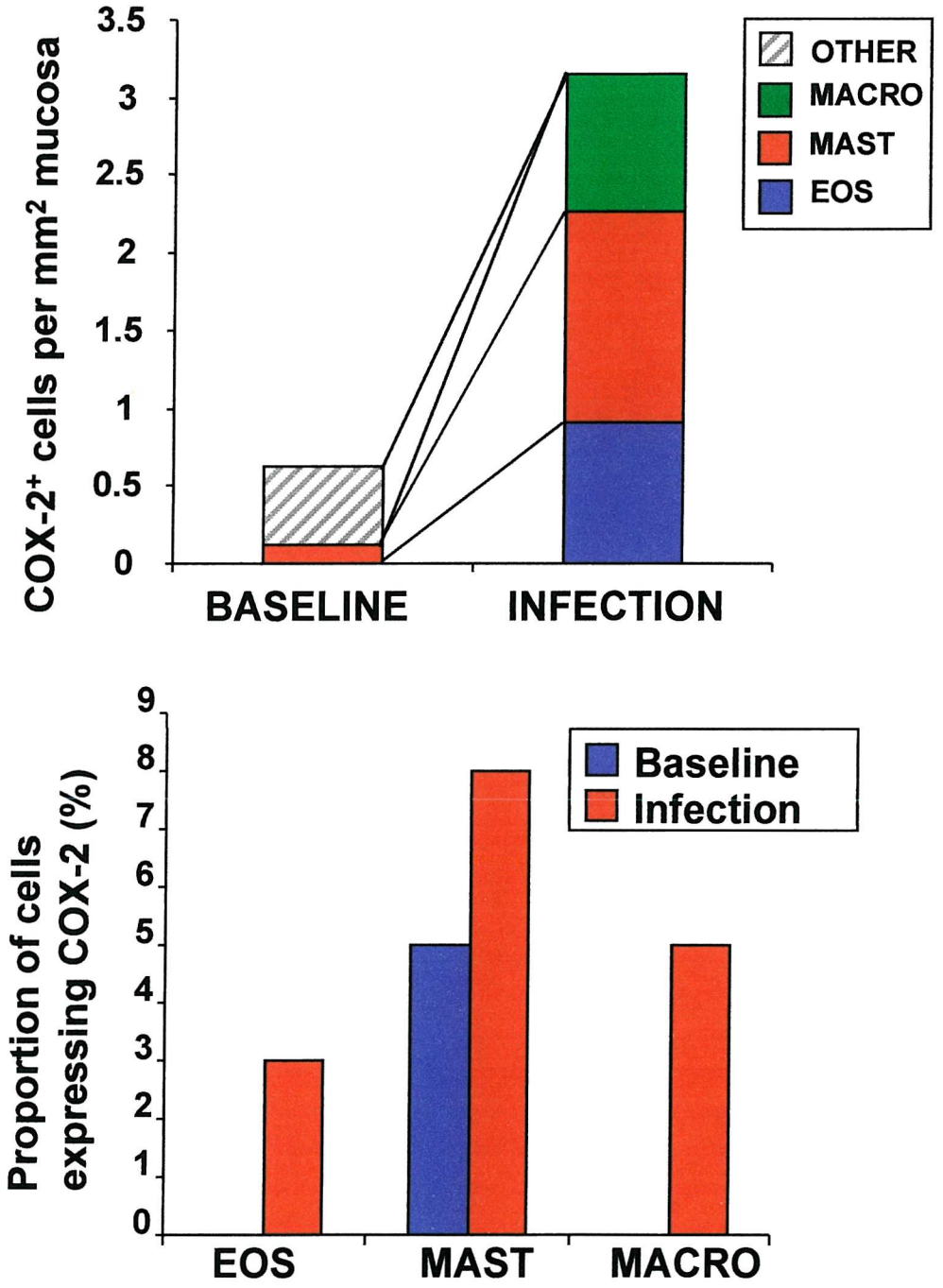


Fig. 4.10 Colocalisation of COX-2 positive cells to eosinophils, mast cells and macrophages in the bronchial mucosa of 6 representative normal subjects before (baseline) and during infection with HRV. ***Top***: At baseline, COX-2 can only be localised to mast cells, but eosinophils and macrophages immunostain for COX-2 with infection. ***Bottom:*** The proportion of all cells expressing COX-2 is increased with HRV infection only mast cells express COX-2 at baseline.

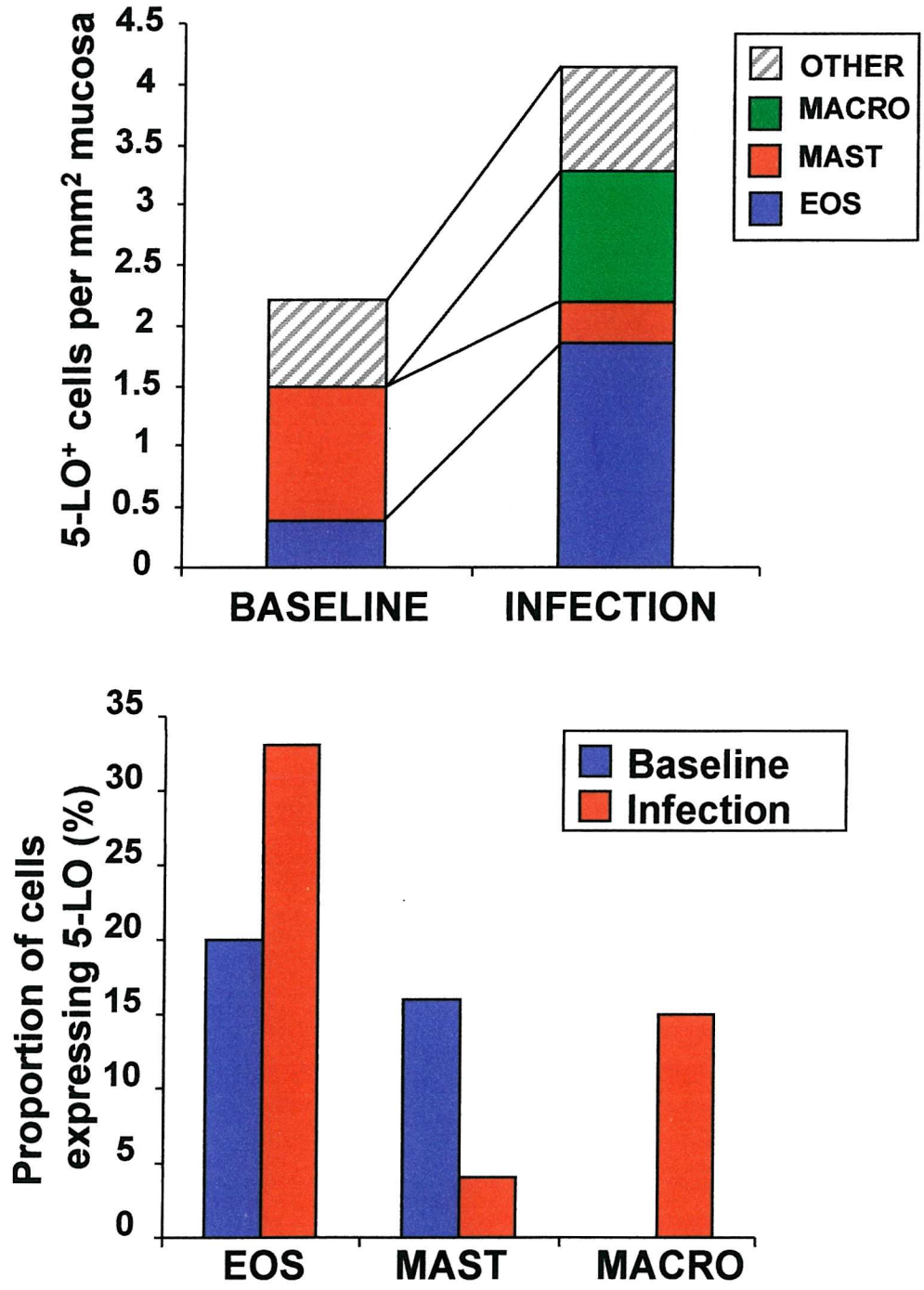


Fig. 4.11 Colocalisation of 5-LO to eosinophils, mast cells and macrophages in bronchial biopsies from 6 representative normal subjects before (baseline) and during infection with HRV. ***Top***: There was an increase in the number of 5-LO$^+$ cells which were eosinophils and macrophages at baseline, but the number of 5-LO$^+$ mast cells decreased. ***Bottom***: This was associated with increases in the proportion of eosinophils and macrophages which immunostained for 5-LO with HRV infection. The proportion of mast cells immunostaining for 5-LO decreased with HRV infection.

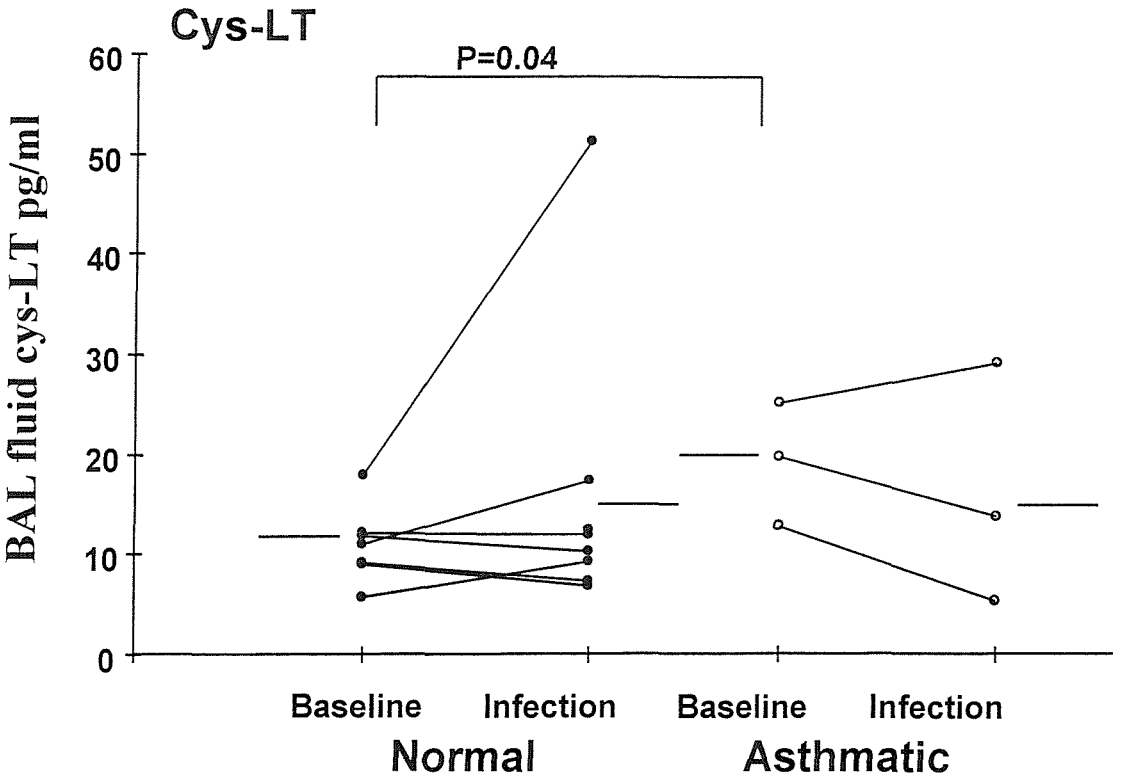


Fig.4.12 Concentration of cys-LTs in BAL fluid from 7 normal and 3 asthmatic subjects before and during HRV infection. The asthmatic subjects had significantly higher BAL fluid cys-LTs than normal subjects at baseline (P=0.04). There was no change in cys-LT levels in the BAL fluid from normal (P=0.5) or asthmatic subjects (P>0.4 Students paired T-test).

cells of 2.96 cells/mm^2 during infection, mast cells represented 1.36 COX-2$^+$ cells/mm^2, macrophages and eosinophils could account for 0.8 cells/mm^2 each (**Fig. 4.10**).

4.8 Relationship between leukotriene pathway enzymes and inflammatory cells.

The distribution of 5-LO in inflammatory cells in normal bronchial mucosa before and during HRV infection was demonstrated using the *camera lucida* technique. At baseline, 20% of eosinophils and 16% of mast cells were 5-LO positive, while 5-LO did not localise with macrophages (**Fig. 4.11**). With HRV infection 15% of macrophages and 33% of eosinophils immunostained for 5-LO, while the proportion of mast cells staining for 5-LO fell 4-fold to 4% (**Fig. 4.11**). The increase in 5-LO$^+$ cells in normal subjects with HRV infection can be attributed to an increase in expression of 5-LO in macrophages and eosinophils. There is an increase from 0.4 to 1.9 5-LO$^+$ cells/mm^2 which were eosinophils and a novel expression of 5-LO in macrophages representing 1.1 cells/mm^2 of a total of 4.14 cells/mm^2 which are 5-LO$^+$. 1.1 5-LO$^+$ cells/mm^2 were mast cells before infection and this fell to 0.33 cell/mm^2 during infection (**Fig. 4.11**).

4.9 Cysteinyl leukotriene measurements in BAL fluid.

An enzyme immunoassay was used to examine cys-LT levels in the BAL fluid from 7 normal and 3 asthmatic subjects before and during infection. The asthmatic subjects had significantly higher BAL fluid cys-LT levels before infection than the normals (p=0.04) (**Figure 4.12**). HRV infection did not change in the mean total BAL fluid cys-LT in normal subjects (11.0 ± 1.4 pg/ml (pre-infection), 16.4 ± 6.0 pg/ml (during infection), p=0.5, n=7) (**Figure 4.12**), or in the asthmatic subjects (19.0 ± 3.47 pg/ml (pre-infection), 16.0 ± 6.8 pg/ml (during infection), p>0.4 (Student's paired T-test), n=3) (**Figure 4.12**).

4.10 Summary

All subjects were successfully infected with HRV 16. Infection did not alter measurements of lung function in normal subjects, but the asthmatic subjects had poorer FEV_1 and increased bronchial responsiveness than the normal subjects. Bronchial responsiveness increased in the asthmatic subjects following HRV infection.

In bronchial biopsies from normal subjects, HRV increased counts of 5-LO$^+$ cells, and FLAP$^+$ cell counts tended to increase. Counts of cells immunostaining for LTC_4 synthase and LTA_4 hydrolase were not changed with HRV. The asthmatic subjects had higher 5-LO$^+$ cell counts than normal subjects at baseline, while counts of cells immunostaining for FLAP, LTC_4 synthase and LTA_4 hydrolase were not different

at baseline. HRV did not change counts of cells immunostaining for these enzymes in asthmatics.

Counts of COX-1^+ cells were unchanged in normal subjects with infection and were not different at baseline. COX-1^+ cell counts tended to increase in the asthmatic subjects with HRV. COX-2^+ cell counts were increased in normal subjects with HRV, and at baseline the asthmatic subjects had significantly higher counts than the normal subjects, but these were unchanged with infection.

Counts of mast cells trended upwards in normal subjects with infection and were significantly higher in asthmatics at baseline. Mast cell counts did not change in the asthmatic group with HRV. In normal subjects, HRV infection significantly increased macrophage counts, and the asthmatic group trended towards more macrophages than normals at baseline, but counts were unchanged in asthmatics with infection. Counts of eosinophils, $CD3^+$ T-cells, and $CD4^+$ T-cells were not different in all groups. Counts of $CD8^+$ cells were higher in the asthmatic group than the normal group at baseline, but did not change in either group with infection.

Immunostaining for activated NF-κB showed increased positive cell counts in asthmatics at baseline compared to normals, and cell counts also increased in the asthmatic group with infection, while there was no change in immunostaining for NF-κB in normal subjects.

There was no change in BAL fluid cys-LT measurements in either the normal or asthmatic group with infection, but the asthmatics had higher BAL fluid cys-LTs at baseline.

4.11 Discussion

This study was performed to extend the study of Fraenkel et al. (1995) which characterised cellular changes in the bronchial submucosa and epithelium during experimental infection with human rhinovirus serotype 16. Fraenkel and colleagues showed that when normal (n=11) and asthmatic (n=6) subjects were considered together, HRV infection induced a significant increase in bronchial responsiveness to histamine. Counts of submucosal $CD3^+$ lymphocytes increased significantly with infection. There were also trends towards increases in $CD4^+$ and $CD8^+$ T-lymphocytes, and all submucosal T-lymphocyte counts fell in convalescence. The increase in $CD3^+$ T-lymphocytes was also seen as a trend in the normal subjects when analysed separately. There were no changes in submucosal mast cells, eosinophils or neutrophils with viral infection although submucosal mast cell counts were decreased in convalescence. Weak

trends towards increased epithelial lymphocyte counts were apparent with HRV, and these fell significantly with convalescence. Epithelial eosinophil counts increased with HRV and persisted into convalescence in the asthmatic group. Peripheral blood lymphocytes were decreased by viral infection in the asthmatics and in the group as a whole and the change correlated with the change in PC_{20} histamine but not with changes in submucosal lymphocyte counts. There was no change in peripheral blood leukocytes (Fraenkel et al. 1995).

The present study investigated nine of the 11 normal, and all 6 of the asthmatic subjects to characterise changes in the expression of enzymes of the eicosanoid pathways, and to link these to inflammatory cell markers.

Macrophage counts were not measured by Fraenkel and colleagues (1995). The present study showed a significant 2-fold increase in $CD68^+$ macrophage counts in the bronchial mucosa of normal subjects during HRV infection, while counts were unchanged in the asthmatic group. Macrophages express ICAM-1, and binding of HRV to ICAM-1 induces macrophage activation and production of TNF-α (Gern et al. 1996). HRV cannot replicate in airway macrophages and by the production of inflammatory cytokines and mediators the macrophage might serve as an anti-viral defence cell and induce an inflammatory response to infection (Gern et al. 1996). Macrophages are phagocytic cells, which scavenge for invading organisms, small particles, ageing and damaged cells. These cells might be increased by HRV in the bronchial mucosa of normal subjects to ingest and remove cells infected with HRV. Macrophages do not increase in asthmatic subjects as their baseline counts are higher than in normals and hence might be better equipped for host defence.

There was no increase in $CD3^+$ T-lymphocytes in either the normal or asthmatic groups with HRV infection. When the data from the two groups were amalgamated in the current study an increase in $CD3^+$ cell counts was seen (35.8 ± 23.2 cell/mm^2 baseline; 50.3 ± 9.6 cell/mm^2 HRV; $P=0.052$, $n=15$) comparable to that seen by Fraenkel and colleagues ($P=0.05$, $n=17$) but the increase was not seen in each group analysed separately. The amalgamation of data from normal and asthmatic subjects is not recommended, as the groups may differ in their response to HRV infection, and also were very different at baseline. In agreement with the cited paper there was no change in counts of $CD4^+$ cells with HRV in either group. The increase in $CD8^+$ cells in Fraenkel et al. (1995) was not seen in the present study in the normal group ($P=0.18$), in the asthmatic group ($P=0.8$), or in both groups combined ($P=0.24$, $n=15$), although there was an upward trend.

Bronchial submucosal eosinophils were unchanged in both studies. The present study did not address epithelial cell counts separately and hence, cannot be compared with the increase in epithelial eosinophil counts observed by Fraenkel et al,. 1995. This increased epithelial eosinophil count was based on very low counts of eosinophils (~0-3.2 cell/mm epithelium) and hence may not be statistically robust.

Counts of mast cells were significantly 4-fold higher at baseline in asthmatic subjects than in normal subjects, and there was a trend towards increased mast cell counts in normal subjects with infection. Fraenkel et al. 1995, however, did not report any increase in mast cell counts with infection although a decrease in mast cell counts was seen in the convalescent phase of normal and asthmatic subjects together, indicating that HRV might indeed induce changes in the mast cell population.

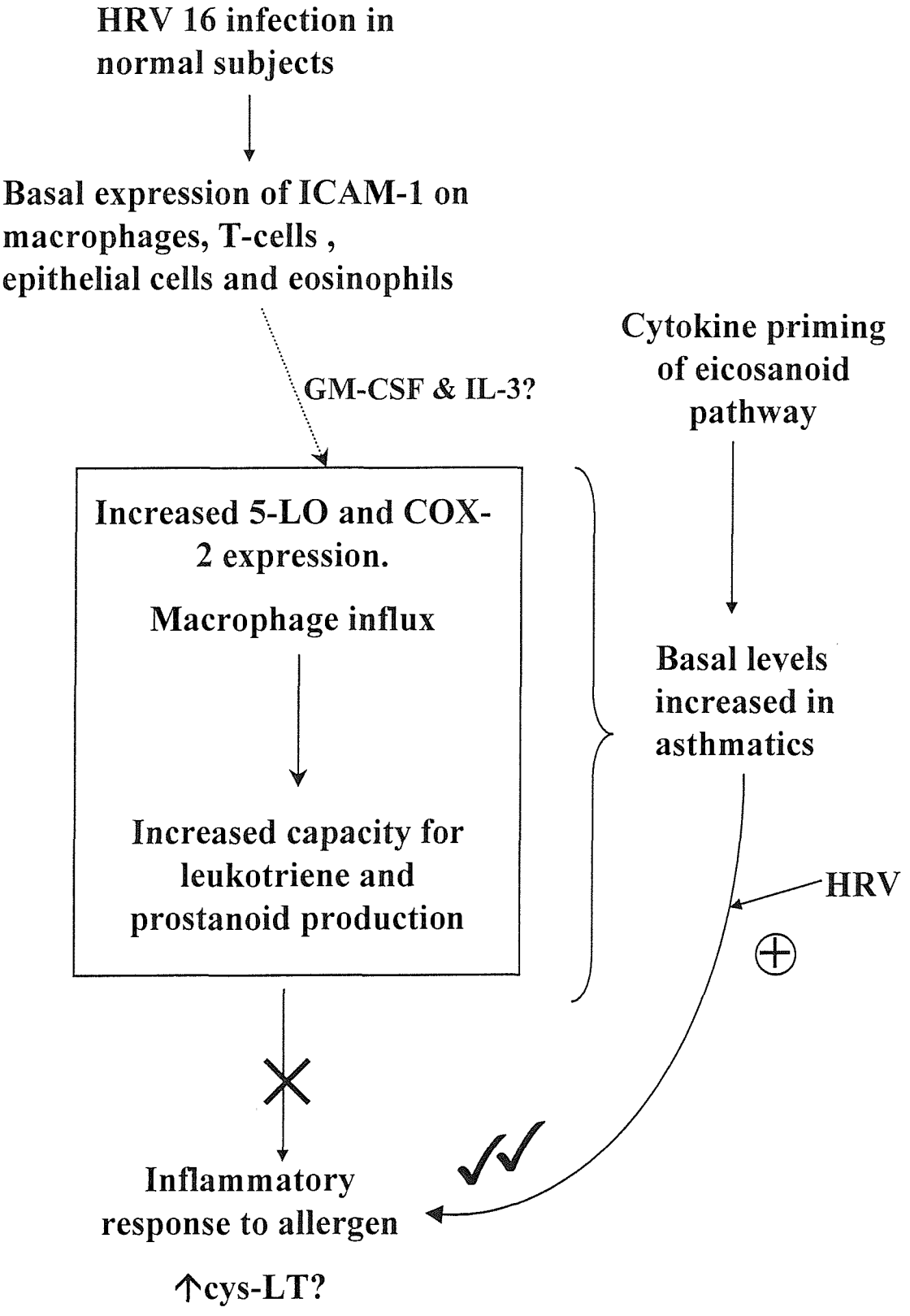
Immunohistochemical analysis of bronchial biopsies from normal subjects showed significant 2 and 3 fold increases in COX-2^+ and 5-LO^+ cell counts respectively during HRV infection with associated trends towards increases in the related enzymes COX-1 and FLAP, but not the downstream enzymes LTC_4 synthase or LTA_4 hydrolase. The correlations between counts of COX-1^+ cell and mast cells in both normal and asthmatic subjects at baseline and with HRV infection, and the observation that the change in mast cell counts correlated with the change in COX-1^+ cell counts in normal subjects with HRV, support the theory that COX-1 is constitutively expressed in the mast cell. The increase in COX-2^+ cell counts can be accounted for by increased expression of COX-2 in eosinophils, mast cells and macrophages, combined with an influx of macrophages and possibly mast cells. Similarly, 5-LO^+ cell counts were increased due to increased expression in eosinophils and macrophages, together with a contribution from macrophage influx. Overall, this suggests that the increases in 5-LO and COX-2^+ cell counts are largely dependent on macrophage influx (**Fig. 4.13**).

Inflammatory mediators in BAL fluid were not measured by Fraenkel et al. (1995) and have not previously been measured after HRV infection. LTC_4 has been shown to be elevated in nasal secretions from both wheezing and non-wheezing children after RSV infection (Volovitz et al. 1988). The increased representation of 5-LO and FLAP in inflammatory cells in normal subjects with HRV was not accompanied by changes in BAL fluid cys-LTs. It is possible that small changes in cys-LTs were missed due to local degradation by the peroxidative pathway (Lee et al. 1983a), but the lack of changes in lung function and bronchial responsiveness does not support this. It is more likely that the enzymes were not activated for the production of cys-LTs. Exposure of human blood monocytes to Epstein-Barr virus (EBV) causes rapid, transient priming of cells for LTB_4 and LTC_4 synthesis which involves stimulatory events at both the level of

5-LO activation and arachidonate release (Gosselin and Borgeat, 1997). EBV does not directly stimulate synthesis of leukotrienes in these cells, but primes them for a second stimulus such as fMLP or calcium ionophore (Gosselin and Borgeat, 1997). EBV is therefore suggested to regulate the LT synthetic pathway at the 5-LO enzyme level, such that the second stimulus enhances activation of 5-LO in a manner analogous to priming of LT synthesis by GM-CSF (Gosselin and Borgeat, 1997). Lymphocyte derived GM-CSF and IL-3 have been shown to increase expression of 5-LO and FLAP in monocytes (Ring et al. 1996). Cytokine priming of macrophages following HRV binding to T-cells or other inflammatory cells might be responsible for the increase in 5-LO in the present study, and a second stimulus may be required for production of cys-LTs (**Fig. 4.13**).

A baseline comparison between normal and asthmatic subjects was not highlighted by Fraenkel et al. (1995) due to the amalgamation of normal and asthmatic data, which suggests that they may have missed the very different characteristics of normal and asthmatic subjects. Compared to normal subjects, biopsies from asthmatic subjects had 5 and 10-fold higher counts of cells immunostaining for 5-LO and COX-2 respectively, and 4-fold higher counts of mast cells, and these immunopathological differences were associated with bronchial hyperresponsiveness and lower FEV_1 at baseline. Levels of expression of 5-LO and COX-2 were increased in normal subjects with HRV infection to the levels seen at baseline in asthmatic subjects. There was no change in counts of cells immunostaining for enzymes of the leukotriene or prostanoid pathways or in counts of inflammatory cells with HRV infection, perhaps because there is a pre-existing inflammation in the asthmatic subjects (**Fig. 4.13**). The pathway activated in normal subjects by HRV to raise counts of 5-LO and $COX\text{-}2^+$ cells might, therefore, already be activated in asthmatics. However, infection with HRV has been shown to potentiate the response of allergic asthmatics to allergen challenge (Calhoun et al. 1994). This suggests that the eicosanoid pathway enzymes in asthmatic subjects might be primed further by HRV to produce an enhanced response to allergen. The effect of HRV on allergen induced changes in eicosanoid pathway enzyme expression and mediator production has not been studied. The asthmatic subjects also had significantly higher basal BAL fluid cys-LT levels than the normals, perhaps because of higher basal expression of 5-LO. Whether allergen induced cys-LT production is increased by HRV infection remains to be seen and would be an interesting follow-up to this study. However, the asthmatic BAL data are based on samples from 3 subjects only and hence, this statistical difference is not robust.

Fig. 4.13 Regulation of the eicosanoid pathway in normal and asthmatic subjects with HRV infection.



In asthmatic subjects there was an increase in cells immunostaining for activated NF-κB with HRV infection. The increase in bronchial responsiveness in asthmatic subjects with

HRV might be mediated by NF-κB activation and transcription of inflammatory genes including IL-1β, IL-6, IFN, TNF-α, GM-CSF, RANTES, IL-8, ICAM-1 or VCAM-1 (Baeuerle and Henkel, 1994) (**Fig. 4.13**). There was no change in immunostaining for activated NF-κB or an increase in bronchial responsiveness in normal subjects, possibly because production of TNF-α and other cytokines produced by HRV binding to the low level of ICAM-1 expression on macrophages in normal subjects is not enough to induce NF-κB activation (**Fig. 4.13**). Increased 5-LO and COX-2^+ cell counts and the trend towards increases in FLAP and COX-1^+ cell counts associated with no change in NF-κB activation suggest that the increase in eicosanoid pathway enzymes are unlikely to be mediated by NF-κB. Even though both 5-LO and COX-2 have NF-κB binding sites on their gene promoter regions, other transcription factors may be operating to produce an upregulation of these enzymes in asthmatic subjects.

In agreement with Fraenkel et al. (1995), there was no change in submucosal eosinophil counts in either subject group with HRV infection. The eosinophil has been shown to be recruited to the bronchial mucosa of allergic asthmatics after allergen challenge (Metzger et al. 1987) and this recruitment is enhanced by HRV 16 infection (Calhoun et al. 1994) but eosinophilia is not directly induced by virus. However, the eosinophil may play a role in HRV induced inflammation. In a recent study by Handzel and colleagues (1998) the eosinophil has been found to bind viral particles and present viral antigen to T-cells. In addition, eosinophils from the BAL fluid of allergic asthmatics have been shown to express higher levels of ICAM-1 than blood eosinophils. This would allow airway eosinophils to bind substantial amounts of virus during natural infections, such that the eosinophil is 'primed' to respond in an enhanced fashion to subsequent allergen challenge (Mengelers et al. 1993) (Fig. 4.13). sICAM-1 is increased both in asthmatics and with viral infection (Louis et al. 1997, Cristensen et al. 1995) and has been reported to activate eosinophils to secrete cationic proteins (Chihara et al. 1995) and cause epithelial damage (Fig. 4.13). The increased epithelial eosinophil counts observed by Fraenkel et al. 1995 might be indicative of such eosinophil derived epithelial damage.

CHAPTER 5

Effect of ozone exposure on leukotriene pathway enzymes and inflammatory cells in healthy subjects

Hypothesis: *An increased representation of eicosanoid pathway enzymes, is associated with changes in inflammatory cell counts and mediator levels in BAL fluid from normal subjects exposed to 0.2ppm ozone.*

RESULTS.

5.1 Background.

This study was performed as an extension of a previously published work (Krishna et al. 1998), which reported increases in the proportion of PMN's and epithelial cells and increased concentrations of IL-8, Gro-α and total protein in BAL fluid from normal subjects following exposure to 0.2ppm ozone .

5.2 Immunohistochemical analysis of leukotriene pathway enzymes in bronchial biopsies.

In contrast to the seasonal allergen exposure study and the rhinovirus infection study presented in chapters 3 and 4, changes in expression of leukotriene pathway enzymes after ozone exposure were limited. In the 9 normal subjects counts of cells staining positive for 5-LO were not significantly different after ozone exposure (6.9 ± 1.5 cells/mm^2) compared to treatment with filtered air (6.22 ± 1.01 cells/mm^2; P>0.9) (Fig. 5.1). FLAP$^+$ cell counts also did not differ (ozone: 5.0 ± 1.0 cells/mm^2; filtered air: 4.7 ± 1.5 cells/mm^2; P>0.4) (Fig. 5.1). As expected, LTC_4 synthase$^+$ cell counts were low (2.7 ± 1.0 cells/mm^2) and remained low after ozone exposure (1.9 ± 0.4 cells/mm^2; P>0.9) (Fig 5.2). In contrast, LTA_4 hydrolase$^+$ cell counts increased from 4.1 ± 0.6 cells/mm^2 to 6.0 ± 0.9 cells/mm^2 after ozone exposure, although this did not reach statistical significance (P=0.09) (Fig 5.2).

5.3 Immunohistochemical analysis of prostanoid pathway enzymes in bronchial biopsies.

Similarly to the allergen exposure study, there were no changes in counts of cells immunostaining for cyclooxygenase iso-enzymes following ozone exposure. COX-1$^+$ cell counts demonstrated a trend towards a small decrease from 10.6 ± 1.4 cells/mm^2 after treatment with filtered air to 7.3 ± 1.6 cells/mm^2 after ozone exposure (P=0.11) (**Fig 5.3**). COX-2$^+$ cell counts were lower than COX-1 at 2.8 ± 0.7 cells/mm^2 after treatment with filtered air and remained unchanged after ozone exposure (2.3 ± 0.7 cells/mm^2; P>0.7) (**Fig 5.3**).

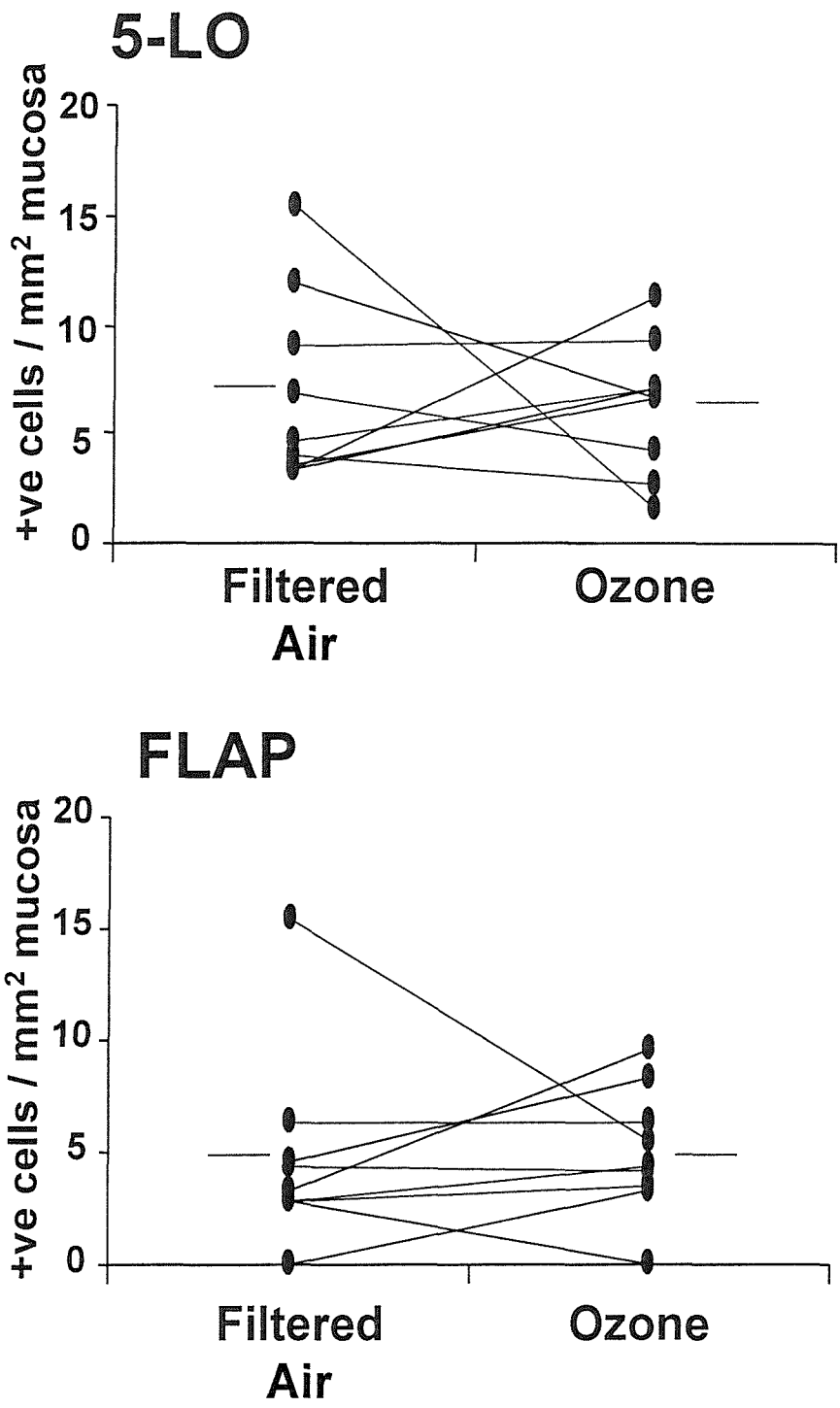


Fig 5.1 Counts of cells immunostaining for 5-LO and FLAP in the bronchial mucosa of 9 healthy subjects 6 hours after treatment with filtered air or 0.2ppm ozone. There were no changes in counts of 5-LO^+ cells or $FLAP^+$ cells with ozone exposure ($p>0.4$ for both). Horizontal lines represent mean cell counts.

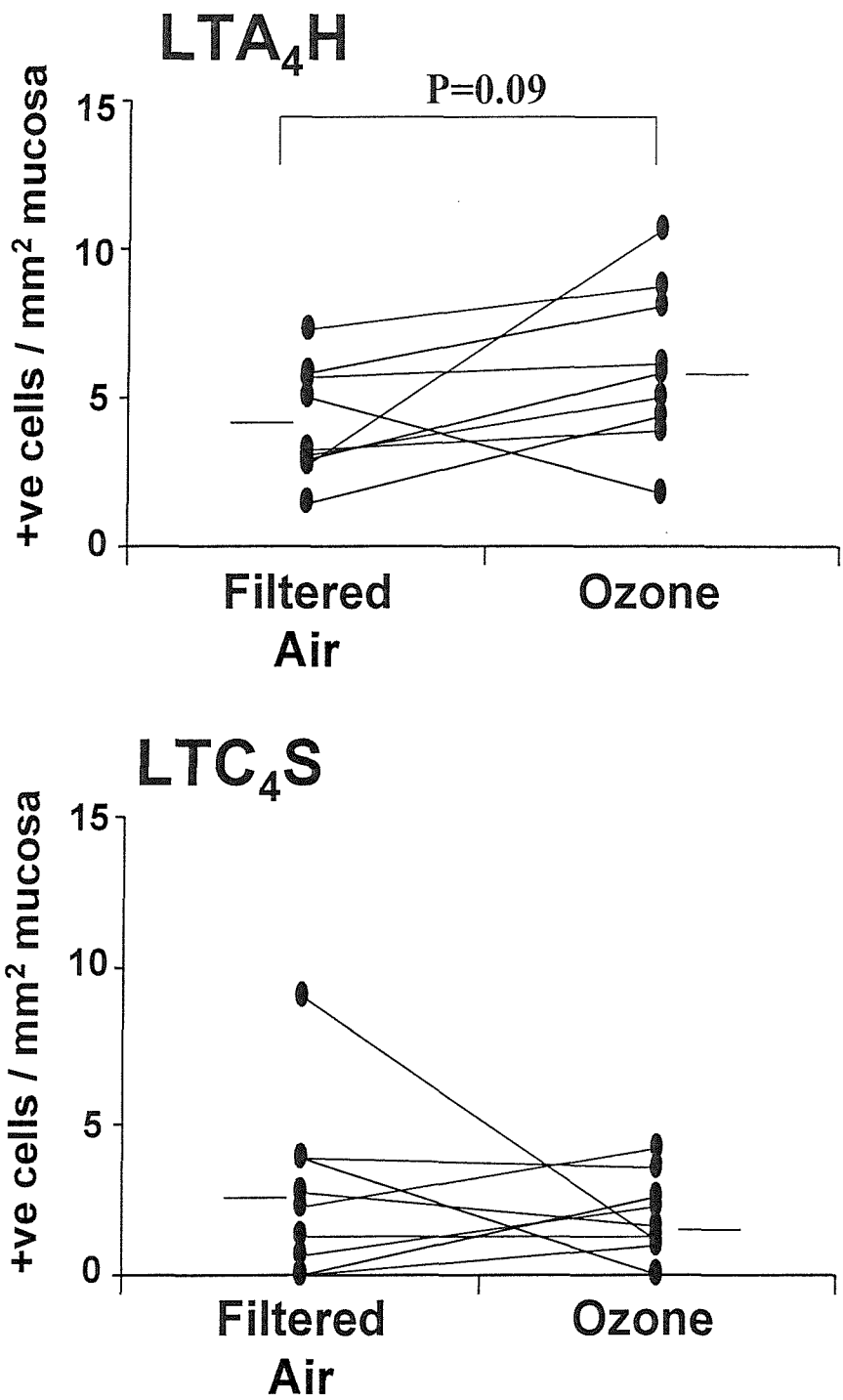


Fig 5.2 Counts of cells staining positively for LTA_4 hydrolase and LTC_4 synthase in the bronchial mucosa of 9 healthy subjects 6 hour after treatment with filtered air or 0.2ppm ozone. There was a trend towards an increase in LTA_4 hydrolase$^+$ cells with ozone exposure ($p=0.09$), with no change in counts of LTC_4 synthase$^+$ cells ($p>0.9$). Horizontal lines represent mean cell counts.

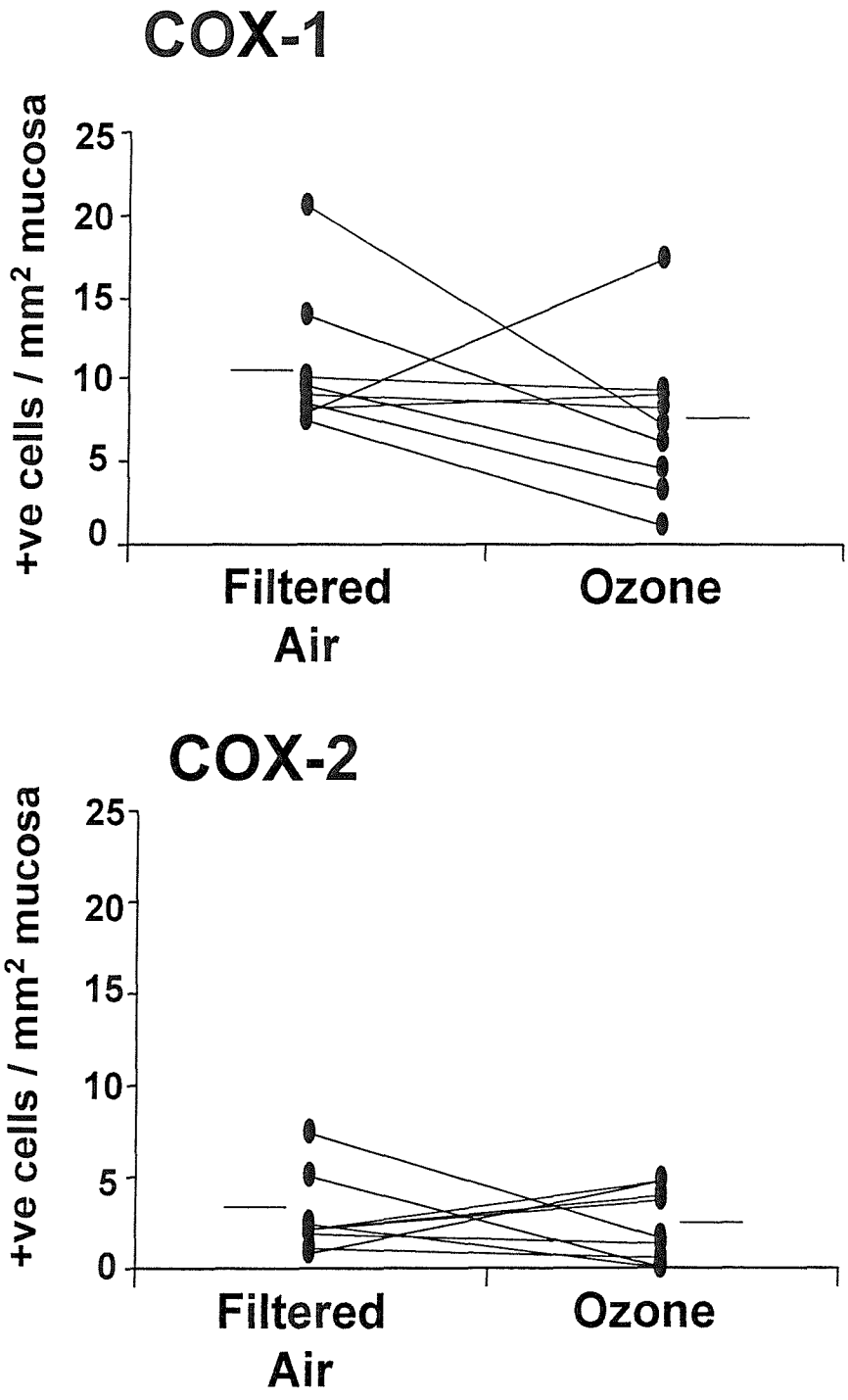


Fig 5.3 Counts of cells staining positively for COX-1 and COX-2 in the bronchial mucosa of 9 healthy subjects 6 hours after treatment with filtered air or 0.2ppm ozone. There were no changes in counts of COX-1^+ cells (P=0.11) or COX-2^+ cells (P>0.7) with ozone exposure. Horizontal lines represent mean cell counts.

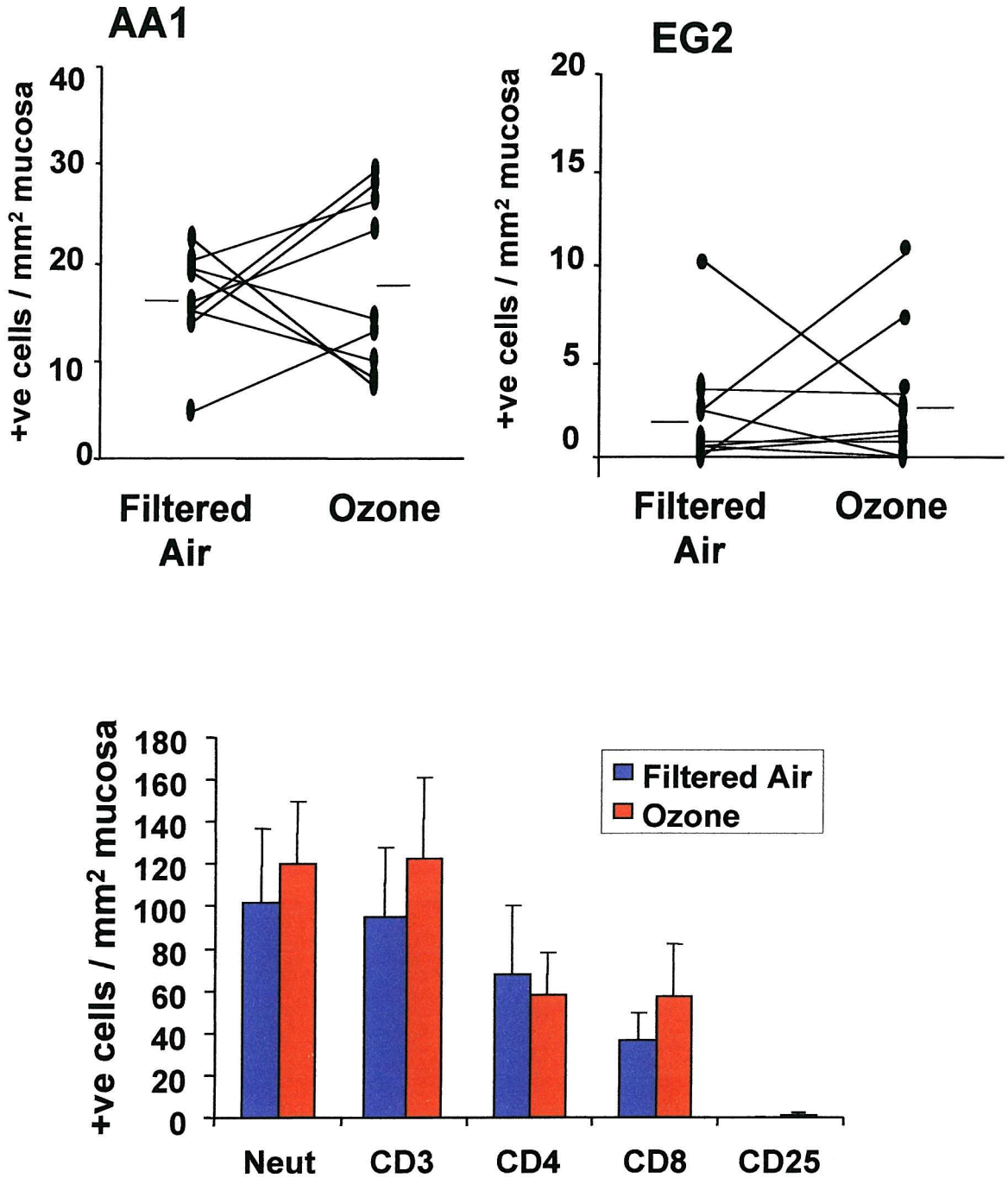


Fig 5.4 (Top) Counts of inflammatory cells in the bronchial mucosa of 9 healthy subjects 6 hours after treatment with filtered air or 0.2ppm ozone. There were no changes in counts of mast cells (AA1) (*top left*) or eosinophils (EG2) (*top right*) with ozone exposure ($p>0.5$ for both). Horizontal lines represent mean cell counts. Counts of neutrophils (Neut), CD3+ Pan-T-cells, CD4+, CD8+ and CD25+ T-cells (*bottom n=8*) were unchanged with ozone exposure ($P>0.15$ for all).

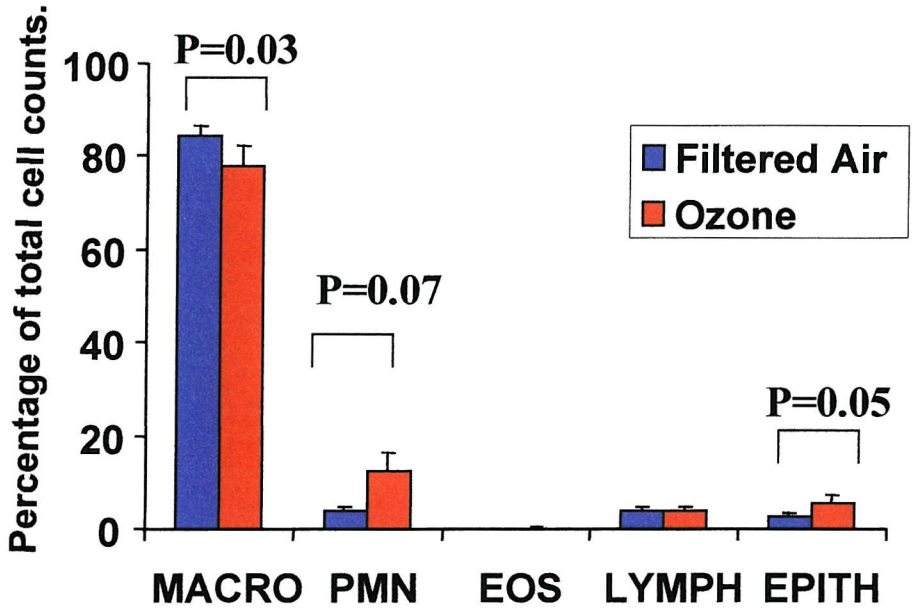


Fig.5.5 Counts of inflammatory cells in bronchoalveolar lavage (BAL) fluid of healthy subjects exposed to either filtered air or 0.2ppm ozone. The percentage of macrophages in BAL fluid decreased with ozone exposure ($p=0.03$). The percentage of PMNs in BAL fluid tended to increase with exposure to ozone ($p=0.07$), reflecting an increase in the percentage of epithelial cells ($p=0.05$). There were no changes in the percentages of eosinophils or lymphocytes ($p>0.6$).

5.4 Immunohistochemical analysis of leukocyte cell markers in bronchial biopsies.

Biopsies were stained for the myeloid cell markers EG2 for eosinophils and AA1 for mast cells. Mast cell counts were stable at 16.1 ± 1.7 cells/mm^2 after filtered air without ozone, and 17.6 ± 3.0 cells/mm^2 with 0.2 ppm ozone (P>0.5) (**Fig. 5.4**). Eosinophil counts were 2.3 ± 1.1 cells/mm^2 in filtered air and 3.0 ± 1.2 cells/mm^2 after ozone (P>0.8) (Fig. 5.4). Ozone exposure did not induce any significant changes in counts of neutrophils, or any T-cell populations in the bronchial mucosa (p>0.5 for all) (Krishna et al. 1998) (**Fig. 5.5**).

5.5 Analysis of BAL fluid inflammatory cells and mediators.

In contrast to findings in the bronchial mucosa the percentage of neutrophils (PMN) in BAL increased 3-fold with ozone exposure, although this did not reach significance (p=0.07) (Krishna et al. 1998) (**Fig 5.5**). There were also 3-fold increases in the percentage of epithelial cells in BAL with ozone exposure (p=0.05) (Krishna et al. 1998) (***Fig. 5.5***) which may be indicative of epithelial shedding. There was an associated reduction in the percentage of BAL macrophages after ozone exposure (p<0.05) (Krishna et al. 1998) (Fig. 5.5). There were no reported changes in the percent of eosinophils or lymphocytes in BAL with ozone exposure (Krishna et al. 1998) (Fig. 5.5).

There was an increase in levels of BAL fluid total protein (p=0.058) (Krishna et al. 1998), accompanied by significant increases in levels of the neutrophil chemoattractants interleukin (IL)-8 and Gro-α (the growth-related oncogene α) (Krishna et al. 1998). After ozone exposure, the levels of IL-8 and Gro-α correlated significantly with the percentage of neutrophils in BAL fluid (Krishna et al. 1998). There were no corresponding changes in the mast cell markers histamine and tryptase, or in the neutrophil marker myeloperoxidase (Krishna et al. 1998).

5.6 Summary

Compared with exposure to filtered air, exposure to 0.2ppm ozone significantly increased airway resistance and decreased peak flow measurements, there was no change in FEV1 or FVC. Bronchial biopsies were immunostained for enzymes of the eicosanoid pathway and inflammatory cell markers, only counts of LTA_4 hydrolase$^+$ cells trended towards an increase, counts of 5-LO, FLAP, LTC_4 synthase, COX-1 and COX-2 remained unchanged with ozone exposure. There were also no changes in counts of mast cells, eosinophils, neutrophils or T-cell populations with ozone exposure. Analysis of BAL cell counts identified increases in epithelial cells, and a trend toward more

neutrophils. Eosinophil and lymphocyte counts were unchanged with ozone exposure. Levels of the neutrophil chemoattractants IL-8 and Gro-α were increased in BAL, but there was no change in BAL fluid histamine, tryptase or myeloperoxidase.

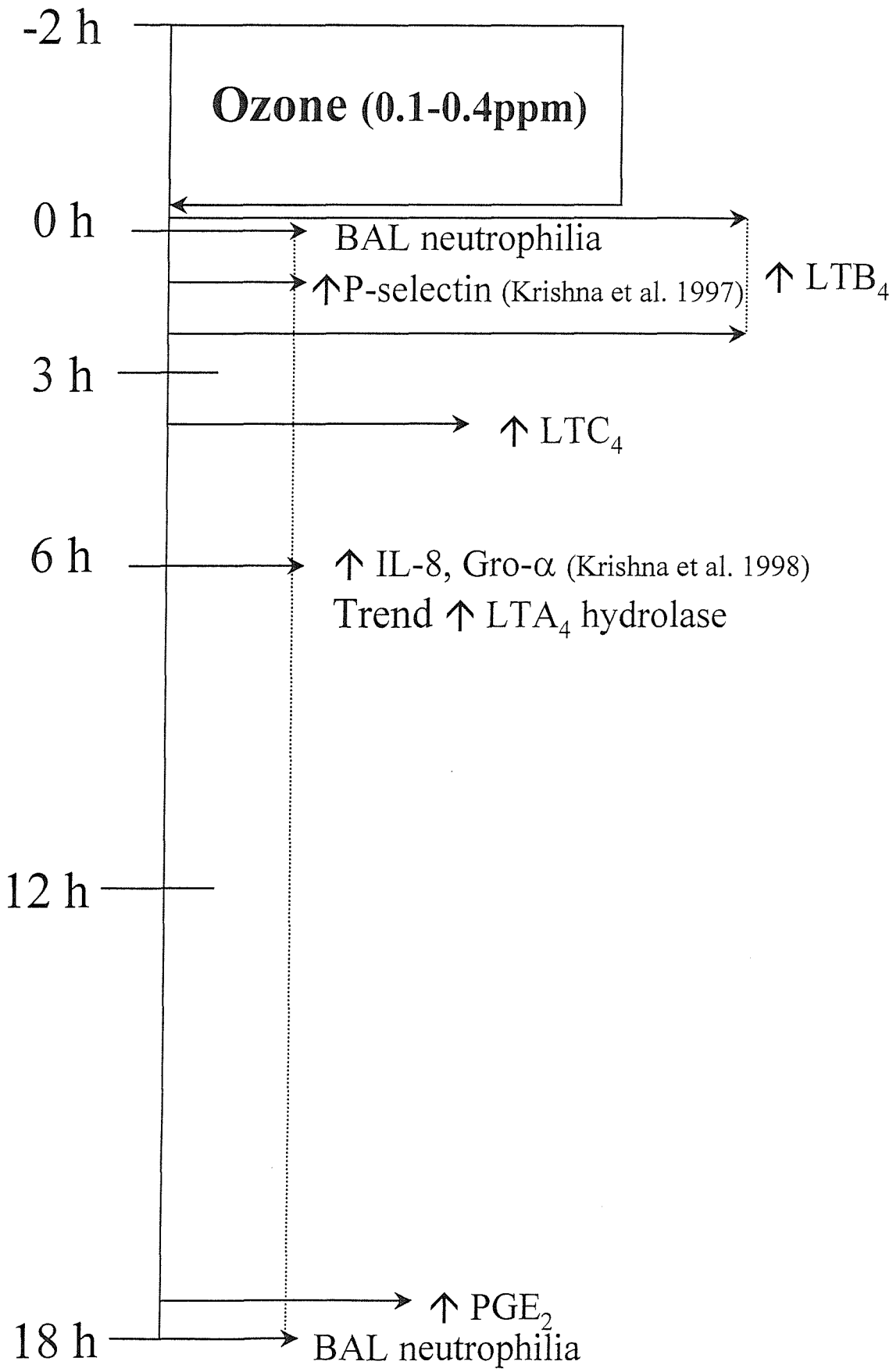
5.7 Discussion.

Ozone is reported to cause exacerbations of asthma (Kreit et al. 1989) and to increase bronchial responsiveness in normal subjects (Golden et al. 1978). In the 9 normal subjects studied, exposure for 2hr to 0.2ppm ozone LTA_4 hydrolase$^+$ cell counts trended towards an increase in the bronchial mucosa ($p=0.09$), and this was associated with significantly increased neutrophil counts, IL-8 levels and Gro-α levels in BAL fluid 6 hours after ozone exposure, compared to a placebo treatment of filtered air.

We detected BAL neutrophilia 6 hours after exposure to ozone (Krishna et al. 1998). Similar studies have demonstrated BAL neutrophilia at earlier and later time points (Coffey et al. 1996, Koren et al. 1989, Devlin et al. 1991). Neutrophilia has been shown to be induced by 1 hour and persists through 6 hours and up to 24 hours (Shelegle et al. 1991). In general, it appears that neutrophil influx occurs early after ozone exposure and persists for over 24 hours (**Fig. 5.6**), and this is associated with early increases in LTB_4 in BAL fluid that might be the cause of the neutrophil influx or a secondary consequence of neutrophilia. P-selectin is upregulated on endothelial cells 1.5 hours after exposure to 1.2ppm ozone for 2 hours (Krishna et al. 1997) (**Fig.5.6**). This increase in adhesion molecule expression might encourage margination and rolling of neutrophils on the blood vessel wall. Our data show a trend towards an increase in cells immunostaining for LTA_4 hydrolase in bronchial biopsies at 6 hours after exposure to 0.2ppm ozone (**Fig. 5.6**). Krishna and colleagues (1998) reported increased levels of IL-8 and Gro-α (**Fig. 5.6**) that correlated significantly with the percentage of neutrophils in BAL fluid. The combined results suggest that the neutrophil chemoattractants IL-8, Gro-α, and possibly LTB_4 may be responsible for the increased percentage of neutrophils observed in BAL fluid after ozone exposure. The neutrophil obviously has an important role in the airway response to ozone as illustrated by experiments in dogs. Ozone exposure causes a neutrophilia associated with airway hyperresponsiveness (Fabbri et al. 1984), which is prevented by neutrophil depletion (O'Byrne et al. 1984).

LTA_4 hydrolase is the committed enzyme for the production of LTB_4, a potent neutrophil chemoattractant (Spada et al. 1994). There were no changes in the counts of cells staining for other eicosanoid pathway enzymes, which remained in the normal

Fig. 5.6 Time course of ozone induced changes in the lung



range for healthy subjects. Despite the lack of change in enzymes producing cys-LTs and prostanoids these mediators may nevertheless be produced by enzyme activation in response to ozone exposure. Previous studies have shown increases in BAL fluid levels of LTB_4 and LTC_4 (Coffey et al. 1996) and PGE_2 (Koren et al. 1989) following ozone exposure. Coffey and colleagues (1996) reported a 1.5-fold increase in LTB_4 in BAL fluid from healthy subjects 2 hours after the end of a 2hr period of exposure to 0.4ppm ozone (**Fig. 5.6**). A 9-fold increase in the percentage of neutrophils in BAL fluid immediately after ozone exposure persisted up to 4 hours. LTC_4 was increased 8-fold after 4 hours but there was no change in PGE_2 or TXB_2 at any time-point up to 4 hours (**Fig. 5.6**). Koren and co-workers performed a similar study exposing healthy subjects to 0.4ppm ozone for 2 hours but then performed bronchoalveolar lavage at 18 hours. Ozone induced an 8.2 fold increase in the percentage of neutrophils in BAL. There was no change in the levels of LTB_4 in BAL, but prostaglandin E_2 increased 2-fold (**Fig. 5.6**). Much of the published literature appears contradictory as regards mediator release, with some groups reporting increased LTB_4 and LTC_4, and no changes in PGE_2 (Coffey et al. 1996). Others report increased PGE_2 with no change in leukotrienes (Koren et al. 1989, Devlin et al. 1991). All groups report increased neutrophils in BAL with exposure to ozone (Coffey et al. 1996, Koren et al. 1989, Devlin et al. 1991). The level of ozone to which the subjects in these studies was exposed was variable, ranging from 0.4ppm ozone for 2 hours (Coffey et al. 1996, Koren et al. 1989) to 0.08ppm for 6.6 hours (Devlin et al. 1991). The time after exposure when samples were taken also varied from 2 hours (Coffey et al. 1996) to 18 hours (Koren et al. 1989, Devlin et al. 1991). However, Shelegele et al. (1991) demonstrated that neutrophilia persists from 1 hour, through 6 hours, and up to 24 hours. FEV_1 was only decreased when measured at 1 hour and did not persist to 6 or 24 hours post exposure, suggesting that different time courses in the response to ozone exist. It is therefore likely that increased leukotrienes (particularly LTB_4) occur early (2-6 hours) and contribute to the BAL neutrophilia observed, and that increased PGE_2 is increased much later (18 hours) (**Fig. 5.6**).

There was no increased neutrophil count in the bronchial mucosa in the present study but the neutrophilia was apparent in BAL. It is possible that this represents the tail end of the response to ozone, as if neutrophils expressing LTA_4 hydrolase are recruited early, the biopsy may have been taken too late to detect increased neutrophils in the bronchial mucosa. However, neutrophilia was observed in BAL. If neutrophils are leaving the tissue in response to the neutrophil chemoattractants LTB_4, IL-8 and Gro-α in the airway lumen, they will be simultaneously transporting neutrophil derived LTA_4 hydrolase from the bronchial mucosa, hence increased levels of LTA_4 hydrolase will no

longer be apparent. A more significant increase in LTA_4 hydrolase$^+$ cells may have been observed at an earlier time point.

We did not observe any increase in cells immunostaining for upstream enzymes of the 5-LO pathway or for LTC_4 synthase. Overall, counts of LTC_4 synthase$^+$ cells were low. The substantial increase in BAL fluid LTC_4 observed by Coffey and colleagues (1996) does not appear to be a result of increased expression of 5-LO or LTC_4 synthase in the bronchial mucosa. However, translocation of 5-LO into the nucleus of neutrophils may contribute to the increase in leukotriene B_4 production observed by Coffey et al. (1996) and by Koren et al. (1989). In a study by Brock and colleagues (1997) 5-LO rapidly moves from the cytosol to the nucleus in neutrophils when they migrate from the blood into sites of inflammation *in vivo*, as well as when they are adhered to various substrates *in vitro*. Adhesion stimulated movement of 5-LO into the nucleus of neutrophils was accompanied by a 3- to 5-fold increase in stimulated LTB_4 production (Brock et al. 1997). We postulate that migration of neutrophils through lung tissue via adhesion molecules such as P-selectin in response to the chemotactic factors IL-8, Gro-α and LTB_4 may cause 5-LO to translocate to the nucleus, the result being increased leukotriene production.

The bronchial epithelium is the first lung tissue to be exposed to inhaled ozone and we have shown an increase in the percentage of epithelial cells in BAL, which may be indicative of epithelial shedding following ozone-induced damage. A recent study has shown that bronchial epithelial cells express 5-LO (Behera et al. 1998). If 5-LO expression was upregulated in epithelial cells after ozone exposure this could lead to increased LT production from epithelial cells. The epithelial cell may also provide LTA_4 for transcellular synthesis (Pace-Asciak et al. 1986), and conversely, may convert neutrophil-derived LTA_4 to LTB_4 via its own constitutive expression of LTA_4 hydrolase.

Counts of cells immunostaining for cyclooxygenase pathway enzymes were unchanged in the bronchial mucosa 6 hours after 0.4ppm ozone. The increase in BAL fluid PGE_2 observed after 0.4ppm ozone by Koren and colleagues (1989) may be indicative of COX upregulation at 18 hours, 12 hours after these patients underwent bronchoscopy. An increase in PGE_2 was also observed 18 hours after exposure to less than 0.1ppm ozone for 6.6 hours (Devlin et al. 1991). However, in subjects who underwent BAL 4 hours after exposure to 0.4ppm ozone, PGE_2 was not increased (Coffey et al. 1996). PGE_2 is therefore produced by the airways at a much later time than the leukotrienes, perhaps as a protective mechanism. Enzymes of the COX pathway have not been examined at this late stage and the increased PGE_2 may be produced by induction of COX-2 or by longer-term upregulation of COX-1.

In summary, ozone, possibly by action on bronchial epithelial cells, causes upregulation of P-selectin and production of the neutrophil chemoattractants IL-8, Gro-α and LTB_4, which promote accumulation of neutrophils in the airway lumen such that it is measured in BAL within 6 hours. Increased LTA_4 hydrolase$^+$ cell counts in the bronchial mucosa might be the source of increased LTB_4. Also elevated in BAL is LTC_4 (Coffey et al. 1996). 18 hours after ozone exposure PGE_2 levels in BAL are increased and neutrophilia persists (Koren et al. 1989).

CHAPTER 6

Expression of eicosanoid pathway enzymes in human inflammatory cells.

Aims and hypothesis: *To characterise the expression of eicosanoid pathway protein expression in human lung mast cells and peripheral blood leukocytes. Culture of purified peripheral blood eosinophils with IL-5 or GM-CSF increased expression of 5-LO, FLAP, COX-1 and/or COX-2.*

RESULTS.

6.1 Expression of prostanoid pathway enzymes in purified human lung mast cells.

Immunocytochemical staining of mast cell cytospin preparations showed that COX-1 immunoreactivity was expressed in 82.7 ± 7.9% of mast cells (n=6) while only 3.8 ± 1.4% expressed COX-2 (n=6) (**Fig. 6.1, Fig 6.2**). This suggests that constitutive COX-1 is the principle source of mast cell derived PGD_2 in normal lung.

6.2 Expression of leukotriene pathway enzymes in purified human lung mast cells.

Mast cell centrifuge preparations were immunostained for 5-LO, FLAP and LTC_4 synthase. The proportion of mast cells immunostaining positively for 5-LO (n=6) was 64.4 ± 14.1%, with 60.52 ± 13.3% being FLAP positive (n=6) and only 0.22 ± 0.2% of cells positive for LTC_4 synthase (n=6) (**Fig 6.1, Fig 6.3**). Mast cells therefore have the capacity for the production of leukotrienes, although the low proportion of LTC_4 synthase positive cells suggests that normal human lung mast cells do not produce significant amounts of cys-LTs at baseline.

6.3 FACS analysis of mixed leukocytes.

Mixed leukocytes from normal subjects were purified by dextran sedimentation and contaminating erythrocytes were removed by hypotonic lysis. Cells were fixed in 4% paraformaldehyde at 4°C and permeabilised with saponin buffer for intracellular fluorescence staining for proteins of the eicosanoid pathway as described in section 2.6.

The mixed leukocyte preparation was run on a FACScan and individual cell types were distinguished by their forward scatter (FSC) reflecting size, and side scatter (SSC) reflecting granularity profile (morphology) and by their natural autofluorescence (**Fig 6.4**). Conventionally, FSC is plotted on the x-axis with SSC on the y-axis. Eosinophils appear high on the SSC axis, as they are the most granular and bi-nucleated; in addition eosinophils have the highest autofluorescence of the leukocytes and can be distinguished from neutrophils on this basis (Carulli et al. 1998). The staining procedure used in this study (paraformaldehyde fixation and saponin permeabilisation) facilitates the identification of eosinophils in a mixed cell population by increasing the morphological

Expression of eicosanoid pathway enzymes in purified human lung mast cells.

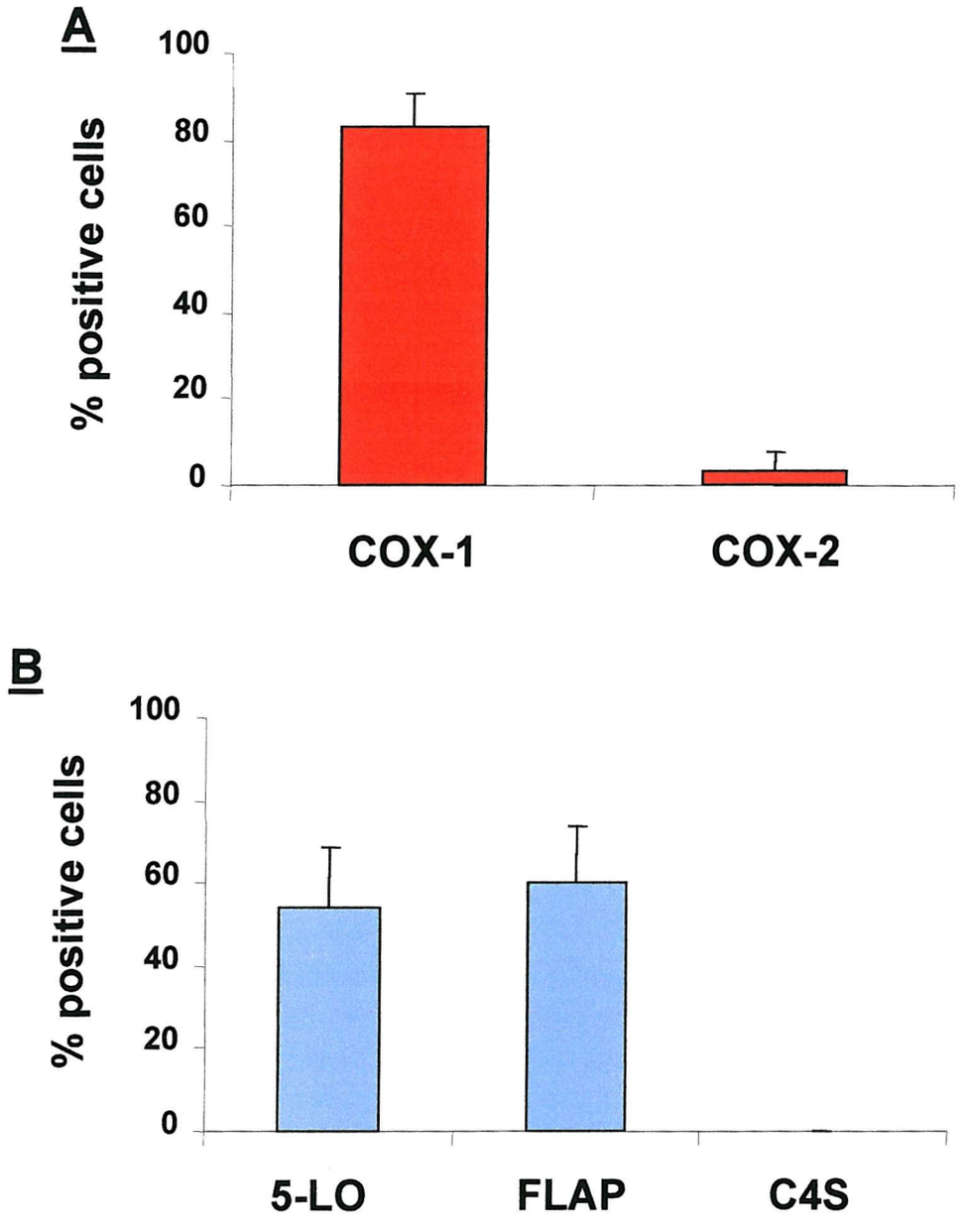


Fig. 6.1. Expression of 5-LO and COX pathway enzymes in human lung mast cells. ***Panel A***. Proportion of mast cells expressing COX-1 and COX-2. 82.7 ± 7.9% and 3.76 ± 1.4% of mast cells immunostained for COX-1 and COX-2 resepctively. ***Panel B***. Proportion of mast cells expressing 5-LO, FLAP and LTC_4 synthase. 54.4 ± 14.1% and 60.52 ± 13.3% of mast cells were positive for 5-LO and FLAP respectively. LTC_4 synthase was only expressed in 0.22 ± 0.2% of mast cells.

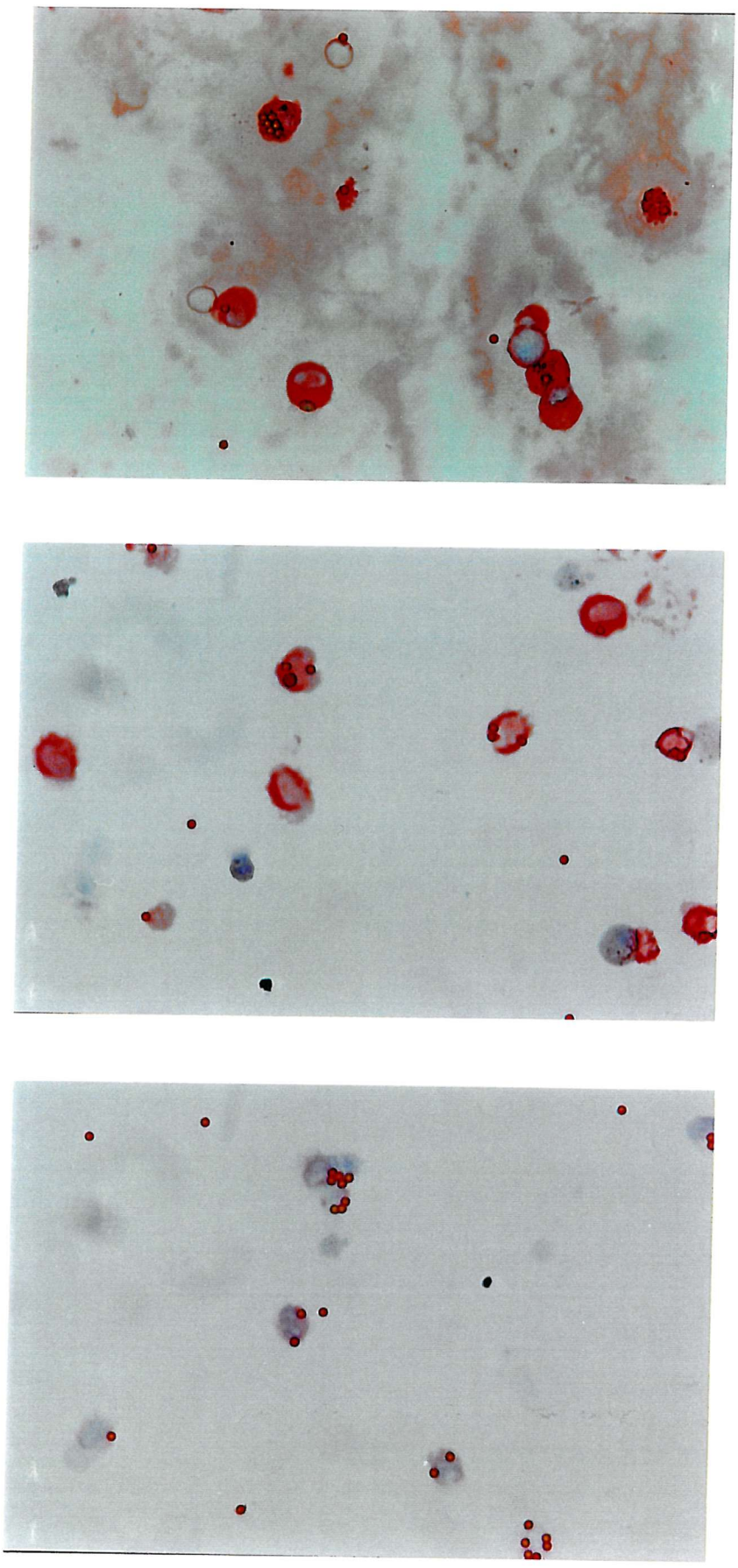


Fig. 6.2. Representative photomicrographs (x400) of cytospin preparations of purified human lung mast cells immunostained for the mast cell marker AA1 (***top***), and COX-1 (***middle***) and COX-2 (***bottom***). Positive cells appear red (AEC) against a blue counterstain of Mayers Haematoxylin.

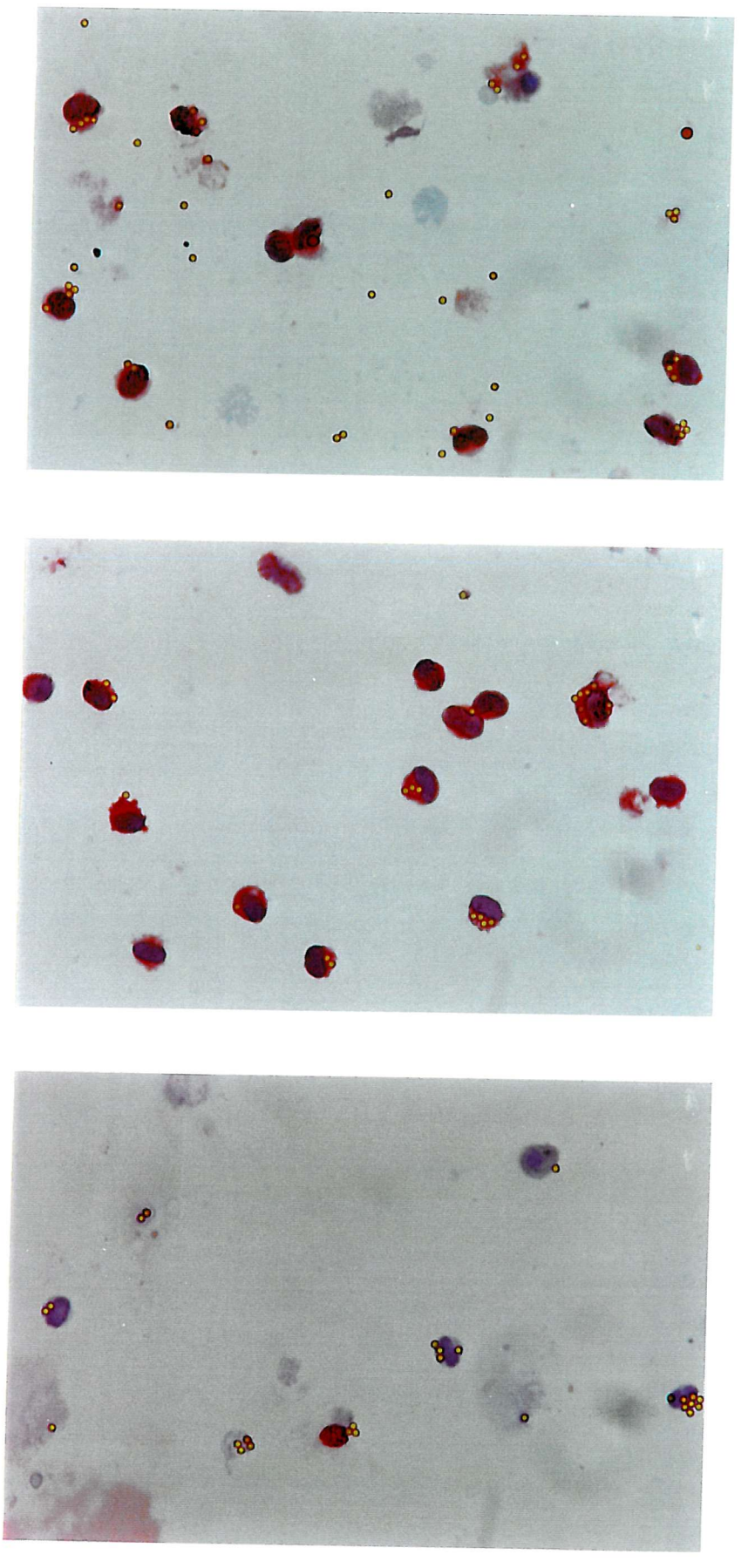


Fig. 6.3. Representative photomicrographs (x400) of cytospin preparations of purified human lung mast cells immunostained for the 5-LO (***top***), FLAP (***middle***) and LTC_4 synthase (***bottom***). Positive cells appear red (AEC) against a blue counterstain of Mayers Haematoxylin.

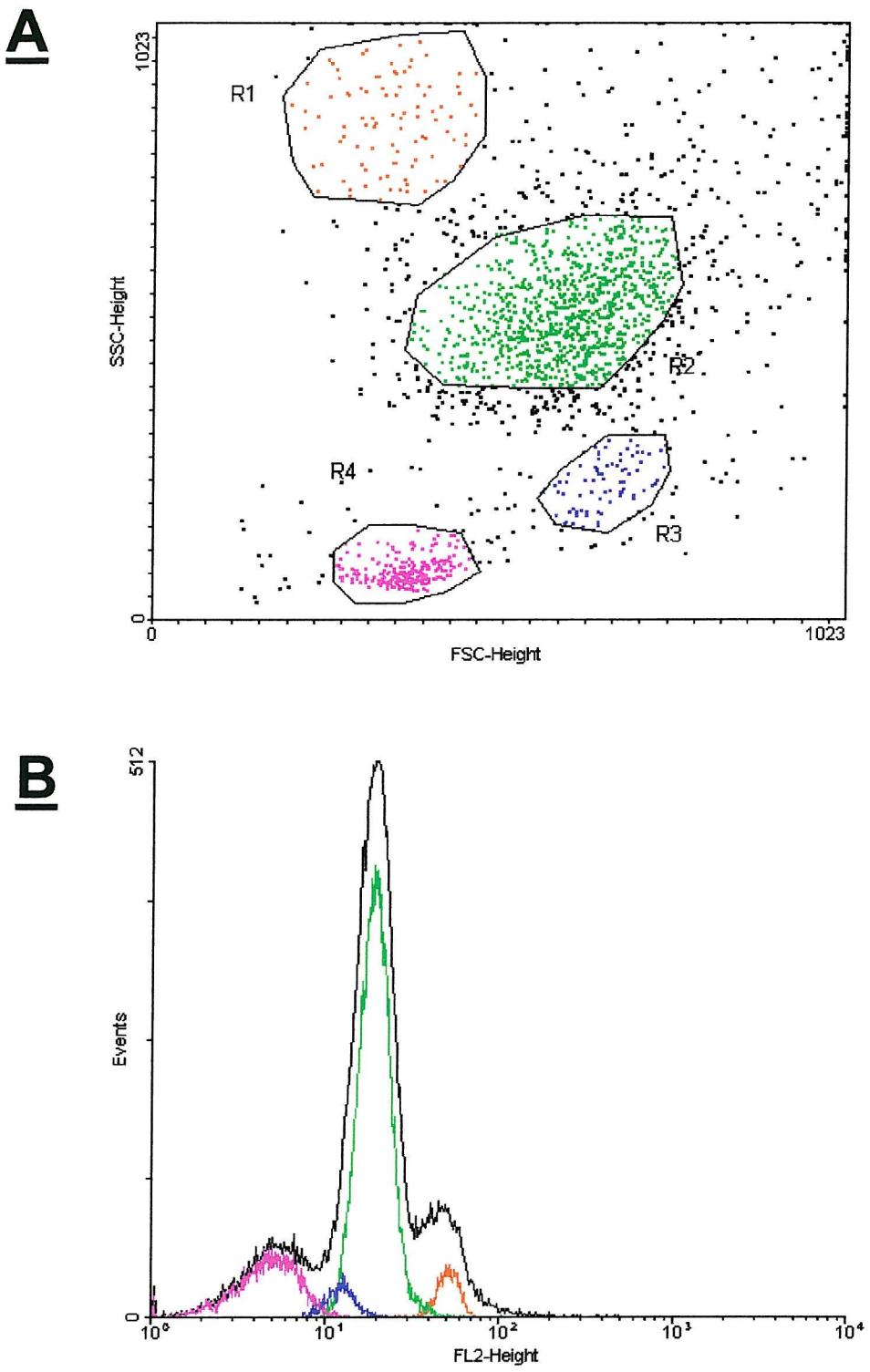


Fig. 6.4 Representative FACScan profile of mixed leukocytes. ***Panel A***: Forward scatter (FSC), side scatter profile of mixed leukocytes. R1(red) = eosinophils, R2 (green)=neutrophils, R3 (blue) = monocytes, R4 (purple) = lymphocytes. ***Panel B***: Red autofluorescence profile of unlabelled mixed leukocytes, colours are the same as panel A. Cell types can be distinguished by scatter signals and autofluorescence.

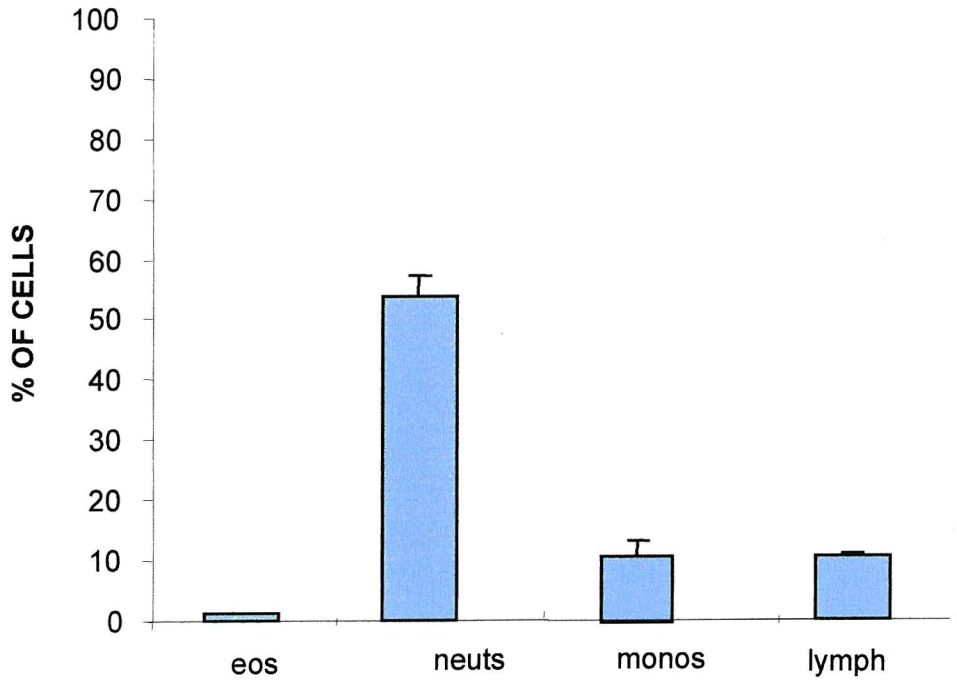


Fig. 6.5 Proportion of cells from normal subjects which are selected for FACScan.

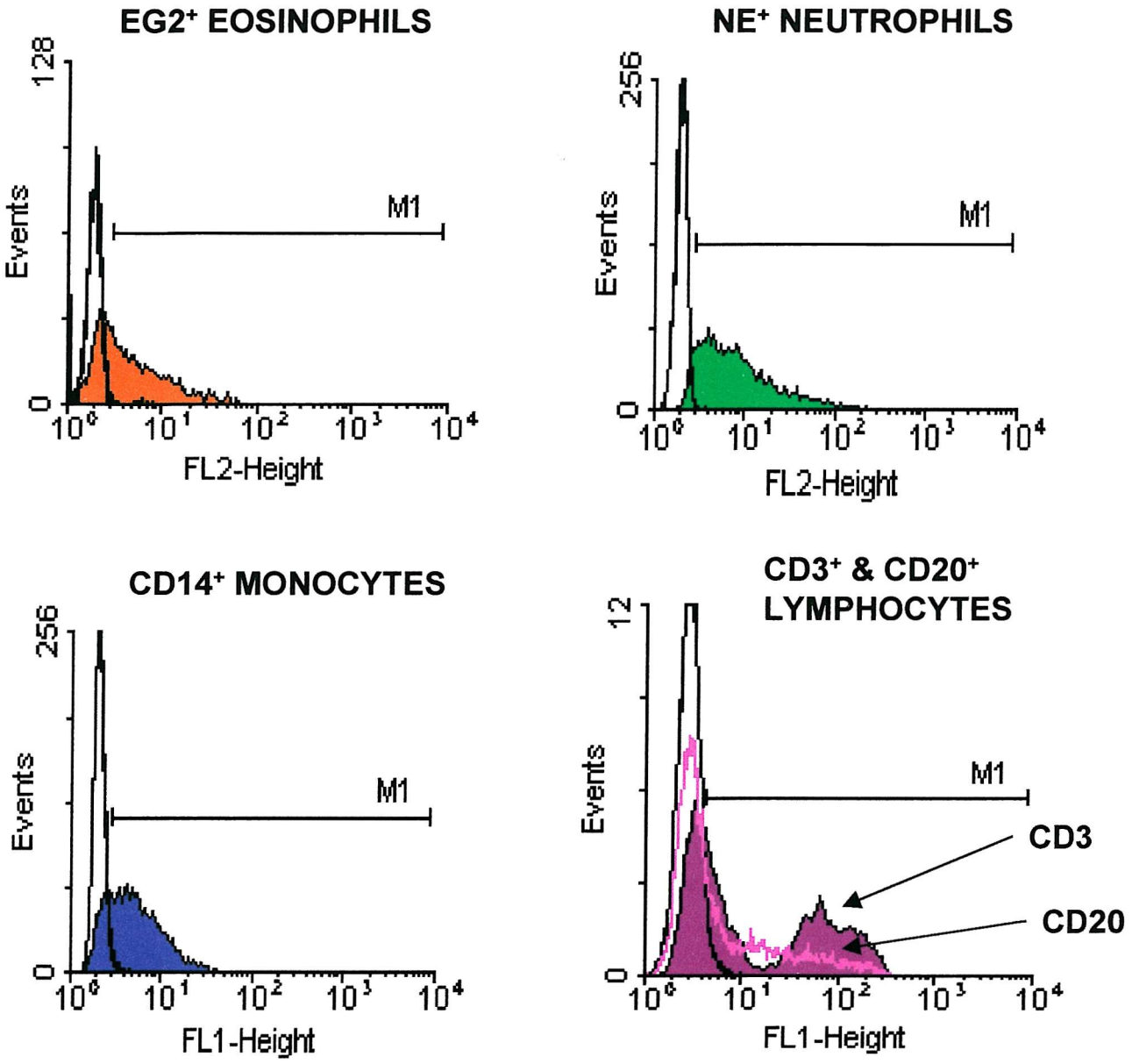


Fig. 6.6. Immunostaining for cell markers (coloured) compared to appropriate isotype control (black) in mixed leukocytes. ***Top left***: eosinophils immunostaining for eosinophil cationic protein (EG2), 56% are positive. ***Top right***: neutrophils immunostaining for neutrophil elastase, 90% are positive. ***Bottom left***: Monocytes immunostaining for CD14, 75% of cells are positive. ***Bottom right:*** Lymphocytes immunostaining for CD3 (purple) or CD20 (pink), 64% of cells are $CD3^+$, 41% of cells are $CD20^+$.

separation between eosinophils and neutrophils (Lavigne et al. 1997). Eosinophils are one of the rarer cell types representing approx. 1-5% of mixed leukocytes in normal subjects (**Fig 6.5**). Neutrophils are multinucleated but less granular and slightly larger and therefore appear below and to the right of the eosinophils on the FSC / SSC scatter plot. Neutrophils have a lower autofluorescence than eosinophils (Carulli et al 1998), but overlapping with monocytes and greater than lymphocytes. These are the most abundant cell-type in the mixed leukocyte preparations typically representing approx. 60-65% of mixed leukocytes in normal subjects (**Fig 6.5**).

Peripheral blood mononulear cells (PBMCs) are not granular and so appear low on the SSC axis. Monocytes are the larger of these cells and are high on the right of the FSC axis, with the smaller lymphocytes appearing to the left of the monocytes on the FSC. Monocytes typically represent approx. 5-8% of mixed leukocytes in normal subjects (**Fig 6.5**). The lymphocyte region contains both T-lymphocytes and B-lymphocytes, which cannot be distinguished on the basis of FSC / SSC profile or immunofluorescence and are therefore considered as one population for the purpose of these initial experiments. Lymphocytes typically represent approx. 30% of the mixed leukocyte population in normal subjects. Regions smaller than the entire cell population were selected for FACScan analysis to reduce contamination by overlapping of morphological regions, particularly in the case of eosinophils and neutrophils.

The identity of the cell populations was confirmed by immunofluorescence with appropriate antibodies to cell markers: eosinophil cationic protein (EG2) for eosinophils, neutrophil elastase (NE) for neutrophils, CD14 for monocytes, and CD3 and CD20 for T-lymphocytes and B-lymphocytes respectively (**Fig 6.6**). The process of fixation can disrupt antigen presentation for many cell surface markers and hence intracellular markers were used where possible. Basophils were too small a population to be quantified effectively by FACScan in mixed leukocyte preparations, but are found in a region higher on the SSC axis due to their granularity, and fall between lymphocytes and monocytes on the FSC axis. For each cell type the percent of cells immunostaining positively and the median fluorescence intensity (MFI) were recorded after subtraction of values for isotype control immunofluorescence. Median fluorescence intensity was considered appropriate due to the skewed distribution of staining.

6.4 Expression of leukotriene pathway enzymes in peripheral blood leukocytes.

5-LO was detected in all cell types (**Fig 6.7**), the greatest expression being in neutrophils with 31.3 ± 7.4% of cells being positive, and with a high MFI (2.94 ± 1.03). The proportion of monocytes and lymphocytes expressing 5-LO was approximately half that

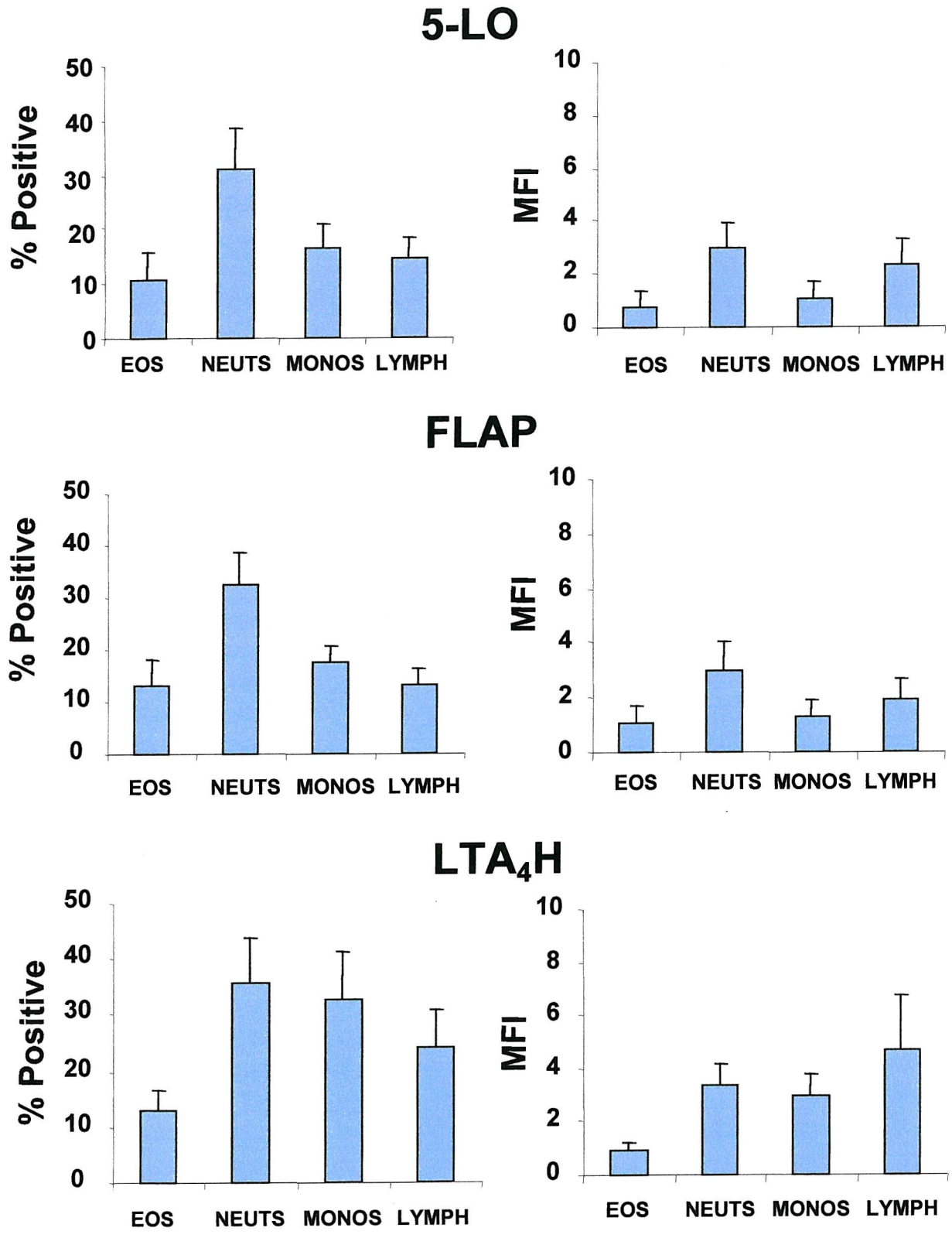


Fig 6.7. Expression of 5-LO, FLAP and LTA_4 hydrolase in eosinophils, neutrophils, monocytes and lymphocytes from normal subjects expressed as % expression (***left***) and median fluorescence intensity (***right***). Bars are mean ± SEM.

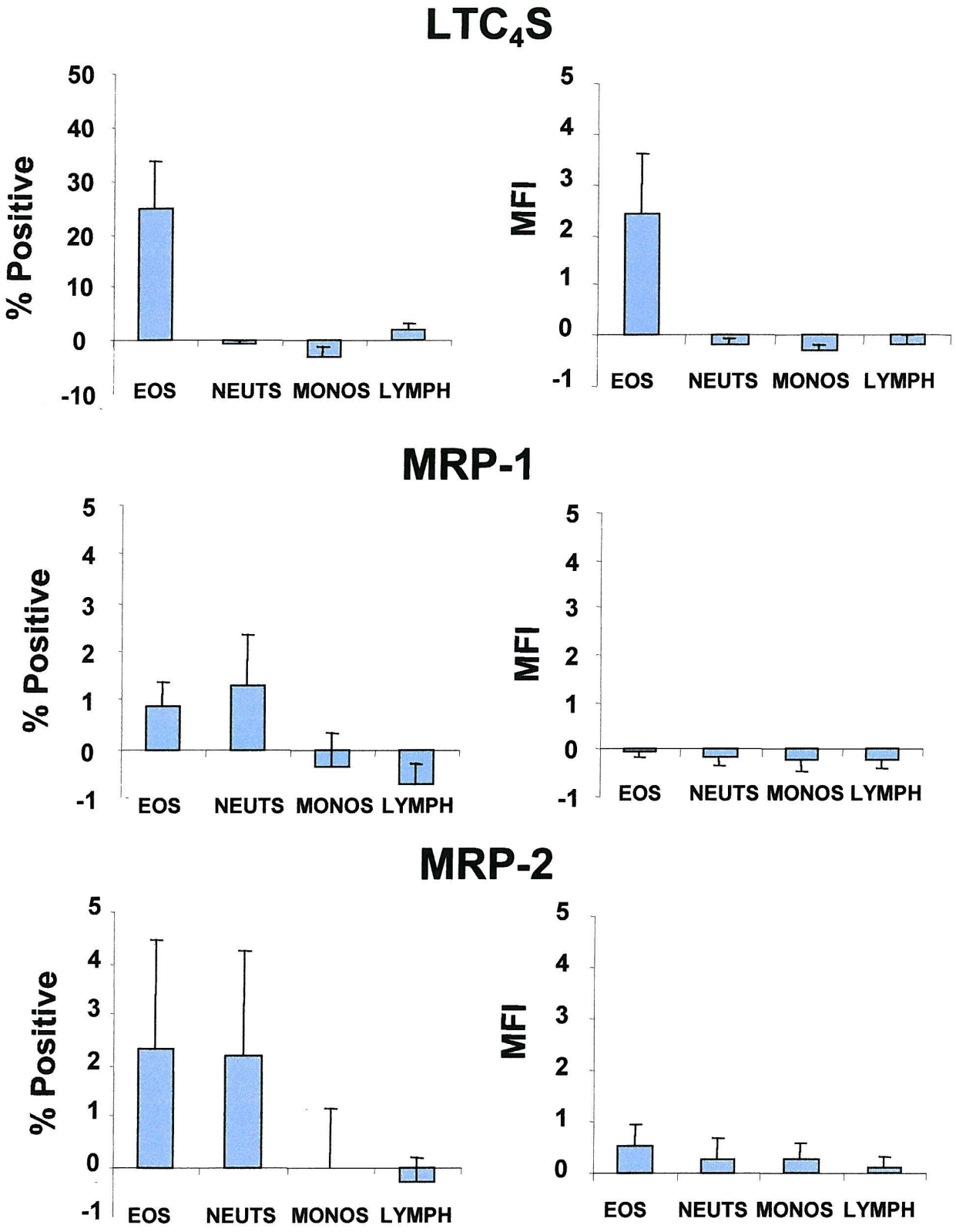


Fig 6.8 Expression of LTC_4 synthase, MRP-1 and MRP-2 in eosinophils, neutrophils, monocytes and lymphocytes from mixed leukocytes from normal subjects expressed as % expression (***left***) and median fluorescence intensity (***right***). . Bars are mean ± sem % expression.

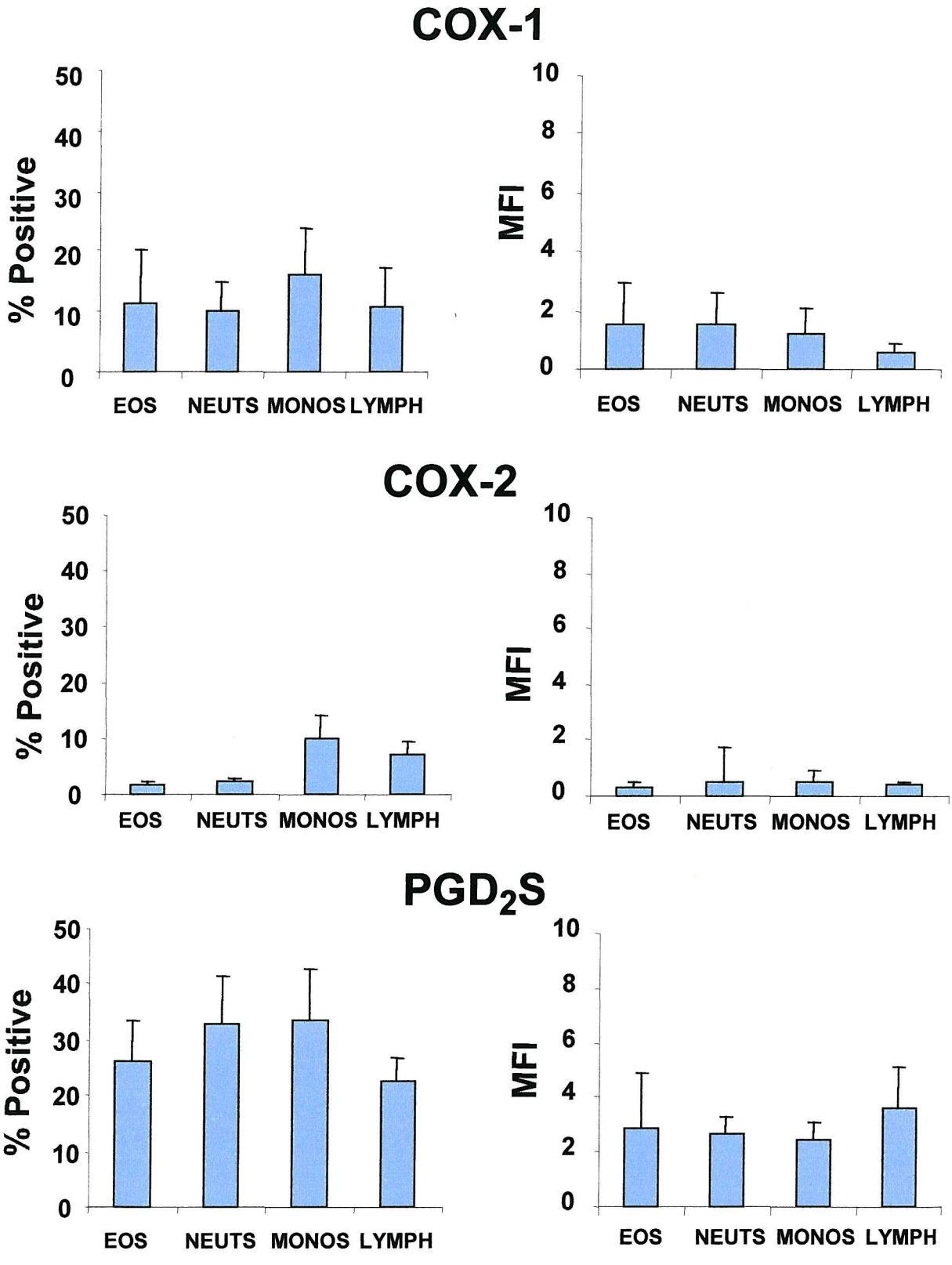


Fig. 6.9 Expression of COX-1, COX-2 and PGD_2 synthase in eosinophils, neutrophils, monocytes and lymphocytes in mixed leukocytes from normal blood expressed as % expression (***left***) and median fluorescence intensity (***right***). Bars are mean ± SEM.

of neutrophils (~15%) however MFI values for 5-LO staining in lymphocytes was high at 2.3 ± 1.0 compared to 1.1 ± 0.6 in monocytes. Eosinophils had the lowest 5-LO expression (10.8 ± 4.8%; 0.8 ± 0.7 MFI). (**Fig. 6.7**).

FLAP expression in mixed leukocytes was similar to that of 5-LO, with neutrophils expressing FLAP in the largest proportion of cells (32.2 ± 6.8%) and at the highest levels (3.0 ± 1.0 MFI), between 10 and 20% of monocytes, lymphocytes and eosinophils were FLAP positive with MFI's ranging between 1-2 (**Fig 6.7**). These data suggest that the capacity to initiate leukotriene synthesis is found in all of the blood leukocytes examined, but only in a subpopulation of each cell type.

Surprisingly, **LTA_4 hydrolase** immunofluorescence was also detected in all blood leukocyte types (**Fig 6.7**). Neutrophils and monocytes had the highest levels of expression with more than 30% of cells from normal subjects staining positive for the enzyme and MFI values above 3 (**Fig 6.7**). Lymphocytes had slightly lower levels at ~25% but MFI was high at 4.7 ± 2.0, and 13% of eosinophils stained positive for LTA_4 hydrolase but with lower 'amounts' of protein (0.9 ± 0.3 MFI).

In normal subjects **LTC_4 synthase** was detected in highest proportions in eosinophils with ~25% of cells staining positive above isotype control and a high MFI value (2.4 ± 1.2) (**Fig 6.8**). The lymphocyte population showed a low proportion of cells expressing LTC_4 synthase (~2%) but without measurable MFI. Neutrophils and monocytes from normal subjects did not stain positive for LTC_4 synthase (**Table 6.8**).

6.5 Expression of MRP in peripheral blood leukocytes.

MRP-1 was not detected in mixed leukocytes from normal subjects (**Fig 6.8**). Less than 2% expression was detected in eosinophils and neutrophils from normal subjects but MFI values were zero for all cell types (**Fig 6.8**).

There was some low percent expression of **MRP-2** in eosinophils and neutrophils (2.3 ± 2.2; 2.2 ± 2.0 % respectively) and positive MFI of MRP-2 (0.13-0.53) in all cells from normal subjects but MRP-2 was not expressed in monocytes or lymphocytes (≤0%) (**Fig 6.8**).

6.6 Expression of prostanoid pathway enzymes in peripheral blood leukocytes.

COX-1 expression was detected in eosinophils, neutrophils and lymphocytes from normal subjects in 10-12% of cells (**Fig. 6.9**). Monocytes expressed COX-1 in the highest proportion of cells (15.9 ± 7.81%). The MFI of COX-1 expression indicated that

eosinophils, neutrophils and monocytes had the highest levels of COX-1 protein (1.2-1.6 MFI) but was lower in lymphocytes (0.6 ± 0.3 MFI).

COX-2 expression was detected in all cell types (**Fig 6.9**). Similarly to COX-1, COX-2 was expressed at the highest proportion in monocytes (10 ± 4.16%; 0.6 ± 0.3 MFI). Eosinophils and neutrophils expressed COX-2 in 1.6 ± 1.0 and 2.3 ± 0.9% of cells respectively but neutrophils appeared to have high 'amounts' of COX-2 protein (0.6 ± 1.2 MFI) compared to lower levels in eosinophils (0.3 ± 0.2 MFI). COX-2 was detected in 7.1 ± 2.21% of lymphocytes with 'moderate amounts' of protein (0.4 ± 0.1 MFI). Expression of COX-2 in blood leukocytes is lower than their COX-1 expression, suggesting that prostanoids produced by leukocytes are predominantly COX-1 derived.

Immunostaining of cells with the antibody against **PGD_2 synthase** suggested high (10-35%; 2.5-3.6 MFI) expression of PGD_2 synthase in all cell types (**Fig 6.9**).

Expression of COX-1, COX-2 and PGD_2 synthase in all cell types suggests that all blood leukocytes examined have the capacity for the production of prostanoids, including PGD_2 but that this may be restricted to sub-populations of each cell type.

6.7 Cytokine modulation of 5-LO, FLAP, COX-1 and COX-2 expression in purified peripheral blood eosinophils.

Immunomagnetically purified eosinophils were cultured with or without 10ng/ml IL-5 or GM-CSF for variable time periods and immunofluorescence of 5-LO, FLAP, COX-1 and COX-2 was quantified by flow cytometry. The FSC/SSC profile identifies three morphological regions (**Fig 6.10**). Region 1 is the eosinophil population, region 2 is predominantly lymphocytes as the purification method allows some contamination by PBMCs, (neutrophils were not a major contaminant as they are removed by magnetic selection of CD16 positive cells), and region 3 is cell debris. The eosinophil region only was selected for the purpose of these experiments (**Fig 6.10**) and their identity was confirmed by immunostaining with EG2 (for eosinophil cationic protein) (**Fig 6.10**). The eosinophils were typically 80-90% pure as determined by Kimura's staining.

6.7.1 Effect of IL-5 and GM-CSF on eosinophil viability.

IL-5 and GM-CSF maintained the viability of eosinophils in culture better than the culture conditions alone over 24 hours. The cells cultured without either cytokine were typically only 60% viable by 24 hours, compared with approximately 88% for both IL-5 and GM-CSF.

The cells cultured with RPMI + 10% FCS showed increasing control sample

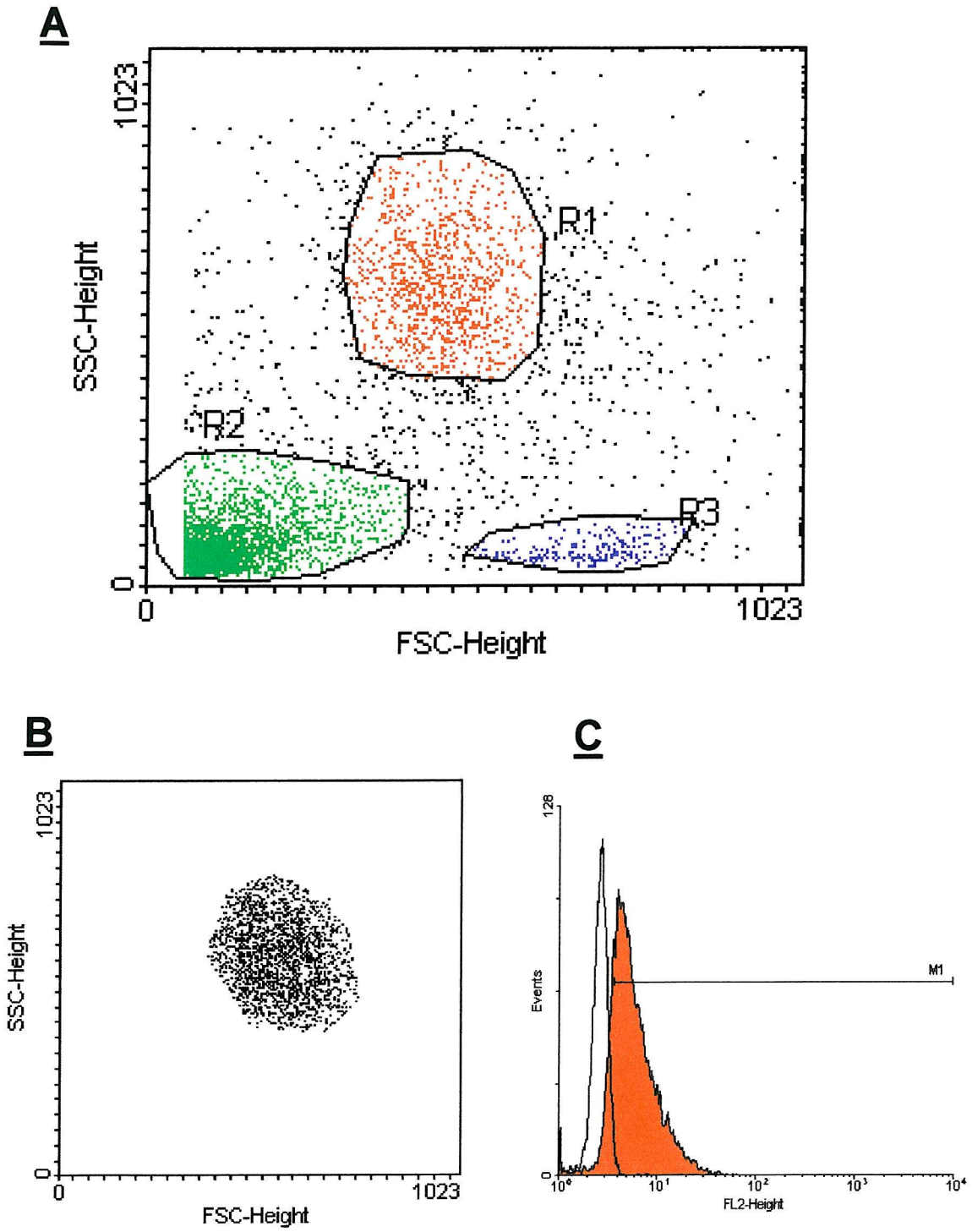


Fig 6.10 ***Panel A:*** Forward scatter, side scatter profile of purified eosinophils, R1=eosinophils, R2=cell debris, R3=contaminating PBMCs. ***Panel B:*** Forward scatter, side scatter profile of gated eosinophils. ***Panel C:*** EG2 staining of gated eosinophils above isotype control (black).

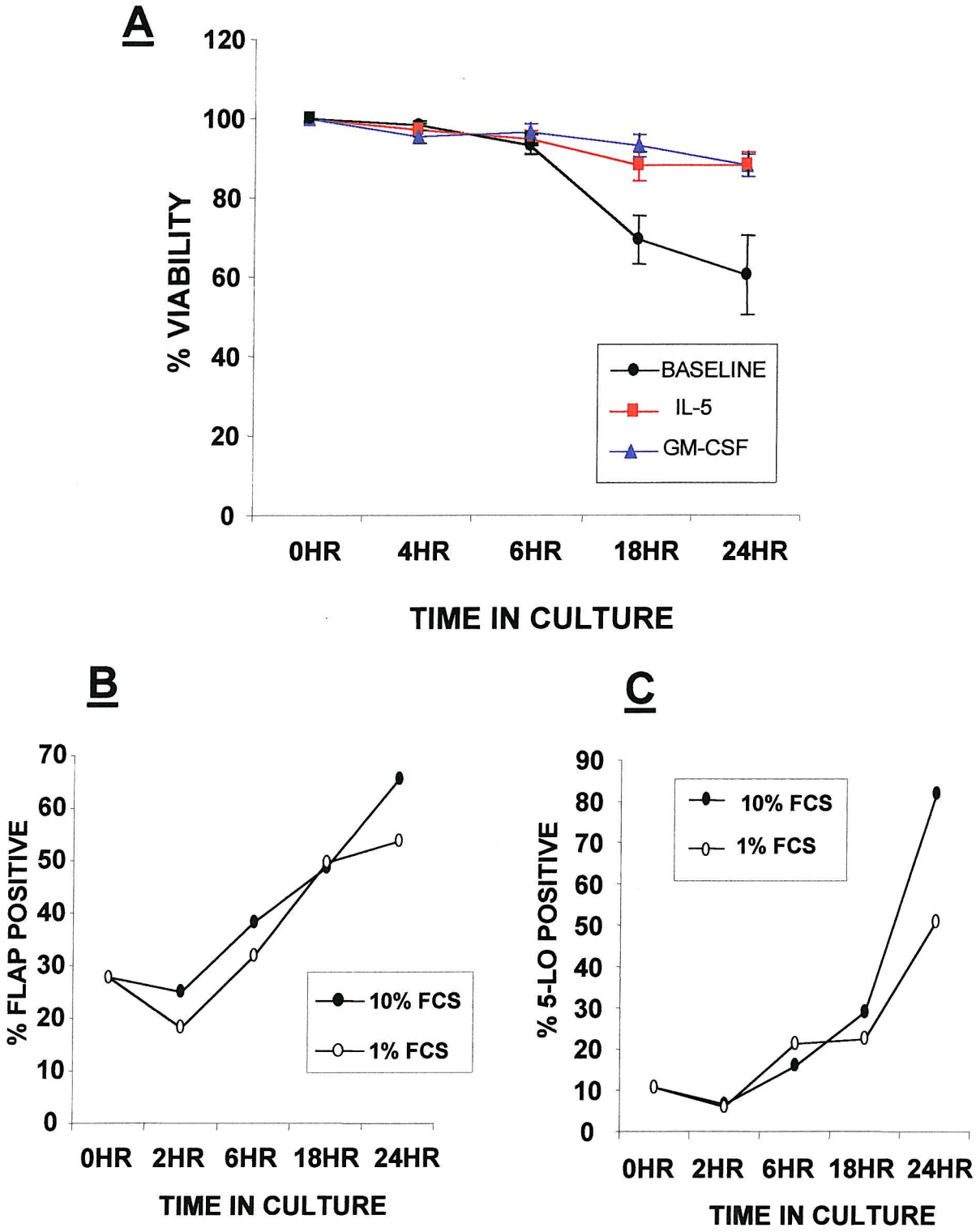


Fig. 6.11 ***Panel A:*** Percent eosinophil viability with or without 10ng/ml IL-5 and GM-CSF as determined by trypan blue exclusion. ***Panel B:*** Effect of culture conditions on baseline % FLAP expression over time with the addition of 10% or 1% FCS. ***Panel C:*** Effect of culture condtions on baseline % 5-LO expression over time with the addition of 10% or 1% FCS.

staining of 5-LO and FLAP over 24 hours (**Fig 6.11**). 5-LO was expressed in 20.6 ± 9.6% of cells at baseline, this expression remains stable up to 6 hours culture time, but rises to 40.6 ± 12.3% and 57.3 ± 12.1% after 18 and 24 hours in culture respectively. FLAP was expressed in 42.1 ± 10.9% of cells immediately before culture and % expression remains stable over 24 hours of culture. Median fluorescence intensity of FLAP staining increases from 2.00 ± 0.56 immediately before culture to 10.78 ± 3.90 after 24 hours of culture. To test whether culture in 10% FCS was stimulating the cells, cells were also cultured with 1% FCS. The reduction of the FCS to 1% did not appear to affect the rise in staining of control samples (**Fig 6.11**). The use of serum-free supplements caused the cells to adhere strongly to the tissue culture plate and was not a suitable replacement. Eosinophils are not naturally adherent cells and it was preferable to avoid the use of trypsin to lift the cells from the tissue culture plate. The increase in control sample staining of 5-LO and FLAP may therefore be a result of reduced viability in cells cultured without IL-5 or GM-CSF. Statistical comparisons are therefore only made between cytokine stimulated cells and the equivalent baseline cells at each time point, and not compared to freshly isolated cells.

6.7.2 Effect of IL-5 and GM-CSF on expression of 5-LO in eosinophils.

Eosinophils were cultured with and without 10ng/ml IL-5 over a 24-hour period and were fixed at 0hr, 2hr, 4hr, 6hr, 18hr, and 24hr time points for FACScan analysis of 5-LO expression.

6.7.2.1 *IL-5 incubation over 24hr.*

Treatment of cells with IL-5 (10ng/ml) did not significantly change 5-LO expression compared to in the absence of cytokine (p=0.549 ANOVA) (**Fig 6.11**).

6.7.2.2 *GM-CSF incubation over 24hr.*

Culture of eosinophils with GM-CSF (10ng/ml) significantly increased % 5-LO expression compared to incubations in the absence of cytokine (p=0.037 ANOVA) (**Fig 6.12**). This increase in was greatest after 6 hours of culture with GM-CSF, rising from 22.7 ± 6.0% (control), to 49.8 ± 12.9% with GM-CSF (p=0.052 T-Test). MFI was not significantly increased by GM-CSF incubation due to the effect of the rising MFI of control samples (p=0.138 ANOVA) (**Fig 6.12**), but when the 18 and 24 hr time points are not included in the ANOVA test a p value of 0.009 was achieved. A significantly higher MFI of 5-LO expression in GM-CSF stimulated cells was confirmed by

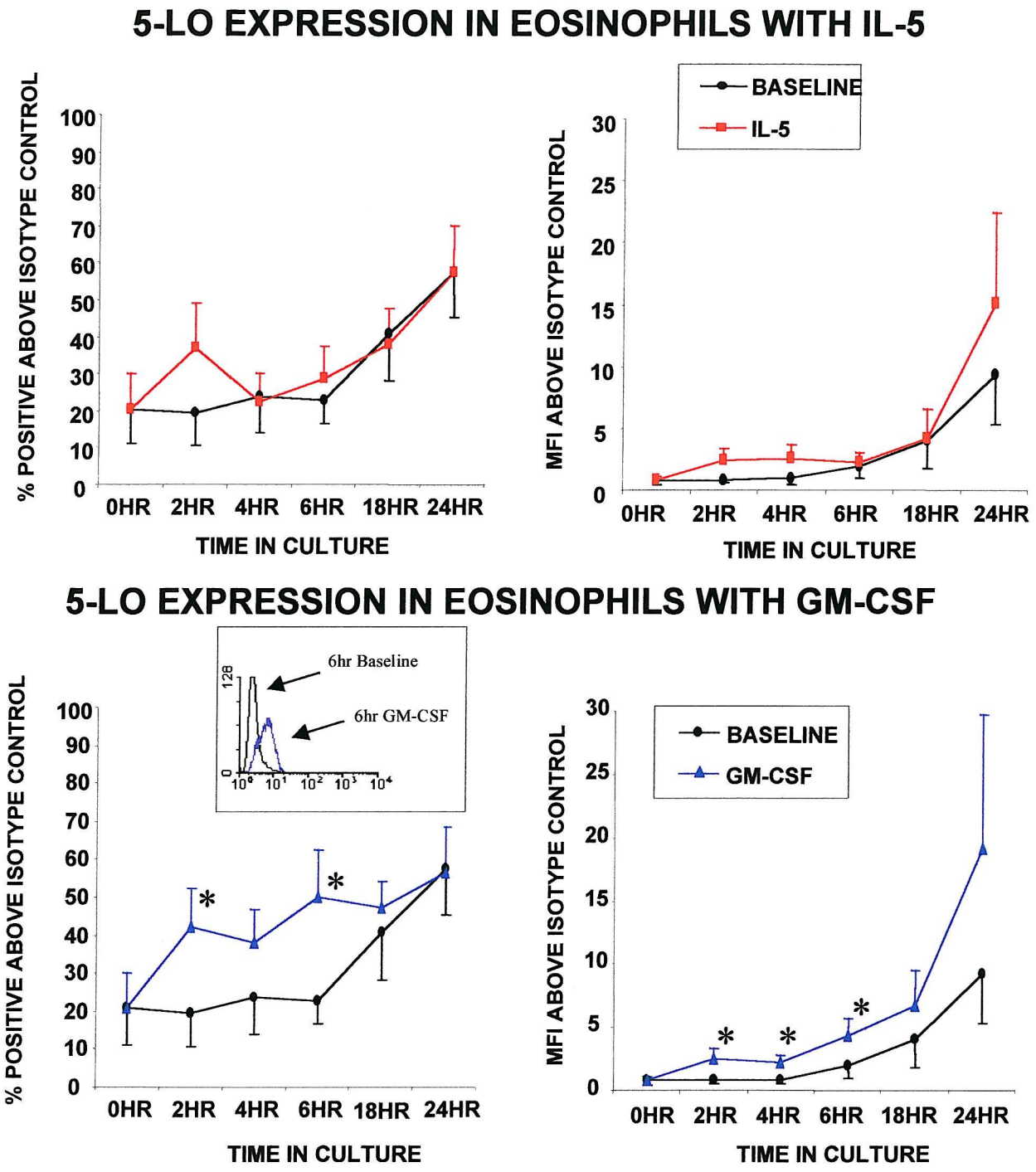


Fig. 6.12 ***Top left***: % 5-LO expression in eosinophils incubated for 24 hours with (■) or without (●) IL-5. ***Top right***: MFI of eosinophils immunostained with anti-human 5-LO antibody with (■) or without (●) IL-5. 5-LO expression was not changed by incubation with IL-5 ($p>0.4$ ANOVA). ***Bottom left***: % 5-LO expression in eosinophils incubated for 24 hours with (▲) or without (●) GM-CSF. Insert: representative FACScan of 5-LO staining after 6 hours incubation with (blue) and without (black) GM-CSF. ***Bottom right***: MFI of eosinophils immunostained with anti-human 5-LO antibody with (▲) or without (●) GM-CSF. GM-CSF increased 5-LO expression in eosinophils compared to baseline over 6 hours ($p<0.02$ ANOVA). (* $p<0.05$ T-test).

DOSE RESPONSE FOR THE EFFECT OF GM-CSF ON 5-LO EXPRESSION IN HUMAN EOSINOPHILS

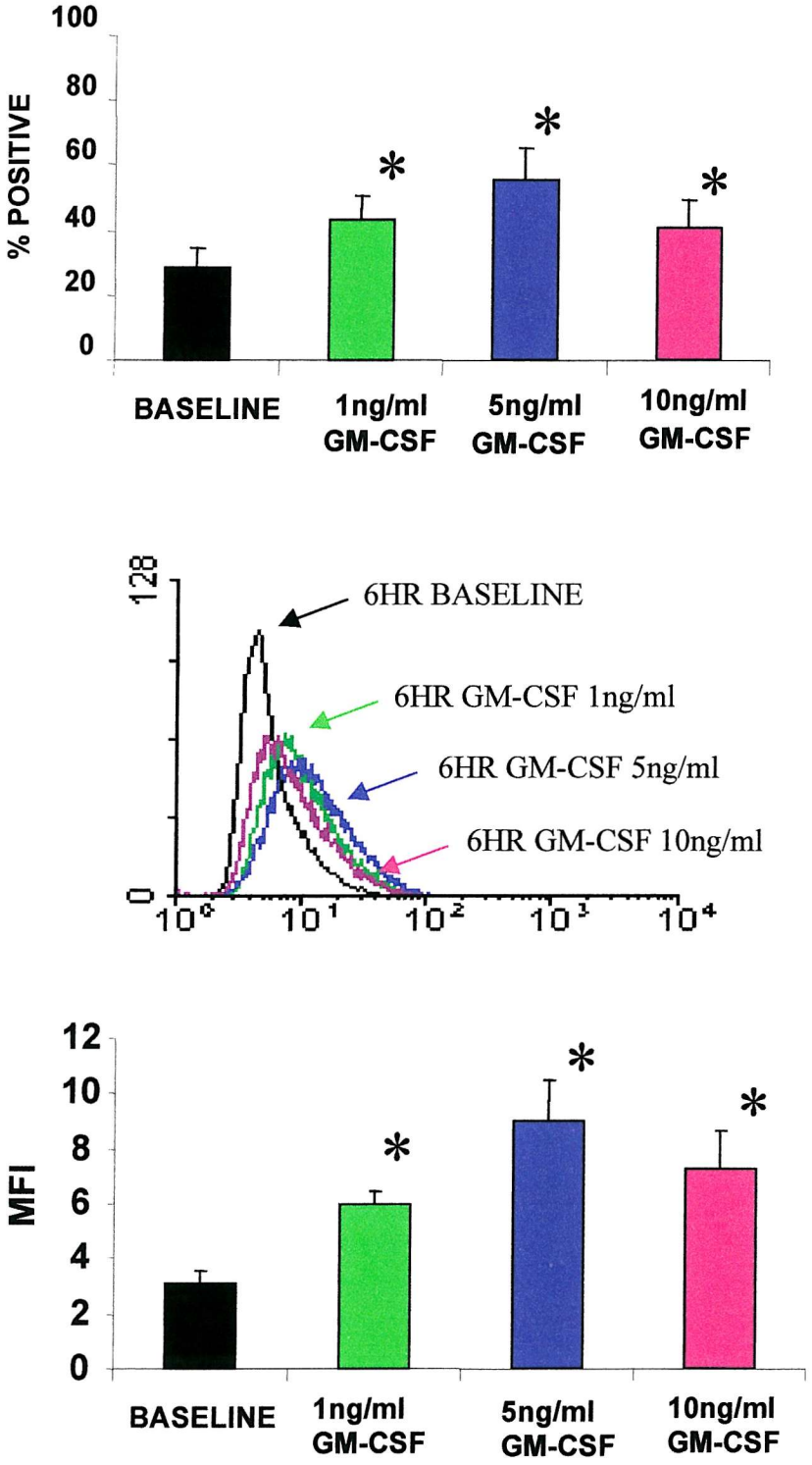


Fig. 6.13 ***Top***: Dose dependent increase in % expression of 5-LO in eosinophils incubated for 6 hours with GM-CSF, reaching a maximum at 5ng/ml GM-CSF. ***Middle***: Representative FACScan of 5-LO expression with increasing doses of GM-CSF. ***Bottom***: Dose dependent increase in MFI of 5-LO expression in eosinophils incubated for 6 hours with GM-CSF, reaching a maximum at 5ng/ml GM-CSF. (* $p<0.05$ T-test of treated vs baseline)

significant T-Tests of individual time points (2 hours (p=0.034), 4 hours (p=0.041) and 6 hours (p=0.035)).

6.7.2.3 ***Dose response of effect of GM-CSF on 5-LO expression.***

To examine the effect of dose of GM-CSF on 5-LO expression eosinophils were cultured with GM-CSF at concentrations of 0ng/ml, 1ng/ml, 5ng/ml and 10 ng/ml for 6 hours (**Fig 6.13**). % 5-LO expression increased from 28.1 ± 6.1% in controls to 43.7 ± 6.6% with 1ng/ml GM-CSF (p=0.025 T-test). Incubation with 5ng/ml GM-CSF produced a 2-fold increase in % 5-LO expression to 55.2 ± 10.7% (p=0.013 T-test). Percent expression did not rise further with 10ng/ml GM-CSF (41.3 ± 8.2%) but still remained significantly higher than control (p=0.012 T-test). Median fluorescence intensity was increased 3-fold from 3.1 ± 0.5 at baseline to 9.0 ± 1.5 with 5ng/ml GM-CSF (p=0.0041) (**Fig 6.13**). 5ng/ml is therefore the optimum concentration of GM-CSF for the upregulation of 5-LO in eosinophils.

6.7.3 Effect of IL-5 and GM-CSF on expression of FLAP in eosinophils.

Eosinophils were cultured with and without 10ng/ml IL-5 over a 24-hour period and were fixed at 0hr, 2hr, 4hr, 6hr, 18hr, and 24hr time points before FACScan analysis.

6.7.3.1 IL-5 incubation over 24hr

The proportion of eosinophils immunopositive for FLAP was not increased by incubation with 10ng/ml IL-5 (p=0.431 ANOVA) (**Fig 6.14**). However an increase in FLAP expression was indicated by an increase in mean MFI (**Fig 6.14**), this was not seen over 24 hours due to the rising level of FLAP staining in control incubations (p=0.198 ANOVA) but was seen over 6 hours (p=0.041 ANOVA). The increase in FLAP MFI was significant after 2 hours (p=0.040 T-test) (3.11 ± 0.78 in controls, to 5.85 ± 1.41 with IL-5) and trending after 4 hours (p=0.060 T-test). The lack of increase in the proportion of $FLAP^+$ cells but the evident increase in MFI suggests that FLAP is upregulated in those cells already expressing FLAP protein.

6.7.3.2 ***Dose response of effect of IL-5 on FLAP expression.***

The MFI of FLAP staining was upregulated after 2 hours of incubation with IL-5 at 1ng/ml (p=0. 0.017 T-test), 5ng/ml (p=0.0039 T-test) and 10ng/ml (p=0.031 T-test) (**Fig 6.15**). The greatest increase was from 4.12 ± 1.12 in controls to 9.43 ± 2.08 with 5ng/ml IL-5. This dose response effect is also seen with % expression which rises from 47.3 ±

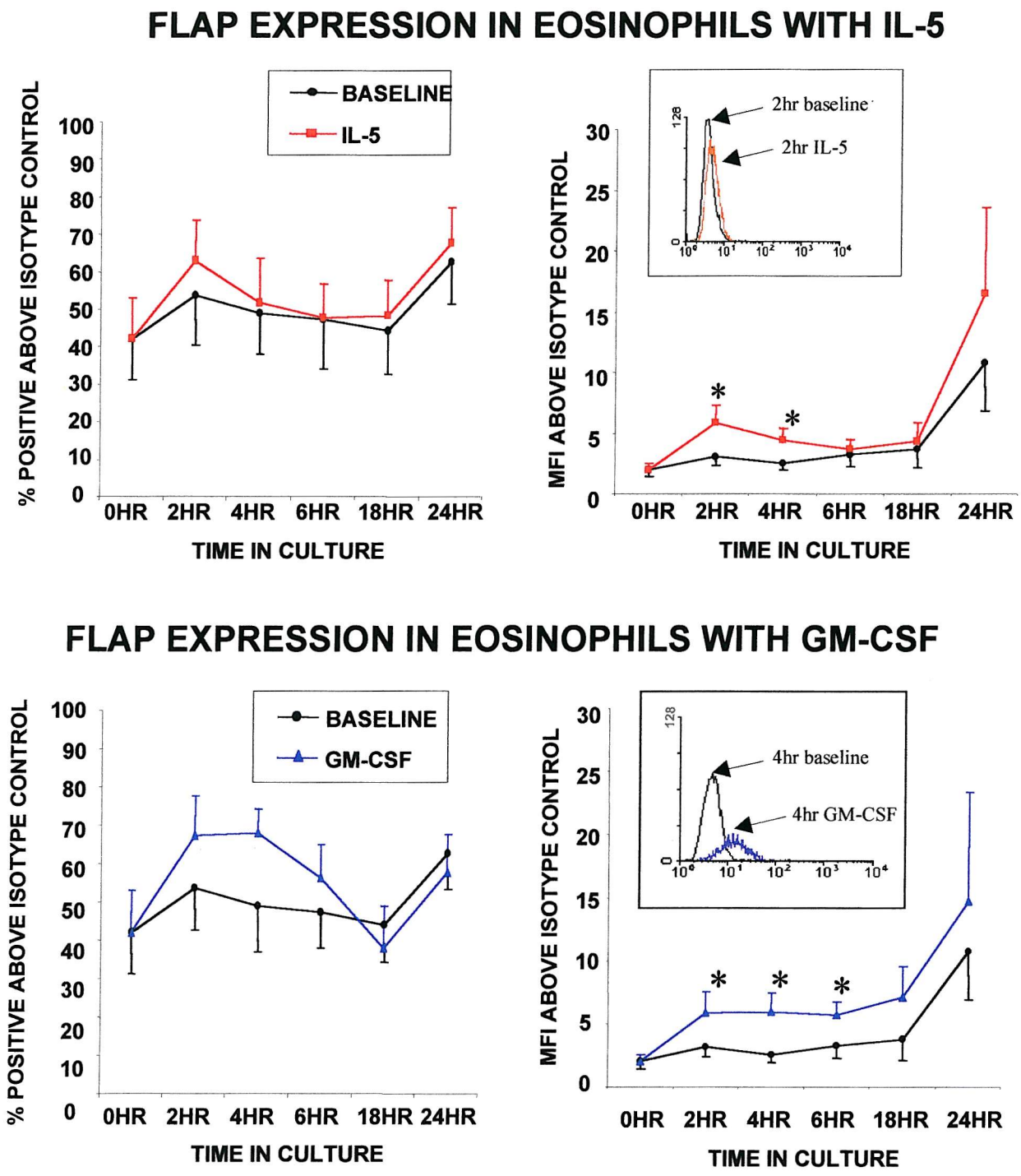


Fig. 6.14 ***Top left***: % FLAP expression in eosinophils incubated for 24 hours with (■) or without (●) IL-5. ***Top right***: MFI of eosinophils immunostained with anti-human FLAP antibody with (■) or without (●) IL-5. IL-5 increased MFI of FLAP staining compared to baseline over 6 hours (p=0.041 ANOVA). Insert: representative FACScan of FLAP staining after 2 hours incubation with (red) and without (black) IL-5 ***Bottom left***: % FLAP expression in eosinophils incubated for 24 hours with (▲) or without (●) GM-CSF. Insert: representative FACScan of FLAP staining after 4 hours incubation with (blue) and without (black) GM-CSF. ***Bottom right***: MFI of eosinophils immunostained with anti-human 5-LO antibody with (▲) or without (●) GM-CSF. GM-CSF increased MFI of FLAP expression in eosinophils compared to baseline over 6 hours (p=0.006 ANOVA). (* p<0.05 T-test).

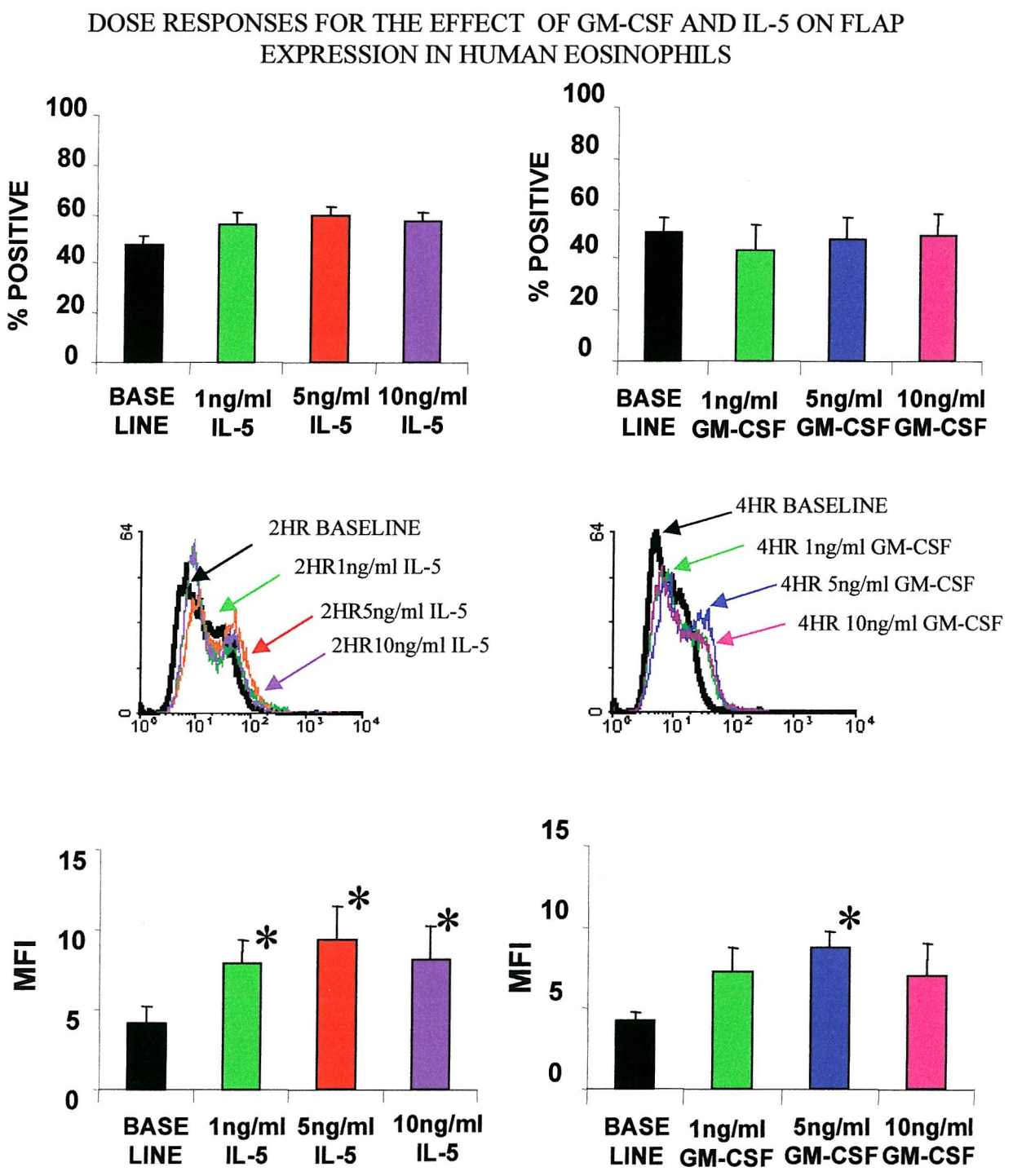


Fig. 6.15 ***Top left***: Dose responsive increase in % expression of FLAP in eosinophils incubated for 2 hours with IL-5. ***Middle left***: Representative FACScan of FLAP expression with increasing doses of IL-5. ***Bottom left***: Dose dependent increase in MFI of FLAP expression in eosinophils incubated for 2 hours with IL-5, reaching a maximum at 5ng/ml IL-5. (* $p<0.05$ T-test of treated vs baseline). ***Top right***: % expression of FLAP in eosinophils incubated for 4 hours with GM-CSF, % expression of FLAP does not change significantly with <10ng/ml GM-CSF. ***Middle right***: Representative FACScan of FLAP expression with increasing doses of GM-CSF. ***Bottom right***: Dose dependent increase in MFI of FLAP expression in eosinophils incubated for 4 hours with GM-CSF, reaching a maximum at 5ng/ml GM-CSF. (* $p<0.05$ T-test of treated vs baseline)

4.3% in controls to 59.9 ± 3.44% with 5ng/ml IL-5 (p=0.025 T-test). 5ng/ml is therefore the optimum concentration of IL-5 for the upregulation of FLAP in eosinophils.

6.7.3.3 GM-CSF incubation over 24hr.

FLAP expression was increased by incubation with 10ng/ml GM-CSF (**Fig 6.14**). In a similar manner to increased FLAP expression with IL-5, this increase is not evident in terms of % expression (p=0.467 ANOVA) but only in terms of MFI. Again due to the rising MFI of control samples of FLAP expression this is not significant when the ANOVA test includes the 18 and 24 hour time points (p=0.132 ANOVA) but becomes significant when these time points are excluded (p=0.006 ANOVA). Hence, culture of eosinophils with GM-CSF accelerates the increase in FLAP expression seen with control cultures. The increase in MFI of FLAP expression with GM-CSF is also significant by T-Test of individual time points (2 hours (p=0.043), 4 hours (p=0.043), and 6 hours (p=0.019) GM-CSF vs. control). There is a trend towards increased % expression of FLAP after 4 hours of incubation with GM-CSF (p=0.081 T-test). The greatest increase in FLAP expression is after 4 hours of treatment with GM-CSF with an increase in % expression from 42.1 ± 10.9% at baseline to 68.0 ± 6.3% with GM-CSF, and a three-fold increase in mean MFI from 2.0 ± 0.6 at baseline to 6.0 ± 1.5 with GM-CSF.

6.7.3.4 Dose response of effect of GM-CSF on FLAP expression.

The proportion of $FLAP^+$ eosinophils does not change with the addition of 1-10ng/ml GM-CSF (p>0.05 T-test). However, the mean MFI of FLAP expression increases from 4.22 ± 0.47 in the absence of cytokine to 7.24 ± 1.45 after 4 hours of incubation with 1ng/ml GM-CSF, reaching a maximum of 8.81 ± 0.88 with 5ng/ml GM-CSF but not rising further with 10ng/ml GM-CSF (7.08 ± 1.81) (**Fig 6.15**). There is a significant difference between FLAP expression in controls and FLAP expression after incubation for 4 hours with 5ng/ml GM-CSF (p=0.011 T-test). These data suggest that those cells already FLAP positive are increasing their levels of FLAP protein but that FLAP expression is not induced *de novo* in cells not previously expressing the protein.

6.7.4 Effect of IL-5 and GM-CSF on expression of COX-1 in eosinophils.

COX-1 was expressed in 10-20% of eosinophils at baseline. Eosinophils were incubated with or without 10ng/ml IL-5 or GM-CSF to determine the effect of these cytokines on COX-1 expression over 24 hours.

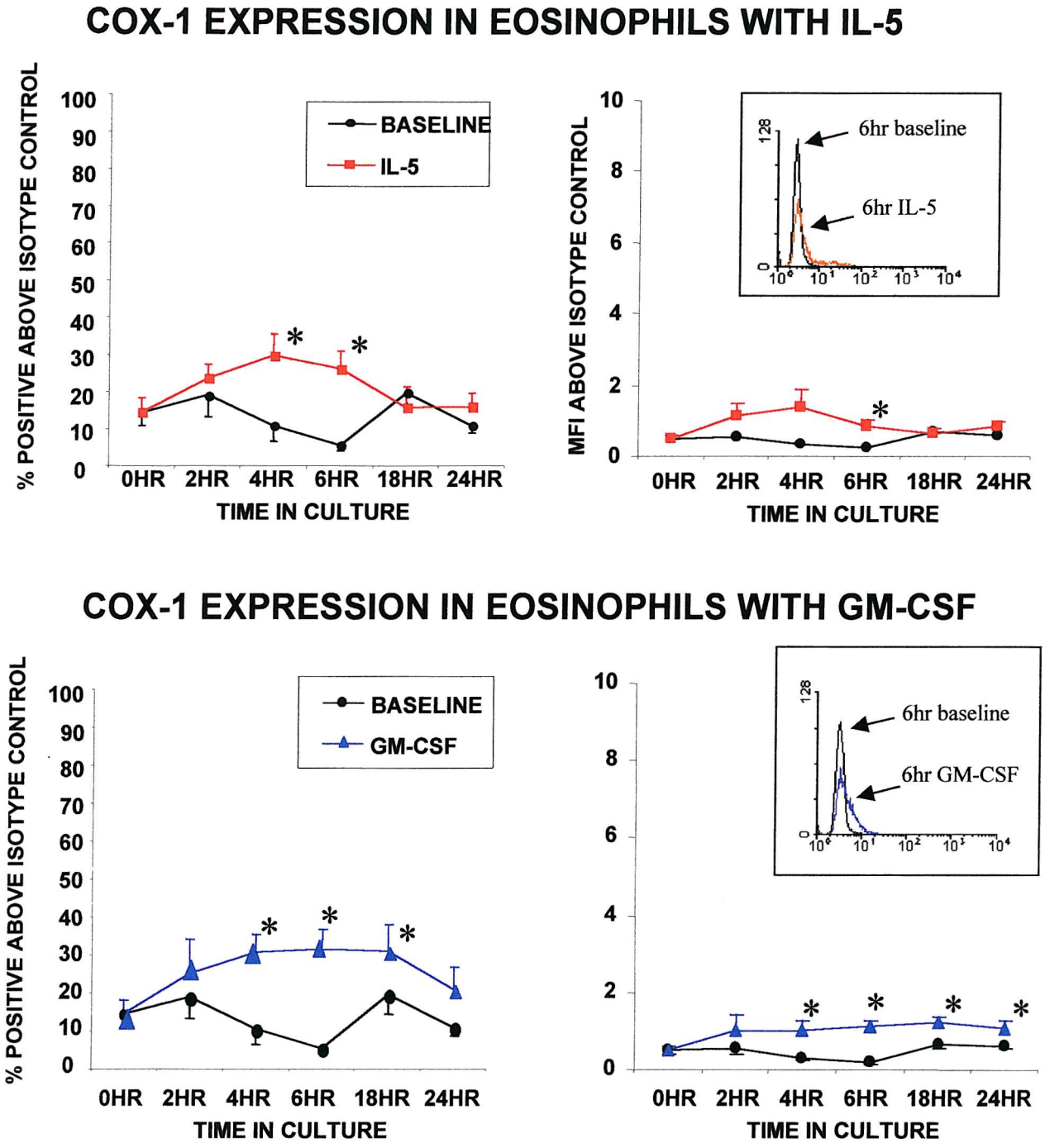
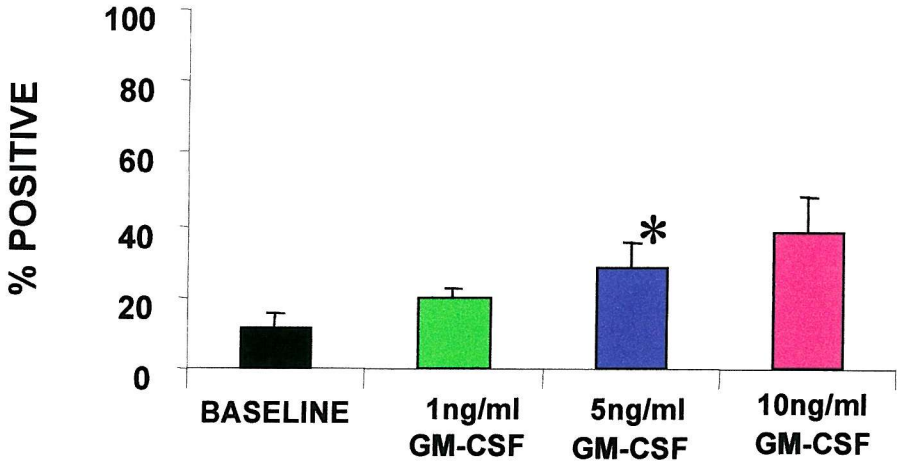
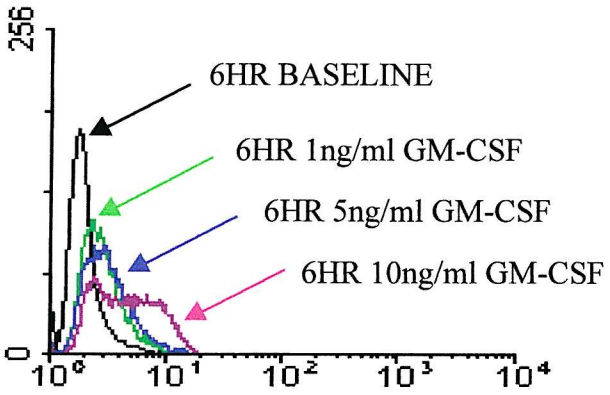


Fig. 6.16 ***Top left***: % COX-1 expression in eosinophils incubated for 24 hours with (■) or without (●) IL-5. ***Top right***: MFI of eosinophils immunostained with anti-human COX-1 antibody with (■) or without (●) IL-5. IL-5 increased expression of COX-1 compared to baseline over 24 hours ($p<0.003$ ANOVA). Insert: representative FACScan of COX-1 staining after 6 hours incubation with (red) and without (black) IL-5 ***Bottom left***: % COX-1 expression in eosinophils incubated for 24 hours with (▲) or without (●) GM-CSF. Insert: representative FACScan of COX-1 staining after 6 hours incubation with (blue) and without (black) GM-CSF. ***Bottom right***: MFI of eosinophils immunostained with anti-human COX-1 antibody with (▲) or without (●) GM-CSF. GM-CSF increased COX-1 expression in eosinophils compared to baseline over 24 hours ($p=0.001$ ANOVA). (* $p<0.05$ T-test).





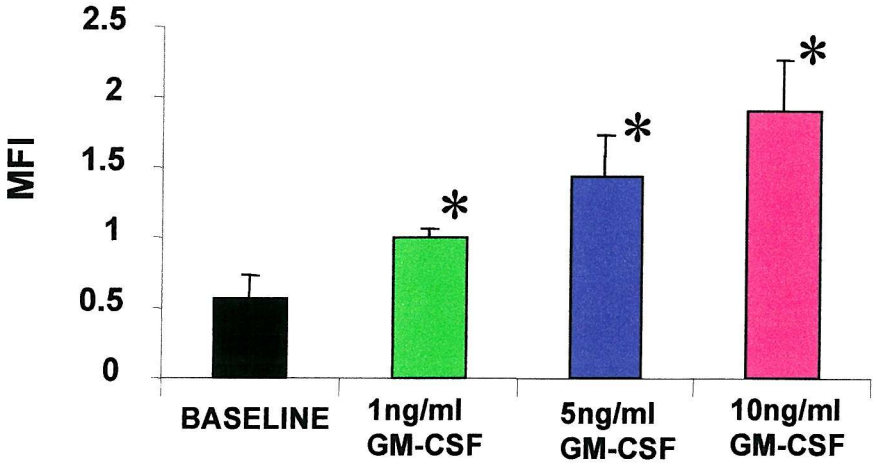


Fig. 6.17 ***Top***: Dose dependent increase in % expression of COX-1 in eosinophils incubated for 6 hours with GM-CSF, reaching a maximum at 10ng/ml GM-CSF. ***Middle***: Representative FACScan of COX-1 expression with increasing doses of GM-CSF. ***Bottom***: Dose dependent increase in MFI of COX-1 expression in eosinophils incubated for 6 hours with GM-CSF, reaching a maximum at 10ng/ml GM-CSF. (* $p<0.05$ T-test of treated vs baseline)

6.7.4.1 IL-5 incubation over 24hr

Percent expression of COX-1 was significantly increased by incubation with 10ng/ml IL-5 compared to baseline (p=0.003 ANOVA), MFI was also increased (p=0.002 ANOVA) (**Fig 6.16**). The proportion of COX-1^+ eosinophils was significantly increased after 4 hours (p=0.024 T-test) and 6 hours (p=0.0077 T-test) of treatment with 10ng/ml IL-5 compared to untreated cells at the same time points. The increase % expression of COX-1 was greatest at 6 hours rising from 5.6 ± 1.5% in controls to 26.2 ± 4.8% with IL-5. COX-1 MFI was also increased at 6 hours from 0.23 ± 0.07 in controls to 0.86 ± 0.18 with IL-5 (p=0.015 T-test). It was not determined whether this response was dose dependent.

6.7.4.2 GM-CSF incubation over 24hr.

COX-1 expression was increased by incubation with 10ng/ml GM-CSF as determined both by % expression (p<0.001 ANOVA) and MFI (p<0.001 ANOVA). This increase in % expression was evident after 4 hours (p=0.0046 T-test), 6 hours (p=0.0032 T-test) and 18 hours (p=0.017 T-test) of GM-CSF incubation. It was also evident in MFI after 4 hours (p=0.016 T-test), 6 hours (p=0.0021 T-test), 18 hours (p=0.022 T-test) and 24 hours (p=0.043 T-test) of GM-CSF incubation. The increase in COX-1 expression was maximal at 6 hours rising from 5.6 ± 1.5% (0.23 ± 0.07 MFI) in controls to 31.6 ± 5.1% (1.10 ± 0.18 MFI) with GM-CSF.

6.7.4.3 Dose response of effect of GM-CSF on COX-1 expression.

COX-1 expression increased steadily with increasing doses of GM-CSF reaching its highest levels at 10ng/ml GM-CSF (**Fig 6.17**). Four-fold increases were seen in COX-1 expression with 10ng/ml GM-CSF compared to in the absence of cytokine (p=0.077 T-test %, p=0.034 T-test MFI). Levels of COX-1 expression rose from 10.7 ± 5.3% (0.58 ± 0.15 MFI) at baseline to 38.5 ± 9.2% (1.9 ± 0.36 MFI). Therefore a concentration of GM-CSF greater-than or equal-to 10ng/ml is optimum for the upregulation of COX-1 in eosinophils.

6.7.5 **Effect of IL-5 and GM-CSF on expression of COX-2 in eosinophils.**

Expression of COX-2 was low to negligible in eosinophils at baseline. Cells were incubated with 10ng/ml IL-5 or GM-CSF to determine whether COX-2 expression could be induced in eosinophils.

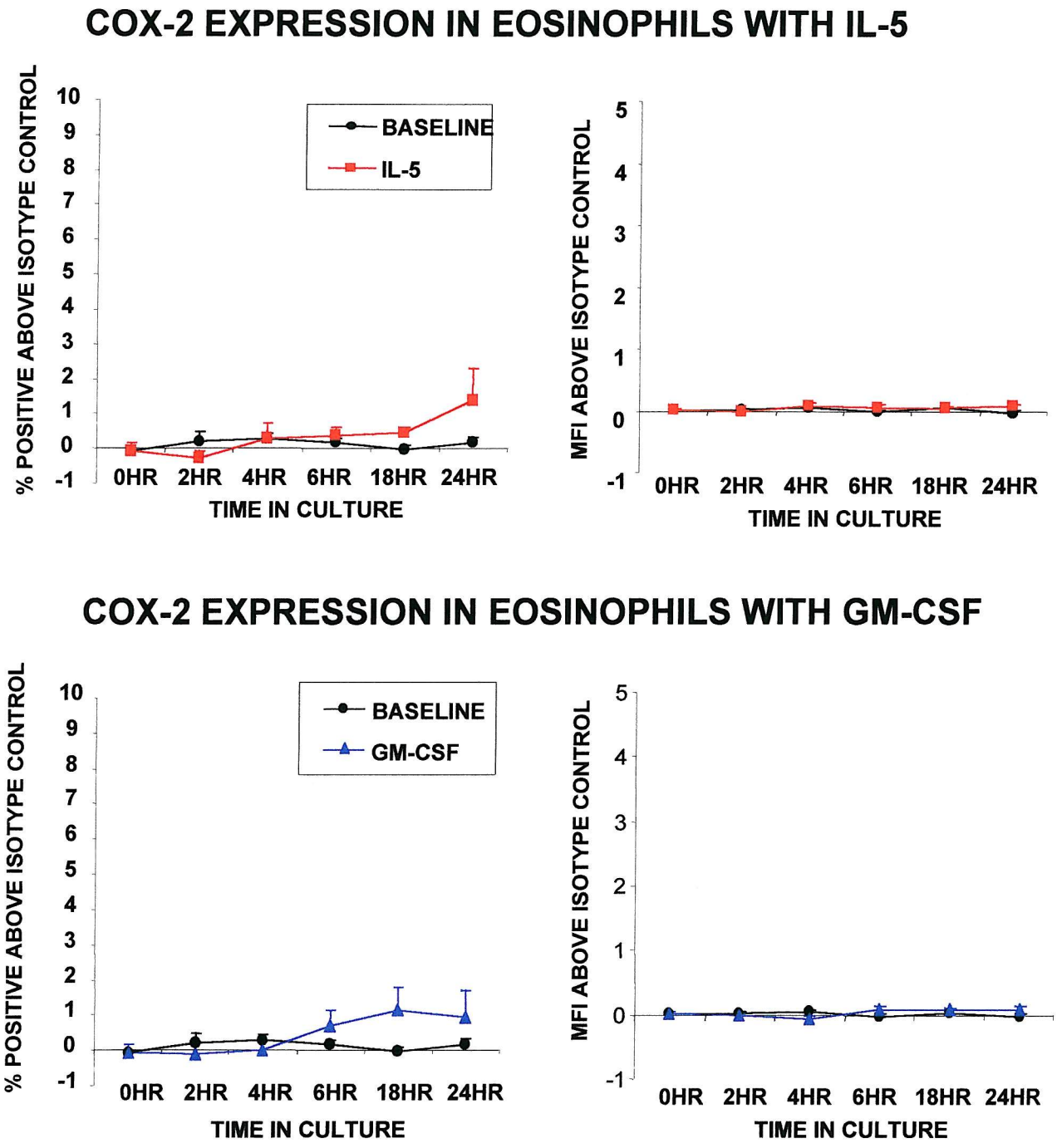


Fig. 6.18 ***Top left***: % COX-2 expression in eosinophils incubated for 24 hours with (■) or without (●) IL-5. ***Top right***: MFI of eosinophils immunostained with anti-human COX-2 antibody with (■) or without (●) IL-5. IL-5 increased MFI of COX-2 expression compared to baseline over 24 hours ($p=0.048$ ANOVA). ***Bottom left***: % COX-2 expression in eosinophils incubated for 24 hours with (▲) or without (●) GM-CSF. ***Bottom right***: MFI of eosinophils immunostained with anti-human COX-2 antibody with (▲) or without (●) GM-CSF. GM-CSF did not alter COX-2 expression in eosinophils compared to baseline over 24 hours ($p>0.1$ ANOVA). (* $p<0.05$ T-test).

6.7.5.1 IL-5 incubation over 24hr

The proportion of COX-2^+ eosinophils was not increased by 10ng/ml IL-5 compared to in the absence of cytokine (p=0.132 ANOVA). COX-2 MFI was significantly increased by IL-5 incubation (p=0.048 ANOVA) (**Fig 6.18**). This increase in COX-2 MFI trended toward significance after 18 hours of IL-5 incubation (p=0.07 T-test) and after 24 hours of IL-5 incubation (p=0.093 T-test), this suggests a late increase in COX-2 expression which may not have reached its maximum by 24 hours.

6.7.5.2 GM-CSF incubation over 24hr.

COX-2 expression was not changed by incubation with 10ng/ml GM-CSF compared to in the absence of cytokine (% p=0.132 ANOVA, MFI p=0.269 ANOVA) (**Fig 6.18**).

6.8 Summary and Discussion.

The basal expression of proteins of the eicosanoid pathway was characterised in purified human lung mast cells, and in peripheral blood eosinophils, neutrophils, monocytes and lymphocytes, and the effect of culture with the eosinophilopoietic cytokines IL-5 and GM-CSF on 5-LO, FLAP, COX-1 and COX-2 expression in purified eosinophils was examined.

Purified human lung mast cells were immunostained for 5-LO, FLAP, LTC_4 synthase, COX-1 and COX-2 as cytospin preparations and the percentages of positive cells were counted by light microscopy. Approximately 60% of mast cells expressed both 5-LO and FLAP. However expression of LTC_4 synthase was low with only 0.2% of cells expressing the enzyme. Baseline production of cys-LTs from human lung mast cells is likely to be low.

5-LO, FLAP and LTC_4 synthase have previously been identified in mouse bone marrow-derived mast cells (BMMC) developed with stem cell factor and IL-10 (Murakami et al. 1995c). 5-LO and FLAP were only detectable after 4 days in culture with IL-3, and LTC_4 synthase expression was not evident until after 4-5 weeks of culture with IL-3 (Murakami et al. 1995c). TNP-BSA stimulated LTC_4 generation was low in BMMC developed in SCF and IL-10 at approx. $8ng/10^6$ cells but rose to over $200ng/10^6$ cells after 2-4 weeks of culture with IL-3 (Murakami et al. 1995c).

Human lung mast cells of less than 20% purity stimulated with anti-IgE are reported to generate approx. 7ng LTC_4 $/10^6$ cells in the absence of SCF and $20ng/10^6$ cells in the presence of 10ng/ml SCF (Bischoff and Dahinden 1992). However Levi-Schaffer and colleagues (1987) were unable to measure significant levels of leukotriene

C_4 or B_4 from freshly isolated (23-68% purity) immunologically activated human lung-derived mast cells. LTB_4 production has also been detected in the human mast cell line (HMC1) (Macchia et al. 1995) and it is estimated that purified human lung mast cells can produce approx. 2ng LTB_4 / 10^6 cells (Freeland et al. 1988). This suggests that despite the low levels of LTC_4 synthase, human lung mast cells may have the capacity for LTC_4 production and that mast cells may also produce LTB_4. Expression of these leukotriene-producing enzymes in human lung mast cells may be upregulated by cytokines.

Expression of 5-LO, FLAP and LTC_4 synthase in BMMC is upregulated by IL-3 (Murakami et al. 1995). However, human mast cells do not have the IL-3 receptor, the equivalent cytokine in human cells is unknown.

Prostaglandin D_2 is the major eicosanoid product of mast cells (Levi-Schaffer et al. 1987) and can be produced in by skin mast cells in response to IgE-dependent stimulation, A23187, and by a number of non-immunological stimuli (Benyon et al. 1989, Holgate et al. 1984). Cyclooxygenase expression has never previously been characterised in purified human lung mast cells. We have shown that COX-1 was expressed in 82% of human lung mast cells, suggesting a large capacity for the production of prostanoids. COX-2 was expressed in only 3% mast cells and therefore likely contributes only a small amount to prostanoids produced by mast cells at baseline. COX-2 is upregulated in IL-3 developed BMMC within 10 hours of incubation with SCF, IL-1β and IL-10, (Murakami et al. 1994), and this upregulation was suppressed by IL-4 (Murakami et al. 1995a). COX-1 was upregulated after a period of 1-2 days of incubation with IL-3, IL-9, and IL-10 (Murakami et al. 1994). Induction of COX-2 increased cytokine-stimulated PGD_2 generation (Murakami et al. 1994), and induction of COX-1 increased IgE-dependent PGD_2 production (Murakami 1994). The early upregulation of COX-2 (hours) and the late upregulation of COX-1 (days) reflects their inducible and constitutive natures respectively. COX-1 and COX-2 may also be upregulated in purified human lung mast cells in a similar fashion by combinations of these or other cytokines.

Expression of eicosanoid enzymes in mixed leukocytes has never been characterised, and the antibodies to these proteins have never previously been studied by flow cytometry, a method that is quicker and more accurate than conventional immunostaining of cytospin preparations and analysis by light microscopy. Flow cytometry has allowed us to study individual cell populations in a mixed leukocyte preparation without the need for lengthy purification procedures.

We have shown that eosinophils, neutrophils, monocytes and lymphocytes from peripheral blood express both 5-LO and FLAP. Eosinophils, neutrophils and monocytes have previously been reported to express 5-LO (Cowburn et al. 1999, Brock et al. 1997, Coffey and Peters-Golden 1993) and FLAP (Cowburn et al 1999, Woods et al. 1993, Coffey and Peters-Golden 1993). 5-LO and FLAP were expressed in 10-15% of lymphocytes. The expression of 5-LO and FLAP in lymphocytes has been contentious. Both B and T lymphocyte cell lines have been reported to express FLAP, B-lymphocytes reportedly express 5-LO but it is not expressed in the T-lymphocyte cell lines studied (Jakobsson et al. 1992). 5-LO and FLAP were detected in 10% and 13% respectively of eosinophils within mixed leukocytes, while immunomagnetically purified eosinophils expressed 20% and 40% 5-LO and FLAP respectively. This variation may be due to the large number of mixed leukocytes immunostained with a given quantity of antibody compared to the smaller numbers of purified eosinophils immunostained with the same quantity. Hence, with proteins highly expressed in cells, the antibody may not be in excess and the detectable level of protein is effectively lowered.

Eosinophils preferentially generate LTC_4 (Shaw et al. 1985), the expression of LTC_4 synthase, the committed enzyme for LTC_4 production, in 25% of eosinophils reflects this capacity for LTC_4 production. Radioimmunoassay (RIA) has shown that LTC_4 is released by eosinophils after incubation with stimuli including A23187, unopsonized zymosan and PAF reaching a maximum between 15 and 30mins (Burke et al. 1990, Mahauthaman et al. 1988, Bruijnzeel et al. 1987). LTC_4 synthase was not detected in neutrophils that exclusively produce LTB4 and related metabolites and isomers when stimulated with A23187 (Verhagen et al. 1984) and LTC_4 synthase expression was also not evident in monocytes from normal subjects. LTC_4 synthase has been detected in the monocytic cell line THP-1 and A23187 stimulation of these cells resulted in LTC_4 synthesis (Riddick et al. 1999). Two percent of lymphocytes immunostained positively for LTC_4 synthase.

LTA_4 hydrolase was detected in 13% of eosinophils. LTB_4 synthesis by eosinophils has been reported (Henderson et al. 1984) but this was measured in an impure population of eosinophils. Bruijnzeel and colleagues (1985) and Verhagen and colleagues (1984) have shown that eosinophils exclusively produce LTC_4 when stimulated with calcium ionophore and therefore the expression of LTA_4 hydrolase in eosinophils may not be a true measurement. The antibody for LTA_4 hydrolase is crude rabbit polyclonal antiserum and may produce a background signal. We have shown LTA_4 hydrolase expression in greater than 30% of neutrophils and monocytes and this corresponds well with their preferential production of LTB_4 (Bray 1983). LTA_4

hydrolase was expressed in 25% of lymphocytes. Lymphocytes are not considered to be a significant source of leukotrienes, but LTB_4 production has been measured in tonsillar B-lymphocytes and peripheral blood T-lymphocytes incubated with the precursor LTA_4 (Odlander et al 1989).

Expression of the LTC_4 export proteins MRP-1 and MRP-2 was low or negligible in all cells. In eosinophils, which produce significant amounts of LTC_4, it was surprising that only 0.8% of cells expressed MRP-1 and only 2.3% of cells expressed MRP-2 since it is known that LTC_4 is exported from eosinophils in an ATP-dependent manner (Lam et al. 1989) and that MRP is a leukotriene C_4 export pump (Leier et al. 1996). MRP-1 and 2 are membrane proteins and the fixation and permeabilisation of these cells for intracellular staining for flow cytometry may have disrupted the MRP antigens. The LTC_4 export system is distinct from the LTB_4 carrier system (Lam et al. 1992); hence neutrophils, monocytes and lymphocytes that produce predominantly LTB_4 would not necessarily express large amounts of the LTC_4 export protein.

COX-1 was detected in 10 & 20% of all leukocyte types, whereas the proportion of cells expressing COX-2 was less than 3% for both eosinophils and neutrophils, but higher in monocytes (10%) and in lymphocytes (7%), suggesting that the capacity for the production of prostanoids is greater in monocytes than the other leukocytes. Peripheral blood monocytes have been reported to produce TXB_2, 6-keto $PGF_{2\alpha}$ and PGE_2 (Hoffman et al. 1987) but it is not known which are COX-1 or COX-2 products. The major prostanoid product from peripheral blood eosinophils is TXB_2, followed by PGD_2, PGE_2 and $PGF_{2\alpha}$ in descending order (Kroegel and Matthys. 1993). Human neutrophils have been shown to produce PGE_2 and TXB_2 when treated with LPS (Niiro et al. 1997). Lymphocytes do not produce any prostanoid products when stimulated with A23187 or TPA (Hoffman et al. 1987). In this study lymphocytes had COX-1 expression comparable with eosinophils and COX-2 expression higher than in both eosinophils and neutrophils, indicating that lymphocytes do have the capacity to produce prostanoids. However, similar to their capacity for LTB_4 production (Odlander et al 1989) they do not release any product with A23187 stimulation and therefore are unlikely to produce either leukotrienes or prostanoids under normal conditions. PGD_2 synthase expression is high in all cells suggesting that all cells have the capacity to produce significant quantities of PGD_2. Prostanoid assays suggest that among myeloid cells only the mast cell produces large amounts of PGD_2, the antibody against PGD_2 synthase is a crude rabbit polyclonal antiserum which is not yet fully characterised and these data therefore indicate that the specificity of the antibody may be questionable. Antibodies against the other committed

prostanoid synthases are currently unavailable and therefore cannot be used to discuss the profile of prostanoid production from these leukocytes.

Purified eosinophils were incubated with the eosinophilopoietic cytokines IL-5 and GM-CSF for 24 hours. IL-5 and GM-CSF are eosinophil survival-promoting cytokines (Walsh et al. 1998); we saw increased viability of eosinophils cultured with 10ng/ml IL-5 or GM-CSF compared with vehicle control.

Cys-LT production by eosinophils is increased by incubation with IL-5 (Takafugi et al. 1991, Cowburn et al. 1999). IL-5 upregulated the expression of FLAP, COX-1 and COX-2, but not 5-LO in the current study. The effect of IL-5 on 5-LO and FLAP has previously been determined in our department by Cowburn and colleagues (1999) by conventional immunostaining of cytospin preparations and analysis by light microscopy, while in the present experiments flow cytometry was used. The proportion of eosinophils expressing FLAP at baseline when analysed by immunocytochemistry was approx. 35% and rose to 65% with the addition of 10ng/ml IL-5, (Cowburn et al. 1999). When analysed by flow cytometry FLAP expression in eosinophils at baseline was approx. 40% and rose to 63% 6 hours after the addition of IL-5.

Expression of 5-LO in eosinophils was approx. 20% when measured by flow cytometry, substantially lower than the 80% expression determined by immunocytochemistry (Cowburn et al. 1999). Expression of 5-LO was, however, unchanged in eosinophils incubated with IL-5 consistent with the data reported by Cowburn and colleagues (1999). This suggests that the two methods are comparable but that absolute numbers of positive cells cannot be considered definitive. The advantage of flow cytometry over light microscopy is that larger numbers of cells can be counted (10,000) reducing the risk of statistical variation when only counting a few hundred cells. In addition, investigator error is reduced as the definition of a 'positive cell' can be strictly defined by analysis of the isotype control. Flow cytometry (MFI) also gives some information about levels of expression of protein in individual cells. The MFI data showed that the amount of FLAP protein is increased by GM-CSF in cells already expressing FLAP rather than *de novo* expression in cells not previously expressing the protein. When cells are analysed by Western blot, information about the total quantity of protein is available but not whether a few cells are expressing the protein at high levels or whether all cells are expressing a moderate amount.

Confocal microscopy showed that 5-LO translocated to the nucleus of eosinophils after exposure to IL-5 (Cowburn et al. 1999), this highlights a disadvantage of analysis of protein expression by flow cytometry as it gives no information about the localisation of the protein within the cell.

Eosinophil expression of both 5-LO and FLAP was upregulated by GM-CSF. This contrasts with the effect of IL-5 on eosinophils where 5-LO expression was unchanged but FLAP was increased. The difference in response may be a result of the greater affinity of GM-CSF for its receptor than IL-5 (Miyajima et al. 1993), or the 'constitutive' characteristics of the 5-LO gene (Funk et al. 1989, Hoshiko et al. 1990) compared to the 'highly inducible' characteristics of the FLAP gene (Kennedy et al. 1991). These data are, however, consistent with observations in neutrophils where FLAP and 5-LO proteins are increased by incubation with GM-CSF (Pouliot et al. 1994a, Pouliot et al 1994b), and also with studies in both monocytes and the monocytic cell line THP-1 where 5-LO and FLAP proteins are upregulated by GM-CSF and IL-3 (Ring et al. 1996, Ring et al. 1997).

Eosinophils cultured in the absence of cytokine show evidence of increased 5-LO and FLAP immunostaining over 24 hours. IL-5 and GM-CSF increase the viability of eosinophils in culture and hence the increase in staining may be an effect of non-viable cells that have not broken up or changed morphologically taking up the antibody in a non-specific manner. These cells cannot be eliminated with the use of propidium iodide (PI) as the cells are fixed for intracellular staining and they would all stain with PI. However, eosinophils incubated with Il-5 do not have significantly different 5-LO expression compared to control cells and both display the rise in 5-LO expression over time (**Fig 6.12** ***Top***). This would indicate that the culture conditions used for these experiments (RPMI + 10% FCS) may be causing an increase in 5-LO expression. Therefore, IL-5 and GM-CSF accelerate the increase in FLAP and/or 5-LO caused by culture of eosinophils.

The effect of IL-5 on cyclooxygenase expression in eosinophils has not previously been investigated. COX-1 expression was increased within 4 hours of incubation with IL-5 and persisted beyond 18 hours. COX-2 expression was increased only after 18-24 hours of incubation with IL-5. This increase was marginal and a larger increase over a longer period of time cannot be excluded. In addition, expression of COX-1 but not COX-2 was upregulated by GM-CSF within 24 hours. In mouse bone marrow-derived mast cells COX-1 and COX-2 are upregulated by pro-inflammatory cytokines, but COX-2 expression is increased within 6-12 hours and COX-1 expression is increased after 1 day in culture and persists for 7 days (Murakami et al. 1994). A similar pattern was seen in PMA stimulated human umbilical vein endothelial cells (HUVEC). COX-2 mRNA was substantially induced between 1-10 hours whereas COX-1 mRNA was induced more slowly (10-24 hours) and to a lesser extent (Hla and Neilson 1992). COX-1 & 2 are regulated by different mechanisms (Pilbeam et al. 1993).

Our results are not consistent with studies in mouse bone marrow-derived mast cells (Murakami et al. 1994), HUVECs (Hla and Neilson 1992), osteoblastic MC3T3-E1 cells (Pilbeam et al. 1993) or in the colonic epithelial cell line HT-29 (Jobin et al. 1998) which show COX-2 to be induced more rapidly and by more stimuli than COX-1. These data suggest that these mechanisms may differ between cell types and/or between stimuli such that COX-2 is upregulated more slowly than COX-1 by IL-5, and only COX-1 is upregulated by GM-CSF in eosinophils.

CHAPTER 7

Modulation of leukotriene production and release from purified peripheral blood eosinophils by leukotriene modifier drugs.

Introduction and aims.

The multi-drug resistance associated protein (MRP) has been identified as a cysteinyl-leukotriene transport protein, and may lead to the development of a new class of leukotriene modifier drug. MRP inhibitors are being developed and are being assessed for their effects on inhibiting leukotriene C_4 release. The MRP inhibitors could potentially have greater anti-inflammatory effects than leukotriene receptor antagonists (LTRA) because the prevention of LTC_4 release from the cell would prevent cys-LT activity at both cys-LT_1 and cys-LT_2 receptors, whereas LTRA only block cys-LT_1 and not the action of cys-LTs at the cys-LT_2 receptor on blood vessels. MRP inhibition may also have benefits over LT synthesis inhibitors, as MRP is specific for the cys-LTs but not LTB_4 (Lam et al. 1992). MRP inhibitors may therefore have important anti-inflammatory effects in asthma. Inhibition of MRP also enhances the effectiveness of cytotoxic drugs by blocking glutathione-dependent transport and prohibiting efflux of these drugs from the target cells. The combination of the anti-inflammatory and pro-cytotoxic effects of MRP inhibition may have important therapeutic consequences in cancer therapy. *In this study we aimed to determine the contribution of MRP to leukotriene C_4 export in eosinophils using a putative MRP-1 inhibitor XR9173, and to compare its effects with those of MK-571, a leukotriene receptor antagonist with MRP inhibition properties (Leier et al. 1994b), and MK-886, a FLAP inhibitor (Vickers et al. 1995).* Leukotriene C_4 synthesis by eosinophils was stimulated by 1μM A23187 for 15min at 37°C and LTC_4 released into the supernatant and intracellular LTC_4 extracted from the cell pellet were measured by enzyme immunoassay.

7.1 Leukotriene production/release from eosinophils.

Unstimulated eosinophils generated small amounts of LTC_4 (0.88 ± 0.7 ng/million cells n=4), 60% of which is released from the cell and 40% retained (**Fig 7.1**). Stimulation of eosinophils with 1μM A23187 caused a 20-fold increase in LTC_4 production (18.9 ± 3.4 ng/million cells n=4), 79% of which was released from the cell and 21% was retained (**Fig 7.1**). The leukotriene modifier drugs used in this study were dissolved as 5mM stock solutions in DMSO, and further diluted in DMSO to working concentrations such that the percentage of DMSO in each experiment was the same (1%). The concentration of DMSO from the addition of A23187 was negligible (0.05%). The addition of 1% DMSO to the culture medium reduced the A23187 stimulated release to 9.0 ± 1.6 ng/million cells of which 85% was released and 15% was retained (n=4) (**Fig 7.1**). This was the appropriate vehicle control.

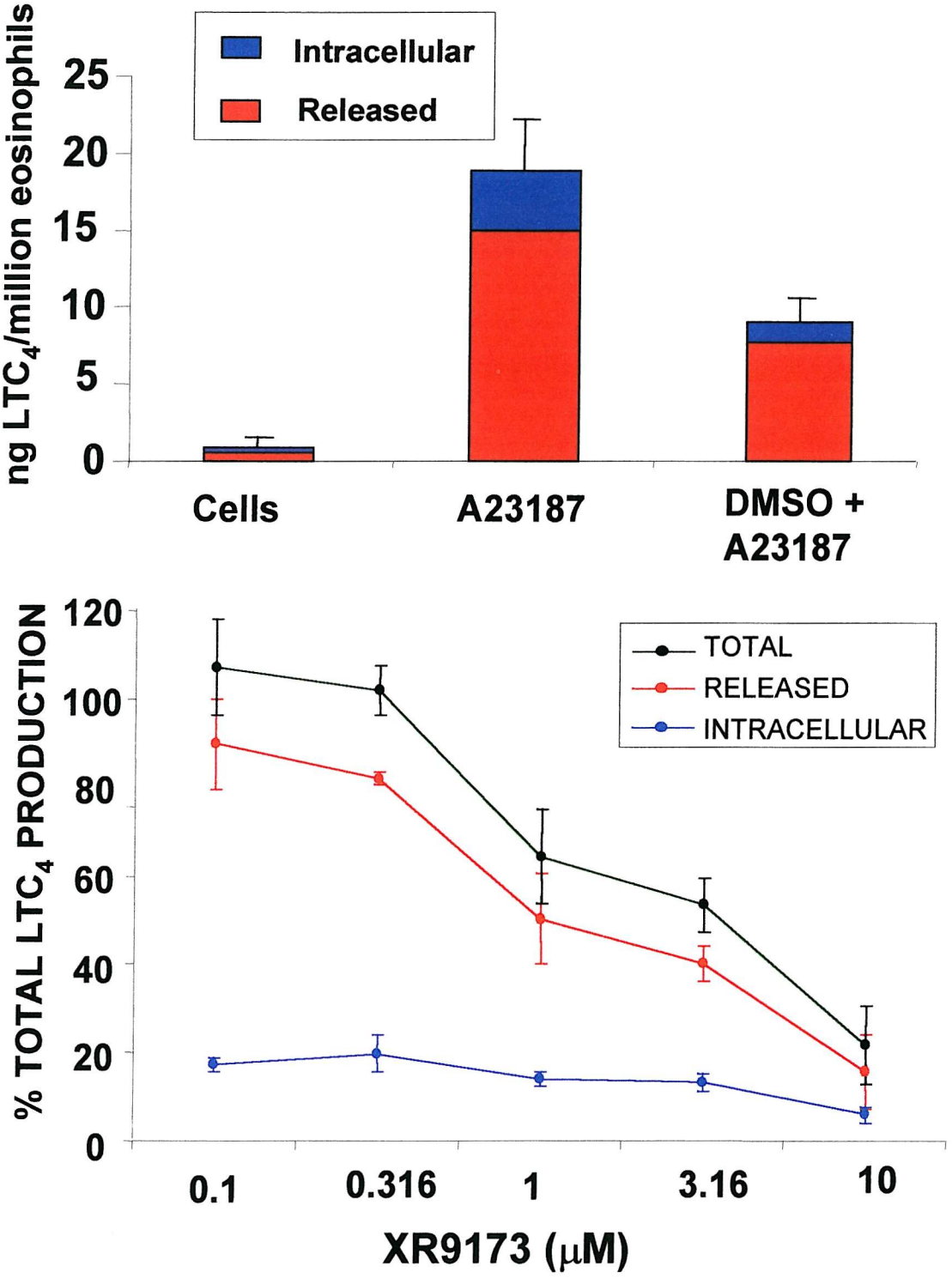


Fig 7.1 ***Top***: Baseline and A23187 stimulated LTC_4 production by eosinophils, and the effect of 1% DMSO (vehicle control) on simulated production. Bars represent total LTC_4 production (released (red) + intracellular (blue)). ***Bottom***: Effect of increasing concentrations (0.1μM-10μM) XR9173 on total, released and intracellular LTC_4 in eosinophils expressed as % of total LTC_4 production by vehicle control. XR9173 10μM inhibited total LTC_4 production by 89% (p=0.000 ANOVA) and significantly inhibited both released and intracellular LTC_4 (p=0.001, p=0.005 respectively, ANOVA).

7.2 The effect of XR9173 on A23187 stimulated leukotriene production/release from eosinophils.

XR9173 was added to eosinophil cultures at concentrations between 0.1 and 10μM (**Fig. 7.1**). At 0.1 μM XR9173, total A23187 stimulated LTC_4 production was 107.1 ± 11% of that produced by the vehicle control and significantly decreased to 21.6 ± 8.8 % with increasing concentrations of XR9173 to 10μM XR9173 ($p<0.001$ ANOVA) (**Fig 7.1**). At each concentration of XR9173, the proportion of total LTC_4 released was consistently between 72 and 84%, and the proportion retained (intracellular) was consistently between 16 and 28%. Both released and intracellular LTC_4 were significantly decreased ($p=0.001$, $p=0.005$ respectively ANOVA) (**Fig 7.1**). The lack of an increase in intracellular LTC_4 associated with decreased LTC_4 release suggests that XR9173 is inhibiting LTC_4 production rather than export.

7.3 The effect of MK-886 on A23187 stimulated leukotriene production/release from eosinophils.

To examine the effects of a known LT synthesis inhibitor in this system the leukotriene synthesis inhibitor MK-886 was added to eosinophil cultures at concentrations between 1 and 100nM (**Fig 7.2**). At 1nM MK-886, total A23187 stimulated LTC_4 production was 116.9 ± 15.6 % of the vehicle control value and this was reduced to 1.4 ± 0.3 % by MK-886 31.6nM ($p=0.004$ ANOVA) (**Fig 7.2**). Released LTC_4 was in the range of 78-86% and the intracellular LTC_4 between 16 and 22% of total production at each concentration of MK-886 (**Fig 7.2**). Both released and intracellular LTC_4 were significantly reduced by the addition of MK-886 over this concentration range ($p=0.008$ for both. ANOVA). The nM range at which MK-886 is effective and the almost complete abrogation of LTC_4 production confirms that MK-886 is a potent and specific inhibitor of LTC_4 synthesis in this system.

7.4 The effect of MK-571 on A23187 stimulated leukotriene production/release from eosinophils.

MK-571 is reported to inhibit LTC_4 export from the cell (Leier et al. 1994b). Total LTC_4 production was significantly decreased from 111.9 ± 8.4 % to 49.9 ± 10.8 % of vehicle control by MK-571 between 0.1 and 10μM ($p=0.001$ ANOVA) LTC_4 synthesis/ release was not fully inhibited by 10μM MK-571 (**Fig 7.2**). Over this range of MK-571 concentrations, released LTC_4 decreases significantly ($p=0.003$ ANOVA) but intracellular LTC_4 does not change significantly with increasing doses of MK-571 ($p=0.668$ ANOVA). LTC_4 release remained in the range 86 to 73% and intracellular

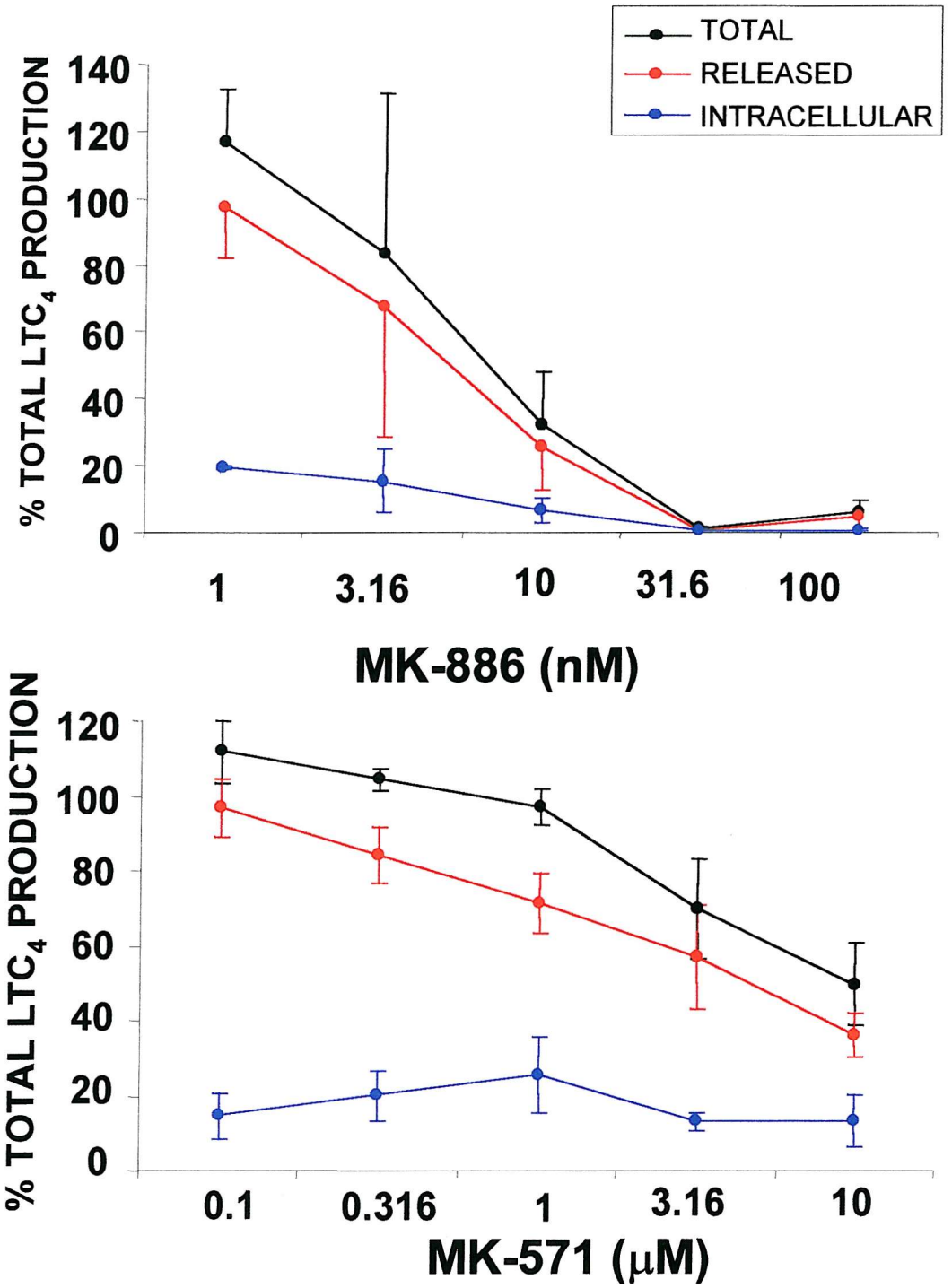


Fig 7.2 ***Top***: Effect of increasing concentrations (1nM-100nM) MK-886 on total (black), released (red) and intracellular (blue) LTC_4 in eosinophils expressed as % of total LTC_4 production by vehicle control. MK-886 100nM inhibited total LTC_4 production by 98.5% (p=0.004 ANOVA), and both released and intracellular LTC_4 were inhibited (p=0.008 for both, ANOVA) ***.Bottom***: Effect of increasing concentrations (0.1μM-10μM) MK-571 on total (black), released (red) and intracellular (blue) LTC_4 in eosinophils expressed as % of total LTC_4 production by vehicle control. MK-571 10μM inhibited total LTC_4 production by 50% (p=0.001 ANOVA). Released LTC_4 was decreased by MK-571(p=0.003 ANOVA) but intracellular LTC_4 was not changed (p=0.668 ANOVA)

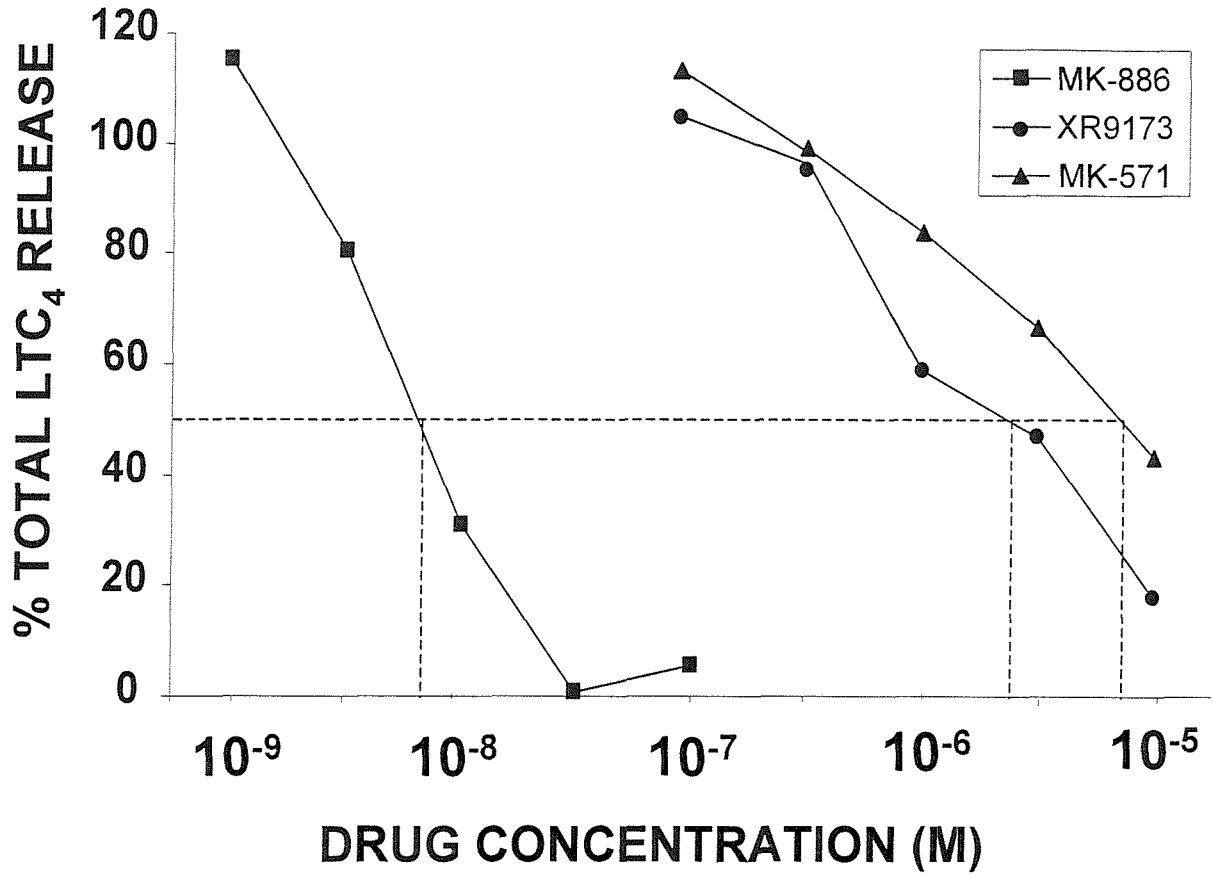


Fig. 7.3. Inhibition curves for the effect of MK-886 (■), XR9173 (●) and MK-571 (▲) on LTC_4 release from eosinophils compared to LTC_4 release from vehicle control. Dashed line represents IC_{50} values of <10nM, <3μM and <10μM respectively.

LTC_4 within the range 14 to 27% of total LTC_4 production with each dose of MK-571 (**Fig 7.2**). These values are within the range seen with the other compounds, but the lack of change in intracellular LTC_4 suggests that LTC_4 export is indeed inhibited by MK-571. However, the decrease in total LTC_4 production also suggests that MK-571 may be inhibiting synthesis of LTC_4.

7.5 Comparison of the effect of compounds XR9173, MK-571 and MK-886 on A23187 stimulated leukotriene production/release from eosinophils.

MK-886 inhibits LTC_4 synthesis at a range of 1-100nM and half maximal inhibition (IC_{50}) occurs between 3 and 10nM (**Fig 7.3**). This indicates that MK-886 is a more potent LT modifier than either MK-571 or XR9173, both of which inhibit LTC_4 release in the μM range (**Fig. 7.3**). LTC_4 release is half maximally inhibited by XR9173 at less than 3μM and by MK-571 at less than 10μM (**Fig. 7.3**), suggesting that XR9173 is a marginally more potent LT modifier than MK-571.

7.6 Discussion.

Total, released and intracellular LTC_4 were decreased by increasing concentrations of XR9173, suggesting that XR9173 is not inhibiting LTC_4 transport but may be a weak inhibitor of leukotriene synthesis by actions on 5-LO, FLAP or LTC_4 synthase.

MK-886 is generally considered a FLAP inhibitor but its effects are not limited purely to FLAP inhibition. At much higher concentrations, MK-886 has also been described to inhibit LTC_4 synthase (Lam et al. 1994) and export of LTC_4 in inside-out plasma membrane vesicles (Schaub et al. 1991) and platelets (Sjolinder et al. 1999). MK-886 inhibits FLAP with an IC_{50} of ~3nM (Vickers et al. 1995), but inhibits LTC_4 synthase with an IC_{50} of <3μM (Lam et al. 1994) and acts as an LTC_4 transport inhibitor with an IC_{50} of <2μM (Schaub et al. 1991). We have confirmed that MK-886 inhibits LTC_4 production with an IC_{50} of ~3nM. The effects of transport inhibition were not evident at micromolar concentrations of MK-886 as LTC_4 production was already maximally inhibited. We cannot determine the contribution of LTC_4 synthase inhibition as the effect will be the same as for FLAP inhibition. For the purpose of this study, however, MK-886 can be considered as a specific synthesis inhibitor as LTC_4 production is almost completely abrogated in the nM range.

MK-571 is a cys-LT_1 receptor antagonist with secondary inhibitory actions on LTC_4 transport (Leier et al. 1994b, Schaub et al. 1991). MK-571 inhibits transport of LTC_4 in plasma membrane vesicles with an IC_{50} of 1μM (Schaub et al. 1991, Leier et al.

1994b). This IC_{50} was confirmed in the present study, where release of LTC_4 from eosinophils was half-maximally inhibited at less than 10μM. The effect of probenecid (inhibitor of organic acid transport) on LTC_4 release from eosinophils preloaded with LTC_4 was studied by Lam and colleagues in 1992. They showed that with increasing concentrations of probenecid (0-20mM) the release of LTC_4 was reduced but that the total LTC_4 (released plus intracellular) was maintained, showing no effect on synthesis. We have shown that with an increasing concentration of MK-571 (0.1μM - 10μM) LTC_4 release is reduced, but if LTC_4 export were inhibited a reciprocal increase in intracellular LTC_4 would be expected. An increase in intracellular LTC_4 was not seen but in contrast to MK-886 and XR9173 there was no decrease in intracellular LTC_4, suggesting that MK-571 may also be inhibiting LTC_4 export. However, total LTC_4 is reduced (by 50%), suggesting that MK-571 may also have LT synthesis inhibition properties.

MK-886 inhibits FLAP, LTC_4 synthase and LTC_4 export; MK-571 is a LT receptor antagonist but has LTC_4 export inhibition properties and possibly may also inhibit LT synthesis. The multiple effects of the anti-leukotriene drugs on different levels of leukotriene synthesis, transport and effect are understandable since the majority of drugs are structural analogues of the natural substrates of these proteins. The structure of XR9173 is based on glutathione, the common moiety of cys-LTs and other substrates of MRP. However, it appears that in the micromolar range XR9173 is acting as a synthesis inhibitor. It may also inhibit LTC_4 export as proposed but this may occur only at concentrations above 10μM, at which LTC_4 synthesis is already inhibited. The characterisation of the LT modifier properties of XR9173 is difficult to achieve, as the comparison with MK-886 and MK-571 was complicated by the multiple properties of these drugs. To further establish the role of XR9173 as a LT synthesis inhibitor its effect on LTB_4 synthesis in neutrophils could be studied to identify possible inhibition of enzymes above LTC_4 synthase in the synthetic pathway. Neutrophils do not express LTC_4 synthase and LTB_4 is their predominant leukotriene product. Inhibition of LTB_4 synthesis in neutrophils by XR9173 would suggest that XR9173 is an inhibitor of 5-LO or FLAP. Direct effects of XR9173 on LTC_4 synthase could be investigated in platelets in which 5-LO is absent. LTC_4 synthesis could be measured following addition of stable LTA_4 methyl esters. Any effects of simultaneous export inhibition could be determined in a cell free system, with the addition of radiolabelled arachidonic acid or LTA_4 and production of labelled LTC_4 measured. Its role as a LTC_4 export inhibitor could be determined using plasma membrane vesicles preloaded with LTC_4, and release of LTC_4 measured. A23187 is a non-specific cell stimulator and a more physiological stimulator of LTC_4 synthesis in eosinophils should also be investigated.

CHAPTER 8

General discussion

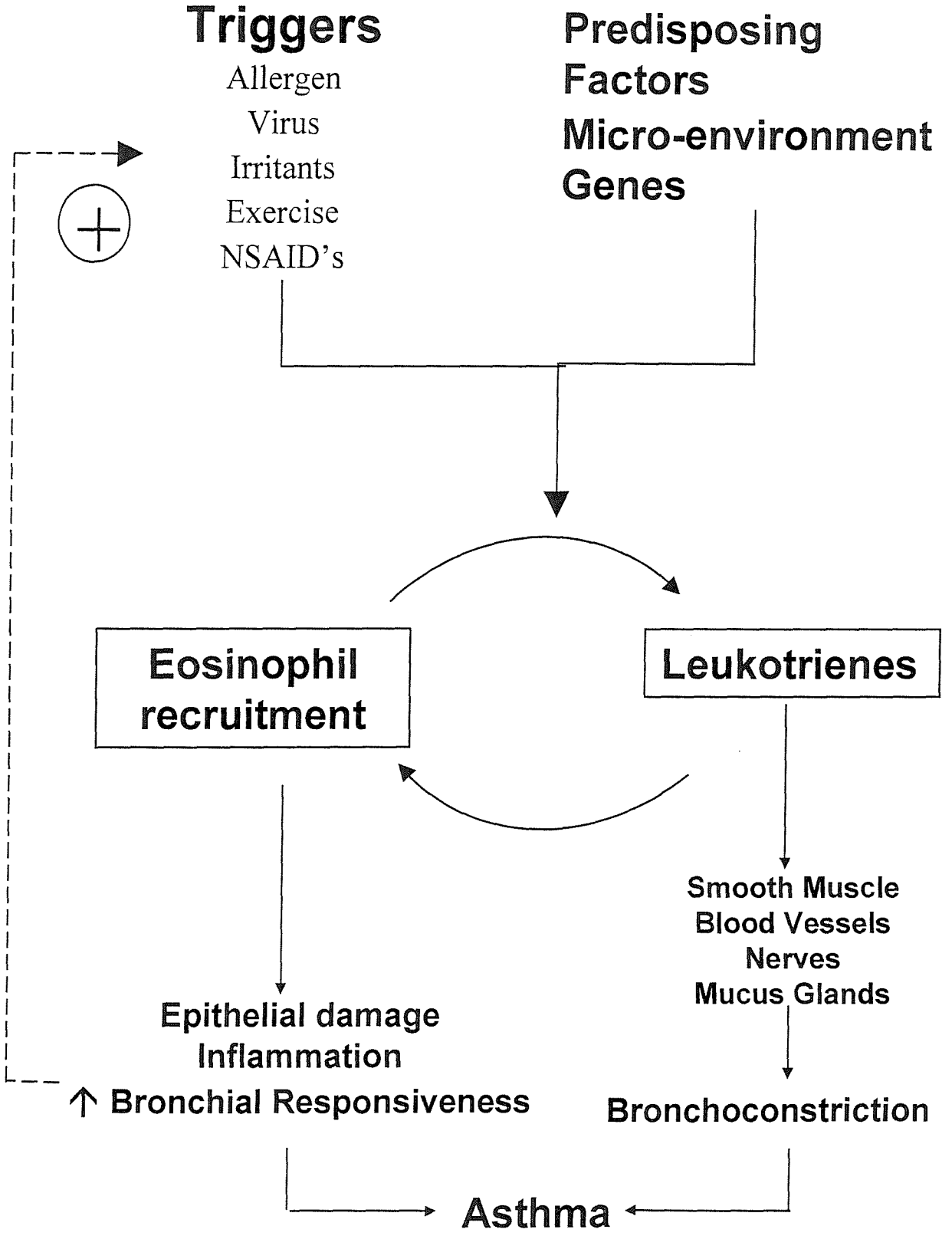
Eicosanoids are derived from arachidonic acid released from membrane phospholipids. The arachidonic acid passes down two pathways for the production of leukotrienes and prostanoids. Leukotrienes are produced by the action of 5-LO and FLAP on arachidonic acid. The unstable product of the lipoxygenase reaction, LTA_4 is then further metabolised either by leukotriene A_4 hydrolase for the production of the potent neutrophil chemoattractant LTB_4, or by leukotriene C_4 synthase for the production of LTC_4. Following export of LTC_4 from the cell by MRP, the other cysteinyl leukotrienes (LTD_4 and LTE_4) are produced by the actions of enzymes ubiquitous in tissue fluids.

The cysteinyl leukotrienes act on cys-LT receptors and are exceptionally potent constrictors of bronchial smooth muscle (Dahlen et al. 1980, Chagnon et al. 1985). They also impair mucociliary clearance (Bisgaard and Pedersen, 1987) and increase mucus secretion (Marom et al. 1982, Coles et al. 1993). In addition, the cys-LTs increase microvascular permeability causing oedema (Woodward et al. 1983), and are specific chemoattractants for eosinophils (Spada et al. 1994, Laitinen et al. 1993). These actions implicate the cys-LTs in the pathogenesis of asthma.

Prostanoids are thought to modulate the production/ actions of the cys-LTs. In aspirin-sensitive asthma, PGE_2 acts as a brake on cys-LT production and prevents aspirin-induced bronchoconstriction and the increase in LTE_4 excreted in urine (Sestini et al. 1996). PGE_2 also has directly opposing actions to the cys-LTs by causing relaxation of bronchial smooth muscle (Walters et al. 1984). The other prostanoids relevant in asthma, PGD_2, $PGF_{2\alpha}$, and TXA_2 are proinflammatory, producing effects including bronchoconstriction, vasodilation and hyperresponsiveness largely via the TP receptor (Coleman et al. 1987, Hamberg et al. 1975). The prostanoids are produced by the action of cyclooxygenase enzymes on arachidonic acid and in a two step process produce PGH_2 (Vane et al. 1994) which by the action of relevant synthase enzymes, or by non-enzymatic isomerisation, produce an array of receptor-active prostanoids (DeWitt and Smith, 1988, O'Banion et al. 1992). Cyclooxygenase enzymes are of two forms, COX-1 and COX-2 which catalyse the same reactions, but differ in their gene promoter regions, COX-2 having transcriptional elements (Appleby et al. 1994) and a TATA box absent from COX-1 (Smith et al 1995), making the COX-2 gene more inducible than COX-1.

A number of factors are required for an individual to undergo an asthmatic response (**Fig 8.1**). The individual must first be exposed to a trigger such as airborne

Fig. 8.1 The inflammatory cascade.



pollen, viral infection or ozone. An asthmatic individual must have a number of predisposing factors that make them react to these triggers, these may include polymorphic genes that increase the production of inflammatory products, and a microenvironment which is primed to respond more vigorously to an inflammatory stimulus. This combination of factors causes a number of cellular and mediator derived changes in the tissue, a key example being the production of cysteinyl leukotrienes, which may then contribute to eosinophil recruitment (Laitinen et al. 1993). A major product of the eosinophil is leukotriene C_4, and thus a vicious cycle of eosinophil recruitment and inflammatory mediator production is started. The leukotrienes and other inflammatory mediators have effects that culminate in the bronchoconstriction and bronchial hyperresponsiveness which are characteristic of asthma. Cytotoxic products of inflammatory cells such as eosinophil cationic protein (ECP) also cause epithelial damage allowing the various asthmatic triggers better access to the underlying tissues resulting in a heightened inflammatory response.

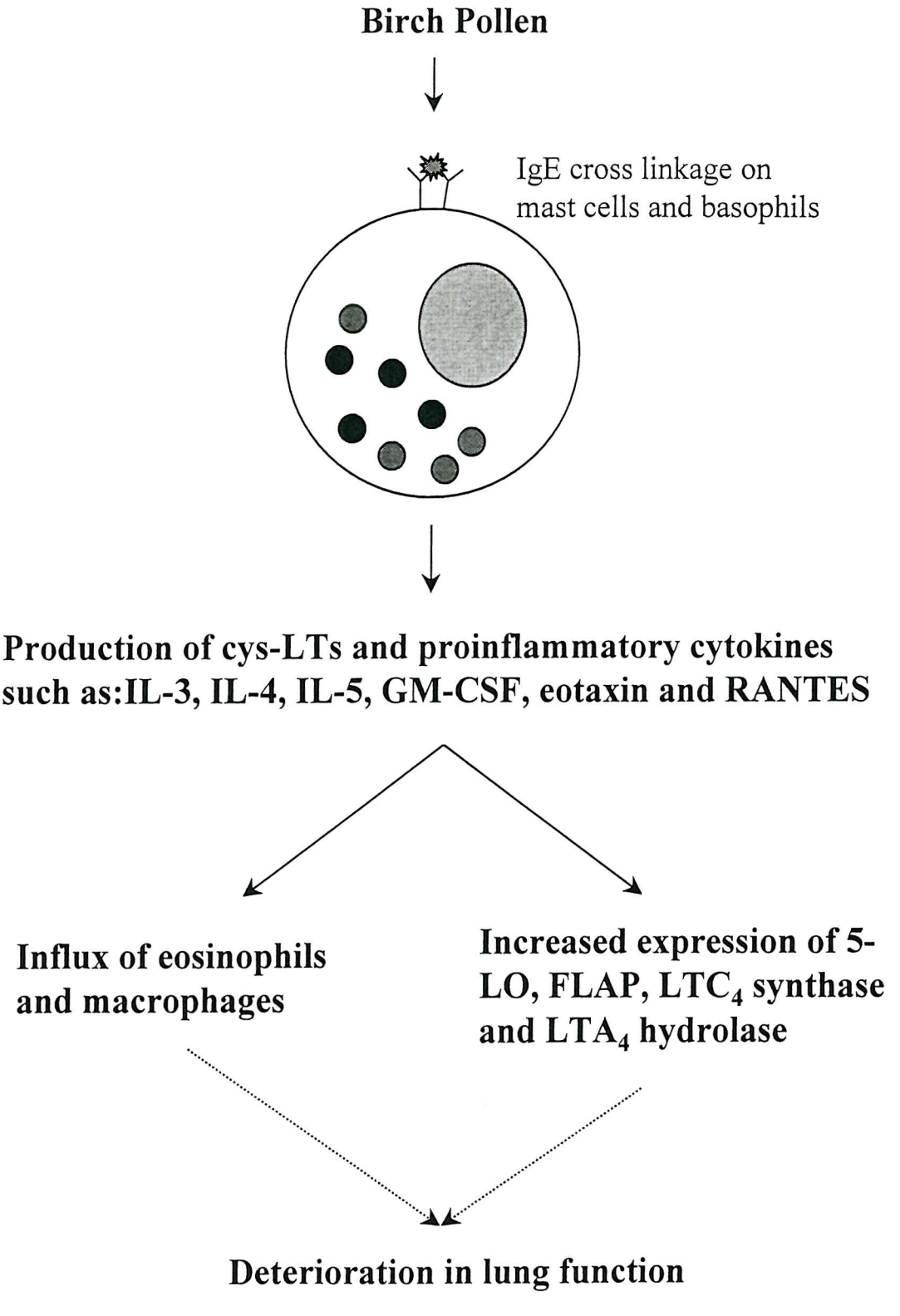
The capacity for lung tissue to generate eicosanoid mediators in response to stimuli depends on a number of factors surrounding the enzymes of the eicosanoid pathway and their related transport proteins e.g. MRP (Leier et al. 1994) or PGT (Kanai et al. 1995). The numbers of cells expressing the enzymes and the levels of expression of the enzymes in individual cells are variable and will influence total eicosanoid mediator production. However, an increase in enzyme expression or representation in the lung, does not necessarily produce an increase in mediator production, as a relevant stimulus is required for activation of the enzymes. In addition, various enzymes within the pathway may be rate limiting for mediator release. All enzymes of the pathway must be present, or there must be a source of an intermediate substrate for transcellular synthesis (Pace-Asciak et al. 1986). The level of expression of an enzyme upstream in the pathway might be at basal levels, and therefore increased expression of an enzyme downstream might have little effect due to a limited availability of substrate. 5-LO can undergo suicide inactivation when highly activated, and hence, the capacity to generate leukotrienes depends on a balance between the degree of activation of the pathway and capacity of cells to synthesise active 5-LO (Malaviya and Jakschik, 1993). The immunochemical methods used in this thesis only give information on the numbers of enzyme positive cells and to some extent changes in expression of enzymes in particular cell types, but not information on the degree of activation of the pathway. Attempts were made to remedy this deficiency where possible by assays of the LT products in BAL fluid.

This thesis aimed to characterise the regulation of eicosanoid enzyme expression in human lung mast cells and peripheral blood leukocytes in vitro and to determine the response to various triggers of asthma (including allergen, ozone and HRV infection) in terms of inflammatory cells, eicosanoid pathway enzymes and related transport proteins, and the eicosanoid mediators themselves in the lung in vivo. In addition, we aimed to confirm the role of XR9173 as an MRP inhibitor by measuring LTC_4 production / release from eosinophils. The responses to seasonal allergen exposure, rhinovirus 16 infection and ozone exposure were markedly different in the subject groups studied. Cellular expression of eicosanoid enzymes varied with cell type and GM-CSF and IL-5 upregulated expression of eicosanoid pathway enzymes in eosinophils. XR9173 inhibited production of LTC_4, suggesting that it acts as a leukotriene synthesis inhibitor.

Few studies have successfully investigated the effect of seasonal allergen exposure on asthmatic subjects, and, furthermore, the effect of a natural pollen season on eicosanoid pathway enzymes in inflammatory cells and the production of eicosanoid mediators have never been investigated. In our study, seasonal allergen exposure induced changes in asthmatic subjects predominantly mediated by the leukotriene pathway with increases in counts of cells immunostaining for 5-LO, FLAP, LTA_4 hydrolase, and LTC_4 synthase, associated with an influx of eosinophils, macrophages, $2D7^+$ basophils and neutrophils to the bronchial mucosa. Patients experienced decreased lung function (morning and evening PEF) and increased bronchial responsiveness, in addition to increased symptom scores and medication use. Lung function measurements showed negative relationships with $5\text{-}LO^+$ cell counts pre- and in-season. In contrast, expression of COX-1 and COX-2 and PGD_2 synthase did not change in-season and were not related to clinical measures of asthma.

The *camera lucida* technique (Bradding et al. 1992) was employed to provide an indication of the localisation of the eicosanoid pathway enzymes in inflammatory cells pre- and in-season and, depending on the change in inflammatory cell counts in-season, whether the change in cell counts immunostaining for the enzymes were due to cell influx or to an increase in expression of the enzyme in resident cells. We observed a 20-fold increase in LTC_4 $synthase^+$ cells identified as eosinophils in-season. Eosinophils represented >60% of LTC_4 $synthase^+$ cells in-season, a figure similar to that seen previously in aspirin intolerant asthmatics (Cowburn et al, 1998), suggesting that the increase in LTC_4 synthase in-season was almost entirely attributable to influx of eosinophils and increased LTC_4 synthase expression in these cells. The influx of eosinophils and macrophages, and an increase in expression in resident macrophages

Fig 8.2 Proposed mechanism by which seasonal allergen exposure increases eosinophil and macrophage counts, and increases expression of leukotriene pathway enzymes.



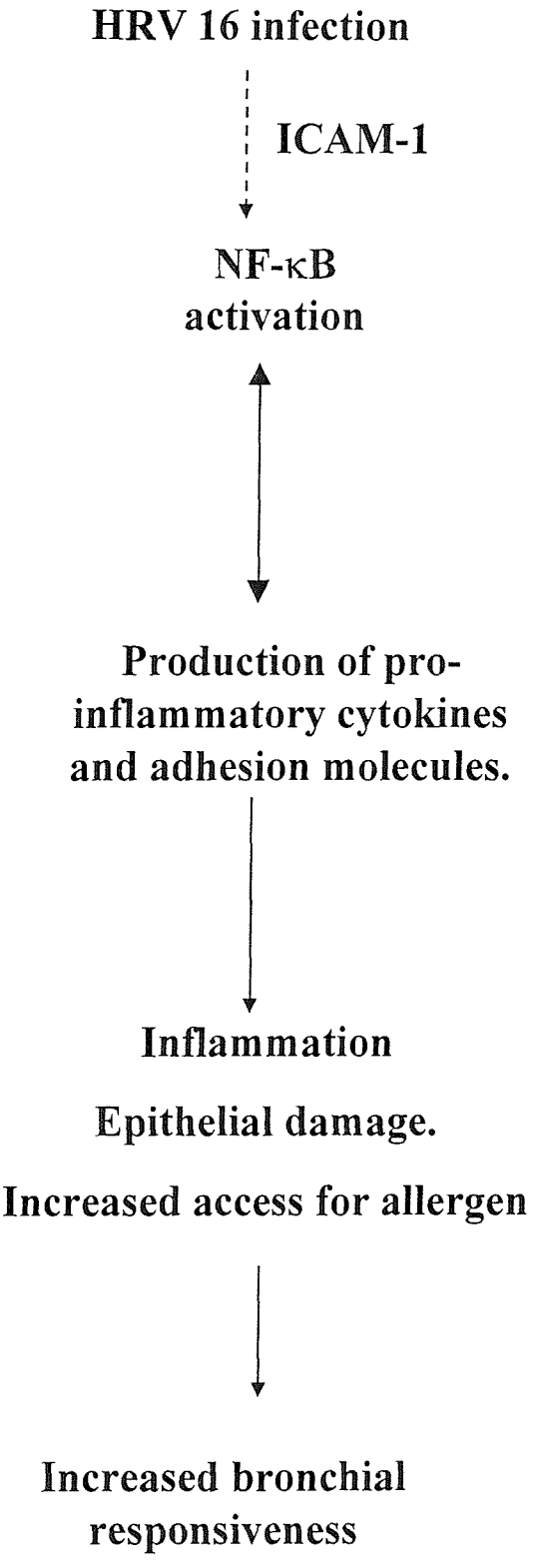
contributed to the increase in 5-LO$^+$ cells, whereas FLAP$^+$ cells were increased due to an increase in expression of FLAP in mast cells and eosinophils with a further contribution from eosinophil influx.

Production of leukotrienes by resident eosinophils or mast cells may start a self-potentiating cycle of leukotriene production, eosinophil influx and upregulation of leukotriene pathway enzymes. In addition, the production of proinflammatory cytokines such as IL-3, IL-4, IL-5, GM-CSF, eotaxin and RANTES might be elevated in-season (Robinson et al. 1993, Venge et al. 1996, Jose et al. 1994, Humbert et al. 1997) and contribute to the influx of inflammatory cells and increased expression of leukotriene pathway enzymes in resident cells (Cowburn et al. 1999) (**Fig. 8.2**).

The response of normal subjects to rhinovirus infection was mainly macrophage driven with increases in counts of 5-LO, FLAP and COX-2$^+$ cells, although they experienced no change in lung function or bronchial responsiveness in response to infection with HRV-16. The asthmatic subjects had poorer lung function, and higher bronchial responsiveness than the normal subjects at baseline, and counts of mast cells, 5-LO and COX-2$^+$ cells and levels of BAL cys-LTs were also raised at baseline. There was no change in inflammatory cell counts, counts of cells immunostaining for eicosanoid pathway enzymes, or cys-LT measurements in the asthmatic subjects, despite increased bronchial responsiveness.

In the asthmatic subjects the increase in bronchial responsiveness was associated with an increase in cells immunostaining for activated NF-κB (2C7), the nuclear factor that activates transcription of many inflammatory genes including those for IL-1β, IL-6, IFN, TNF-α, GM-CSF, RANTES, IL-8, ICAM-1 and VCAM-1 (Baeuerle & Henkel 1994). TNF-α and interleukin-1β can also activate NF-κB (Barnes and Adcock, 1997; Newton et al. 1997). ICAM-1 is itself a receptor for HRV 16 (Couch, 1985; Abraham and Colonno, 1984; Staunton et al. 1989) and is upregulated on eosinophils, T-cells and epithelial cells in asthmatics compared to normals; hence these cells have the capacity to bind more virus (Hansel et al. 1991; Derose et al. 1994; Bentley et al. 1993; Vignola et al. 1993; Chanez et al. 1993). The increase in bronchial responsiveness in asthmatics with HRV infection might partly be due to an increase in activated NF-κB causing an increase in proinflammatory cytokine production and adhesion molecule expression (Baeuerle and Henkle, 1994) (**Fig.8.3**). These factors may increase the activation of inflammatory cells increasing the release of substances toxic to the bronchial epithelium. The resulting epithelial damage might increase access of allergen, or other irritants to the tissue, and hence promote bronchial hyperresponsiveness (**Fig. 8.3**). No increase was

Fig. 8.3 Proposed mechanism of HRV induced bronchial hyperresponsiveness in the asthmatic airway



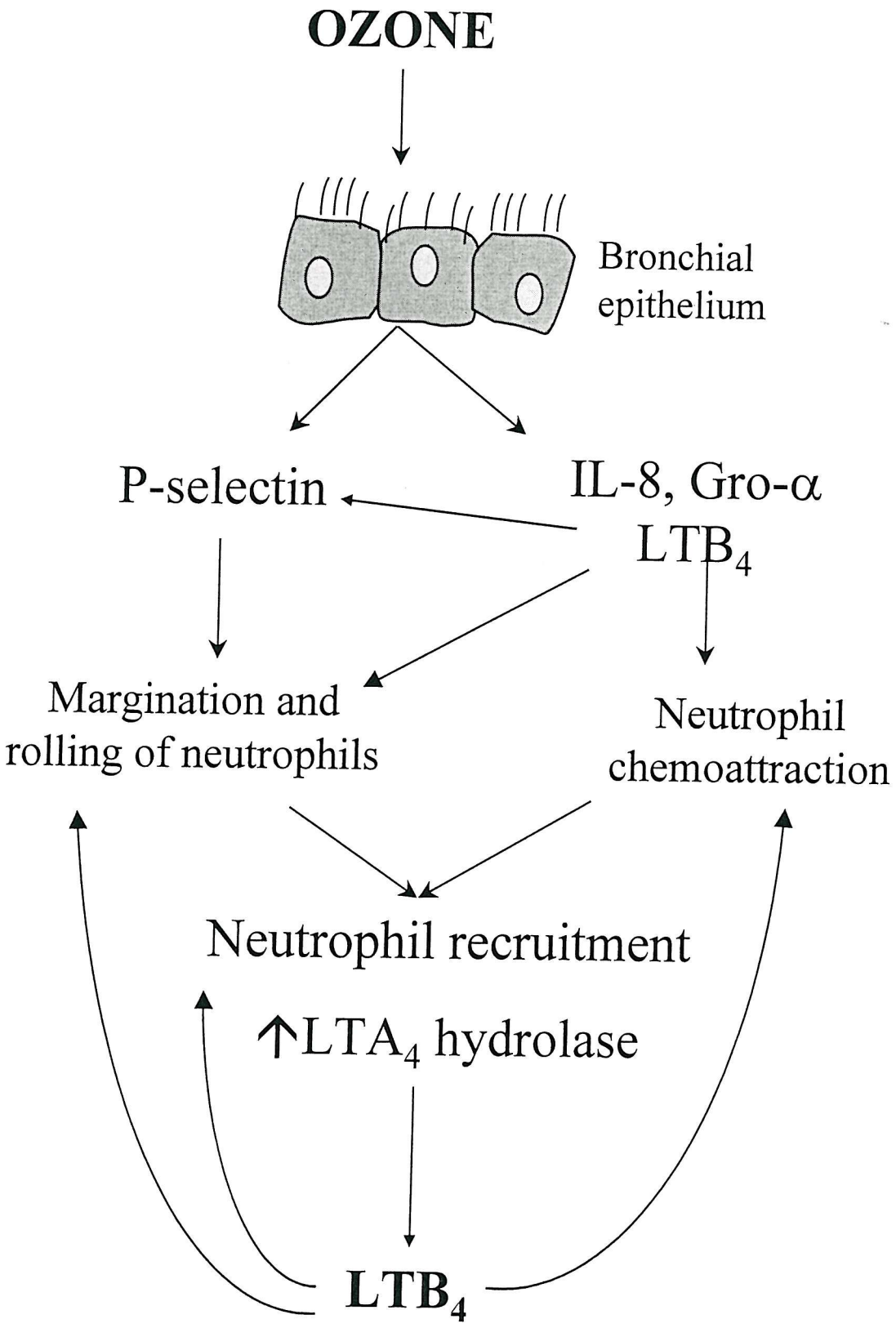
seen in inflammatory cell counts or enzyme expression in the asthmatic subjects as these pathways may already have been induced in the asthmatic state.

In the normal subjects, upregulation of 5-LO and COX-2 occurred without a change in NF-κB activation, so a different signalling pathway may be utilised for the induction of these enzymes.

Ozone exposure caused a response centred on neutrophil and neutrophil chemoattractants. Neutrophilia is a consistent characteristic of the response to ozone exposure, being induced early, before 1 hour, and persisting at least up to 24 hours (Schelegle et al. 1991). However, lung function changes have a much shorter time scale, not being evident after 6 hours (Schelegle et al. 1991). Production of LTB_4 and cys-LTs in the BAL fluid is reported to be elevated early in the response to ozone (before 6 hours) (Coffey et al. 1996). Cys-LTs are potent bronchoconstrictor mediators and hence might be associated with the early decrement in lung function. Also elevated at an early time point is P-selectin expression on endothelial cells (Krishna et al.1997) and the neutrophil chemoattractants IL-8 and Gro-α in the BAL fluid (Krishna et al. 1998) which in combination with elevated LTB_4 might contribute to ozone induced neutrophilia (**Fig 8.4**). The bronchial epithelium is the first cellular structure to be exposed to inhaled ozone. Hence, it is possible that the elevated IL-8, Gro-α and LTB_4 might be epithelium derived and that the epithelium is driving the ozone-induced neutrophilia (**Fig. 8.4**). Bronchial epithelial cell lines produce LTB_4 but their production of LTC_4 has not been determined. BAL fluid PGE_2 is elevated by ozone at 18 hours (Koren et al. 1989, Devlin et al. 19991) and this may modulate the production of cys-LTs and hence reduce a cys-LT induced bronchoconstriction.

In the present study, counts of submucosal cells immunostaining for LTA_4 hydrolase trended towards an increase 6 hours after ozone exposure. LTB_4 is reported to be elevated 2 hours after exposure (Coffey et al. 1996) and might be produced by an increase in LTA_4 hydrolase expression, suggesting the role of a cell type in addition to the epithelium in the production of LTB_4. This might be the neutrophil or macrophage (**Fig. 8.4**). Epithelial immunostaining was not analysed, and an increase in LTA_4 hydrolase in the epithelium cannot be ruled out. Further work should include measurement of eicosanoid pathway enzymes in the epithelium of these bronchial biopsies, and also in vitro studies on enzyme expression in cultured epithelial cells (cell line and/or primary epithelial cells) exposed to ozone.

Fig 8.4 Mechaism of ozone induced neutrophil accumulation in the human lung.



Analysis of peripheral blood leukocytes has allowed the characterisation of eicosanoid pathway enzymes in these cells. This analysis has confirmed that eosinophils have the capacity for cys-LT production with ~25% of cells expressing LTC_4 synthase and having high levels of 5-LO and FLAP. The monocyte/macrophage population is reported to primarily synthesise LTB_4, this is corroborated by the high levels of LTA_4 hydrolase expression in normal blood monocytes. Analysis of the leukotriene pathway enzymes in leukocytes also confirmed that neutrophils have the capacity for the production of LTB_4 with neutrophils having the highest levels of LTA_4 hydrolase and 5-LO and FLAP.

Lymphocyte leukotriene production is contentious, however we have provided further evidence that lymphocytes have the capacity for the production of leukotrienes particularly LTB_4. Lymphocytes do not produce leukotrienes with many physiological stimuli (Odlander et al 1989), so the contribution of lymphocytes to leukotriene production in the asthmatic lung is likely to be limited. However, they may be involved in transcellular synthesis.

All cells have the capacity for the production of prostanoids as COX-1 is expressed in 10-15% of peripheral blood leukocytes from normal subjects. PGD_2 synthase was expressed in >20% of peripheral blood leukocytes suggesting that PGD_2 is a major prostanoid product from these cells, however, the PGD_2 synthase antibody is a new and not well-characterised crude antiserum (Mahmud et al. 1997). The enzyme expression may therefore be lower than it appears in these experiments. COX-2 expression was low in eosinophils and neutrophils (1-3%), but higher in monocytes and lymphocytes (7-10%), suggesting that COX-2 derived prostanoids are of importance in these cells and may contribute to pro-inflammatory prostanoid production in the lung with reference to asthma. Since COX-1 and COX-2 catalyse the same reaction, it is difficult to determine the source of the different prostanoids. It may depend on the colocalisation of the downstream enzymes. Further work should include localisation of COX-1 and COX-2 to the same or different cells, and determining whether particular downstream enzymes are localised to a particular COX isotype. This can be achieved by double staining of cells for flow cytometry, but the availability of antibodies to the down stream enzymes will be inhibitory to this study.

We have also characterised baseline expression of 5-LO, FLAP, LTC_4 synthase, COX-1 and COX-2 in purified human lung mast cells by immunocytochemistry. COX-1 was expressed in greater than 80% of mast cells and COX-2 in less than 5%. COX-1 is therefore the primary source of prostanoid products in mast cells. Expression of 5-LO

and FLAP were high (~50-60%) in mast cells suggesting that they have the capacity to produce leukotrienes, but LTC_4 synthase expression was low indicating that at baseline mast cells do not produce significant amounts of cys-LTs.

Initial experiments with human lung mast cells of 80-98% purity failed to detect any leukotriene C_4 production when stimulated with goat anti-human IgE (data not shown) which is in agreement with Levi-Schaffer and colleagues (1987) who also were unable to measure LTC_4. Leukotriene C_4 has previously been measured in mast cell preparations of varying purity after immunologic stimuli (Bischoff and Dahinden 1992, Benyon et al. 1989, MacGlashan et al. 1982), but few experiments have measured LTC_4 release from a >95% pure mast cell preparation. LTC_4 production is increased in human lung mast cells when cocultured with fibroblasts for 1 week (Levi-Schaffer et al. 1987) and when incubated with SCF for 20min (Bischoff and Dahinden 1992). This, therefore, raises the question of whether highly purified mast cells synthesise LTC_4 without an accessory cell type or whether they require exposure to an additional stimulus e.g. SCF for leukotriene C_4 production.

Further work should include measurement of the basal, calcium ionophore-stimulated and anti-IgE-stimulated levels of production of PGD_2 and LTC_4 by enzyme immunoassay to confirm predictions regarding mediator production by these cells. Purified human lung mast cells cultured in SCF will be stimulated with IL-1β or IL-10 to determine their effect on PGD_2 and LTC_4 production, and on expression of the eicosanoid pathway enzymes. Addition of the COX inhibitor indomethacin inhibits the production of PGE_2 in eosinophils (Tenor et al. 1996), and increases PAF stimulated LTC_4 production. Replacement of PGE_2 returns LTC_4 production to its previous levels. This may be a mechanism relevant to aspirin-induced asthma as these patients produce high levels of cys-LTs during aspirin challenge (Cowburn et al. 1998). It is not yet known if LTC_4 production is increased by COX inhibition in human lung mast cells, and the effect of indomethacin on LTC_4 production will also be measured.

GM-CSF is reported to enhance 5-LO gene transcription and protein synthesis (Stankova et al. 1995, Pouliot et al. 1994b) and expression of FLAP in human neutrophils (Pouliot et al. 1994a). In eosinophils, GM-CSF and IL-5 upregulate LT synthesis (Scoggan et al. 1995, Cowburn et al. 1999). This increase in cys-LT production by IL-5 is associated with increases in the expression of FLAP and translocation of 5-LO to the nucleus (Cowburn et al. 1999).

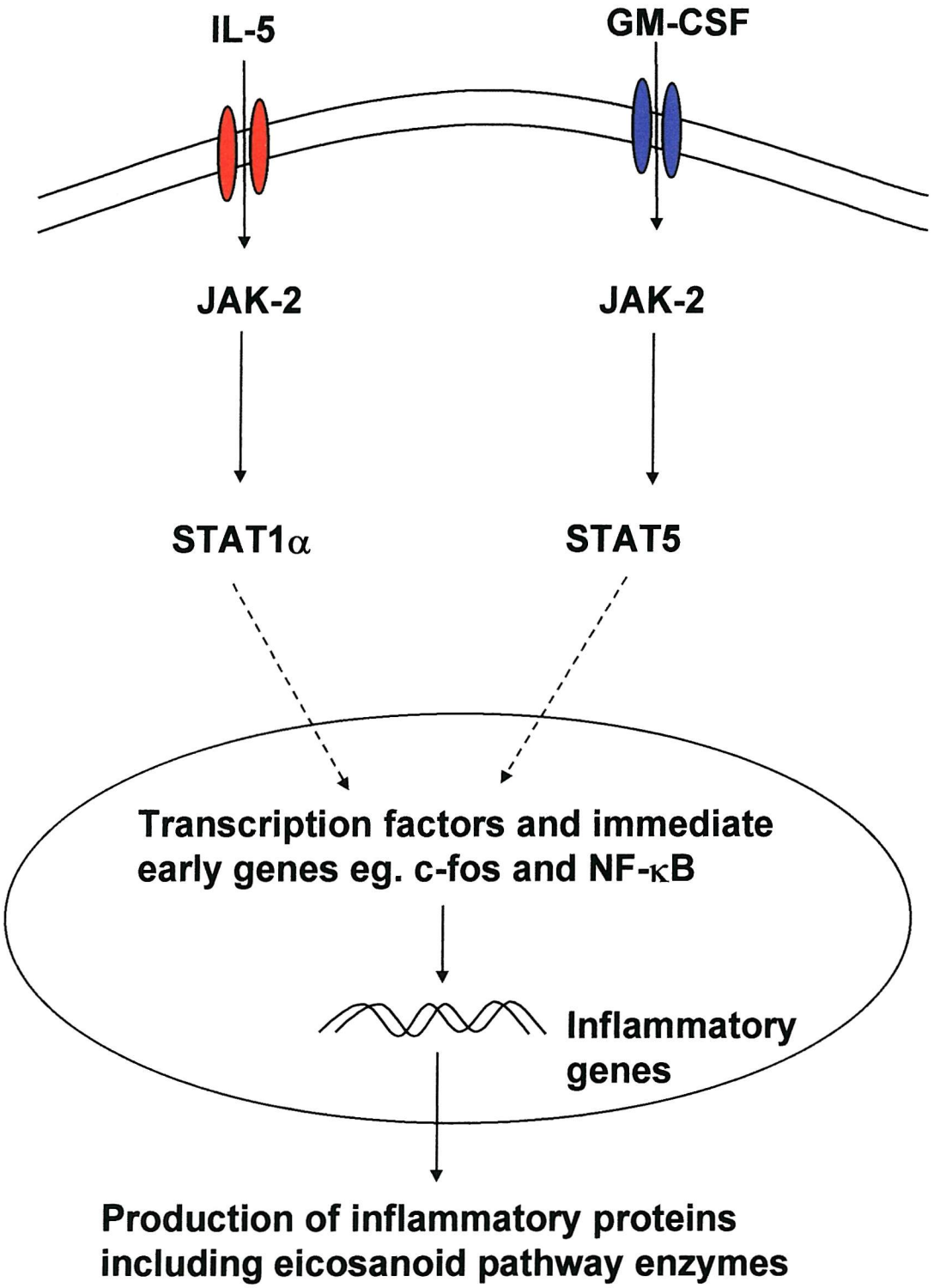
The regulation of eicosanoid pathway enzymes by GM-CSF and IL-5 has been further investigated using FACS analysis; we have confirmed that in eosinophils IL-5

upregulates FLAP but not 5-LO expression as seen by Cowburn et al. (1999). We have extended the studies in eosinophils to include the effect of GM-CSF on 5-LO and FLAP expression and the effect of both IL-5 and GM-CSF on COX-1 and COX-2 expression. Expression of both 5-LO and FLAP was increased in eosinophils incubated with GM-CSF but only FLAP expression was increased when incubated with IL-5. COX-1 expression was increased by treatment with both IL-5 and GM-CSF, and COX-2 expression was increased by IL-5.

We have demonstrated that exposure of asthmatics to seasonal allergen causes increased bronchial counts of cells immunostaining for both 5-LO and FLAP, and that asthmatics have higher counts of 5-LO and COX-2 positive cells compared to normal subjects. 5-LO and COX-2 positive cell counts are also increased in the lung of normal subjects during infection with HRV 16. These studies suggest that cytokines such as IL-5 and GM-CSF may be elevated under these conditions *in vivo* and may highlight a role for cytokine priming in increasing cellular enzyme expression in the bone marrow, circulation or lung in asthma and infection. This may produce a marked increase in eicosanoid pathway activity leading to impaired lung function and leukocyte migration. Further work should include analysing cytokine expression in bronchial biopsies and peripheral blood leukocytes and measuring cytokine levels in BAL fluid samples of the above subjects in an effort to relate cytokine production / priming to changes in eicosanoid enzyme expression. Levels of cytokine receptor expression may vary in cells. For example, the GM-CSF receptor is elevated in intrinsic asthmatics compared to atopic asthmatics (Kostimbos et al. 1997). Levels of IL-5 and GM-CSF receptor should be examined on eosinophils and related to the magnitude of effect of the cytokines on enzyme expression. Binding of IL-5 to its receptor activates tyrosine kinases including JAK2, which in turn causes the activation of the nuclear transcription factor STAT1α (Van der Bruggen et al. 1995, Hiraguri et al. 1997). GM-CSF similarly uses the JAK2-STAT5 signalling pathway (Hiraguri et al. 1997, Wheadon et al. 1999) resulting in the induction of c-fos (Itoh et al. 1996) (**Fig 8.5**). IL-5 and GM-CSF cause the production of additional transcription factors which interact with NF-κB to amplify the expression of some genes (reviewed in Barnes and Adcock 1997) (**Fig 8.5**). (The intracellular signalling pathways by which IL-5 and GM-CSF increase enzyme expression should be investigated using compounds which inhibit tyrosine kinases, MAP kinases, transcription factors and NF-κB etc.

The techniques used in this thesis have successfully measured expression of the eicosanoid pathway enzymes in inflammatory cells from human lung and peripheral

Fig 8.5 Signalling pathway by which IL-5 and GM-CSF upregulate eicosnaoid pathway enzyme expression.



blood. Immunohistochemical analysis of inflammatory cells and eicosanoid pathway enzymes in GMA embedded bronchial biopsies is advantageous over paraffin embedded or frozen as very thin sections can be cut allowing for localisation of enzymes to inflammatory cell markers using the *camera lucida*. An alternative to this technique is double-immunostaining; such that two markers can be localised to cells on a single section. However, previous attempts at double immunostaining produced unreliable quantitative results when the order of application of the antibodies was reversed (A.S. Cowburn 1999, PhD Thesis, University of Southampton).

The three immunohistochemical studies in the present thesis could further be enhanced by analysis of mRNA expression by *in situ* hybridisation using nucleic acid probes for eicosanoid enzymes. This would give information about changes in mRNA levels as a protein may persist in the cell long after its translation and long after the mRNA encoding it has declined.

Enzyme expression in mast cells was carried out by immunocytochemistry on cytospin preparations; this was the preferred method as it is undesirable to run tissue preparations through the FACScan. However, when analysing enzyme expression in blood leukocytes flow cytometry was an advantageous technique as it allows large amounts of data to be analysed, and in addition larger numbers of cells can be counted (10,000) reducing the risk of statistical variation when only counting a few hundred cells. Investigator error is reduced with the use of flow cytometry as the definition of a 'positive cell' can be strictly defined by analysis of the isotype control. Flow cytometry also gives some information about levels of expression of protein in individual cells. When cells are analysed by Western blotting, information about the total quantity of protein is available but not whether a few cells are expressing the protein at high levels or whether all cells are expressing a moderate amount. Western blotting is a useful technique for the measurement of protein, but large numbers of cells are required to achieve a strong signal from a weakly expressed protein (e.g. COX in eosinophils) and this is not always possible when analysing a rare cell type. The polymerase chain reaction (PCR) could also be employed to measure changes in specific mRNA levels in the cells as this would give further information about the time frame of changes in enzyme expression.

Cys-LTs and PGD_2 were measured in BAL fluid samples from normal and asthmatic patients and from eosinophil supernatants by enzyme immunoassay after methanol extraction. This provides a fairly crude but reliable measure of mediator level in the samples and some limited information about the activation-state of the relevant

enzymes. This measurement could be further improved by solid-phase extraction of the sample, which removes any contaminating components. The remaining non-polar sample may then be separated into individual eicosanoids by High Performance Liquid Chromatography (HPLC) and then assayed. This prevents other related compounds cross-reacting with the antibody and producing false positive results. The antibodies supplied with the enzyme immunoassay kits have very low cross reactivities with related eicosanoids and therefore the long procedure of solid phase extraction and HPLC is not considered essential. Mass spectrometry can also be used to measure eicosanoid products in a range of samples.

The proportions of eosinophils immunopositive for 5-LO in normal subjects compare favourably when measured in bronchial biopsies by the camera lucida technique (20%) and by FACS on both purified eosinophils (20.6%) and eosinophils in a mixed leukocyte preparation (10.8%). COX-2 expression was not detected in eosinophils in bronchial biopsies, this is corroborated by study of purified eosinophils, when 0.25% were COX-2 positive and in eosinophils of a mixed leukocyte population when 1.6% expressed COX-2. This consolidates the validity of these techniques to successfully measure enzyme expression in a given cell type.

The diverse cellular responses observed in the clinical studies suggest that exacerbations of asthma associated with these triggers are mediated by different mechanisms, and may suggest the need for different treatment regimes.

Corticosteroids are one of the most commonly used drug treatments for asthma, and have been shown to decrease mast cell and eosinophil counts in bronchial biopsies (Djukanovic et al. 1992) and decrease the production of cytokines e.g. IL-4, IL-5, GM-CSF (Robinson et al. 1993, Wang et al. 1994). However, corticosteroids have been reported to enhance the production of leukotrienes by upregulating 5-LO and FLAP expression (Riddick et al. 1997, Pouliot et al. 1994a, Cowburn et al. 1999) and in combination with SCF increase COX-1 expression in mast cells (Samet et al. 1995). Steroid treatment also does not diminish allergen-induced increases in BAL eicosanoid levels or urinary LTE_4 (O'Shaughnessy et al. 1993, Dworski et al. 1994). In the light of the results discussed in chapter 3, corticosteroids while effective, may exacerbate some aspects of allergic asthma as they might further increase the levels of leukotriene pathway enzymes above already elevated levels.

Drugs that block the effect of, or the production of, the leukotrienes, have been shown to be successful in treating allergic asthma, preventing the bronchoconstrictor response to diverse triggers of asthma, including allergen, cold air, exercise, NSAIDs

and PAF (Holgate, Bradding and Sampson, 1996). Leukotriene synthesis inhibitors, such as the 5-LO inhibitor zileuton, and the leukotriene receptor antagonists montelukast and zafirlukast improve baseline lung function measurements by 10-20% and improve symptom scores and medication use by 30-60% (Sampson and Holgate, 1998). They are reported to prevent exacerbations of asthma during reduction of high-dose inhaled corticosteroids (Tamaoki et al. 1997) and to have additive effects with corticosteroid therapy (Smith et al. 1996). The increased expression of 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase particularly in eosinophils in our study of seasonal asthma would suggest that 5-LO blockade would serve better than a cys-LT receptor antagonist as allergen might also stimulate the production of LTB_4 from the already elevated LTA_4 hydrolase. The 5-LO inhibitor zileuton and both LTB_4 and LTD_4 antagonists have been shown to inhibit eosinophil migration in response to fMLP in guinea-pig tracheal explants (Munoz et al. 1997). 5-LO inhibition would therefore block both arms of leukotriene production and abolish the effects of both the cys-LTs on bronchial responsiveness and eosinophil influx, and LTB_4 on eosinophil influx.

The effect of ozone on leukotriene production and neutrophilia might be inhibited by loratadine. It was shown that loratadine inhibits LTB_4 production by neutrophils in vitro, a production of LTB_4 that is promoted by coculture of neutrophils with airway epithelial cells (Amsellem et al. 1998). Nedocromil sodium has also been shown to inhibit ozone induced inflammatory responses by reducing IL-8 and GM-CSF production in cultured human bronchial epithelial cells (Rusznak et al. 1996). LTB_4 production by macrophages isolated from patients with nocturnal asthma is decreased by prednisone, associated with decreased neutrophil influx (Wenzel et al. 1994). These drug types may all have an inhibitory effect on ozone induced inflammation, but another candidate would be a leukotriene B_4 receptor antagonist LY293111, which has been shown to reduce allergen-induced neutrophilia (Evans et al. 1996). Assuming a pivotal role for LTB_4 in the neutrophilia characteristic of the response to ozone, this receptor blockade might have beneficial effects.

In LTC_4 synthesis / release experiments in eosinophils the leukotriene receptor antagonist and MRP inhibitor MK-571 inhibited the quantity of LTC_4 synthesised and released, but the intracellular quantity was not reduced. This is an indication (but not definitive proof) of inhibition of LTC_4 export. It may also suggest that MK-571 is acting as a leukotriene synthesis inhibitor. MK-886 at low concentrations, inhibited total LTC_4 released as well as both released and intracellular LTC_4, confirming its effects as a potent synthesis inhibitor. These drugs were compared with the effect of the developmental compound XR9173. The effect of XR9173 was comparable to that of MK-886 with

decreases in total, released and intracellular LTC_4, although with lower potency. This suggests that XR9173 may be a leukotriene synthesis inhibitor and not an MRP inhibitor as proposed.

Analysis of MRP expression in eosinophils by flow cytometry indicated that expression of both MRP-1 and MRP-2 was low. The effect of MK-571 MRP inhibition on released LTC_4 suggests that MRP expression is higher than reported. A recent report has indicated that the chloride channel cystic fibrosis transmembrane conductance regulator (CFTR) may also be involved in glutathione transport (Gao et al. 1999) and hence may contribute to export of LTC_4. Inhibitors of MRP may, therefore, inhibit other glutathione transporters including CFTR thereby having more effect on LTC_4 transport than the low levels of MRP would support. Substrates of MRP have also been reported to inhibit chloride transport (Lindsell and Hanrahan, 1999) which would result in secretion of thick mucus.

The role of MRP inhibitors as effective treatments for asthma has not been studied due to the lack of a specific MRP inhibitor. Current compounds with MRP inhibitor activities also have effects on other levels of the leukotriene pathway. It is possible that a specific MRP inhibitor will be beneficial to asthma patients and may present an alternative drug regime based on the current success of existing leukotriene modifier drugs. MRP inhibitors may also be useful in cancer therapy, since they prevent the export of glutathione-based drugs from the cell and hence prevent drug resistance. The possible effect of CFTR inhibition would have to be considered when using these drugs as an asthma treatment as mucus secretion is already elevated in asthmatic patients and a thickening of this mucus would be undesirable.

The diverse effects of individual leukotriene modifier drugs on different levels of the leukotriene pathway (FLAP, MRP, cys-LT_1) are predictable in hindsight since the structure of the drug is based on the structure of the leukotriene itself. A similar crossover of effects is seen with NSAIDs. In human and murine cell lines which overexpress MRP and show increased efflux of drugs (thereby characterising a multidrug resistant cell type) the addition of indomethacin decreased the accumulation of vincristine (Draper et al. 1997) suggesting that indomethacin is an inhibitor of MRP. MRP is also amenable to modulation by indomethacin (Roller et al 1999). It is not known whether indomethacin is functioning by inhibition of glutathione-S-transferase or by direct competition of the drug at the transport site (Draper et al. 1997). It also remains undetermined whether NSAIDs have the same inhibitory effect on transport of LTC_4 by MRP, or whether this phenomenon is relevant to asthma. A relationship certainly exists between the two branches of the eicosanoid pathway. FLAP transfected

cells display increased PGE_2 production compared to those cells not naturally expressing FLAP but with no variation in levels of 5-LO products (Battu et al. 1998). This increase in PGE_2 synthesis was associated with an increase in COX-2 expression compared to non-transfected cells (Battu et al. 1998). The mechanism by which this occurs is not determined. 5-LO has been shown to interact with ΔK12H4.8 a protein related to a hypothetical helicase. This protein has an RNase III motif and a double-stranded RNA binding domain (Provost et al. 1999). Thus, the interaction of 5-LO with ΔK12H4.8 suggests that 5-LO may have a non-catalytic role in the cell (Provost et al. 1999). The actions of the eicosanoid pathway enzymes, their products and modulatory drugs are, therefore, not as straightforward as previously thought and interactions between the pathways and nuclear / intracellular roles for eicosanoids should be further considered.

In summary, eicosanoids are lipid mediators with a wide range of actions upon bronchial and vascular smooth muscle activity, mucus secretion, vascular endothelium, leukocyte migration, and proliferation of structural airway cells such as epithelium and bronchial smooth muscle. In particular, the cysteinyl-leukotrienes have a critical role in asthma as the predominant mediators of bronchoconstriction in response to diverse environmental triggers and as important mediators of eosinophil recruitment, and they may also play a role in airway remodelling. These activities are apparent from the clinical pharmacology of new leukotriene modifier drugs that are currently used as oral prophylactic therapy by about four million asthmatics worldwide.

The overarching aim of this thesis was to explore the mechanisms by which cys-LTs are over-produced in patients with asthma. Although extensive work through the 1980s had broadly characterised the extracellular release of cys-LTs by inflammatory leukocytes in vitro, the relative contribution of various cell-types to eicosanoid production within the asthmatic lung was obscure. During the early 1990s, elucidation of the molecular biology of the leukotriene synthetic pathway provided the necessary antibodies and other tools for this thesis to localise and quantify the requisite enzymes of the 5-LO pathway within the lung. The bronchial biopsy studies in this thesis were the first to show that airway dysfunction during seasonal allergen exposure is linked to increased expression of 5-LO pathway, but not COX pathway, enzymes, and that this is a product both of leukocyte influx (eosinophils and macrophages) and of the induction of their expression within infiltrating and resident cells (eosinophils and mast cells). Distinct patterns of altered eicosanoid enzyme expression were observed after exposure to human rhinovirus and ozone, suggesting that inflammatory responses to these triggers may rely upon diverse pathophysiological pathways leading to upregulation of

eicosanoid activity, including influx of stimulus-specific leukocyte cell-types and induction of enzyme expression by NF-kB and other mechanisms.

A consistent outcome of the bronchial biopsy studies was the finding that individual eicosanoid enzymes may be expressed by only a relatively small proportion of cells of a particular leukocyte phenotype. This finding was confirmed by immunocytochemical and FACS analysis of their expression within immunomagnetically purified populations of mast cells from human lung and of eosinophils, neutrophils, monocytes, and lymphocytes from venous blood. In eosinophils, upregulation of 5-LO pathway enzymes was demonstrated in response to eosinophilopoietic cytokines (IL-5, GM-CSF) that are known to be generated by mast cells and T-cells within the asthmatic lung. This evidence is supportive of the view that blood eosinophils and other short-lived cell-types previously regarded as 'end-stage', may in fact be matured and differentiated by the cytokine environment within the airway mucosa into a leukotriene-generating phenotype, analogous to the recognised differentiation of long-term resident cell-types such as mast cells. Conversely, evidence that the capacity of mast cells to generate cys-LT may be dependent on transcellular synthesis with accessory cells provides a novel insight into the complex integration of cellular function *in situ* within the airway wall.

The thesis also extended its examination of the distribution and regulation of 5-LO pathway enzymes to the proteins (MRP-1 and MRP-2) that are thought to be responsible for the export of LTC_4 from inflammatory leukocytes. In particular, it provided preliminary efficacy data on a compound (XR9174) that may be the precursor of a new class of leukotriene modifier drugs with potential applications not only in combating airway inflammation in asthma, but also in combining anti-inflammatory activity with enhancement of the efficacy of cytotoxic drugs in cancer chemotherapy. The study highlighted the relative lack of selectivity for 5-LO pathway proteins not only of this compound but also of other anti-leukotriene drugs, including a potent FLAP inhibitor and a $CysLT_1$ receptor antagonist.

Overall, a combination of techniques including GMA immunohistochemisty, FACS analysis, immunocytochemistry, immunomagnetic purification, short-term cell culture, and immunoassays, was used to show that a multiplicity of mechanisms may underlie abnormal metabolism of a group of molecules of central importance in diverse phenotypes of asthma. Better understanding of these mechanisms at the genetic, biochemical, cellular, pharmacological and clinical levels may lead not only to novel therapies but also to better targeting of current and new therapies to those patients most likely to demonstrate clinical benefit.

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Appendix

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Effect of natural exposure to seasonal allergen on the expression of eicosanoid pathway enzymes in bronchial biopsies of atopic asthmatics[1].

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ABSTRACT.

Cysteinyl-leukotrienes (cys-LT) are implicated in bronchoconstriction and airway eosinophilia in asthma, and their synthesis may be modulated by prostanoids. To characterise the cellular sources of leukotrienes and prostanoids in the allergic asthmatic airway, we performed immunohistochemical analysis of 5-lipoxygenase (5-LO) and cyclooxygenase (COX) pathway enzyme expression and leukocyte markers in bronchial biopsies obtained from 12 birch pollen-sensitive asthmatic patients before and during natural exposure to environmental birch pollen. In the pollen season, asthmatics had bronchial hyperresponsiveness ($P=0.004$), reduced peak expiratory flow (PEF; $P\leq0.02$), and higher symptom scores ($P<0.005$) compared with pre-season. In-season bronchial biopsies had 2-fold more cells immunostaining for 5-LO ($P=0.02$), 5-LO activating protein (FLAP; $P=0.04$), and LTA_4 hydrolase ($P=0.05$), and 4-fold more for the terminal enzyme for cys-LT synthesis, LTC_4 synthase ($P=0.02$). Counts of cells immunostaining for COX-1, COX-2, and prostaglandin D_2 synthase were unchanged. The increased prevalence of LTC_4 synthase was partly due to increased eosinophil counts ($P=0.035$) and partly to an increased proportion of eosinophils expressing the enzyme ($P=0.047$). There was also a significant increase in macrophage counts ($P=0.019$), but not in mast cells or $CD3^+$, $CD4^+$, $CD8^+$ or activated ($IL\text{-}2R^+$) T-lymphocytes. Before and during the pollen season, PEF correlated inversely with $5\text{-}LO^+$ cell counts. We demonstrate that increased expression of leukotriene pathway enzymes in eosinophils within the bronchial mucosa is linked to deterioration of lung function during natural exposure of allergic asthmatics to environmental allergen.

[249 words]

INTRODUCTION.

The predominant mediators of bronchoconstriction in asthma in response to diverse stimuli are the cysteinyl-leukotrienes (cys-LTs) LTC_4, LTD_4, and LTE_4, products of the 5-lipoxygenase (5-LO) pathway (**1,2**). In asthmatic patients, inhaled cys-LTs are potent bronchoconstrictors and cause specific influx of eosinophils into the bronchial mucosa (**3,4**). Specific LT synthesis inhibitors and cys-LT receptor antagonists reduce symptoms, improve lung function and suppress eosinophilia in the airway and blood of patients with persistent asthma (**2,5-7**). Synthesis of cys-LTs may be regulated by prostanoid products of cyclooxygenase-1 (COX-1), particularly PGE_2 (**8,9**), while prostanoids such as PGD_2 generated by the sequential action of cytokine-inducible COX-2 and PGD_2 synthase (**10**) may contribute to bronchoconstriction.

The common substrate of the 5-LO and COX pathways is arachidonic acid. During stimulus-specific cell activation, arachidonic acid released by phospholipases including cytosolic phospholipase A_2 (**11**) is translocated to the 5-lipoxygenase activating protein (FLAP) (**12**) and converted in two steps to leukotriene (LT)A_4 by 5-LO (**13**). LTA_4 is converted to the dihydroxy-leukotriene, LTB_4, by cells expressing LTA_4 hydrolase (**14**) and/or to LTC_4, by cells expressing LTC_4 synthase, which conjugates LTA_4 to reduced glutathione (**15**). After carrier-mediated cellular export of LTC_4 (**16**), the sequential cleavage of glutamate and glycine residues provides the extracellular receptor-active metabolites LTD_4 and LTE_4 respectively (**17,18**). In prostanoid biosynthesis, the released arachidonic acid is directly converted by constitutive COX-1 (prostaglandin endoperoxide synthase-1) or by induced COX-2 in two steps to the intermediate prostaglandin (PG)H_2, which is the common substrate of the terminal prostanoid synthases (**19,20**), including PGD_2 synthase (**21**).

The cellular sources of cys-LTs in the asthmatic lung are poorly understood. In our previous investigation in bronchial biopsies from patients with aspirin-intolerant asthma (AIA) (**22**), immunohistochemical analysis demonstrated significantly more cells expressing LTC_4 synthase than in biopsies from aspirin-tolerant asthma patients or normal subjects, without increased immunodetection of other 5-LO or COX pathway proteins. The increased prevalence of LTC_4 synthase$^+$ cells in AIA biopsies was partly accounted for by a higher proportion of eosinophils staining for the enzyme (50% vs 21%), irrespective of exposure to aspirin. The selective increase in LTC_4 synthase-positive cells correlated with persistently-elevated cys-LT levels in BAL fluid and with bronchial hyperresponsiveness to inhaled lysine-aspirin (**22**).

Most asthma is associated with atopy, and in atopic asthmatics concomitant rises in the levels of LTC_4, histamine, tryptase and PGD_2 during the early bronchoconstrictor response to inhaled allergen challenge suggest that degranulating mast cells are the principal source of cys-LTs in this response (**23-25**). However, blockade of the late response to allergen challenge by oral cys-LT receptor antagonists (**26**) suggests that a secondary phase of cys-LT release is associated with influx of eosinophils, basophils, or monocytes (**27-30**). We have shown that blood eosinophils from mild allergic asthmatics have increased expression of FLAP compared to normal eosinophils, and that this is mimicked by *in vitro* culture of normal eosinophils with IL-5 for 6 h (**31**), leading to enhanced cys-LT synthesis (**31,32**). Sequential upregulation of the expression of $cPLA_2$, 5-LO, FLAP, and LTC_4 synthase has been demonstrated in cord blood progenitor cells forced to differentiate into eosinophils by culture with IL-5 and IL-3 (**33**). We hypothesised that in allergic asthmatics, upregulation of the expression of 5-LO pathway enzymes in eosinophils or other cells by the cytokine microenvironment within the bronchial mucosa may be linked to enhanced capacity for cys-LT synthesis and poorer lung function.

Inhalation or endobronchial instillation of a large quantity of allergen in the laboratory does not effectively mimic natural exposure to low levels of environmental allergen over an extended period. This study aimed to determine the profile of expression of eicosanoid pathway enzymes in inflammatory leukocytes *in situ* in the bronchial mucosa of allergic asthmatic patients before and during natural exposure to a seasonal aeroallergen. For the first time, we show that seasonal exposure of birch-sensitive asthmatics to airborne birch pollen significantly increases bronchial mucosal counts of eosinophils and macrophages. This is associated with significant increases in the counts of cells expressing enzymes of the leukotriene synthetic pathway (5-LO, FLAP, LTA_4 hydrolase, and LTC_4 synthase), but not in those expressing COX-1, COX-2, or PGD_2 synthase. In-season, 62% of cells staining for LTC_4 synthase, the requisite enzyme for cys-LT synthesis, were eosinophils. Moreover, the proportion of eosinophils immunostaining for LTC_4 synthase increased from 1.5% to 9% ($p=0.047$), suggesting induction of the enzyme in infiltrating eosinophils by the cytokine microenvironment. Increased numbers of cells expressing 5-LO pathway enzymes during the pollen season, particularly eosinophils and macrophages, correlated significantly with deterioration in morning and evening peak expiratory flow (PEF), and were associated with an increase in asthma symptom scores and enhanced bronchial responsiveness. Our results show that airway dysfunction in seasonal asthma is associated with an increased potential for cys-LT synthesis in the bronchial wall, linked to an influx of 5-LO^+ eosinophils and macrophages and to induction of LTC_4 synthase expression within eosinophils.

METHODS.

Patient Recruitment.

Approval for the study was obtained from the Ethics Committee at the Sahlgrenska University Hospital, Gothenburg, Sweden, and patients gave informed written consent. Twelve non-smoking mild asthmatic adults (mean age 30.6 yrs, range 23-43 yrs) with a clinical history of seasonal birch pollen-related asthma were recruited from the Gothenburg area. They all had a positive skin-prick test (wheal >3mm) to birch pollen. None of the patients had positive skin-prick tests to house-dust mite or molds. Six patients tested positive for cat or dog dander but had no daily contact with pets. Seven subjects allergic to grass pollen were not excluded as the grass pollen season follows the birch pollen season.

Patients were studied pre-season in February/March and in-season in May, 1997. On neither occasion had they experienced a respiratory infection in the previous two months. Patients had not used inhaled or systemic corticosteroids for at least one year before the study, but were using inhaled β_2-agonist bronchodilators for asthma symptoms and anti-histamines (oral acrivastine and topical levocabastine) for rhinoconjunctival symptoms as required.

Seven days before pre-season and in-season bronchoscopies, monitoring of lung function was initiated with twice-daily peak expiratory flow (PEF) measurements using Wright mini peak flow meters, and patients were asked to complete diary cards recording their medication use (β_2 agonist puffs/day and antihistamines) and scores for asthma and rhinitis symptoms. Symptom scores were on a scale of 0-3 where 0 = no symptoms, 1 = mild, 2 = moderate, and 3 = severe symptoms. Two days before each bronchoscopy, total serum IgE was assayed by ELISA and bronchial responsiveness was measured as the provocation concentration of inhaled methacholine required to decrease FEV_1 by 20% from baseline (methacholine PC_{20} FEV_1).

Bronchoscopy and bronchial mucosal biopsy.

On the pre-season and in-season study days, patients underwent flexible fiberoptic bronchoscopy according to American Thoracic Society guidelines (**34**). After pre-medication with morphine-scopolamine and inhaled salbutamol (0.2 mg), and under local anaesthesia with 2% xylocaine, an Olympus BF1 bronchoscope (Lake Success, NY, USA) was used to obtain a bronchial mucosal biopsy from the subcarina of an upper lobe segment.

Biopsy specimens were immediately added to ice-cold acetone containing the protease inhibitors iodoacetamide (20mM) and PMSF (2mM)(Sigma Chemical Co., Poole, UK) and fixed at -20°C overnight. Biopsies were transferred to fresh acetone and then to methyl benzoate for 15 minutes each at room temperature, before infiltration with glycol methacrylate (GMA) resin (Polysciences,

Northampton, UK) as described (**35**). Polymerisation of the GMA resin took place in airtight capsules with methyl benzoate at 4°C overnight. The polymerised resin block was stored desiccated at -20°C.

Immunohistochemistry.
Immunohistochemical staining for leukocyte markers and for 5-LO and COX pathway enzymes was performed as described (**22,35**). Serial semi-thin sections (2 μm) were cut by ultramicrotome with a glass knife and floated onto 0.2% ammonia solution for 90s, before adherence to microscope slides coated with poly-L-lysine (Sigma). Sections were pre-treated with sodium azide (0.1%) and hydrogen peroxide (0.3%) for 30 minutes to irreversibly inhibit endogenous peroxidases, confirmed by the absence of colour development when aminoethylcarbazole (AEC) substrate was added directly to control slides. Sections were treated with Dulbecco`s modified Eagle`s medium containing 20% FCS and 1% BSA (Sigma) for 30 minutes to block non-specific binding sites. Primary Abs to the target proteins were added to separate sections for one hour (polyclonals) or overnight (monoclonals) at room temperature.

Mouse mAbs to leukocyte markers were AA1 (Dako, High Wycombe, UK) for mast cell tryptase (dilution 1:1000)(**36**), EG2 (Pharmacia Biosystems Ltd., Milton Keynes, UK) for eosinophil cationic protein (ECP; 1:200) (**37,38**), UCTH1 (anti-CD3) for pan-T-lymphocytes (1:100)(Dako), PG-M1 (1:15)(Dako) against the macrophage-specific form of CD68 (**39**), NP57 (Dako) against neutrophil elastase (NE) for neutrophils (1:1000), anti-CD4 against T-helper cells (1:10)(Becton Dickinson Ltd., Oxford, UK), OK25 (anti-CD8) against cytotoxic/suppressor T cells (1:100)(Becton-Dickinson), and ACT-1 (anti-CD25) against activated (IL-2R-positive) T-lymphocytes (1:50)(Dako). Rabbit polyclonal Abs against human 5-LO (LO-32; 1:300), FLAP (H4;1:300), and LTA_4 hydrolase (1:500) (**40**) were generous gifts from Dr Jilly Evans (Merck & Co., West Point, PA, USA). Rabbit polyclonal IgG to human lung LTC_4 synthase (1:50) and rabbit polyclonal antiserum to PGD_2 synthase were as described (**41,42**). Mouse mAb raised against ovine COX-1, but which cross-reacts with the human enzyme, was used (1:150), and the mouse Mab to COX-2 was directed against a 19 amino acid peptide sequence from the human enzyme (1:150)(Cayman Chemical Inc., Ann Arbor, MI, USA).

For monoclonal primary antibodies, biotinylated rabbit anti-mouse IgG secondary antibody (1:300)(Dako) was applied for 2 hours at room temperature, followed by streptavidin-biotin horseradish peroxidase (SAB-HRP) complex (1:200)(Dako) for 2 hours. For polyclonal primary Abs, biotinylated swine anti-rabbit IgG (1:300)(Dako) was applied for 1 hour followed by SAB-HRP complex for 1 hour. SAB-HRP forms a red reaction product from the substrate AEC (0.03%) in acetate buffer, incubated at 37°C for 25-30 min. Sections were counterstained with Mayer`s hematoxylin for 60-90 s before coverslips were mounted.

Positively-stained nucleated cells in the total mucosal area (excluding epithelium, mucus glands, blood vessels and areas of forceps damage) of at least two non-adjacent sections from each biopsy were counted by Zeiss light microscope at x400 magnification. The counting was performed blind on coded sections. Submucosal areas in each section were determined using an image analysis system (ColorVision 164SR; Analytical Measurement Systems, Cambridge, UK). Cell counts are expressed as mean cells per mm^2 of mucosa. Control sections were routinely immunostained in the absence of primary Ab or in the presence of an unrelated isotype-matched mouse IgG (for mAbs) or non-immune rabbit serum (for polyclonal Abs). In all cases there was no colour development.

To characterise the distribution of 5-LO and COX pathway enzyme expression in inflammatory cell types, adjacent 2μm sections were immunostained in parallel and optically superimposed using the *camera lucida* system (Leica UK Ltd., Milton Keynes, UK) as described (**22,43**). Populations of cells immunopositive for each enzyme were identified as eosinophils, mast cells, and macrophages. In addition, to examine the degree of expression of enzymes within a specific inflammatory cell-type, colocalisation data were also expressed as the proportion of that cell-type immunopositive for individual eicosanoid enzymes.

RESULTS.

Effect of seasonal allergen on clinical measures of asthma severity.

Airborne birch pollen counts in Gothenburg were monitored daily during 1997 with a Burkard volumetric trap (**Figure 1**). Asthmatic patients (n=12) experienced significant deteriorations in bronchial responsiveness during the birch pollen season compared to pre-season values, with median PC_{20} FEV_1 for methacholine falling from 14.0 mg/ml to 2.7 mg/ml (P=0.001, n=12, Wilcoxon) (**Figure 2**), accompanied by significant falls in morning PEF (P=0.023) and evening PEF (p=0.014) (**Figure 2**). Asthma symptom scores rose from 0.18 ∀ 0.09 to 0.68 ∀ 0.10 (P=0.004) and there was a trend to increased use of inhaled β_2-agonists from 0.04 ∀ 0.04 to 0.23 ∀ 0.07 puffs/day (P=0.19). Seasonal allergen exposure also significantly increased rhinitis symptom scores from 0.23 ± 0.07 to 1.07 ± 0.10 (P<0.0001), accompanied by a significant rise in the use of oral and topical anti-histamines (P=0.017).

Leukocyte markers in bronchial biopsies.

Immunohistochemical analysis of bronchial biopsies obtained during the pollen season showed that mean counts of $EG2^+$ eosinophils more than doubled from 15.5 ± 3.3 cells/mm^2 to 35.0 ± 10.4 cells/mm^2 (n=12, P=0.035 Wilcoxon), and $CD68^+$ macrophage counts rose from 8.3 ± 1.8 cells/mm^2 to 14.9 ± 2.6 cells/mm^2 (P=0.019), compared to pre-season biopsies (**Figure 3**). Neutrophil counts showed a trend toward a rise that did not reach significance (pre-season: 41.6 ± 11.9, in-season: 79.1 ± 28.2 cells/mm^2; P=0.08), while there were no changes in the number of tryptase-positive (AA1) mast cells (**Figure 3**).

There was a non-significant trend towards a rise in cytotoxic (CD8) T-cell counts (P=0.085), but no changes in pan-T-lymphocyte (CD3) or T-helper cell (CD4) counts (**Figure 4**). The sub-population of 'activated' T-cells expressing the IL-2 receptor (CD25) also did not change, either when expressed as cell counts per mm^2 (**Figure 4**), or as a percentage of CD3 T-cell counts (pre-season: 0.9 ± 0.4%, in-season: 2.2 ± 1.0%; p>0.05).

Eicosanoid pathway enzyme expression in bronchial biopsies.

Immunohistochemistry of bronchial biopsies revealed clear cell-associated staining for the eicosanoid pathway enzymes. During the pollen season, mean counts of 5-LO^+ cells almost doubled from 31.7 ± 7.9 cells/mm^2 to 58.2 ± 8.2 cells/mm^2 (P=0.02, n=12), and $FLAP^+$ cell counts more than doubled from 8.7 ± 2.9 cells/mm^2 to 18.1 ± 3.7 cells/mm^2 (P=0.04)(**Figure 5**). There was a smaller, but significant, rise in the counts of LTA_4 hydrolase$^+$ cells (P=0.05), and counts of cells expressing LTC_4 synthase nearly quadrupled from 1.06 ± 0.25 cells/mm^2 to 3.87 ± 1.03 cells/mm^2 (P=0.021)(**Figure 5**). In contrast, COX-1^+ cell counts remained stable (pre: 37.1 ± 3.4 cells/mm^2; in: 41.0 ± 4.6 cells/mm^2) as did COX-2^+ cell counts (pre: 25.0 ± 4.3, in: 21.0 ± 4.0 cells/mm^2; n=12)(**Figure 6**). Counts of cells expressing PGD_2 synthase also did not change (pre:11.4 ± .0 cells/mm^2; in: 10.5 ± 2.2 cells/mm^2; n=10)(**Figure 6**).

Cellular localisation of eicosanoid pathway enzymes.

Using the *camera lucida* technique (**22,43**) on adjacent thin sections of biopsies from a representative sub-group of 6-8 asthmatics, immunostaining for 5-LO, FLAP, LTC_4 synthase, COX-1, and COX-2 was colocalised to eosinophils, mast cells, and macrophages both before and during the birch pollen season. In pre-season biopsies, 25.7 ± 7.6% of 5-LO^+ cells were eosinophils, 36.5 ± 5.9% were mast cells (n=8), and 21.0 ± 3.9% cells were macrophages (**Figure 7**). FLAP expression also appeared evenly distributed, with 12.4 ± 5.3% of $FLAP^+$ cells being identifiable as eosinophils, 20.7 ± 5.2% as mast cells, and 11.0 ± 3.6 % as macrophages (n=8). Similarly, 19.5 ± 10.0% of LTC_4 synthase$^+$ cells were identifiable as eosinophils, 20.8 ± 10.0% as mast cells, and 20.0 ± 10.0 % as macrophages (n=6). COX-1^+ cells in pre-season biopsies were predominantly mast cells (53.8 ± 3.9%), with only 13.8 ± 5.0% being eosinophils. Similarly, 53.2 ± 6.7 % of COX-2^+ cells were mast cells and only 22.5 ± 6.7 % were eosinophils, with the remainder mostly macrophages (*data not shown*).

During the birch pollen season, mean 5-LO^+ cell counts rose in the paired biopsies from eight patients from 39.7 ± 10.5 cells/mm^2 to 59.8 ± 9.7 cells/mm^2 (P=0.08)(**Figure 7**), close to the values obtained in the complete group of 12 patients (Figure 5). Three-quarters of the increase could be accounted for by increases in the counts of 5-LO^+ eosinophils (from 9.1 ± 3.2 to 17.9 ± 4.7 cells/mm^2; P=0.18, n=8) and

of 5-LO$^+$ macrophages (from 6.6 ± 1.6 to 12.7 ± 2.8 cells/mm^2; P=0.05) (**Figure 7**), with no significant change in the numbers of 5-LO$^+$ mast cells. There was also a trend for the proportion of macrophages staining positive for 5-LO to increase from 19.5 ± 4.2% to 34.1 ± 4.6% (P=0.11), while there were no changes in the proportion of eosinophils staining for 5-LO (pre: 38.4 ± 10.5%; in: 32.7 ± 3.9%) or in the proportion of mast cells staining for the enzyme (pre: 35.5 ± 4.3%; in: 34.7 ± 7.2%).

In paired biopsies from eight patients, mean counts of FLAP$^+$ cells rose from 10.9 ± 4.1 cells/mm^2 pre-season to 22.3 ± 4.7 cells/mm^2 in-season (P=0.08, n=8)(**Figure 7**), close to the values observed in the complete group of 12 patients (Figure 5). The rise could be entirely accounted for by increases in the numbers of FLAP$^+$ eosinophils (from 1.4 ± 0.8 to 7.8 ± 2.9 cells/mm^2; P=0.09) and FLAP$^+$ mast cells (from 1.5 ± 0.4 to 7.7 ± 2.6 cells/mm^2; P=0.03) (**Figure 7**). Since total mast cell counts did not change in-season, the increase in FLAP$^+$ mast cells may indicate induction of FLAP expression within the mast cell population. This is supported by a strong trend to an increased proportion of mast cells immunostaining for FLAP in-season (17.4 ± 4.4%) compared to pre-season (8.4 ± 1.7%; P=0.06). There was a similar trend for an increased proportion of eosinophils to express FLAP in-season (14.2 ± 3.9 %) compared to pre-season (7.9 ± 3.1 %; P=0.09), but no changes in FLAP expression in macrophages.

In paired biopsies from six patients, mean counts of LTC_4 synthase$^+$ cells rose from 1.0 ± 0.2 cells/mm^2 pre-season to 5.0 ± 1.8 cells/mm^2 in-season (P=0.04, n=6) (**Figure 7**), similar to the values observed in the complete group of 12 patients (Figure 5). This rise was matched by a significant increase in LTC_4 synthase$^+$ eosinophils from 0.2 ± 0.1 to 4.2 ± 2.0 cells/mm^2 (P=0.048) (**Figure 7**), such that 62.4 ± 15.1% of total LTC_4 synthase$^+$ cells were eosinophils in-season, compared to only 19.5 ± 10.0% pre-season (P=0.047). There were no changes in the numbers of LTC_4 synthase$^+$ macrophages or LTC_4 synthase$^+$ mast cells. The proportion of eosinophils staining for LTC_4 synthase rose significantly from 1.5 ± 1.1% to 9.0 ± 2.6% (P=0.047), while the proportions of mast cells and macrophages staining for LTC_4 synthase did not change (**Figure 7, *lower panel***).

In paired biopsies from six subjects, counts of cells expressing COX-1 and COX-2 did not change significantly in-season compared to pre-season values, and their distribution remained predominantly (45-55%) in mast cells, the total counts of which also did not significantly change (**Figure 3**). However, there was a significant decrease in the proportion of mast cells expressing COX-1, falling from 71.6 ± 4.6 % pre-season to 46.1 ± 7.1 % in-season (P<0.0001), and a trend for the proportion of mast cells expressing COX-2 to fall from 38.7 ± 7.0 % to 21.2 ± 5.0 % (P=0.18)(*data not shown*). These changes in COX expression within the mast cell populations were not seen in eosinophils or macrophages.

<u>Relationship of leukocyte counts and eicosanoid enzyme immunostaining to clinical measures of asthma severity</u>.
Among clinical measures of disease severity, only PEF values showed meaningful relationships to leukocyte markers in bronchial biopsies. In-season PEF values correlated inversely with macrophage counts (PEFam: ρ=-0.78, P=0.003; PEFpm: ρ=-0.76, P=0.004; n=12), but not significantly with counts of other cell-types.

Significant relationships were observed between PEF and the counts of cells immunostaining for leukotriene enzymes. Pre-season PEFam and PEFpm values correlated inversely with counts of 5-LO$^+$ cells (ρ=-0.59, P=0.04, and ρ=-0.61, P=0.036, respectively; n=12)(**Figure 7**), particularly with counts of 5-LO$^+$ eosinophils (ρ=-0.80, P=0.017, and ρ=0.78, P=0.023). Pre-season PEFam and PEFpm values also correlated inversely with FLAP$^+$ cells (ρ=-0.62, P=0.032, and ρ=-0.64, P=0.026).

During the pollen season, PEFam and PEFpm values correlated significantly with counts of 5-LO$^+$ cells (ρ=-0.65, *p*=0.023, and ρ=-0.66, *p*=0.021, respectively, n=12)(**Figure 7**), particularly with counts of 5-LO$^+$ macrophages (ρ=0.64, P=0.09 and ρ=0.62, P=0.1) and weakly with 5-LO$^+$ eosinophils (ρ=-0.40, P=0.2, and ρ=-0.43, P=0.16). There were no significant relationships during the pollen season between PEF values and counts of cells immunostaining for COX-1 or COX-2.

DISCUSSION.

This study is the first to show that increased asthma symptom scores, bronchial hyperresponsiveness, and impaired lung function in atopic asthmatics during natural exposure to an environmental allergen are associated with increases in bronchial mucosal counts of eosinophils and macrophages, but not of mast cells or T-lymphocytes. Increased asthma severity in the birch pollen season is also associated with relevant increases in the numbers of cells expressing enzymes of the cysteinyl-leukotriene synthetic pathway, but not of those expressing enzymes of the prostanoid synthetic pathway. Both before and during the birch pollen season, counts of cells expressing 5-lipoxygenase (5-LO) showed marked inverse correlations with peak expiratory flow, suggesting that airway dysfunction in seasonal asthma is linked to an increased capacity of the airway to generate leukotrienes.

The 12 birch pollen-sensitive asthmatics recruited to the present study were investigated before and during the well-defined birch pollen season in Sweden (Figure 1), and were not allergic to house-dust mite or exposed to animal danders. The seasonal impact of allergic inflammation on airway hyperreactivity was demonstrated by the increased responsiveness to methacholine and reduction in morning and evening peak expiratory flow measurements (Figure 2).

While the mucosal biopsies showed no overall changes in T-cells in these mild asthmatics (Figure 4), they did demonstrate significant increases in mucosal eosinophil and macrophage counts in the pollen season compared to pre-season (Figure 3). These data extend to the bronchial mucosa the previous finding of elevated eosinophil counts in BAL fluid during a season of high airborne birch pollen counts, compared to a year of low pollen counts (**44**). In BAL fluid and bronchial mucosal biopsies of symptomatic asthmatics, there is increased expression of cytokines and chemokines that can enhance migration and adhesion and reduce apoptosis of eosinophils and/or monocytes *in vitro*, including IL-3, IL-4, IL-5, GM-CSF, eotaxin and RANTES (**43,45-48**). Both IL-5 and cys-LTs elicit eosinophilia in the bronchial mucosa when inhaled by asthmatics (**4,49**), and leukotriene modifier drugs reduce eosinophil counts in the BAL fluid and blood of asthmatics (**5,7**). Since the eosinophil is itself a source of LTC_4 *in vitro* (**50**), and also of basic proteins that can damage bronchial epithelial cells (**51**), a cycle of cys-LT synthesis and eosinophil recruitment may contribute to sustained airway obstruction.

During the pollen season, highly significant increases were observed in bronchial mucosal counts of cells immunostaining for LT pathway enzymes (Figure 5). Pre-season, immunostaining for 5-LO, FLAP, and LTC_4 synthase was distributed evenly among relatively low numbers of mast cells, eosinophils, and macrophages. Increases in 5-LO^+ and $FLAP^+$ cells during the season were mostly attributable to increased eosinophils and macrophages, while for LTC_4 synthase, the increase was entirely due to eosinophils (Figure 7), such that 62% of LTC_4 $synthase^+$ cells in biopsies obtained in-season were eosinophils, compared to only 19% pre-season. This suggests that increased prevalence of LTC_4 synthase-positive eosinophil may be the most important factor increasing the potential of the allergic asthmatic airway to generate cys-LTs.

Furthermore, an increased proportion of eosinophils immunostained for LTC_4 synthase in-season (9%) compared to pre-season (1.5%; Figure 7), with a trend also to an increased proportion expressing FLAP (14.2% versus 7.9%; $p=0.09$). *In vitro*, 5-LO, FLAP, and LTC_4 synthase expression are induced sequentially during the development of eosinophils from cord blood progenitors in the presence of IL-3 and IL-5 (**33**). In human blood eosinophils *in vitro*, IL-5 enhances the expression of FLAP and increases synthesis of LTC_4 (**31,32**). At baseline, 5-LO expression was higher than that of FLAP, and IL-5 did not increase 5-LO expression, but did cause translocation of 5-LO to the nucleus (**32**), analogous to the lack of increase in the proportion of eosinophils immunostaining for 5-LO in the present study. Increased expression of eosinophilopoietic cytokines and chemokines has been demonstrated in T-cells, mast cells and eosinophils in the asthmatic airway (**43,45,46,48,52**). Our data may indicate that the cytokine microenvironment induces the expression of FLAP and LTC_4 synthase in eosinophils in the seasonal allergic asthmatic lung.

PEF values showed significant inverse correlations with the numbers of 5-LO^+ cells in bronchial biopsies both before and during the pollen season (Figure 8). In-season, the relationship was particularly

influenced by the numbers of 5-LO$^+$ eosinophils and macrophages. The possibility that activation of the LTC_4 synthetic pathway in infiltrating eosinophils and macrophages is a principal factor leading to airway dysfunction during the pollen season is suggested by the significant increases in eosinophil and macrophage counts; these cell types represent the overwhelming majority of those cells immunostaining for LTC_4 synthase and possess the other requisite enzymes for cys-LT synthesis. It is also supported by the significant inverse correlations of 5-LO$^+$ macrophages with PEF values in-season, and by a similar correlation of 5-LO$^+$ eosinophils with PEF values pre-season.

The significant increase in counts of cells immunstaining for LTA_4 hydrolase is probably related to the significant rise in macrophage counts and the trend towards increased neutrophil counts. Increased potential for the synthesis of LTB_4 by these cells may contribute to influx of leukocytes, including eosinophils, in the seasonal asthmatic airway.

In contrast, counts of cells immunostaining for COX-1 and COX-2 did not change in the birch pollen season (Figure 6). Pre-season, immunostaining for both COX isoenzymes was predominantly in mast cells. In-season, the proportion of mast cells immunostaining for COX-1 fell significantly from 72% to 46% and the proportion expressing COX-2 also tended to fall from 39% to 21%. In contrast to COX-1 and COX-2, the proportion of mast cells immunostaining for FLAP tended to rise from 8% to 17% (P=0.06). Downregulation of COX isoenzyme expression within mast cells may reflect changes in the cytokine microenvironment within the airway during the pollen season. In murine bone-marrow-derived mast cells *in vitro*, stem cell factor (SCF; *c-kit* ligand) induces the short-lived expression of COX-2 and a longer-lived induction of COX-1 and PGD_2 synthase (**53**), while IL-3 instead promotes the expression of 5-LO, FLAP, and LTC_4 synthase (**54**). Our biopsy data suggest that the cytokine microenvironment in the seasonal asthmatic lung may reduce the relative potential of mast cell populations to generate prostanoids and promote their potential to generate cys-LT.

The predominant prostanoid produced by human lung mast cells is PGD_2, generated from COX-derived PGH_2 by PGD_2 synthase, and the mast cell is the likely source of allergen-induced PGD_2 synthesis in the human airway. PGD_2 acts at thromboxane (TP) receptors to cause bronchoconstriction in human lung. Counts of cells immunostaining for PGD_2 synthase did not change significantly in-season (Figure 6), and the counts of mast cells also did not change (Figure 3). The lack of change in PGD_2 synthase immunostaining, the COX-1 and COX-2 colocalisation data in mast cells, the lack of meaningful relationships between COX immunostaining and clinical measures of asthma severity, and the relative lack of efficacy of TP receptor antagonists in clinical asthma suggest that mast cell-derived PGD_2 may not contribute significantly to airway dysfunction in seasonal asthma.

The profile of inflammatory leukocyte changes observed in the airways of asthmatics naturally exposed to seasonal allergen is distinct in important aspects from that observed after allergen bronchoprovocation in the laboratory. Our data confirm and extend the findings of an influx of eosinophils, neutrophils, and monocyte-macrophages (**27-29,52,55,56**) in bronchial biopsies and BAL fluid after allergen challenge, but they contrast with reports of increases in mast cells and in CD3$^+$, CD4$^+$, and CD25$^+$ T-cells (**52,57,58**). The lack of significant changes in T-cell populations (Figure 4) may reflect the mild disease experienced by the asthmatics in this study, but is nevertheless noteworthy since the seasonal pollen response did include rises in eosinophil and macrophage counts, increased airway reactivity, and decreased pulmonary airflow (Figures 2 and 3).

The increased immunodetection of each protein of the 5-LO/FLAP/LTC_4 synthase pathway in this study also contrasts with our previous investigation in bronchial biopsies from patients with aspirin-intolerant asthma (AIA) (**22**). In that study, significantly more cells expressing LTC_4 synthase were observed in AIA biopsies than in biopsies from aspirin-tolerant asthma (ATA) patients or normal subjects, without increased immunodetection of other 5-LO pathway proteins. The increased prevalence of LTC_4 synthase$^+$ cells was accounted for by higher counts of eosinophils and by a higher proportion of eosinophils staining for the enzyme in AIA biopsies (50%) compared to biopsies of ATA patients with asthma of comparable severity (21%), irrespective of exposure to aspirin. The selective increase in LTC_4 synthase-positive cells correlated with elevated BAL fluid cys-LT levels and with bronchial hyperresponsiveness

to inhaled lysine-aspirin (**22**). In the ATA patients, half of whom were atopic and all of whom had severe disease, eosinophil (EG2) counts were not markedly different from those in the mild asthmatics in the present study either pre-season or in-season. However, the proportion of eosinophils immunostaining for LTC_4 synthase was markedly higher in ATA patients (21%) than in the mild birch-pollen sensitive asthmatics pre-season (1.5%) and in-season (9%) in the present study. Although eosinophil counts may be suppressed by corticosteroid therapy in the ATA patients, the present data suggest that the proportion of eosinophils expressing LTC_4 synthase may be closely related to disease severity. Overall, our present data and those of our previous study (**22**) argue strongly that clinical and pathophysiological sub-types of asthma can be traced back to mechanisms involved in lipid mediator generation.

In summary, we have demonstrated increases in the number of cells immunostaining for enzymes of the 5-lipoxygenase pathway, but not of those studied in the cyclooxygenase pathway, in the bronchial mucosa of allergic asthmatics during natural exposure to an environmental aeroallergen. The occurrence of 5-LO^+ cells, particularly eosinophils and macrophages, was predictive of PEF both before and during the pollen season, suggesting that 5-LO pathway products have a central role in regulating airway caliber in seasonal asthma. Changes in immunopositivity for 5-LO pathway enzymes were linked to increased numbers of eosinophils and macrophages in the bronchial wall, the first time that this has been demonstrated following natural exposure to seasonal allergen, and to increased expression of FLAP and LTC_4 synthase within the eosinophil population. In contrast, this study of natural allergen exposure did not observe the changes in mast cells or T-lymphocytes predicted from allergen challenge studies. Influx of eosinophils during the pollen season may itself be partly be a consequence of cysteinyl-leukotriene chemoattraction (**4**), and upregulation of 5-LO pathway enzymes within infiltrating eosinophils by cytokines such as IL-5 in the lung may result in an enhanced capacity for cys-LT synthesis in response to allergic stimuli.

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FOOTNOTES.

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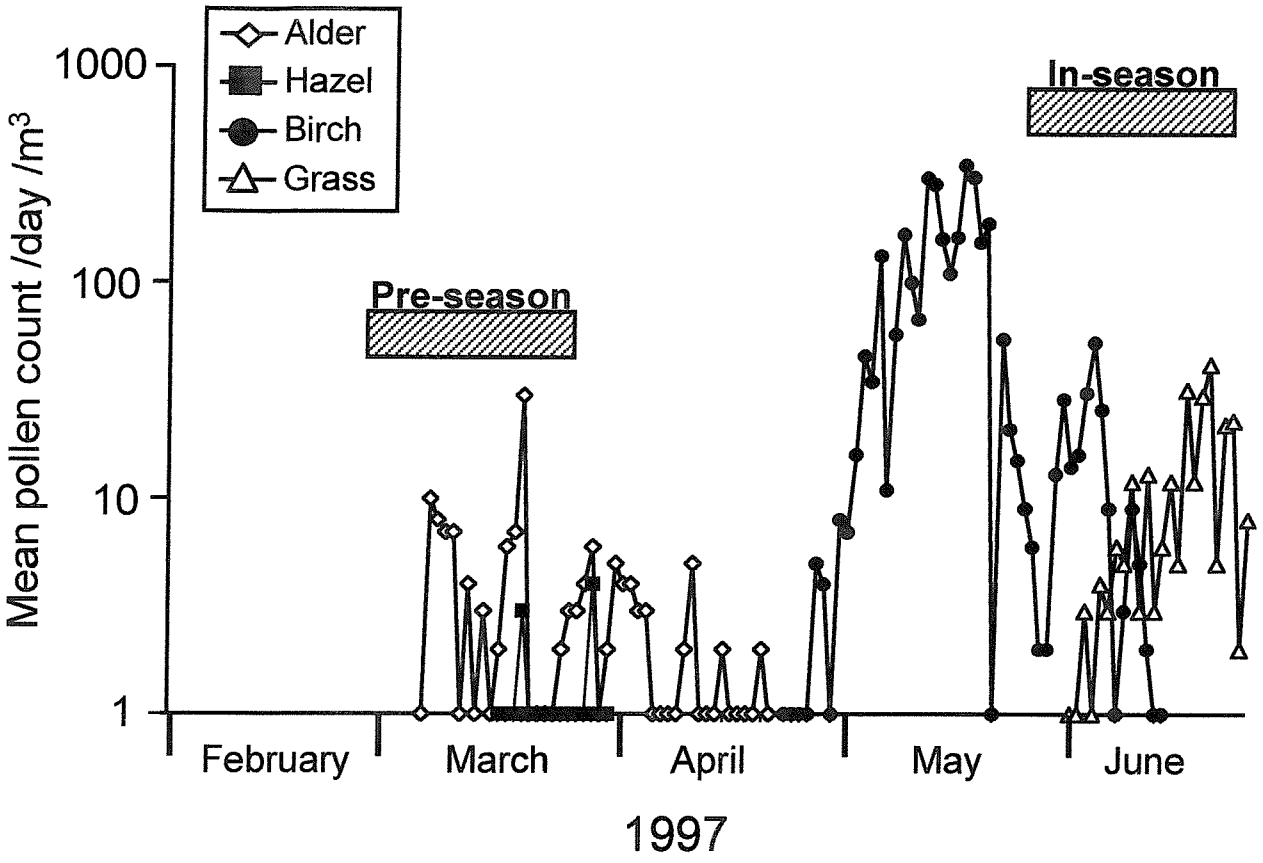
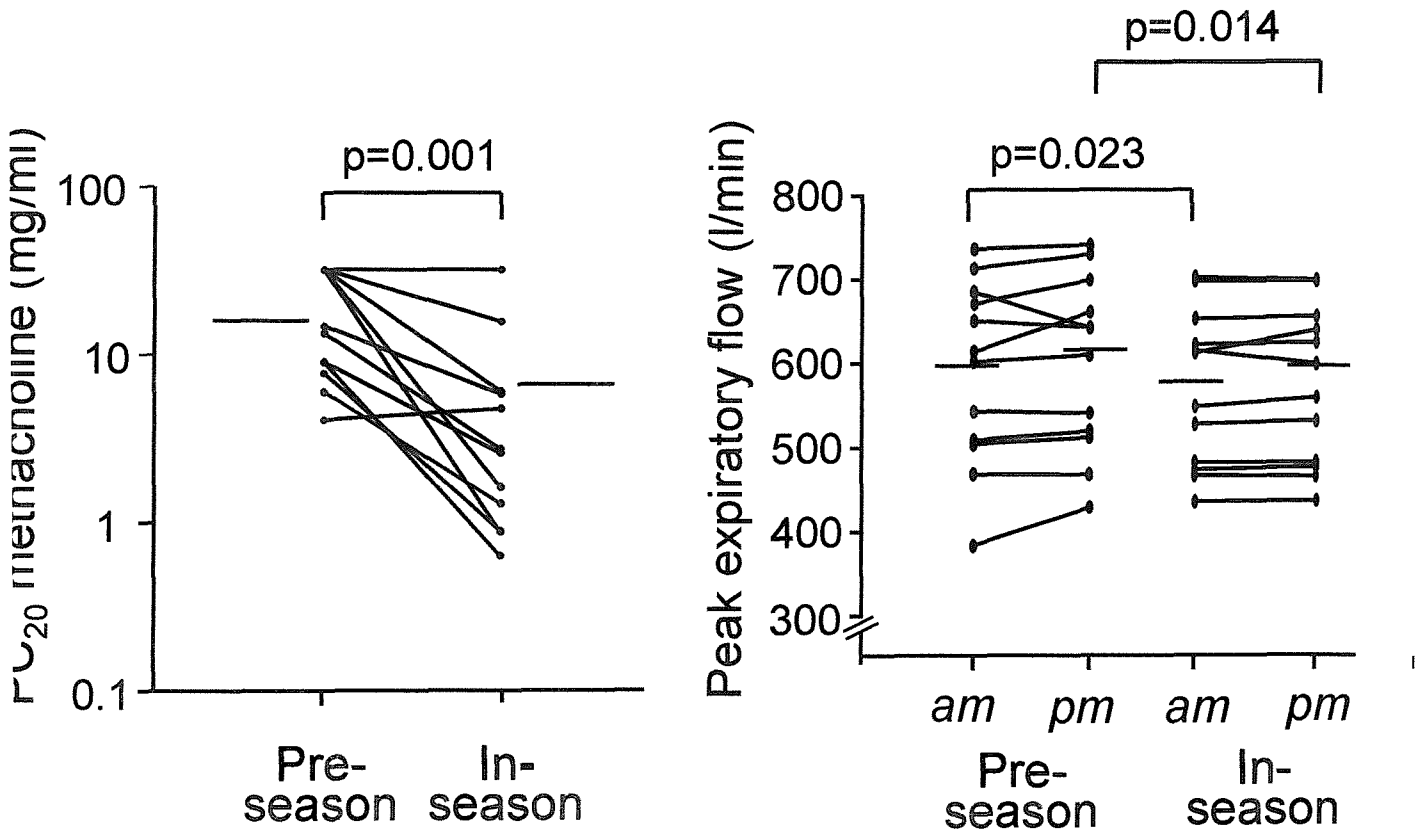
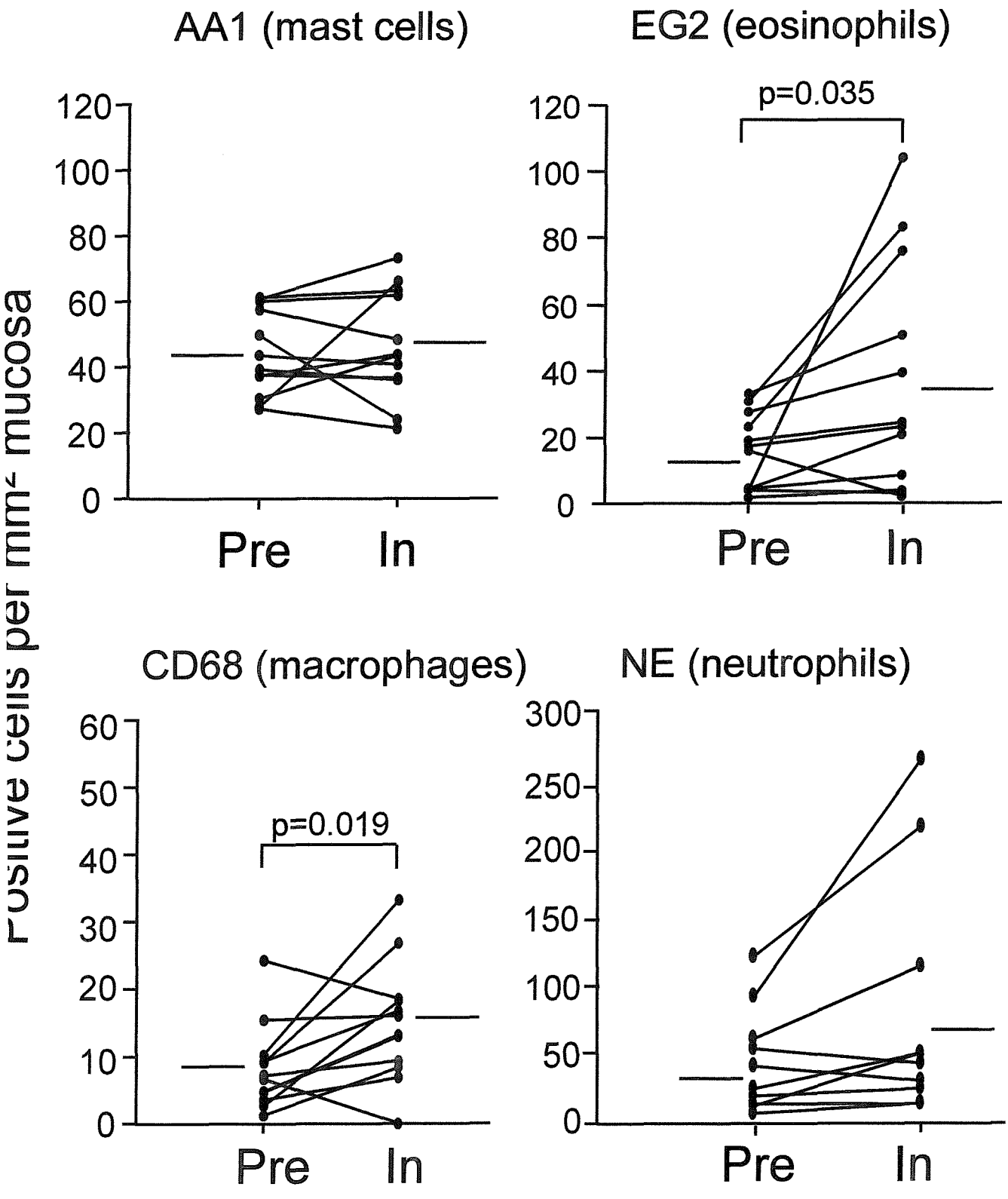
[3]*Abbreviations used in this paper:*
5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase activating protein; COX, cyclooxygenase (PGH synthase); LT, leukotriene; cys-LT, cysteinyl-leukotrienes (LTC_4, LTD_4, LTE_4); PG, prostaglandin; GMA, glycol methacrylate; AEC, aminoethylcarbazole; SAB-HRP, streptavidin-biotin horseradish peroxidase conjugate; BAL, bronchoalveolar lavage; PEF, peak expiratory flow; PC_{20} FEV_1, bronchoprovocation concentration required to reduce forced expiratory volume in 1s by 20%; AIA, aspirin-intolerant asthma;

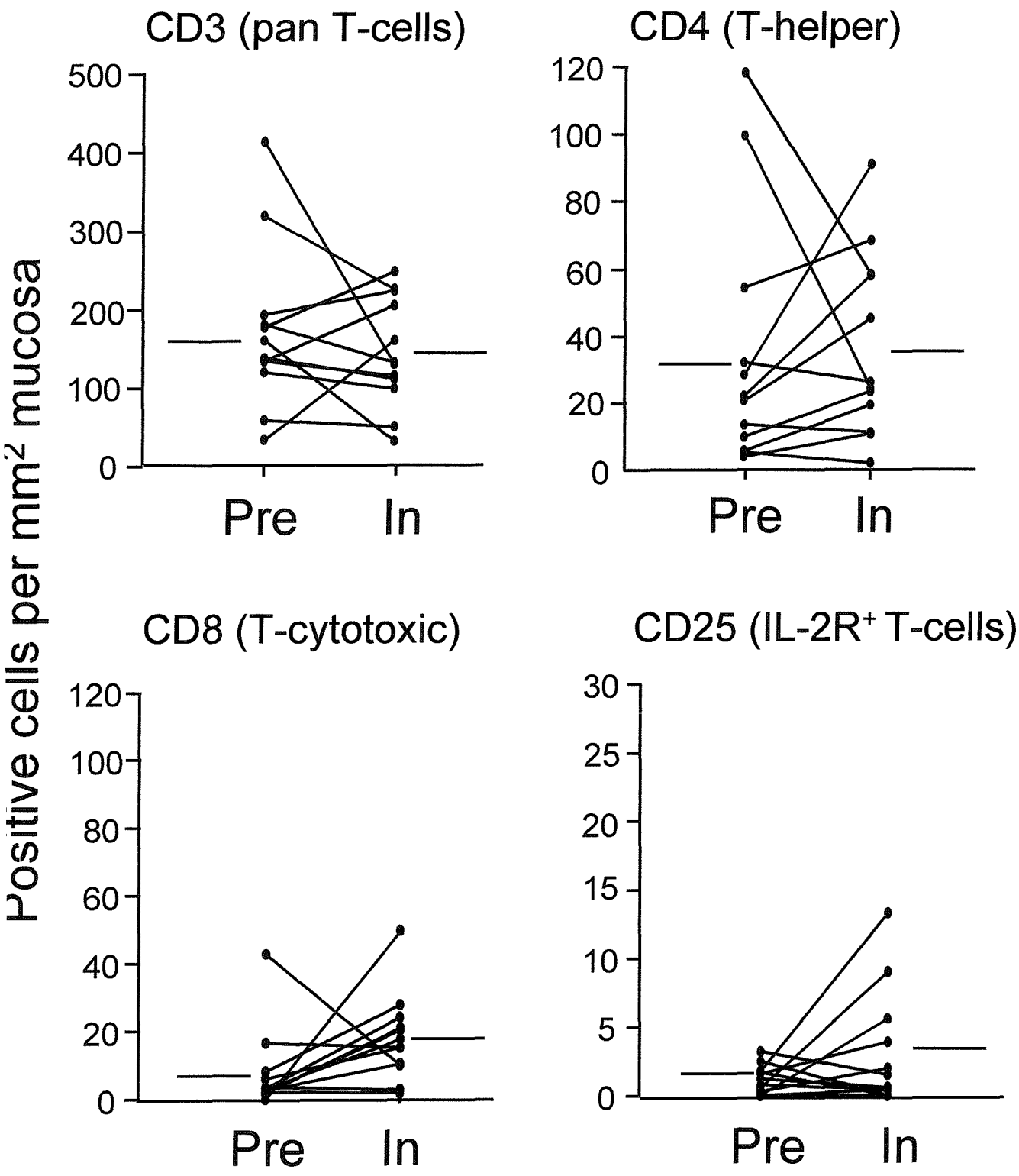
LIST OF FIGURES

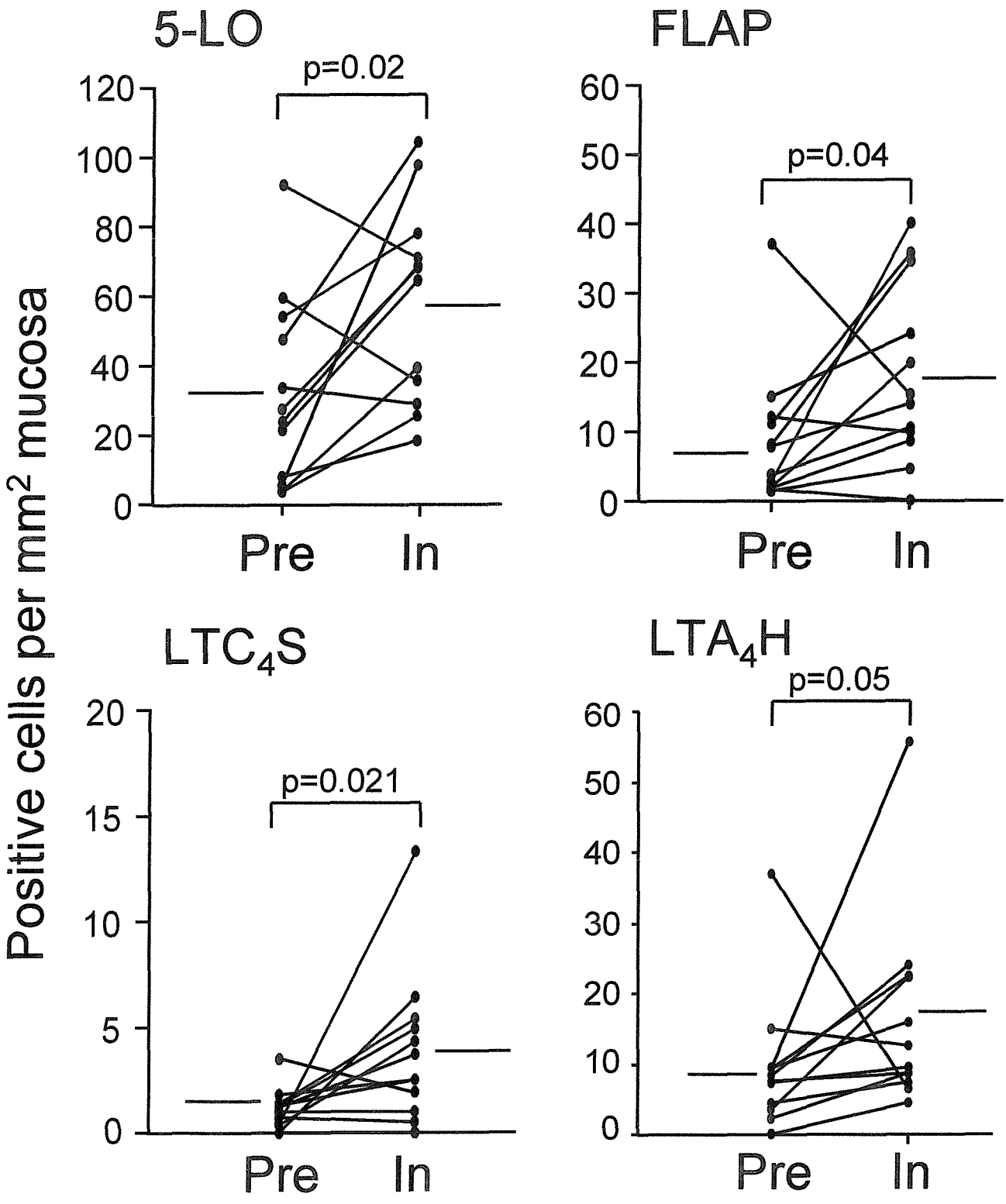
Figure 8. Relationship between 5-LO expression in bronchial biopsies and lung function. Morning peak expiratory flow (PEFam; litres/min; *y-axes*) correlates with bronchial mucosal counts of cells (per mm^2) immunostaining for 5-lipoxygenase (5-LO) in biopsies collected before (*left*) and during (*right*) the birch pollen season. There were also significant correlations both pre-season and in-season between 5-LO^+ cell counts and evening peak expiratory flow (PEFpm) (*not shown*).

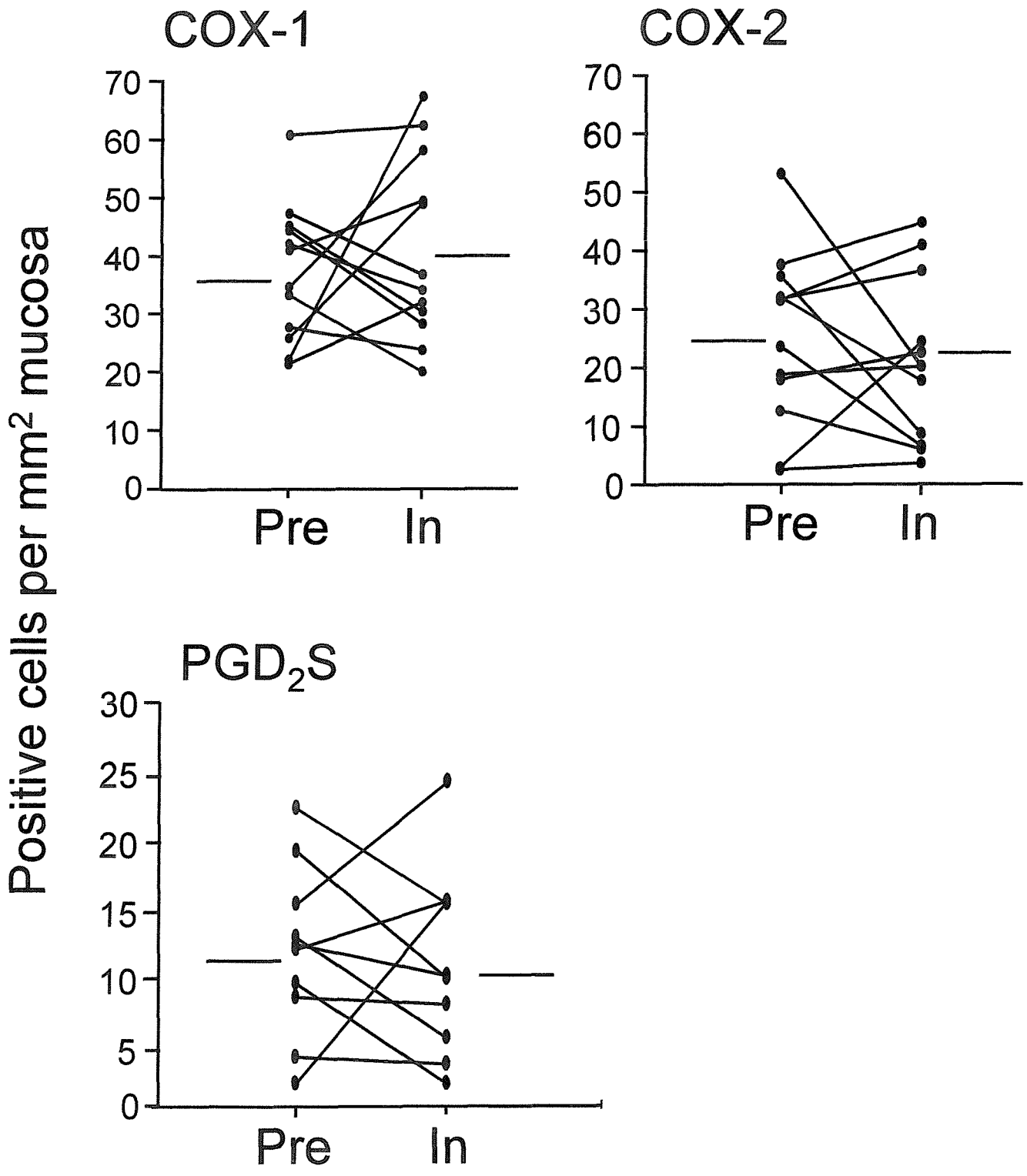

Alder
Hazel
Birch
Grass
1000
100
10
1
Mean pollen count /day /m3
Pre-season
In-season
February
March
April
May
June
1997

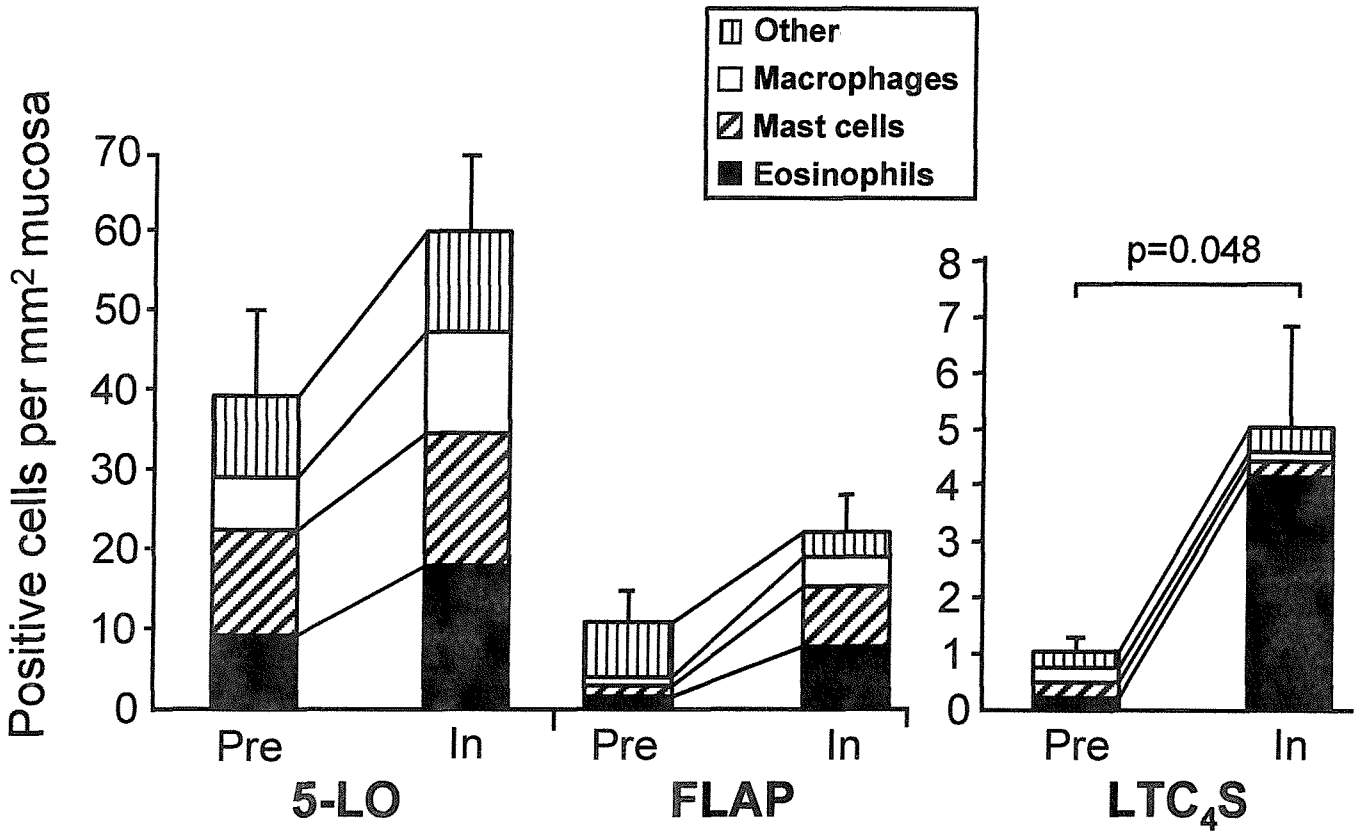

p=0.001
100
10
1
0.1
Pre-
season
In-
season
p=0.014
p=0.023
Peak expiratory flow (l/min)
800
700
600
500
400
300
am
pm
am
pm
Pre-
season
In-
season

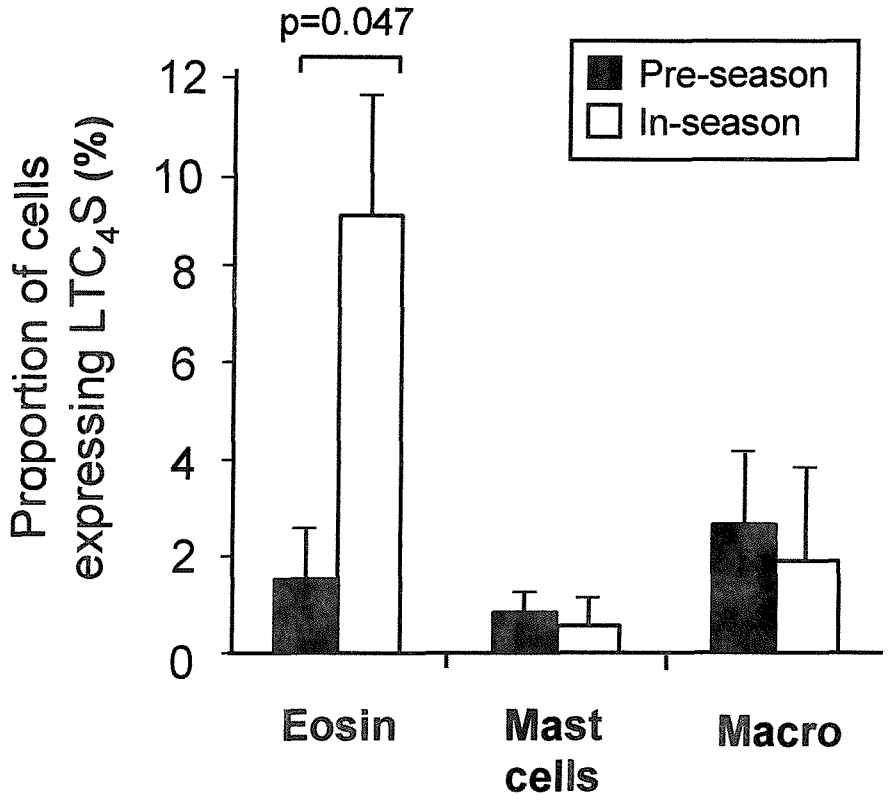

AA1 (mast cells)
EG2 (eosinophils)
p=0.035
CD68 (macrophages)
p=0.019
NE (neutrophils)
Positive cells per mm² mucosa
120
100
80
60
40
20
0
300
250
200
150
50
30
10
Pre
In

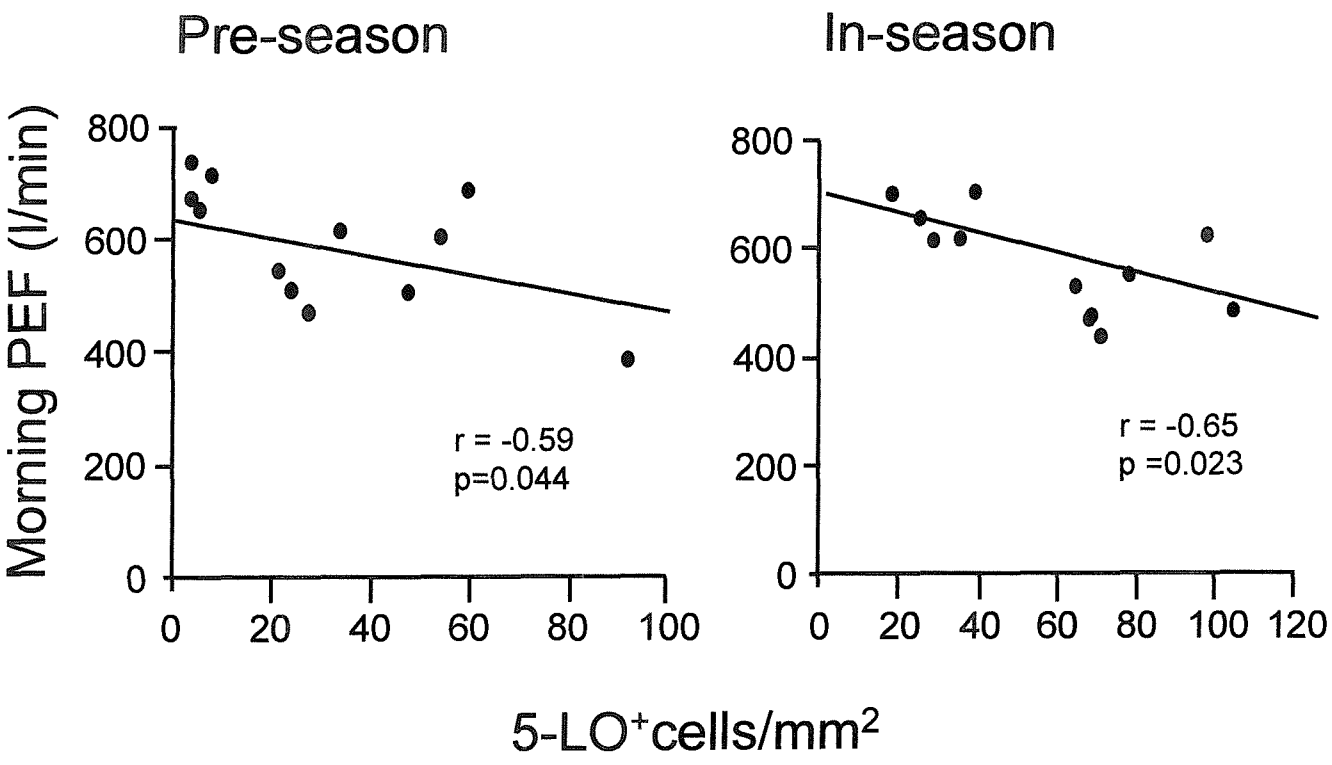

CD3 (pan T-cells)
CD4 (T-helper)
CD8 (T-cytotoxic)
CD25 (IL-2R+ T-cells)
Positive cells per mm2 mucosa
500
400
300
200
100
0
120
100
80
60
40
20
0
30
25
20
15
10
5
0
Pre
In


5-LO
p=0.02
120
100
80
60
40
20
0
Pre
In
FLAP
p=0.04
60
50
40
30
20
10
0
Pre
In
LTC4S
p=0.021
20
15
10
5
0
Pre
In
LTA4H
p=0.05
60
50
40
30
20
10
0
Pre
In
Positive cells per mm2 mucosa


COX-1
COX-2
PGD2S
Positive cells per mm2 mucosa
70
60
50
40
30
20
10
0
25
15
5
Pre
In


Other
Macrophages
Mast cells
Eosinophils
Positive cells per mm² mucosa
70
60
50
40
30
20
10
0
Pre
In
5-LO
Pre
In
FLAP
p=0.048
8
7
6
5
4
3
2
1
0
Pre
In
LTC4S


p=0.047
Pre-season
In-season
Proportion of cells expressing LTC4S (%)
12
10
8
6
4
2
0
Eosin
Mast cells
Macro


Pre-season
In-season
Morning PEF (l/min)
800
600
400
200
0
0
20
40
60
80
100
120
r = -0.59
p=0.044
r = -0.65
p =0.023
5-LO+cells/mm2

Publications List

1. Seymour, M.L., Åberg, D., Riise, G., Rak, S., Holgate, S.T. and Sampson, A.P. (1998) Seasonal allergen exposure increases expression of leukotriene pathway enzymes and induces eosinophil influx in bronchial mucosa of atopic asthmatics. *J.Allergy Clin. Immunol.* **101**, 1 pt2 711 (abstract).

2. Cowburn, A.S., Seymour, M.L., Holgate, S.T. and Sampson, A.P. (1998) Interleukin-5 enhances expression of 5-lipoxygenase activating protein (FLAP), but not 5-lipoxygenase (5-LO) , in human blood eosinophils. *J.Allergy Clin. Immunol.* **101**, 1 pt2 957 (abstract).

3. Seymour, M.L., Gilby, N., Bardin, P.G., Fraenkel, S.T., Johnston, S.L. and Sampson, A.P. (1998) Human rhinovirus infection increases 5-lipoxygenase and cyclooxygenase-2 expression in normal airways. *Eur. J. Clin. Invest.* **28**, A52 (Abstract)

4. Seymour, M.L., Rak, S., Åberg, D., Riise, G., Penrose, J.F., Kanaoka, Y., Austen, K.F., Holgate, S.T. and Sampson, A.P. (2000) Effect of natural allergen exposure to seasonal allergen on expression of eicosanoid pathway enzymes in bronchial biopsies of atopic asthmatics. (Submitted to J. Immunol.)

5. Seymour, M.L., Gilby, N., Bardin, P.G., Fraenkel, D.J., Holgate, S.T., Johnston, S.L. and Sampson, A.P. (2000) The effect of human rhinovirus infection on eicosanoid enzyme expression in normal and asthmatic subjects. (Paper in preparation)