Errata

Chapter 1
P1, para 1, line 6. "affects…many sufferers" for "effects…many suffers"
P1, para 2, line 2. "can be defined as allergic" for "can be defined allergic"
P3, para 1, line 6. "free IgE" for "free IgG"
P13, last line. "a proposition that" for "a preposition that"
P14, para 1, line 3. Delete "by airway epithelial cells"
P25, para 2, line 8. "eosinophil's role" for "eosinephils role"
P25, para 3, line 4. "eotaxin has been shown" for "eotaxin has been show"
P26, para 1, line 1. "CC chemokine" for "CC chemokines"
P29, para 1, line 11. "airway smooth muscle" for "smooth airway muscle"
P29, para 1, line 17. "after infection with" for "after expression with"
P37, para 2, line 1. "potential to affect" for "potential to effect"
P43, para 1, line 3. Delete "in serum"
P43, para 1, line 4. "small concentrations" for "small does"
P44, para 2, line 1. "Whilst most" for "Whist most"

Chapter 2
P52, para 1, line 9. Replace last sentence with "Sections were de-waxed and rehydrated through histoclear and graded ethanols."
P53, para 2, line 7. "horseradish-peroxidase" for "horse-raddish-peroxidase"
P56, para 1, line 13. "temperature, given 4 washes…capture layer which" for "temperature, 4 washes…capture layer was"
P78, para 2, line 6. "Tukey post-test" for "Tukey posttest"

Chapter 3
P79, Heading. "ICS Treated and Untreated" for "ICS Treated an Untreated"
P80, para 2, line 3. "expect that a subject" for "expect that subject"
P89, para 3, line 2. "ratio of activated to total" for "ratio of total to activated"

Chapter 4
P101, para 2, line 5. "FVC ANOVA" for "FVC AVOVA"
P111, para 2, line 2. "after cessation" for "after succession"
P112, para 3, line 6. "did not increase" for "did not decrease"
P114, para 2, line 3. "see figure 4.14" for "see figure 4.12"

Chapter 5
Figure 5.17 legend. Delete "activated" from "activated BAL IL-5"

Chapter 6
P141, line 4. Delete second "vs"
P143, para 1, line 2. "which may reflect that" for "which may refect that"
P143, para 4, line 5. "where most correlations" for "were most correlations"

Chapter 7
P153, para 1, line 7. "chemokine unique in its ability" for "chemokine unique is its ability"
P153, para 2, line 1. "prominence for their" for "prominence in their"

Addendum

Chapter 3
The protocol for the serum cytokine measurements used in section 3.3.4 is fully described in section 2.4.3, this was omitted from the materials and methods section of chapter 3.

Chapter 5
Due to tissue and staining quality, full data was not available for biopsy eosinophils in this section, thus figures 5.8 and 5.9 have fewer continuous lines than data from other samples in this study.

Chapter 6
The actually steroid dose each subject was receiving, prior to randomisation, in this study was approximately 400µg/day.
Figure 6.1. The means for each column are: 89.6, 91.4, 93.4, 96, 84.5, 87.1
Figure 6.2. The means for each column are: -1.9, -1.6, -1.7, -1.2, -1.7, -1.4

Bibliography
Add: **Glare EM, Divjak M, Bailey MJ, Walters EH.** beta-Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels. *Thorax 2002 Sep;57(9):765-70*

A note of statistics: The author believes that the use of non-parametric statistics throughout this thesis provided a more conservative and cautious approach to the data, requiring larger differences to achieve significant p values, as opposed to log transformed parametric statistics. This was especially important in the analysis of BAL cytokine data, which, measured in the picogram range, has a higher degree of error than other measures.

A note on referencing: The structure of this thesis was such that discussion chapters tend to refer back to previous sections as opposed to repeating the same material in each chapter. References to previous work are concentrated mainly in sections 1.3.2 for IL-5, 1.3.4 for eotaxin and 1.5.2 and 1.5.4 for the effects of steroids on these cytokines in asthma. Chapter 7 (particularly section 7.3) summarises and relates the findings of this thesis to current research and thinking in asthma.

Eosinophils and Eosinophilic Chemokines in Asthma and the Effect of Inhaled Corticosteroids

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Department of Medicine,
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Submitted to Monash University for the Degree of Doctor of Philosophy.

January, 2003.

To those who came before:
It is on your toil and insight,
that all our work stands.

To those that come after:
May you find something here,
that leads you forward.

Acknowledgements

I would like to thank my supervisor Professor Haydn Walters, for his assistance and guidance throughout this thesis, as well as his patience with our somewhat long-distance drafting process.

I would also like to thank Professor Napier Thompson for the opportunity to study in the Department of Medicine.

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To Dr. Chris Ward, for his advice and help though the mechanics of the project, and his friendship throughout.

The rest of the group, David Reid, Mike Pais, Dharshini Wignarajah and Bernadette Orsida for their support, friendship and helping to make the time go faster during those long incubations.

To my parents for their love, continual support and encouragement.

And finally (and by no means last or least), to my darling Emma, for far too many things than I could list!

Summary

Asthma is characterised, at a cellular level, by an eosinophilic inflammation of the airways. These eosinophils contain damaging mediators that, when released, may contribute substantially to the ongoing pathology of asthma. Current treatments with inhaled corticosteroids are able to control asthma symptoms and reduce these cells significantly in number, but they are never completely abolished. More importantly, when the drugs are removed, the eosinophils return very quickly to the airways, along with the asthmatic symptoms. The therapeutic reduction and subsequent return of these potentially important cells is thought to be mediated by a number of soluble factors produced by the immune system. These cytokines are both responsible for the growth, function and homing of eosinophils to the airways and if properly understood, might form a basis from which eosinophils could be manipulated through targeted therapies. This thesis sought to explore a part of this still somewhat hypothetical underlying network, by determining how several of these cytokines relate to airway eosinophils and corticosteroid treatment, and how these together relate to asthmatic symptoms.

We undertook a series of bronchoscopic studies of asthmatic patients treated with different corticosteroid regimes, over periods of up to 12 months. From each patient we harvested lavage fluid from the airways, took bronchial biopsies, and blood and serum in some cases. Patients also received thorough clinical and physiological testing, to determine the activity of their asthmatic disease at the time of bronchoscopy. From the bronchial lavage supernatant we were able to measure two important cytokines: IL-5 responsible for the development and activation of eosinophils, and eotaxin, a very strong chemotactic (tissue-homing) factor for eosinophils. We also counted eosinophil numbers both for the lavage cells and in bronchial biopsies. Eosinophils and cytokines were also assessed in the serum for some studies. All these factors were compared and contrasted to asthmatic symptom scores, lung function and airways hyperreactivity data, which were recorded for all patients.

The thesis includes four results chapters. The first was a cross-sectional analysis of cytokines and eosinophils for asthmatic subjects on a range of steroid dosages, which are compared to steroid-free asthmatic and non-asthmatic control subjects. This section

showed that both IL-5 and eotaxin were reduced with steroid treatment, though IL-5 was more sensitive to treatment than eotaxin. This chapter also supported other studies, showing that eosinophils were reduced, but not completely ablated after steroid treatment.

The second and primary longitudinal study took asthmatics receiving high-dose inhaled steroid treatment and reduced their dosage, in an attempt to measure the change in the airways as symptoms just recurred. In this study we found that although patients symptoms did recur and their lung function was reduced, no significant changes occurred in eosinophils or our measured cytokines. In addition, airway hyperreactivity did not change. This could be a very important finding is asthma, as it shows that eosinophils are not intrinsically responsible for the initiation of asthmatic symptoms or deterioration in disease activity.

The next longitudinal study took steroid-free asthmatic patients and placed them on a high steroid dose for 12-months. Here, we found that IL-5 was affected within 3-months, whereas eotaxin remained unchanged over the entire period. Eosinophils also showed a gradual improvement over the 12-months. Similarly to what was observed in the treatment withdrawal study above, lung function and airways hyperreactivity diverged after 3-months of treatment. Lung function improvement plateaued after 3-months, whereas airways hyperreactivity continued to improve for the entire period.

The final study was a follow-up of previous work, which had showed reductions in airway biopsy eosinophils after combined steroid and bronchodilator treatment in symptomatic asthmatics already receiving steroid treatment. Cytokine measurements in this study again showed no changes in eotaxin levels. However, IL-5, which was at near normal levels at the beginning of the study, actually increased dramatically after supplemental bronchodilator treatment. From this we have hypothesised that IL-5 could play a role in clearance of eosinophils from the airway wall.

In this thesis we were able to show that IL-5 in the airways is far more closely associated with eosinophils than eotaxin. IL-5 is also rapidly and greatly reduced by steroid treatment, whereas eotaxin is less susceptible. We have also shown in several studies that eosinophils and their associated cytokines are strongly related to airways

hyperreactivity, but that lung functional changes and patient symptoms are not as strongly related to these cells. Very importantly, changes in these measures of asthma activity occur before changes in these "classic" asthmatic inflammatory cells. The implications of this for understanding asthma pathogenesis are potentially great, as it offers evidence that the eosinophil, thought to be centrally important in asthma pathology, is not responsible for the initiation of asthmatic symptoms in the airways.

The work embodied in this thesis was performed in the Department of Medicine, Monash University, during 1997-2002. It contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, no material previously published or written by another person except where reference is made in the text.

[redacted]

Bryce Nathan Feltis

Part of this thesis or methods developed herein have been published or presented at meetings as follows:

Publications:

Bryce N Feltis, David W Reid, Chris Ward, Ros Bish, and E Haydn Walters. BAL eotaxin and IL-5 in asthma, and the effects of inhaled corticosteroid and long acting β_2 agonist. *Submitted to Thorax.*

Ward C, Pais M, Bish R, Reid D, Feltis B, Johns D, Walters EH. Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma. *Thorax 2002 Apr;57(4):309-16*

Ward C, Johns DP, Bish R, Pais M, Reid DW, Ingram C, Feltis B, Walters EH. Reduced airway distensibility, fixed airflow limitation, and airway wall remodeling in asthma. *Am J Respir Crit Care Med 2001 Nov 1;164(9):1718-21*

Meeting Presentations:

B N Feltis, C Ward, D W Reid, E M Glare, R Bish, R L Boyd and E H Walters. A Cross Secional Study of Eotaxin and IL-5 in the Bronchoalveolar Lavage (BAL) of Asthmatics on Different Levels of Inhaled Corticosteroids. Thoracic Society of Australia and New Zealand Annual Meeting, Melbourne, 2000.

B N Feltis, C Ward, D W Reid, E M Glare, R Bish, R L Boyd and E H Walters. A Cross Secional Study of Eotaxin and IL-5 in the Bronchoalveolar Lavage (BAL) of Asthmatics on Different Levels of Inhaled Corticosteroids. Determinants and targets in progressive airway disease satellite meeting, Melbourne, 2000.

B N Feltis, C Ward, D W Reid, E M Glare, R Bish, S Gollant, R L Boyd and E H Walters. Eotaxin and IL-5 levels in steroid-naïve vs steroid-treated asthmatics. World Congress on Lung Health, Florence, Italy, 2000.

B N Feltis, C Ward, D W Reid, R Bish, R L Boyd and E H Walters. Salmeterol and fluticasone on BAL eotaxin and IL-5 levels in asthma. World Congress on Lung Health, Florence, Italy, 2000.

Abbreviations

AHR:	Airways hyper-reactivity
ANOVA:	Analysis of variance
APC:	Antigen presenting cell
BAL:	Broncho-alveolar lavage
BDP:	Beclomethasone dipropionate
BHR:	Bronchial hyper-responsiveness
C#:	Complement factor #
CCR:	CC chemokine receptor
CFU:	Colony forming unit
EBBx:	Endobronchial biopsy
ECP:	Eosinophil cationic protein
EDN:	Eosinophil-derived neurotoxin
EG:	Eosinophilic granule
ELISA:	Enzyme Linked Immunosorbent Assay
Eo/B:	Eosinophil bone marrow progenitor
EPO:	Eosinophil peroxidase
FEF_{25-75}:	Force mid-expiratory flow rate
FEV_1:	Forced expiratory volume over one second
FP:	Fluticasone propionate
FVC:	Forced Vital Capacity
GC:	Glucocorticoid
GMA:	Glycol methacrylate
GM-CSF:	Granulocyte-monocyte colony stimulating factor
GR:	Glucocorticoid receptor
GRE:	Glucocorticoid response element
ICAM:	Intercellular adhesion molecule
ICS:	Inhaled corticosteroid
Ig:	Immunoglobulin
IL:	Interleukin
LABA:	Long-acting beta agonist
LTB4:	Leukotriene B4
MBP:	Major basic protein
MCP:	Monocyte chemotactic protein
MIP:	Macrophage inhibitory protein
PAF:	Platelet activating factor
PBMC:	Peripheral blood mononuclear cell
PD_{20}:	Provocative bronchoconstrictor dose
RANTES:	Regulated on activation, normal T cell expressed and secreted
RLU:	Relative light units
T_H:	T helper cell
TNF:	Tumour necrosis factor
VCAM:	Vascular cell adhesion molecule

Table of Contents

Chapter 1: Literature Review

1.1 Introduction

Asthma has become one of the most important and most prevalent diseases in the Western world. Whilst asthma is still far more common in developed nations, the incidence of asthma is increasing worldwide. As developing countries become more urbanised the prevalence of asthma increases and the patterns of incidence already apparent in many regions begin to form (Beasley, *et al*, 2000). Asthma is now a major health problem that significantly effects the lives of its many suffers and has a substantial economic impact on public health spending. The public profile of asthma provides significant motivation for the funding of research into new drugs and treatments as well as basic scientific research into the pathology of the disease.

It is now well established that asthma is an inflammatory disease of the airways. Asthma can be defined allergic (atopic) or non-allergic (non-atopic), usually involving a later onset and more greatly influenced by occupational factors. Whilst the pathology between these is very similar, asthma is most commonly allergic and defined as reversible airways obstruction with bronchial hyperresponsiveness. Allergic asthma is a condition where the introduction of an allergen into the airways causes an immediate constriction of the airway smooth muscle, resulting in a drop in lung function (Anderton, *et al*, 1979). Whilst asthma may be a distinct disease, it seems equally likely that asthma may be a syndrome, a collection of symptoms that share a common pathophysiological pathway and outcome (Leff, 1997). Though the aetiology of asthma can vary greatly between patients and direct cellular causal

factors have not been determined, several pathologic features seem common to most cases.

A large number of bronchoscopic studies have shown that the airways in asthma contain an increased number of eosinophils, mast cells, and T lymphocytes, with evidence for activation of all these cell types (Djukanovic, *et al,* 1990; Ying, *et al,* 1997). These cells present in the airways most likely have individual roles in maintaining the inflammation and preventing the condition from resolving. Thus it has been postulated that by interfering with these cells, it may be possible to lessen the severity or resolve asthma.

Whilst there are a number of factors in the airways and throughout the body that control the differentiation, proliferation, chemotaxis and activation of these leukocytes, the "allergic" inflammatory nature of the cells present in the airways leads to a specific immunologic hypothesis. Thus inflammatory cells within the airways are coordinated by a network of chemoattractants, cytokines, chemokines (chemoattractive cytokines), growth factors and mediators. These interleukins and chemokines are directly involved in the recruitment, activation and survival of immune and inflammatory cells in airway tissue and have the potential to be manipulated to affect the balance between the maintenance and resolution of the inflammatory response and thus have a key role in the natural history of asthma.

A large proportion of asthmatic immunopathology can be described as "allergic". From an immunologic standpoint, this type of asthma can be regarded as a T-helper

cell (specifically T_H2) driven, IgE mediated hypersensitivity. The common allergic pathway involves a number of processes. Allergen is processed by antigen presenting cells (APCs), which interact with local T cells. These T cells release an array of cytokines, such as IL-3, IL-4 and IL-5, which promote B cell activity, primarily the production of IgE or IgG, as well as drawing other effector cells into the airways. If free IgG antibody is present, then binding and cross-linkage of local mast cells, via antibody (F_c) receptors, occurs causing damaging degranulation along with further mediator release, which may result in asthma "attacks".

Where asthma differs from other manifestations of airways disease is that in the asthmatic airways, an increased number of eosinophils, cells usually associated with parasitic defence but also present in allergy, are present. These eosinophils are not present in normal airways and contain granules consisting of several potent and potentially damaging mediators (Gleich, *et al*, 1988). This eosinophilia seems to be particularly important for the final common pathway into established clinical asthma, and the chronic phase of its pathophysiology. An increase in airway eosinophil numbers has been a constant feature of broncho-alveolar lavage (BAL) and airway biopsy findings in both atopic (allergic) and non-atopic asthma (Booth, *et al*, 1995). The later condition is poorly understood – but IL-5 is probably important without allergic sensitisation or IgE switching of B cells. These eosinophils are thought to be "activated", and stain for the eosinophilic granular marker of activation, EG2. This is positive in the presence of the cleaved form of eosinophil cationic protein (ECP) (Djukanovic, *et al,* 1990), with increased amounts of ECP, major basic protein (MBP) and eosinophil-derived neurotoxin (EDN) in the BAL fluid of symptomatic asthmatics (Schmekel, *et al*, 1991). The release of so many damaging and inflammatory

compounds into the airway tissues is believed to contribute greatly to the exacerbation of asthma.

The current central principle of treatment for asthma is the use of "preventative" anti-inflammatory glucocorticoids (GCs), particularly in the form of inhaled corticosteroids (ICS). These have some effect on regulation of the pathogenic cytokine networks. GCs are able to bind to key promoter-associated DNA sequences, and downregulate production of key cytokines, such as IL-5, while upregulating other markers, such as beta adrenoreceptors (Drouin, *et al*, 1989). Steroids are of obvious benefit to asthmatic patients, but their precise actions on the immune system *in vivo* are not well understood. In fact most evidence for the effects of GCs on cytokines is drawn from *ex vivo* cell culture (Kelly, *et al*, 2000) or animal model work (Trifilieff, *et al*, 2000).

One of the principal dilemmas in asthma management is that despite rapid and vast improvements in asthma severity that can be shown after ICS treatment, once treatment is removed, the clinical condition and airway eosinophilia usually revert to their pre-treatment levels (Ward, *et al*, 2002). It may be that key chemokines within the lung are not adequately suppressed by GCs and immediately resume the inflammatory process when treatment is withdrawn. Cell culture work is vital in establishing the precise effects of GCs on particular cell types, but does not adequately represent the complex microenvironment of the airways. Likewise animal models allow for GC type and dosage manipulations that cannot be used in humans, but asthma is a condition unique to humans and cannot be precisely modelled in animals. Because human intervention studies using steroids and airway sampling are

challenging to organise and their results are by their nature variable, there is a distinct lack of human *in vivo* steroid studies, especially those that examine cytokines. In such a uniquely human and symptomatically spectral disease, it may only be in the affected airways themselves that we can begin to understand the underlying pathology of asthma.

The following sections describe current knowledge on the nature of the eosinophil, the major cytokines and chemokines related to the eosinophil, the known pathology of asthma and the function and use of glucocorticoids in asthma. In order to understand the potential role of eosinophils in asthma it is necessary to understand the cells themselves (section 1.2) and how they are closely linked to several key cytokines that may be of great importance in asthma (section 1.3). How these cells affect the airways relies on an understanding of both the clinical and pathological aspects of asthma (section 1.4). A description of steroid structure and function both at the receptor and genomic level and their effect on cytokines and eosinophils in asthma (section 1.5) is followed by a summary of this projects' motivations and objectives (section 1.6).

1.2 The Eosinophil

Eosinophils are terminally differentiated leukocytes that are thought to share a common myeloid progenitor along with neutrophils, basophils, mast cells and monocytes (Verma, *et al*, 1980). They are cytotoxic effector cells whose role in immunity is still somewhat controversial, though it is clear that they play a role in countering parasitic infections (esp. helminthic infections) and mediating "type I" hypersensitivity reactions (McEwen, 1992). The destructive nature of the eosinophil's granules, against large extra-cellular bacteria and parasites and host-tissues is well

established. They can also produce leukotrienes, platelet activating factor and lipoxins, all of which may play some role in allergic inflammation (Weller, 1993). However, more recently the regulatory function of eosinophils, evidenced in their abilities to produce a range of cytokines, chemokines and growth factors, is only beginning to be understood (Lacy, *et al,* 1997).

1.2.1 Eosinophil Characteristics

The eosinophil is characterised by its highly positively charged cytoplasmic granules. It is this strong positive charge which gives the granules their distinctive red stain, and the cells their name, when dyed with eosin. These granules contain four primary strongly cationic proteins; major basic protein (MBP), eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO). Though these factors are also present to some extent in basophils and neutrophils, their levels range from four to over one hundred times greater in eosinophils than in these other cell types (Abu-Ghazaleh, *et al,* 1992).

Eosinophils express numerous cell surface antigens, many of which are upregulated in allergic disease (Frick, 1988). It has been shown that expression of CD69, VLA-4, CD11b, CD18, CD16, CD54, HLA-DR, and CD66b (Hansel, *et al*, 1991; Mawhorter, *et al*, 1996; Julius, *et al*, 1999) all increase upon eosinophil activation, usually achieved by priming the cells with selected cytokines *in vitro* (see 1.2.3) or extracting cells from allergic tissues. This variability in phenotyping has led to mass screening of antibody panels (Matsumoto, *et al*, 1998) in an effort to isolate definitive markers for activated eosinophils. These studies have only further reinforced the fact that expression of eosinophil surface markers is largely determined by specific cytokine

priming and antigen exposure. In the complex, largely unknown microenvironment of the airways, eosinophilic granular mediators remain consistent and reliable markers of eosinophil activation.

1.2.2 Development and Differentiation of the Eosinophil

Eosinophils are derived from a bone marrow pre-cursor, a hemopoietic cell, classed as the eosinophil-basophil progenitor (Eo/B-CFU). Such cells are identified as positive for CD34 and IL-3R (interleukin-3 receptor) alpha or by their responsiveness to IL-5 (Denburg, *et* al, 1997). The responsiveness of these bone marrow precursors to IL-5 invariably leads to eosinophil production, though within these colonies a small number of basophils, macrophages and neutrophils are always found (Enokihara, *et al*, 1988). This established IL-5 as the primary terminal differentiation factor for eosinophils (Yokota, *et al*, 1987).

Though T-lymphocytes are the classic source of IL-5, studies of bone marrow stromal cells indicate high levels of production of IL-5 mRNA and protein and further that this production is upregulated by exposure of stromal cells to the inflammatory cytokines IL-1alpha and IL-1beta (Hogan, *et* al, 2000). A further breakdown of IL-5 mRNA expression in (Balb/c) mice shows production predominantly by CD34-positive (CD34+) progenitor cells. Following sensitisation and allergen challenge, CD3-positive (CD3+) T lymphocytes become the major source of IL-5 (Minshall, *et al,* 1998). This may suggest a time-dependant process in which eosinophils are exposed to IL-5 from various sources over the course of their development. Although some work has been done to verify this (Denburg, *et al*, 1999), there is currently no concise model of the process. Other groups have

suggested that IL-3 and GM-CSF may play a role in earlier stages of development (Sonoda, *et al*, 1989). More recent work suggests that these factors enhance the rate of eosinophil production, more specifically that IL-3 and GM-CSF act synergistically with IL-5 in *in vitro* eosinophil differentiation (Takamoto, *et al*, 1995). The eosinophil developmental process has been illustrated in figure 1.1.

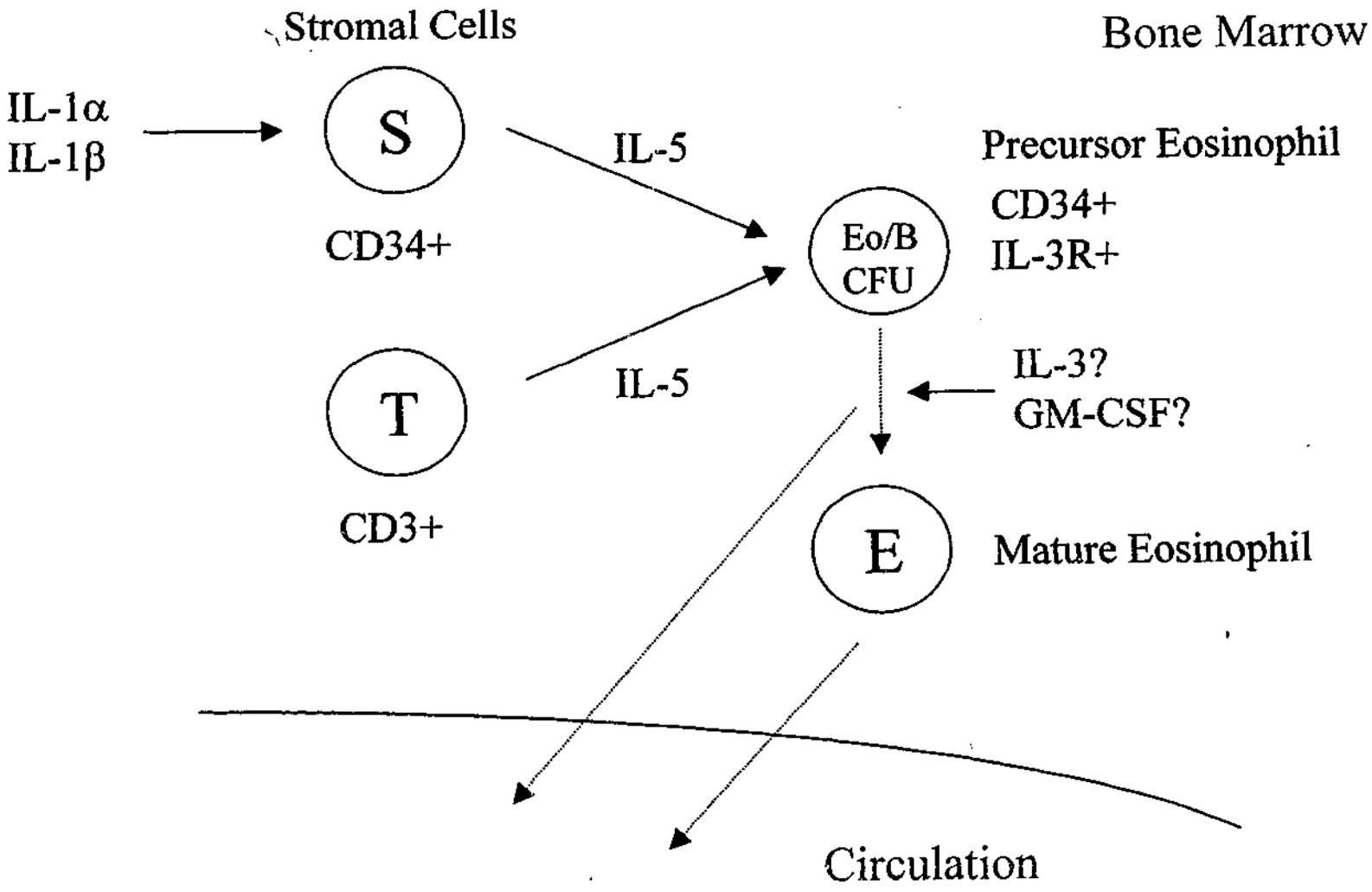
The model for eosinophil differentiation has been further complicated by some quite recent work suggesting eosinophil pre-cursors are found at sights of inflammation and undergo terminal differentiation at these sites (Cameron, *et al*, 2000). Though little additional work has been done to support this hypothesis as yet, it has been known for some time that eosinophil development can be influenced, at least in the bone marrow and in bone marrow cell culture, by direct exposure to allergen (Murali, *et al*, 1992; Milbourne, *et al*, 1993).

1.2.3 Eosinophil Chemotaxis

In order for eosinophils to reach sites of inflammation they must undergo directed movement along a chemical gradient (chemotaxis), taking them through the circulation to the inflammatory site where they must migrate through the endothelium to the specific origin of the chemotaxic signal. Both these processes have been the subject of much recent research.

The factors that cause eosinophil chemotaxis are many and varied. Early work isolated a number of agents including complement products C5a and C3a and the peptide products of mast cells and basophils activated by IgE-mediated reactions, called eosinophil chemotactic factor of anaphylaxis (ECF-A) (Goetzl, 1976). Other

Figure 1.1. The developmental process of the eosinophil in the bone marrow, including the major cells and cytokines affecting differentiation.


Stromal Cells
Bone Marrow
IL-1α
IL-1β
S
CD34+
IL-5
Precursor Eosinophil
CD34+
IL-3R+
Eo/B
CFU
T
CD3+
IL-5
IL-3?
GM-CSF?
E
Mature Eosinophil
Circulation

groups found leukotriene B4 (LTB4) to show eosinophil chemotaxis (Taylor, *et al*, 1989) which would later lead to clinical treatment of asthma with LTB4 antagonists.

It was about this time that T-cells were shown to express eosinophil chemotaxic products, platelet-activating factor (PAF) being one of the first of these. PAF was shown to modulate eosinophil accumulation in rat models (Etienne, *et al*, 1989) and also human inflammatory skin diseases (Morita, *et al,* 1989). Other cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) were found to be chemotactic in high doses, but more importantly, were found to greatly enhance the chemotactic effect of other factors, such as PAF and interleukin-8 (IL-8) (Warringa, *et al*, 1991). The most effective of these eosinophil-priming agents was found to be IL-5 (Coeffier, *et al*, 1991), which though greatly enhancing the chemotactic effects of PAF and IL-8, was shown to be inhibitory for GM-CSF (Warringa, *et al*, 1992).

Perhaps unique in the eosinophil activation and chemotaxis model was the cytokine RANTES (Regulated on Activation, Normal T cell Expressed and Secreted), which could prime eosinophils for chemotaxis by other agents, but was in itself highly chemotactic (Alam, *et al*, 1993). Further redundancy in eosinophil chemotactic ability was further illustrated by the monocyte chemotactic proteins (MCP-2 and MCP-3), which although found to be strongly chemotactic for eosinophils, acted in opposition to each other and also to RANTES (Noso, *et al*, 1994). This was one of the first indications of a common eosinophil chemotactic cytokine (later termed chemokine) receptor, which was later defined as CCR3 (CC chemokine receptor 3). This was further reinforced by demonstrations that many chemotactic cytokines, classified as

CC chemokines, due to sharing common structural motifs, specifically an adjacent pair of cysteine residues at the amino terminal end (Wells, *et al*, 1999), are chemotactic for eosinophils (Lukacs, *et al*, 1996).

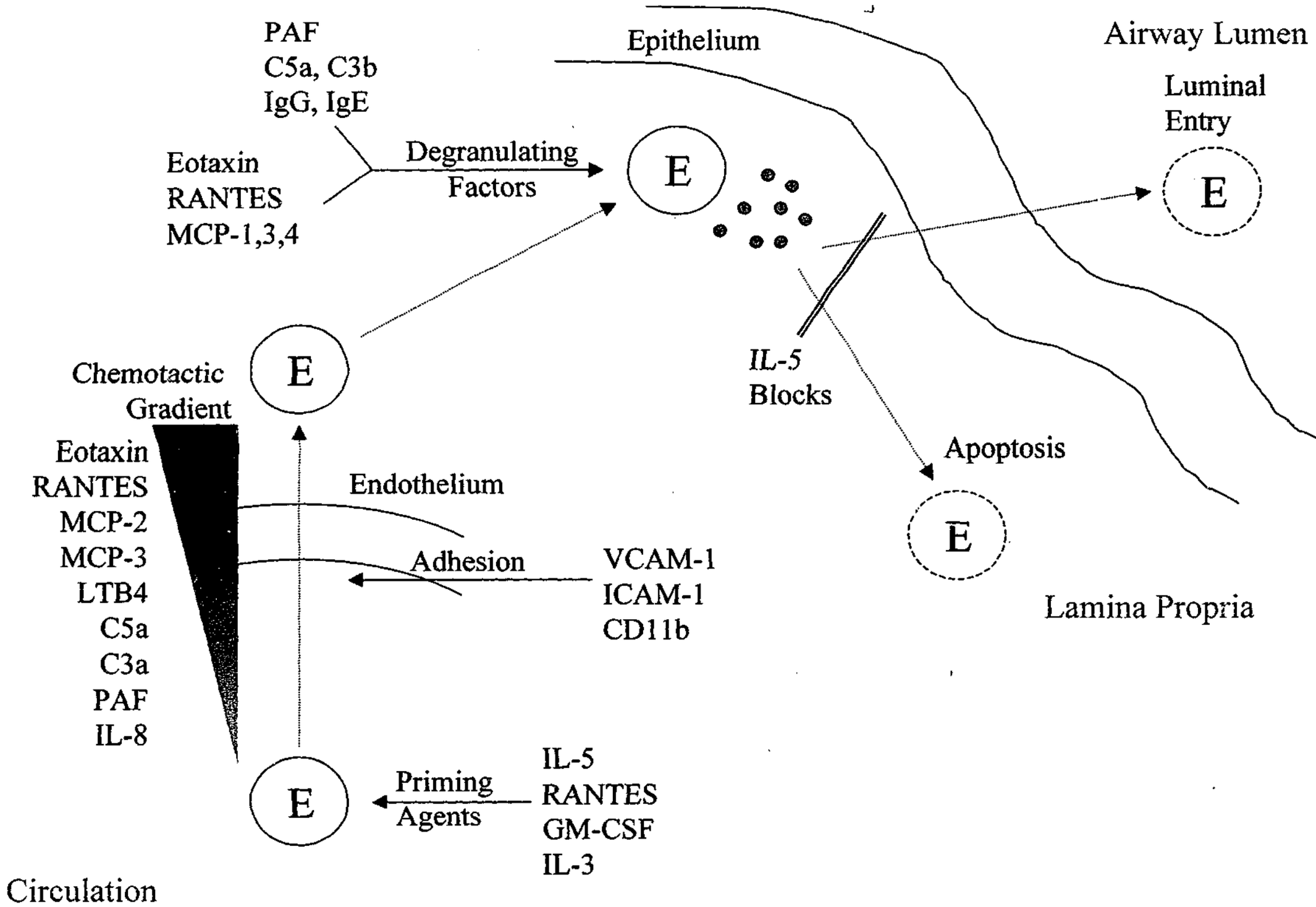
In spite of the plethora of eosinophil chemokines, most of which are active on multiple cells types, only one specific chemokine subfamily, namely the eotaxins function exclusively on eosinophils and has no effect on neutrophils or other myeloid cells (Jose, *et al*, 1994). Whilst all three eotaxins (eotaxin, eotaxin-2 and eotaxin-3) may be of importance in asthma, only the first described eotaxin has undergone any significant amount of research. The major eosinophil chemokines and priming agents, in order of potency are illustrated in figure 1.2.

1.2.4. Eosinophil Transendothelial Migration

The mechanisms for eosinophil transendothelial migration are still unclear. Eosinophils utilise several pathways to adhere to vascular endothelial cells, including binding to intercellular adhesion molecule-1 (ICAM-1), E-selectin, and vascular cell adhesion molecule-1 (VCAM-1). Eosinophils express a range of adhesion markers, including L-selectin, ligands for P and E-selectin, and the integrins LFA-1 (CD11a/18) and Mac-1 (CD11b/18) (Resnick, *et al*, 1993). However, this range of adhesion factors is also expressed by many other leukocytes, and thus they alone are not specific enough to ensure eosinophil accumulation.

It has been suggested that cytokines may be linked to controlling specificity of eosinophil adhesion. Thus, it has recently been shown in rat models that VCAM-1 expression on eosinophils is modulated by the T-cell produced cytokine IL-4 (Larbi,

Figure 1.2. Eosinophil chemotactic factors, adhesion and activation factors. Degranulation and survival factors are also illustrated.


PAF
C5a, C3b
IgG, IgE
Eotaxin
RANTES
MCP-1,3,4
Degranulating
Factors
Epithelium
Airway Lumen
Luminal
Entry
E
E
Chemotactic
Gradient
E
IL-5
Blocks
Apoptosis
E
Eotaxin
RANTES
MCP-2
MCP-3
LTB4
C5a
C3a
PAF
IL-8
Endothelium
Adhesion
VCAM-1
ICAM-1
CD11b
Lamina Propria
IL-5
RANTES
GM-CSF
IL-3
Priming
Agents
E
Circulation

et al, 2000). However, it has been noted that *in vivo* endothelial cells taken from bronchial biopsies express VCAM-1 weakly and strongly express ICAM-1, an adhesion pathway highly dependant on Mac-1 (CD11b) (Wardlaw, 1993). In support of this, other work has shown that CD11a and CD11b are upregulated on eosinophils when they are treated with eotaxin in a dose dependant manner (Jia, *et al*, 1999), suggesting an exclusive pathway for the transendothelial migration of eosinophils. Indeed work that supports VCAM-1 as the primary modulator for migration of eosinophils also suggests that CCR-3, the eosinophil chemokine receptor that is also the only eotaxin receptor, can be blocked to prevent VCAM-1 expression on eosinophils (Nagata, *et al*, 2001). Conversely and of particular interest to this project is the suggestion that the inhaled corticosteroid budesonide is able to downregulate ICAM-1 and Mac-1 (CD11b) expression on human bronchial epithelial cells (HBEC). Although this has only been shown in cell culture (Gonzalez Rodriguez, *et al*, 2000) it demonstrates a potential pathway for corticosteroid action in the reduction of eosinophilia.

1.2.5 Degranulation and the Fate of Eosinophils

As mentioned previously (1.2.1), products released from activated eosinophils include basic proteins, lipid mediators and reactive oxygen species. These products are likely to be responsible for damage to surrounding tissues at the site of degranulation, including the airway epithelium in asthma. A large number of factors are able to induce eosinophil degranulation; these include common eosinophil activators such as platelet-activating factor (PAF) (Kroegel, *et al*, 1989) and complement factors C3a, C3b and C5a (Calson, *et al*, 1993, Takajuji, *et al*, 1994). This triggering of degranulation by opsonins (antibody and complement factors which coat targets such

as bacteria), part of the eosinophils role in parasitic defence, can also be affected by immunoglobulin. Thus eosinophils sensitised to parasites can be made to degranulate when exposed to high levels of IgE (Gounni, *et al*, 1994), whereas cells exposed to common lung allergens, such as grass pollens, react to specific IgG subtypes but not IgE (Kaneko, *et al*, 1995), which may suggest a close relationship between eosinophil activation and degranulation conditions. Furthermore, IgG-stimulated eosinophils have been shown to degranulate when exposed to the beta 2 integrins, CD11b/18, which are vital to eosinophil adhesion and migration (Kaneko, *et al*, 1995) with some work suggesting that adhesion to target cells may be vital to eosinophil degranulation and physical disruption. This may be facilitated by stirring cell suspensions, thus preventing eosinophil-to-target contact, which is sufficient to prevent degranulation no matter which activating factor is applied to the eosinophils (Horei, *et al*, 1994). It is therefore not surprising that eosinophil degranulation can be augmented by the presence of activated fibroblasts (Takafuji, *et al*, 1998) or endothelial cells expressing CD11b (Munoz, *et al*, 1999), both of which are present in vast quantities in the lung and associate directly and constantly with eosinophils.

A second associated pathway of eosinophil degranulation appears to be cytokine dependant. It is clear that the CCR3 specific chemokines, such as eotaxin, RANTES and the monocyte chemotactic proteins (MCP-1,3,4) directly induce degranulation and that blockage of this receptor prevents eosinophils from degranulating (Fujisawa, *et al*, 2000). While IL-5 has some enhancing effect, it remains controversial as to wether IL-5 and other non-CCR3 cytokines, such as IL-3 and TNF-alpha can directly cause degranulation without the influence of other factors (Horie, *et al*, 1996).

The ultimate fate of eosinophils, post-activation and degranulation is still subject to some debate. It was assumed until recently that eosinophils underwent necrosis and were subsequently phagocytosed by alveolar macrophages (Hurley, 1983). However, necrosis of the eosinophil would result in the release of undesirable inflammatory mediators, possibly restarting the inflammatory process that should end with the removal of eosinophils. A more controlled and less destructive method of disposing of unwanted eosinophils is apoptosis, where cells or sub-components remain membrane bound, thus not releasing their damaging enzymes and inflammatory mediators before undergoing phagocytosis. In the airways, delay of apoptosis may contribute to the pathology caused by the long-term continual degranulation of eosinophils during eosinophilia (Simon, *et al*, 1997). It would appear that IL-5 has the ability to prolong eosinophil survival within the airways and that glucocorticoids, widely used in the treatment of bronchial asthma, negate this protective effect of IL-5 (Zhang, *et al*, 2000). Much of the apoptosis work in eosinophils has been performed *in vitro* and the limited amount of *in vivo* evidence for apoptotic eosinophils is a primary source of debate. Adding to the controversy, a recent *in vivo* biopsy study has demonstrated by induction of apoptosis via steroids a lack of apoptotic eosinophils in airway tissue, but a marked increase in apoptotic cells in the lumen (Uller, *et al*, 2001), work that is backed up to some extent by evidence in cell culture that airway epithelial cells are able to engulf apoptotic eosinophils (Walsh, *et al*, 1999). Taken together with other work, this model suggests that macrophage phagocytosis of apoptotic eosinophils in the airway wall is of small consequence, with most eosinophils moving through the airway epithelium into the lumen and then undergoing apoptosis (Erjefalt, *et al*, 2000). Although it may be that macrophages become more important in the phagocytosis of eosinophils that are already in the lumen, a preposition that has yet to

be established. A strong association between eosinophil apoptosis and the airway epithelium could explain the constitutive expression by airway epithelial cells of eosinophil specific chemokines such as cotaxin by airway epithelial cells (see 1.3.4). Eosinophil adhesion degranulation and survival factors are illustrated in figure 1.2.

1.3 Cytokine Cascades and the Eosinophil

The eosinophil is classically associated with parasitic defence and allergic disease. In both cases the eosinophil is activated by a variety of cytokines, many of which are T cell derived. The eosinophil, under such stimulation, then moves along a chemical gradient toward the site of inflammation where it degranulates, as well as releasing cytokines potentially capable of enhancing the response. These processes of activation and chemotaxis are specific to the inflammatory site, specifically the airways in the context of this study, and the cell types present at that site. Discussed below are the relevant cytokines and chemokines specific to eosinophilia in the airways in asthmatic disease.

1.3.1 Cytokines, Chemokines and the Eosinophil in the Airways

A number of eosinophil chemokines and other chemattractants are present in the airways. These include interleukin-3 (IL-3), granulocyte-macrophage colony stimulating factor (GM-CSF), platelet activating factor (PAF), leukotriene B4 (LTB4), complement factor C5a, and the CC chemokine family (characterised by two adjacent cysteine residues), macrophage inhibitory proteins (MIP) and monocyte chemoattractive proteins (MCP), all of which are active to an extent on eosinophils, but also affect a variety of other cell types. There are, however, several cytokines which appear to be vital to eosinophil chemotaxis and function. These are IL-5, IL-8,

RANTES and eotaxin. Inhibition of any of these chemokines is sufficient to hamper eosinophil chemotaxis to some extent. A second chemotactic pathway associated with IL-4 and IL-13 is also able to abolish tissue eosinophilia, but unlike IL-5/eotaxin, inhibition of these cytokines does not seem to resolve airway inflammation, most likely due to the higher dependence of eosinophils on the primary chemotactic mechanisms (Foster, *et al*, 2001). IL-8 is still somewhat controversial, and while normally associated with the activation and chemotaxis of neutrophils, some groups have claimed IL-8 to be highly effective on eosinophils taken from symptomatic asthmatic subjects (Lampinen, *et al*, 1999). However, other groups have demonstrated no such activity in pure eosinophil cell cultures, even after priming with a variety of cytokines, suggesting the supposed effect of IL-8 on eosinophils is due to neutrophil contamination (Petering, *et al*, 1999).

RANTES and eotaxin are similar in function and structure, though the potency of eotaxin is greater. They are both C-C chemokines, acting through the same receptor on eosinophils, however only eotaxin is exclusively active on eosinophils, whereas RANTES is non-selective, acting on monocytes and lymphocytes in addition to eosinophils (Meurer, *et al*, 1993). IL-5 is the weakest chemokine. It shows only very limited chemotactic effect on eosinophils and none on neutrophils. Priming with IL-5 does, however, greatly enhance the eosinophil chemotactic response to other chemokines (Shemi, *et al*, 1992). IL-5 also has a great range of effects on eosinophils, and is able to affect all stages of growth, function and survival. IL-5 influences eosinophils in very different ways depending on their location in the body and state of activation, and like eotaxin, is selective for eosinophils, though it is able to act in a limited fashion on other cells, primarily T-cells and mast cells.

Clearly, of the known cytokines in the asthmatic airway, IL-5 has the greatest potential role in regulation of eosinophils. Likewise eotaxin is the only chemokine that exclusively causes strong chemotactic activity in eosinophils. Whilst there are many other cytokines, chemokines and mediators and cellular effectors that influence asthmatic disease, the eosinophil still remains a central element, and there seems little doubt that IL-5 and eotaxin are likely to be vital in the recruitment and function of the eosinophil in the asthmatic airways.

1.3.2 The Functions and Activity of IL-5

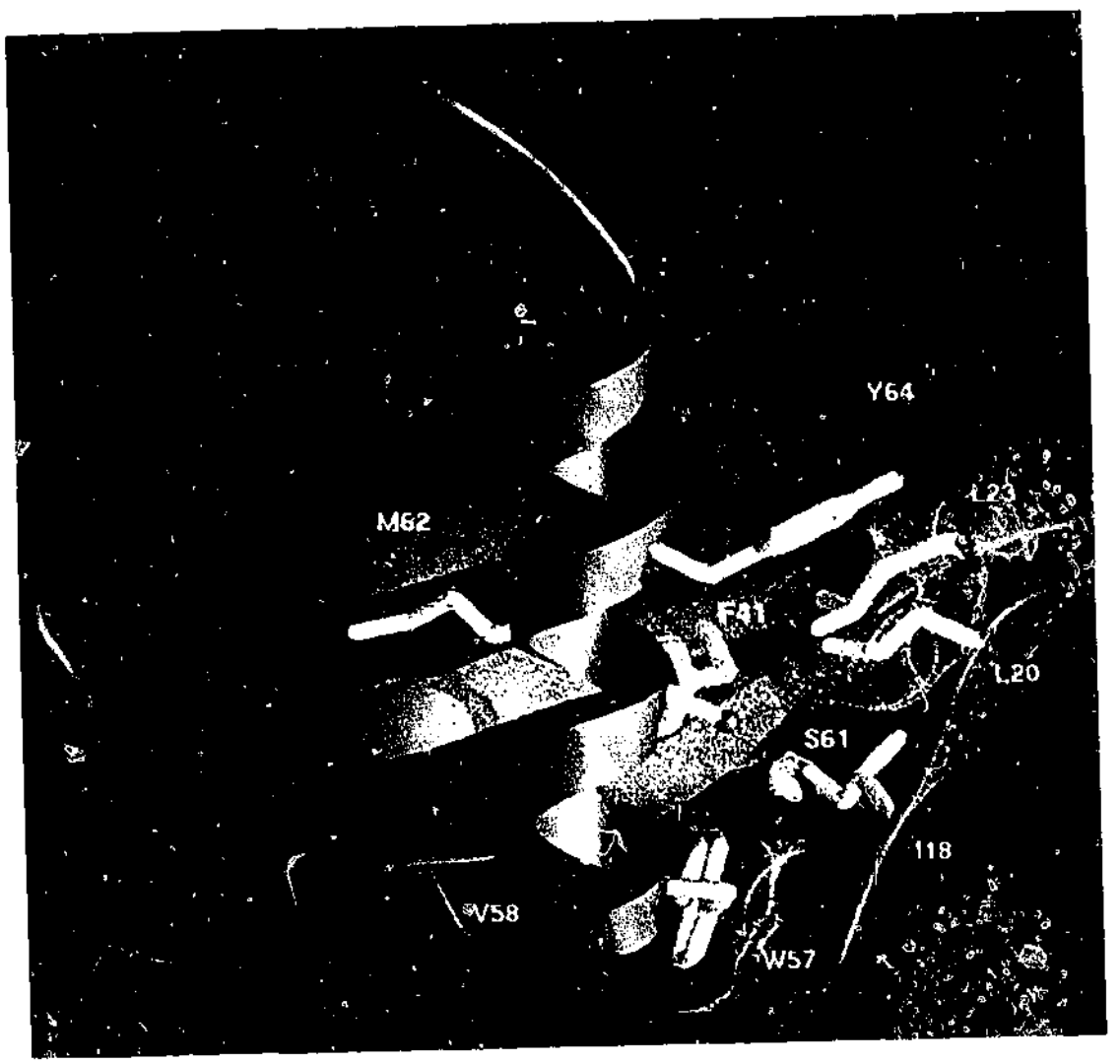
IL-5 was originally designated as a T cell-replacing factor (TRF) that caused B cell growth and differentiation and was termed BCGF II (Swain, *et al*, 1983). It was later found that a cytotoxic T cell activator, the killer helper factor (KHF) was in fact the same mediator as TRF (Takatsu, *et al*, 1987), and due to its pleiotropic effects on multiple leukocytes, it was redesignated as interleukin 5. However, IL-5 has become better known for its wide-ranging biological activity on the eosinophil. Unlike other eosinophilic mediators such as GM-CSF and IL-3, IL-5 has the ability to influence almost all eosinophil functions, from development in the bone marrow to apoptosis and survival at an inflammatory site.

1.3.2.1 IL-5 Structure

Structurally, IL-5 is a 30 kD disulphide-linked homodimeric glycoprotein, with each chain consisting of 115 amino acids, the tertiary structure consisting of two four-helix bundles and two opposing beta-sheets (See figure 1.3, Milburn, *et al*. 1993). Interestingly, both chains are biologically active and able to bind to the IL-5 receptor, though at a much lower affinity than the two combined (~40-fold lower, Li, *et al*,

Figure 1.3. Macromolecular structure of the IL-5 protein (from Edgerton, *et al*, 1997).

Figure 1.4. Macromolecular structure of the eotaxin protein (from Crump, *et al*, 1998).


Y64
M62
F41
L20
S61
118
V58
W57

1997). Human IL-5 shares 70% sequence homology with its murine equivalent and in both species the C terminus end of the protein is responsible for biological activity and receptor binding (McKenzie, *et al*, 1991). At a genetic level IL-5 is related to GM-CSF, IL-4, IFN-γ and IL-2, although at the protein sequence level they share few similarities (Tanabe, *et al*, 1987). While allelic variation in the IL-5 gene could cause a predisposition to conditions such as asthma, comprehensive studies comparing the IL-5 locus in normal human and asthmatic subjects have not revealed any such polymorphisms (Pereira, *et al*, 1998).

1.3.2.2 The IL-5 Receptor

The IL-5 receptor is composed of two chains; the alpha chain is a low affinity receptor specific for IL-5, and the beta chain is identical to the high affinity receptor for GM-CSF and IL-3 and appears to be responsible for signal transduction. This similarity in structure may explain some of the overlap of activity between IL-5, GM-CSF and IL-3 (Tavernier, *et al*, 1991). Specific binding of both IL-5 chains occurs via the alpha-receptor chain, and then receptor triggering is effected via the beta chain (Tavernier, *et al*, 1995).

1.3.2.3 IL-5 Developmental Influences

IL-5 selectively induces and regulates eosinophil proliferation, differentiation and growth in the bone marrow and appears to have identical effects to GM-CSF in the same environment (Clutterbuck, *et al*, 1989). Mouse models have shown not only a constitutive expression of IL-5 in the bone marrow, but also that stimulation with antigen causes an increase in IL-5 mRNA in bone marrow CD34 positive stromal cells (Minshall, *et al*, 1998). This finding has been validated and expanded in human

asthmatic subjects. demonstrating increases in both IL-5 protein expression and IL-5R expression by CD34 positive cells in the bone marrow after allergen challenge (Denburg, *et al*, 1999). It is well established that IL-5 also has the ability to activate or prime eosinophils and prolong their survival *in vitro* (Ohnishi, *et al*, 1993), while glucocorticoids are able to inhibit this survival, possible by the downregulation of IL-5 (Wallen, *et al*, 1991). IL-5 can also function to increase eosinophil adhesion to endothelial surfaces (Lopez, *et al*, 1986), and has now been shown to be produced by human airway epithelial cells (Salvi, *et al*, 1999), and to also enhance adhesion to these cells (Sanmugalingham, *et al*, 2000). This further emphasises the important role the airway epithelium may have in asthma pathology, and especially so for the potential interactions between IL-5 and the epithelium that may assist in direct clearance of eosinophils by luminal entry (see 1.2.4).

1.3.2.4 IL-5 in the Serum

In the circulation intravenous recombinant human IL-5 has been shown to be functional in guinea pig models, inducing a maximal 7% rise in blood eosinophils after 1 hour at doses of 5μg/kg (Portanova, *et al*, 1995). Higher doses had no appreciable effect and the eosinophilia was rapidly cleared after 6 hours. Similar experiments have been performed in mice, showing the same blood eosinophilia, but over a much longer period, requiring 5 days to reach maximal blood eosinophilia which then resolved after 7 days; the doses of IL-5 required were also in the order of 100 fold greater in mice than in guinea pigs (Fattah, *et al*, 1990). Though these differences could be a result of varied kinetics between the two species, it is more likely to be a result of relative homology between the animal and human IL-5 proteins. In human asthmatic subjects, introduction of recombinant IL-5 into the

serum (at doses of 0.1-2μg) appeared to induce an initial rapid reduction of blood eosinophils, for the first 30 minutes, followed by a prolonged eosinophilia peaking at 3-5 hours and had returned to baseline by 24 hours. IL-5 levels returned to baseline by 4 hours (Stirling, *et al*, 2001). Whilst the dosage in the human study is at least 100 fold less than the guinea pig model, the potency and kinetics of the eosinophilia are very similar.

1.3.2.5 Asthma and IL-5

In asthmatics free from systemic corticosteroids, serum levels of IL-5 protein have been shown to be elevated above healthy subjects (Motojima, *et al*, 1997). This is supported by findings of elevated IL-5 mRNA-expressing CD4 positive cells taken from asthmatic patients (Lai, *et al*, 1996) and increased IL-5 protein in the peripheral blood mononuclear cells of asthmatic subjects (Endo, *et al*, 1993). The latter two studies use relatively indirect *ex-vivo* cell culture measurements rather than direct quantitation, whilst direct relationships between lung function and IL-5 levels in the serum in clinical asthma have not been found. However, in mild asthmatics serum eosinophils did show a direct correlation to serum IL-5, and in the same patients after allergen provocation via bronchial inhalation both serum IL-5 and eosinophils increased and remained high for at least 24 hours (Halldén, *et al*, 1999). IL-5 in the serum and eosinophils were both raised after 6 hours in the human allergen challenge model, remained raised over 24 hours and returned to normal levels by 1 week or less (van Der Veen, *et al*, 1999). This latter study, while finding no directly relationship between serum IL-5 and eosinophils the authors, did find a weak correlation between lung function (FEV_1) and serum IL-5 (r^2=0.19, p=0.04). *Ex-vivo* studies also demonstrated that allergen-stimulated T cells from the blood and BAL of asthmatic

patients produce more IL-5 in culture than allergen-stimulated T cells from healthy subjects. Interestingly, the BAL and serum T cells from each patient produced very similar amounts of IL-5 when stimulated *ex-vivo*, even though this varied considerably from patient to patient (Till, *et al*, 1998). The number of IL-5-expressing T cells in the blood has been measured more directly by flow cytometry which showed that IL-5 positive T cells were present in a higher percentage in asthmatic subjects than non-asthmatic controls (Shiota, *et al*, 2002).

1.3.2.6 IL-5, Asthma and Allergen Challenge

The allergen challenge model has also helped to understand the potential role of airway IL-5. The popular ovalbumin-sensitised mouse model has shown that IL-5 knockout mice do not develop an airway eosinophilia, airway damage or BHR after allergen challenge (Foster, *et al*, 1996). Conversely, IL-5 over-production in transgenic mice, although causing a marked rise in blood eosinophils, does not cause tissue accumulation or organ damage (Dent, *et al*, 1990). In human studies, 24 hours after allergen challenge BAL IL-5 protein levels are substantially raised above baseline and can be correlated significantly with BAL eosinophils, which are also raised (Sur, *et al*, 1995). Further exploration of the kinetics of this model has show that IL-5 increased by 3-fold 4 hours after allergen challenge and continued to rise, until reaching 20-fold by 24 hours. During this time eosinophils also rise proportionally, but no direct relationship was observed between BAL IL-5 protein levels and the number of BAL eosinophils (Teran, *et al*, 1999). BAL T cells taken from allergen challenged subjects have increased mRNA expression of IL-13, GM-CSF, IL-3, IL-4 and IL-5, which are the classic "type 2" cytokines and interestingly, in contrast with the profile of IFN-γ, TNF-α, IL-13 and GM-CSF mRNA which is

expressed by T cells from mild, unstimulated, asthmatics. The study also noted, however, that these mRNA profiles did not always match protein expression by the cells and that the asthmatic cytokine phenotype is probably more intermediate in nature (Bodey, *et al*, 1999).

Whilst little work has been done in bronchial biopsies for the allergen challenge model, it has been demonstrated that EG2-positive eosinophils and cells expressing IL-5 mRNA (via *in situ* hybridisation) are upregulated 24 hours after allergen challenge (Bentley, *et al*, 1993). The relevance of allergen challenge models in simulating asthmatic disease is still open to debate, but it is clear that a number of factors, such as IL-5, change drastically after "insults" of this nature. Whist it is difficult to estimate how close this is to the *in vivo* human situation, it does provide a basis for further work to examine some of these changes, which in clinical asthma, may be much smaller and thus much more difficult to detect.

1.3.2.7 IL-5 *in vivo*

The data for IL-5 in *in vivo* asthma is more comprehensive than most other cytokines and chemokines associated with the disease. The importance of IL-5 in the maintenance of eosinophils has ranked the cytokine highly both as a research and clinical therapeutic target. IL-5 mRNA has been detected in greater levels in the bronchial biopsies of asthmatic subjects when compared to non-asthmatic controls, though in this initial work only 60% of asthmatics had measurable levels of IL-5 mRNA, though these were anecdotally the most severe (Hamid, *et al*, 1991, Glare, *et al*, 2002). More recent studies have found strong correlations between biopsy IL-5 mRNA and lung function, but recent concerns about the variability in β-actin, a so-

called "stable" marker or "denominator" for semi-quantitative molecular biology of this sort bring some of these data into question (Humbert, *et* al, 1997). It was later found that 70% of the IL-5 mRNA-expressing cells in bronchial biopsies were activated, CD3 positive, T cells; with the remaining 30% made up of mast cells and activated eosinophils (Ying, *et al*, 1995). This distribution of IL-5 expression was also found to be identical in the BAL.

The same group went on to further define IL-5 mRNA expression in activated T cells to be primarily (70%) comprised of CD4 positive cells, though CD8 positive cells also occasionally expressed IL-5 mRNA. At a protein level, however, IL-5 expression was confined to mast cell and eosinophil staining, contrary to the mRNA findings (Ying, *et al*, 1997). The lack of immunohistochemistry studies in IL-5 research using bronchial biopsy work makes it difficult to determine if some level of translational control is at work in the airways, or whether the immunoreactive staining system is merely less sensitive in detecting IL-5 expressing T cells. However, *ex-vivo* cell culture work on BAL cells has shown increased IL-5 protein production of T cells by ELISA (Till, *et al*, 1995).

Expression of mRNA for the IL-5 receptor has also been found to be elevated in the bronchial biopsies of asthmatics, with expression being confined almost exclusively (93%) to activated eosinophils. Unlike IL-5 expression, IL-5 receptor mRNA expression was very strongly related to lung function (Yasruel, *et al*, 1997). This suggests a possible stability in the receptor that could be a more reliable and relevant measure than the more variable IL-5 protein expression.

1.3.2.8 BAL, Sputum and IL-5 in Asthma

In the BAL fluid IL-5 has similarly been shown to be elevated in asthmatic subjects, and differences between symptomatic and asymptomatic asthmatics have also been found, with symptomatic asthmatics expressing more IL-5, IL-4, IL-3 and GM-CSF positive cells than subjects with asthma but without current symptoms (Robinson, *et al*, 1993). These findings are supported by other groups who also have found increased IL-5 protein in the BAL of symptomatic asthmatic patients and that those with increased BAL IL-5 levels also have increased BAL eosinophils (Sur, *et al*, 1995).

Induced sputum has also been used as a more readily obtainable sample for IL-5 assessment. IL-5 mRNA has been successfully measured in sputum via PCR and found to be elevated in asthmatic subjects relative to non-asthmatic controls, findings which are consistent with BAL, bronchial biopsy and serum (Gelder, *et al*, 1995). IL-5 protein, despite concern regarding degradation by the sputum or sputum processing procedures, is at least partially measurable and also elevated in asthmatic subjects. Those whose IL-5 could be measured showed an inverse relationship between IL-5 and FEV_1, and again higher eosinophils but without a direct correlation between sputum IL-5 and eosinophil numbers (Shoji, *et al*, 1998).

1.3.2.9 "Treatment" of Asthma through Targeting IL-5

The control that IL-5 exerts on all levels of eosinophil development, recruitment, activation and survival makes it a prime target for treatment of chronic asthma. Administration of recombinant human IL-5 by nebulization to asthmatic subjects causes a significant serum eosinophilia after 24 hours, which remains for at least 48

hours (Shi, *et al*, 1999). Sputum eosinophils also increase after IL-5 inhalation, but lung function of asthmatic subjects has not been shown to change (Shi, *et al*, 1998). Whilst insight can be gained from increasing IL-5 concentrations, the primary focus of IL-5 therapeutic research is to reduce airway IL-5 levels. The construction or selection of a suitable IL-5 protein or receptor antagonist has been discussed for some time (Devos, *et al*, 1995), but it is only recently that such drugs have become available and suitable for trials on human subjects.

Anti-IL-5 monoclonal antibody administered intranasally to ovalbumin-sensitised mice causes a significant reduction in BAL and lung tissue eosinophil counts and also reduced airway hyperresponsiveness. The same results were also achieved via intraperitonally administered anti-IL-5 antibody (Shardonofsky, *et al*, 1999). Analogous, but clonally distinct, anti-IL-5 antibodies have shown similar results not only in mice, but also in allergic guinea pigs, rabbits and cynomolgus monkeys. Whilst eosinophilia was inhibited in all these models, a reversal of airway hyperresponsiveness could not be demonstrated in the rabbit and monkey models (Egan, *et al*, 1999).

These results foreshadowed recent work performed with an anti-human IL-5 antibody, targeted for use with human asthmatic subjects. The antibody was able to almost completely remove serum eosinophils, prevent any eosinophilia in the blood following allergen challenge and lower sputum eosinophils by a factor of ten. These effects persisted for at least 30 days after administration of a single dose of the anti-IL-5 antibody. However, during this period there was no measurable change in the late asthmatic response after challenge or airway hyperresponsiveness to histamine

(Leckie, *et al*, 2000). However, further work with the same IL-5 antibody has shown that while serum eosinophils were completely removed, bone marrow and airways eosinophils persist (Flood-Page, *et al*, 2002). What this work does not address is that IL-5 may also be essential for clearance of eosinophils into the airway lumen (Erjefalt, *et al*, 2000), thus anti-IL-5 may not be an appropriate anti-eosinophilic strategy. While this work raises the possibility that the eosinophil's role in asthmatic disease may not be as important in asthma as it has historically been believed to be, more thorough anti-eosinophilic treatments will be needed to determine this.

However, this body of work does show that IL-5 is the definitive eosinophilic cytokine in asthma. It influences all functions of the eosinophil, even growth in the bone marrow does not occur without IL-5. Release of eosinophils into the blood requires IL-5 endothelial transmigration and eosinophil degranulation requires priming with IL-5 to fully function and eosinophil survival is dependent on the presence of IL-5. As long as asthma is defined as an eosinophilic disease, IL-5 will remain important in the ongoing elucidation of the disease process, and the eosinophils role in it. It seems hardly feasible that such a prominent cell type in the asthmatic airway is playing no pathological role.

1.3.3 The Eotaxin Sub-family

Many cytokines and chemokines can cause chemoattraction of eosinophils, primarily through binding to CCR3, the eosinophil CC chemokine receptor. These include eotaxin, RANTES, MCP-2, MCP-3 and MCP-4 (see 1.2.3). In *in vivo* animal allergy models eotaxin has been show, above the other CCR3 specific chemokines, to be the most potent in attracting eosinophil numbers (Teixeira, *et al*, 1997).

1.3.3.1 Eotaxin Structure

Eotaxin is a recently described CC chemokines, first isolated in guinea pig and murine models. Eotaxin was found to be a 73 amino acid, 8.3 kDa protein (see figure 1.4) extracted from BAL fluid of allergen challenged, allergically sensitised guinea pigs. It is similar in sequence to human MCP-1 (53%) and somewhat similar to human RANTES (26%) (Jose, *et al*, 1994). Eotaxin appeared to be unique among the chemokines since it causes the selective infiltration of only eosinophils and not neutrophils into the lungs and skin of guinea pigs (Rothenberg, *et al*, 1995). It was later found in the human airways (Ponath, *et al*, 1996). However, very recently other CC chemokines, which also function through the CCR-3 receptor and are apparently selective for only eosinophils, have been isolated. Eotaxin-2 (Forssmann, *et al*, 1997), while only sharing 39% homology with eotaxin shows near identical function at similar levels of potency as eotaxin (Elsner, *et al*, 1998). A third distinct eosinophilic chemokine, eotaxin-3, was also recently characterised (Shinkai, *et al*, 1999). Eotaxin-3, however, appears to have a chemotactic potency of approximately 10-fold less than eotaxin and eotaxin-2, and shows a markedly different expression pattern, being constitutively expressed in the heart and ovary (Kitaura, *et al*, 1999). While eotaxin-2 would certainly have been of great interest to this study, the novel nature of the chemokine restricted the availability of reagents and eotaxin-2 was not within the timeframe of this project, which therefore deals only with eotaxin-1.

1.3.4 The Action of Eotaxin

Eotaxin mRNA is constitutively expressed in guinea pig and murine lung in contrast to most other organs, suggesting eotaxin may regulate normal eosinophil homing and turnover in the respiratory tract (Rothenberg, *et al*, 1995). Eotaxin mRNA in the lung increased approximately 6-fold after allergen challenge, with mRNA and protein levels peaking at 3 hours. This suggests eotaxin up-regulation is associated with the late phase response, which is characterised by eosinophilia (Rothenberg, *et al*, 1995), with neutrophilia occurring earlier (Sampson, *et al*, 2000). Similarly, in guinea pigs, eotaxin has been shown to peak at 6 hours after allergen challenge, and fall back to constitutive levels by 12 hours, though persistence of eosinophils in these animals lasted at least 24 hours (Humbles, *et al*, 1997). The same study showed that eosinophil accumulation in the airways could be arrested by a neutralising antibody to eotaxin.

Eotaxin was found to elevate intracellular calcium and induce chemotactic responses in human and guinea pig eosinophils *in vitro* (Jose, *et al*, 1994). Eotaxin also has a role in priming eosinophils and can directly increase eosinophilic oxidative metabolism in a dose dependant manner, suggesting a more direct role of eotaxin on tissue pathogenesis (Honda, *et al*, 1999).

1.3.4.1 Eotaxin in Animal Models

Eotaxin has been demonstrated to be a potent chemoattractant, facilitating the local accumulation of eosinophils at picomole doses, in synergy with IL-5 (Collins, *et al*, 1995). In guinea pigs, locally administered IL-5 had little eosinophil chemoattractant activity by itself. However, low doses of intravenous IL-5 dramatically enhanced local eosinophil accumulation in the skin in response to intradermal eotaxin injection

(Rothenberg, *et al*, 1995). Other than local chemotaxis of eosinophils to sites of inflammation, eotaxin is able to directly promote release of eosinophils from the bone marrow, which suggests a further synergism between eotaxin and IL-5 at the level of eosinophil hematopoiesis (Palframan, *et al*, 1998).

The mouse eotaxin gene was cloned and murine eotaxin has been found to have similar direct chemoattractant properties for eosinophils. A similar synergism between IL-4 and murine eotaxin was also observed (Rothenberg, *et al*, 1995). Human eotaxin was found to share 61.8% and 63.2% sequence homology to guinea pig and mouse eotaxin respectively, and was found to be functionally equivalent to the animal eotaxins. However, optimal eotaxin dosage for eosinophil accumulation was found to be substantially higher, at approximately 1000 pM, than guinea pig models, which were optimal with 1-2 pM of eotaxin (Ponath, *et al*, 1996). This once again illustrates similar inter-species potency differences, much like that which was seen with IL-5.

1.3.4.2 Cellular Expression of Eotaxin

Cellular expression of eotaxin mRNA in the lung has been found to be quite broad, being found primarily in bronchial epithelial cells but also present in macrophages, T cells and to a lesser extent mast cells, neutrophils and eosinophils. Unlike the pattern of cytokine expression, the eotaxin receptor CCR3 is far more restricted, being found almost exclusively on $EG2^+$ (ie. activated) eosinophils. CCR3, although also a receptor for other CC chemokines such as RANTES and MCP-3, shows highest affinity for eotaxin. Blockage of CCR3, though not completely eradicating eosinophilia, causes a significant reduction of eosinophil numbers in the lung (Ying, *et al*, 1997).

1.3.4.3 Eotaxin in Human Cells

In human *in vitro* studies, bronchial epithelial cells show a marked increase in eotaxin mRNA and protein expression when stimulated with IL-5, IL-4 and the less well characterised IL-13 (Li, *et al*, 1999). It has been suggested that most Th2-type cytokines, inducers of humoral immunity, have some enhancing effects on eotaxin. Furthermore, adoptive transfer of Th2 cells, when stimulated with antigen, has been shown to induce asthma-like inflammatory responses, along with strong expression of eotaxin, a property not duplicated by the adoptive transfer of Th1-type cells (Li, *et al*, 1998). Cell cultured fibroblasts have also been shown to express eotaxin after interacting with mast cells (Hogaboam, *et al*, 1998), and human airways smooth muscle expresses eotaxin *in vitro* after priming with TNF-α or IL-1β, while constitutive expression of eotaxin was observed *in vivo* in smooth airway muscle tissue of asthmatic subjects (Ghaffar, *et al*, 1999). While the causal link between bronchial asthma and respiratory viral infection is not completely clear, infection of cultured human epithelial cells with influenza virus A increases both eotaxin mRNA and protein expression (Kawaguchi, *et al*, 2000). While this is the first viral work done in human cells exploring eotaxin expression, a similar result was found in guinea pigs after expression with parainfluenza-3 (PI3) virus. Animals infected with PI3 showed a significant elevation (4-6 fold) in BAL eotaxin 2-4 days after infection (Scheerens, *et al*, 1999).

1.3.4.4 Eotaxin in Asthma

Human *in vivo* eotaxin data are more rare, especially longitudinal clinical studies. However, some comprehensive cross-sectional work has been done. Eotaxin in human BAL has been shown to be elevated approximately 6 fold in asthmatic subjects when compared to human controls, though study numbers were small (n=11). In fact the quantity of eotaxin in the BAL was sufficient for the use of BAL supernatant as an *in vitro* chemotactic source (Lamkhioued, *et al*, 1997). Other studies confirm an elevation in eotaxin in the BAL of asthmatic subjects, and go on to show that BAL eotaxin increases in atopic asthmatics after allergen challenge, and that this increase correlates with the increase in eosinophil numbers in the BAL (Lilly, *et al*, 2001). The kinetics of such allergen challenge models shows that eotaxin and eosinophils are not significantly raised above diluent controls until 4 hours after allergen application and that eotaxin but not eosinophil numbers return to normal by 24 hours (Brown, *et al*, 1998).

The kinetics of long-term allergen exposures on airway eosinophils and eotaxin have not been adequately explored in human airways nor has the clinical relevance of single dose allergen models. However, recent work with animal models suggests that there are essential differences in eotaxin expression and eosinophil recruitment between acute and chronic allergen exposure models. The most striking distinction is that a single high antigen dose caused delayed eosinophil recruitment which did not correlate with eotaxin expression, whereas chronic low dose exposure increased eosinophils in line with eotaxin expression (Kumar, *et al*, 2002).

Other techniques such as *in situ* hybridisation in resected lung have shown elevated eotaxin mRNA in the epithelium and inflammatory cells of asthmatic subjects, and significant relationships observed between eosinophils and eotaxin mRNA expression (Taha, *et al*, 1999). In human serum, in an exceptionally large study (n=1034), eotaxin levels have again been shown to be increased in asthmatic subjects, and plasma eotaxin showed an inverse relationship to mean percent predicted FEV_1 (Nakamura, *et al*, 1999). Furthermore, recently, eotaxin in induced sputum has been correlated with both ECP and eosinophil numbers in the sputum. These relationships were not seen in asthmatic subjects who were undergoing steroid treatment (Yamada, *et al*, 2000).

1.3.4.5 Roles of Eotaxin

Eotaxin is thought to be vitally important in the recruitment of eosinophils into the airways. Although its primary role is chemotaxis, it also plays a part in eosinophilic degranulation and release of eosinophils from the bone marrow. Its constitutive expression in the lung and consistent elevation in asthmatics mark it as another potential target for therapeutic intervention for asthmatic treatment. However, the blockage of eotaxin via monoclonal antibody *in vivo* in rat models causes, as well as a reduction in eosinophil numbers, a substantial increase in MIP-2 and cytokine-induced neutrophil chemoattractant (CINC). This results in a substantial increase in neutrophil numbers and also increased vascular leakage in the lung. Application of recombinant eotaxin has the exact opposite effect, raising eosinophil numbers whilst suppressing neutrophils (Guo, *et al*, 2001). These data not only suggests an indirect role for eotaxin in neutrophil regulation, but also may suggest some limitations or potential confounders for intervention therapy directed at eotaxin regulation. This does not suggest that eotaxin blockage is completely non-viable, but that eotaxin

expression is closely tied reciprocally to mediators of neutrophil recruitment. As eotaxin expression, kinetics and mediators become fully understood in the human airways the importance of eotaxin will become more clear. However, at the current level of understanding, it would seem that eotaxin is potentially vital to processes driven through eosinophil recruitment in lung diseases such as asthma.

1.4 Asthma

Asthma has been historically defined as a paroxysonal and reversible airways obstruction, with underlying bronchial hyperresponsiveness to specific and non-specific stimuli. Whilst this clinical definition remains accurate empirically to even current understanding, it gradually became clear that to gain some understanding of asthma as a disease a more pathologic, cellular definition was required (Holgate, *et al*, 1991). Whilst bronchoscopic studies had defined asthma as an eosinophilic condition, and other consistent trends such as elevated serum IgE have been described, in the majority, many patients with asthma did not conform even to these patterns (Pride, 1992). What had become clear through a great deal of epidemiological research over the past decade was that the prevalence of asthma was greater in urban than in rural areas and in developed countries (Burney, 1992), a trend that has become more pronounced and potentially important as the incidence of asthma increases presently.

The current understanding of the aetiology and epidemiology of asthma are summarised below, followed by the most common cellular hypotheses and a brief review of asthma genetics. The section concludes with a summary of the literature on the role of the eosinophil in asthma and its contribution to asthmatic disease. While the emphasis of this thesis is not clinical, nor does the author have a clinical

background, it would still seem important to place the literature and our cytokines of interest into at least a basic clinical context.

1.4.1 Airway Inflammation in Asthma

There are several hypotheses that attempt to explain the underlying asthmatic process. In the first, related to "classic" allergic asthma, cross-linkage of IgE on mast cells by allergens triggers degranulation and release of mediators onto airways smooth muscle, triggering bronchospasm, mucosal oedema and possibly release of other cytokines from the smooth muscle itself (Leff, 1990). The primary weakness of this allergic hypothesis is that the majority of allergic people do not have asthma, and conversely not all asthmatics have an identifiable allergy. Even so, such mechanisms are undoubtedly important in many asthma patients.

The second hypothesis treats asthma as a neurogenic disorder. In this model asthma is caused by a parasympathetic overactivity or a relative β-adrenoreceptor deficiency. Activation of the β-adrenoreceptor has been shown to inhibit mast cell degranulation (Orange, *et al*, 1971) and is a relaxant of smooth muscle. Beta-blockers are highly likely to precipitate an asthma attack in active disease. Investigations into parasympathologic drugs have unfortunately shown no improvement in asthma status overall and thus the role of the parasympathetic nervous system in asthma is now thought to be less important than it was 2-3 decades ago, although it does provide extra bronchodilator effect in acute attacks of asthma (van der Velden, *et al*, 1999).

The most accepted and current hypothesis is that the hyperreactive state of the airways found in asthma is primarily caused by "inflammation", resulting from the migration

of non-resident cells into the airways from the circulation, as well as activation of resident cells. These cells react with both the airway smooth muscle and epithelium, causing increased reactivity in the smooth muscle and release of other potentially damaging mediators and chemokines. In particular, after eosinophil migration into the airways damaging eosinophilic granules are released, which have themselves been shown to induce smooth muscle hyperresponsiveness (Aizawa, *et al*, 1990). While this hypothesis does offer valid explanations for the presence of activated T cells and eosinophils in the airways, it offers no details regarding the triggering mechanisms that begin the inflammatory process. Many exacerbations in asthma could be precipitated by upper respiratory track viral infections rather than allergen exposure (Papadopoulos, *et al*, 2000). Until the initiating insults that lead to asthma, be they cellular, allergic or neurogenic, are defined, it will be very difficult to intervene in the disease process in any permanent curative way.

1.4.2 Clinical Definitions of Asthma

Asthma is a difficult condition to define comprehensively. Classically, asthma is identified by underlying airway hyperresponsiveness, typically with reversible airways narrowing. Inhalation of non-specific agents can cause bronchoconstriction: Methacholine or histamine is commonly used, which cause a degree of bronchoconstriction at a given dosage in asthmatic patients that is not observed in normal controls (Sands, *et al*, 1985). The dose causing a 20% fall in forced expiratory volume over one second (FEV_1), referred to as PD_{20}, is usually taken as the "unit" of bronchial hyperresponsiveness. Whilst the majority of asthmatics respond to methacholine or histamine, not all do, and to further complicate this method of assessment, there have been doubts raised about the daily repeatability of

bronchoconstriction based measures. A small number of studies have indicated a standard deviation of up to 2 doubling doses, a measure which should be exceeded in order to accurately assess a significant worsening or improvement in condition of the patient (Trigg, *et al*, 1990). In some cases, especially where asthma is more active, exposure to occupational or environmental irritants (eg. Hyperventilation of cold air or exercise) can also cause bronchoconstriction probably through mast cell degranulation. In addition to bronchoconstriction, the most common symptoms of asthma are chest tightness, breathlessness and cough, which are complex phenomena related to mediator release, neurogenic stimulation and physiological changes such as air trapping and overinflation.

Certain trends in the prevalence of asthma have been observed. It is now clear that in countries of improved socio-economic status, the incidence of asthma is greatly increased. However, within those regions, poverty seems an important factor in both the incidence and the severity of asthma (Poyser, *et al*, 2002). Between rural and urban areas within the same region, the incidence of asthma is far higher in the cities (Chan-Yeung, *et al*, 2002). It has also been recently demonstrated that asthmatic children from urban areas react positively on skin prick tests more frequently to a larger range of allergens, than asthmatic children from rural areas (Bibi, *et al*, 2002). The global incidence of asthma is steadily increasing, with Britain and Oceania having the highest rate (Miralles-Lopez, *et al*, 1999), and New Zealand having the highest rate of asthma mortality in the world (Crane, *et al*, 1995), especially among the poorer Maori community.

Whilst it is clear that asthma is related to a spectrum of airway hyperreactivity, which itself is related to underlying inflammatory and structural changes (Ward, *et al*, 2002), it remains true that the underlying cause or defect that begins the inflammatory cycle remains elusive. It may be that asthma is actually a series of related diseases that, as yet, we do not have the tools or knowledge to differentiate. Why asthma is increasing in Western countries and within those countries, why the poor are especially affected is not really understood. Although many hypotheses have been put forward, those are beyond the scope of this thesis.

1.4.3 Asthma Pathology and Genetics

Asthma can, pathologically, be viewed as a T cell disease, or more specifically, a disease resulting from a disorder biased towards an excessive Th2 lymphocyte response. Though it has not been effectively demonstrated, the tissue eosinophilia that is seen in the airways is most likely due to the production of IL-5 by activated T cells, though the role that other eosinophilic cytokines such as eotaxin have in asthma pathology is unclear.

There is no single gene for asthma. However, the many candidate genes and positional cloning genes that have been studied have produced several possibly important relationships to asthma. The most common loci for these correlations occur on chromosomes 5, 6, 12 and 13 (Cookson. 2002). The chromosome 5 loci are primarily associated with IgE production and contain a cluster of genes associated with allergic disease such as IL-3, IL-4, IL-9, IL-13, IL-5 and GM-CSF. Importantly the region has also been linked to eosinophil levels in the blood and serum IgE levels. Linkages were

also found between the IgE associated region of chromosome 5 (5q) and a region on chromosome 11 (11q, Palmer, *el al*, 1998).

Chromosome 6 contains the MHCs, which has the potential to effect many aspects of both innate and adaptive immunity. While linkages have again been found to this region and total serum IgE levels and specific IgE related to antigens such as house dust mite and cat allergen Fel di (Moffatt, *et al*, 2001). As yet no direct relationship has been found between MHC gene expression and current clinical measures of lung function and airway hyperresponsiveness.

Chromosome 12 has been linked more directly to both asthma and allergic rhinitis (Barnes, *et al*, 1999). Chromosome 12 contains genes for stem cell factor (SCF), leukotriene A4 hydrolase (LTA4H), thyroid receptor 2 (TR2), and signal transducer and activator of transcription 6 (STAT6) and IFN-γ. An analogous region to human chromosome 12q on the mouse chromosome 10 has been identified to be strongly linked to airway hyperresponsiveness in the mouse asthmatic model (Zhang, *et al*, 1999). This association with allergic reactions on chromosome 12 marks it as a potential location for a major atopy locus, though further mapping of the region will be necessary to identify specific gene locus linkages. Finally, chromosome 13 may also be linked to atopy and IgE (Kimura, *el al*, 1999). The same regions of chromosome 13 (13q14) also seem to be linked to high IgA and possibly infantile eczema (Bhattacharyya, *et al*, 2000). These genetic findings support the hypothesis that asthma instability in a spectrum of genes can lead to the asthmatic condition, rather than alteration of any key disease locus. It is thus very likely that that complex asthma phenotype is itself affected by a number of genetic determinants.

1.4.4 Eosinophils in Asthma

After allergen challenge in atopic asthmatics, there is a transient peripheral blood eosinopenia at 6 hours, followed by an eosinophilia up to 24 hours post challenge (Durham, *et al,* 1985). This secondary peripheral eosinophilia was most marked in those who develop a late asthmatic response, and correlated with AHR. In similar studies involving bronchoscopy post allergen challenge, eosinophils and their products increased in the airways at 2-24 hours. Similarly, airway hyperreactivity (AHR), a central feature of the pathophysiology of asthma, has been found to correlate with airway eosinophilia (Bradley, *et al,* 1991). Severity of clinical human asthma has been found to correlate with airway eosinophilia or eosinophil products in BAL fluid.

The recruitment of eosinophils from blood to airways involves several processes that have already been examined in earlier sections, but to summarise: Initial stimulation and generation of eosinophils occurs in the bone marrow followed by the cytokine controlled subsequent release. Movement of eosinophils through the circulation is facilitated by chemotactic gradients generated in the airways which is followed by adhesion to airways endothelium, via complementary pairs of adhesion molecules on eosinophils and activated endothelial cells. Transendothelial migration and movement of eosinophils through the interstitial matrix, both of with are influenced by eosinophil priming agents and chemattractants within the airways, such as IL-5 and eotaxin (Resnick, *et al,* 1993). The disappointing effects of anti-IL-5 therapy in controlling asthma has put into question the importance of the eosinophil in asthma, which still needs to be answered. It may be that the biology of IL-5 is complex and

removal of IL-5 prevents clearance of eosinophils from the airway wall (see section 1.2.5). Recent clinical work indicates that clinical management of asthma by monitoring sputum eosinophilia (Green, *et al*, 2002), emphasises the still central importance of the cell type, which if not central is at least closely related to the asthmatic disease process.

1.5 Corticosteroids

Anti-inflammatory glucocorticosteroids have been used in the treatment of asthma for the past forty years. Oral steroid gave way to safer inhaled drugs with less systemic side effects (Kravis, *et al*, 1966). The primary function of such steroids in the airways is to reduce inflammation and by doing so, reduce airway hyperresponsiveness both specific and non-specific. It may take several months for the response to become maximal (Barnes, 1990). The recent advent of more potent inhaled steroids such as fluticasone propionate (FP) along with better inhaler delivery systems has allowed for more aggressive prophylactic treatment regimes.

Inhaled steroids are safer than oral steroids, primarily due to fewer systemic side effects. There are, however, still some concerns regarding some aspects of long-term steroid use and there has been a move away from the use of high dose inhaled corticosteroids. While it has been accepted for some time that oral glucocorticoids can inhibit growth in very young children by interfering with growth factor secretion and directly inhibiting new collagen synthesis (Allen, 2002), it remains unclear if long term ICS usage in children can have similar effects. Recent data suggest that high-dose inhaled FP in a small percentage of children causes unexpectedly low growth and adrenal suppression (Wong, *et al*, 2002). Concerns have also been raised

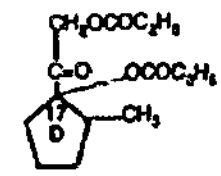
regarding the effects of steroids on bone mineral densities. Some studies have shown reductions in bone mineral densities and increased bone resorption marker expression after long term treatment with budesonide (Ebeling, *et al*, 1998). Conversely, other studies only confirm these findings with oral steroid use and have not found significant reductions in bone mineral density after inhaled beclomethasone dipropionate usage of up to four years (Matsumoto, *et al*, 2001).

It is only in recent years that glucocorticiod function has come to be better understood at the cellular level. The following sections detail how glucocorticoids affect transcription of genes and cytokines, downregulating specific mediators in the inflammatory response and upregulating others. Of particular importance in asthma and in this thesis is how glucocorticoids affect the cytokine mediators of eosinophilia, IL-5 and eotaxin.

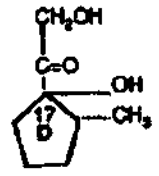
1.5.1 Glucocorticoid Structure and Function

The glucocorticoid (GC) family of drugs are synthetic derivatives of the adrenal steroid cortisol. The GC family of inhaled drugs includes beclomethasone dipriopionate (BDP), budesonide, and fluticasone priopionate (FP), and while they are all structurally similar, alteration of certain key side chains (see figure 1.5) in each has resulted in different levels of potency and subtle differences in pharmokinetics. The primary mechanism by which this is achieved is alteration of the side chain structure to increase lipid solubility of the GC thus greatly increase the drugs' half-life in the body (Umland, *et al*, 2002).

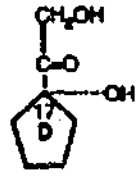
Figure 1.5. The chemical structure of the most common GCs used in the treatment of asthma, the table represents changes at the X1 and X2 positions of the cortisol molecule (from Umland, *et al*, 2002).



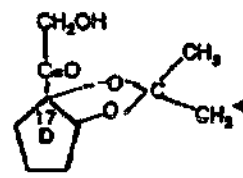
Beclomethasone dipropionate



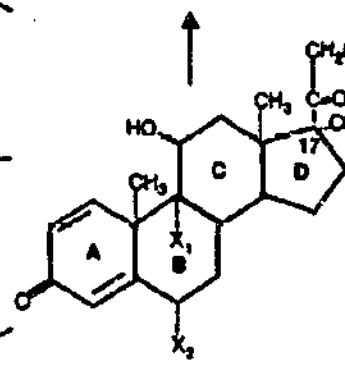
Dexamethasone



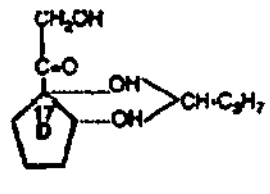
Prednisone



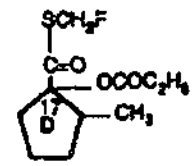
Triamcinolone acetonide



Cortisol

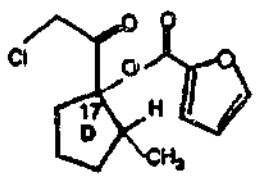


Budesonide



Fluticasone propionate

	X1	X2
Beclomethasonedipropionate	Cl	/
Budesonide	/	/
Cortisol	/	/
Dexamethasone	F	/
Fluticasone propionate	F	F
Mometasone furoate	Cl	/
Prednisone	/	/
Triamcinolone acetonide	F	/



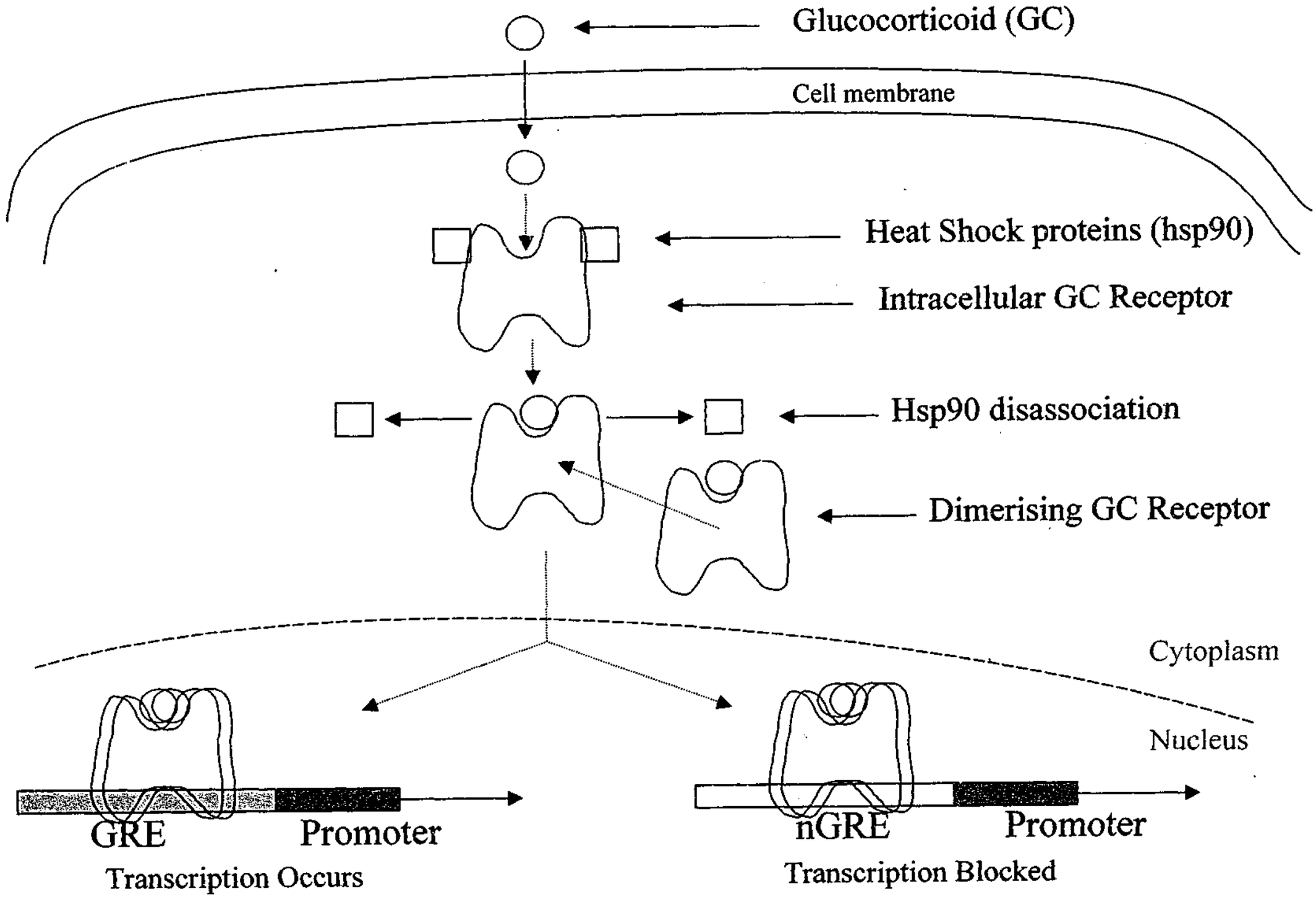
Mometasone furoate

The primary function of GCs is to mimic endogenous hormones involved in modulation of the inflammatory process, in order to therapeutically reduce inflammation. This occurs through a process affected by binding of GCs to cytoplasmic GC receptors (GRs). The GR is part of the steroid/thyroid/retinoic acid receptor superfamily (Oakley, *et al*, 1997). It is intracellular, thus GCs must be lipophilic to cross the cell membrane and bind. The receptor has several variants; a form expressed across most cell types (Type II GR), a specific form expressed in the kidneys, colon and salivary glands (Type I GR) and a non-functional silent receptor (GR_{β}) which may be implicated in steroid resistance in asthmatic patients (Oakley, *et al*, 1999).

After binding of GC to GR, heat shock proteins (hsp90) that hold the GR in the cytoplasm are dissociated, allowing the GC-GR complex to transmigrate to the nucleus. In the nucleus the GR complex dimerises with a second GR complex, this dimer, containing two zinc finger structures is able to bind specific sequences of DNA. This causes activation of specific GC response elements (GRE), which occur in close proximity to the promoter regions of certain genes for example stimulating production of more β receptors. More relevant to anti-inflammatory effects is repression of select genes (eg. switching off of inflammatory cytokines), with target sequences being associated with negative GREs (nGRE) (Drouin, *et al*, 1989). This process is illustrated in figure 1.6

Binding affinities of the GR complex to the GRE directly affects the potency of the GC. Fluticasone proprionate has a very high binding affinity, approximately 3 fold higher than beclamethosone, which corresponds with the clinical efficiency of the two

Figure 1.6. The major features of the intracellular pathway for GC function via gene promotion or suppression.


Glucocorticoid (GC)
Cell membrane
Heat Shock proteins (hsp90)
Intracellular GC Receptor
Hsp90 disassociation
Dimerising GC Receptor
Cytoplasm
Nucleus
GRE
Promoter
Transcription Occurs
nGRE
Promoter
Transcription Blocked

GCs. A newer GC, Mometasone furoate has the highest affinity, about 1.5 times that of FP, but as yet is not approved for clinical use in asthma, though it is used for the treatment of rhinitis (Smith, *et al*, 1998).

The GR complex is also able to directly bind to several transcriptional factors, such as AP-1 and NF-κB, which are vital to cytokine and chemokine transcription. This form of transcriptional inhibition, completely independent of GRE, can be demonstrated in GRE knockout mice, which are still sensitive and can be treated effectively with GCs (Reichardt, *et al*, 1998). GCs can also act in a post-transcriptional manner to either stabilise or destabilise mRNA (Almawi, *et al*, 2002), especially that of inflammatory cytokines such as GM-CSF, IL-1 and IL-3.

1.5.2 Glucocorticoid Effects on Cells *in vitro*

In vitro work into GCs has been quite extensive, and much has been elucidated about not only the function of GCs themselves, but also their effects on inflammatory cells and cytokines in a controlled context. In terms of potency, several studies have found that fluticasone priopionate, which was used in the clinical studies undertaken in this thesis, has a greater effect in both inhibition of cytokine production (Powell, *et al*, 2001) and induction of apoptosis (Hagan, *et al*, 1998), than budesonide and beclomethasone dipriopionate. Whilst the differences in relative effect vary from 4 to 100 fold, FP is consistently more potent, which corresponds with the increased binding affinity (see 1.5.1) and clinical improvement seen with FP over budesonide and BDP.

As mentioned above (see 1.5.1), GCs are able to interact with a variety of genes to selectively inhibit production of cytokines. It has been known for some time that dexamethasone, is able to significantly reduce IL-5 mRNA production in serum PBMC cell culture, even when applied in extremely small does (10^{-9} M) and in the presence of strong mitogens such as phorbol myristate acetate (PMA) and IL-2 (Rolfe, *et al*, 1992). Examining the effects of dexamethasone on IL-5 in *ex vivo* PBMC from atopic asthmatics and normal controls has shown that there is no intrinsic difference in GC function or potency between cells from asthmatic or normal patients' blood (Okayama, *et al*, 1994), although some studies have reported that *ex vivo* T cells from asthmatics on long term GC treatment show decreased production of IL-5 and IFN-γ (Kelly, *et al*, 2000). In pure T-cell lines, dexamethasone also prevents IL-5 production in PMA, testosterone or progesterone stimulated cells (Wang, *et al*, 1993). IL-5 production in mast cells is also sensitive to GCs, mast cell culture IL-5 production can be greatly reduced by addition of dexamethasone both before and after priming of the cells with ionophore and phorbol ester (Sewell, *et al*, 1998).

Much like IL-5, corticosteroids have an effect on eotaxin mRNA and protein secretion and production in human airway epithelial cell cultures, inhibiting the chemokine's production in a dose dependant fashion (Lilly, *et al*, 1997). Similarly, in human airway smooth muscle cell culture, an excellent source of eotaxin when stimulated with TNF-α, the GCs dexamethasone and fluticasone are able to partially inhibit eotaxin production and completely suppress production when applied in combination with the β_2-agonist rolipram (Pang, *et al*, 2001).

GCs have also been found to affect several of the following other cellular responses; though the mechanism remains unclear. GCs are able to directly down regulate the chemotactic response of eosinophils *in vitro*; budesonide lessened chemotaxis of eosinophils to complement factor C5a, though only at quite high doses (10^{-8} M). In contrast, ECP release and H_2O_2 production seem unaffected by direct GC application to eosinophils (Lantero, *et al*, 1999).

Whist most research into the effect of GCs in cell culture indicates suppression of cellular functions; GCs are also able to selectively upregulate cytokine production. Suppression of IL-5 occurs in both $CD4^+$ and $CD8^+$ T cells in culture in the presence of antigen presenting cells with the application of dexamethasone but within the same cultures IL-10 has been found to be greatly elevated (Richards, *et al*, 2000). Under other conditions, however, GCs have been known to suppress IL-10 in cell culture (Brinkmann, *et al*, 1995), and it has been suggested that these discrepancies are due to different populations or stages of activation of the cultured T cells, making *in vivo* extrapolation of such work difficult.

1.5.3 Clinical Treatment with Corticosteroids

Inhaled corticosteroids (ICS) in asthma, in very low doses, seem to have relatively little effect upon airway eosinophilia and even at very high dose a small degree of eosinophilia remains in some individuals, despite great clinical improvement (Bootsma, *et al*, 1995). Furthermore, there is remarkably little data in the literature on the dose-response relationship between inhaled corticosteroids and eosinophilia; on the prospective relationship between eosinophilia and clinical manifestations of asthma including AHR and symptoms; or on the relationship between eosinophilia

and other related manifestations of asthmatic airway pathology. Some groups suggest that there is an apparent lack of a dose-response for ICS when compared to lung function and inflammatory cell numbers in bronchial biopsy specimens (O'Sullivan, *et al*, 2002). There has also been the suggestion that there is an increased frequency of respiratory infections in patients on long term ICS (Oehling, *et al*, 1997), and have found reduced levels of antibody and a reduced potential for stimulation of PBMC from these long-term patients. A recent report from our lab has observed an increase in airway neutrophils and IL-8 in BAL fluid, suggesting activation of the innate immune system with aggressive ICS therapy (Reid, *et al*, in press).

1.5.4 Cellular Events After *in vivo* ICS Application

Much of the *in vivo* work done on GCs and cytokines has been performed on patient serum, mainly due to the relative ease of acquiring blood over BAL. The initial studies into serum cytokines showed that although asthmatic subjects had higher IL-5 levels than normal controls, after 1 week of treatment with oral GCs, despite improved lung function, IL-5 in the serum was not significantly reduced (Corrigan, *et al*, 1993). Asthmatic patients receiving long term oral GCs also continued to show elevated levels of IL-5 (Alexander, *et al*, 1994). However, in both these studies IL-5 could not be detected in normal controls and a proportion of asthmatics, suggesting limitations in the sensitivity of the assays. More recent studies have examined peripheral blood $CD4^+$ and $CD8^+$ T cells by flow cytometry and found that IL-5 expression is significantly reduced after 5 months of increased GC treatment (Gemou-Engesaeth, *et al*, 2002).

Steroid modulation studies using human BAL for outcome measures are very rare, especially those that examine cytokines. Early work on BAL found that after 2 weeks of predisolone treatment, the presence of IL-4 and IL-5 mRNA became difficult to detect, similar to what was found in normal subjects (Robinson, *et al*, 1993). This work was later supported by findings that in endobronchial biopsies IL-4, IL-5 and IFN-γ mRNA is decreased after prednisolone treatment, examined by *in situ* hybridisation (Bentley, *et al*, 1996).

Some groups have suggested that, particularly in mild asthmatics, corticosteroid resistance may be a potential confounder in the measurement of blood and BAL cytokines (Matsuse, *et al*, 1999). Asthmatics who do not respond to GC treatment after 2 weeks, defined by failure of BHR reduction in response to methacholine, do not show reductions in serum ECP or IL-5 mRNA. Whilst these findings are important in understanding steroid function *in vivo*, thorough clinical monitoring over the course of a longitudinal study should assess the presence and effect of this phenomenon.

1.6 Conclusion

It is clear that the eosinophil is prominent in asthma pathology although its pathologenic importance is currently under some degree of revision. Not only are increased eosinophils found in the airways and peripheral blood of asthmatic patients, but also allergen-challenge in sensitised animal models which seek to simulate asthma have found a similar level of eosinophilia. The eosinophils themselves release powerful mediators such as MBP and ECP, which are capable of damaging tissues and activating both elements of the immune system (innate and adaptive) and

"constitutive" structural cells such as local fibroblasts and endothelial cells. The cytokines predominately associated with eosinophils are also found to be elevated in asthma. IL-5 is associated with all aspects of eosinophil development and function, controlling eosinophil bone marrow development, as well as eosinophil priming, chemotaxis, activation and survival in the airways. Likewise, the eotaxins, chemokines that are constitutively expressed within the airways, are also the only chemotactic factors that are specifically active only on eosinophils.

In limited studies, both IL-5 and eotaxin have been found to be elevated in asthma. Despite a large number of investigations into IL-5 in asthma and a somewhat smaller number into eotaxin (due to its novelty), very little data exist on how either of these cytokines is affected by steroids *in vivo*. Glucocorticosteroids are now widely prescribed for the treatment of asthma, and their effects on cell culture suggest they can directly modulate the expression of specific cytokines at a genetic level and may have the ability to alter the balance between classic Type 1 and Type 2 immune responses. What is unclear is how long-term use of ICS affects the complex cytokine cascades in the airways and what happens to these cytokines when steroids are withdrawn. The importance of these omissions in the current understanding of asthma may relate to the failure of conventional steroid therapy to cure asthma, or to fully control it in many patients and the rapid recurrence of symptoms when treatment is withdrawn.

This project has examined the numbers of eosinophils and levels of IL-5 and eotaxin in peripheral blood and the airways themselves in asthmatic subjects across a range of

steroid doses cross-sectionally and compared them to steroid free asthmatics and normal controls. The same issues were then examined across a number of longitudinal studies involving varied steroid regimes. The primary aims of these projects were to examine the relationships or lack thereof between eosinophils, IL-5, eotaxin and ICS treatment, and between these indices of airway inflammation and physiological outcome measures.

More specifically, the thesis attempted to measure airway eosinophilia cross-sectionally in a group of normal and asthmatic human volunteers who underwent bronchoscopy, BAL and endobronchial biopsy. These asthmatics were on a range of ICS doses. Cytokine levels in the airways and blood, as either protein and/or mRNA in these patients were measured for eotaxin and IL-5 and eosinophils counted. Assessments of the relationship between any measured eosinophilia and cytokine levels in regards to established clinical markers of asthma, including symptom scores and physiological indices (including flow rates and BHR) were performed.

Whilst the cross-sectional data measured have hopefully shown some of the effects of ICS on IL-5 and eotaxin, the primary emphasis of the project was designed to relate all of the above measures to longitudinal steroid studies. Data were gathered from three separate longitudinal studies. The first study was of asthma patients already undergoing relatively low-dose ICS treatment who then either had their steroid dosage increased or their steroid treatment supplemented with additional long acting β agonist (LABA) for a period of 3 months. The purpose of this study within the context of this thesis was to determine if there was a dose response relationship between ICS,

eosinophils and cytokines, and whether LABA had any additional effect. In the next study, patients not receiving ICS but nevertheless symptomatic, were placed on a relatively high ICS dose for a period of 12 months. In this study the effects over 3 months of ICS treatment on IL-5 and eotaxin levels were examined, as well as any continuing or additional effects apparent after 12 months of treatment. The final study involved initially symptomatic patients, made asymptomatic by receiving high-dose ICS whose treatment was then reduced. Measurements were made both at the end of the high dose treatment and after partial withdrawal of treatment in order to examine the changing cytokine and eosinophil profiles as the patients' symptoms recurred – or at least that was the intention when the study was designed. It was hoped that these three studies along with the initial cross-sectional data would provide a comprehensive insight into the human *in vivo* interactions and relationships between IL-5, eotaxin, eosinophils and ICS treatment beyond anything that has been previously observed. It was hoped that these studies would lead to a better understanding of how glucocorticoid treatment modulates this aspect of the immune system in the airways, and perhaps give some indication as to why withdrawal of ICS treatment frequently results in the rapid recurrence of symptoms and clinical disease activity.

Chapter 2: Materials and Methods

2.1 General Materials

Water used for preparation of ELISA and Immunohistochemical reagents was Milli-Q-H_2O obtained from a Milli-Q Type I Reagent Grade Water system (Millipore Pty. Ltd., Sydney, Australia). For molecular biology commercially available RNase free PCR water or Diethyl pyrocarbonate (DEPC) treated water was used.

Phosphate buffered Saline (PBS), was prepared at pH 7.2 as follows:

NaCl	8.5 g/l
$NaH_2PO_4.2H_2O$	0.4 g/l
$Na_2HPO_4.12H_2O$	2.7 g/l (or $Na_2HPO_4.2H_2O$ at 1.3 g/l)

2.2 Sample Collection and Processing

2.2.1 Patient Recruitment and Clinical Measures

Patients underwent physiological and clinical assessment including: laboratory measurement of flow-volume characteristics and BHR to methacholine (see section 1.4.2); home monitoring of peak-flow readings for the duration of each study; and a home diary of morning and night symptom scores also for the duration of each study.

2.2.2 Bronchoscopy and specimen collection

Bronchoscopy was performed a minimum of 2 days after physiological challenge, before and at the end of treatment periods for asthma patients. The last dose of study medication was taken 12 hours before bronchoscopy. One bronchoscopy only was performed for controls. Subjects were pre-medicated with intravenous 0.4mg atropine and 5-15mg midazolam. Inhaled salbutamol (200µg) was administered 15 minutes

prior to bronchoscopy. Topical anaesthesia was obtained via 4% lignocaine spray applied to the pharynx and larynx and 2% lignocaine below the cords in 2ml aliquots as required, up to a maximum dose of 6ml. Subjects were monitored throughout the procedure with pulse oximetry and oxygen was administered at 4 litres per minute during the procedure to all volunteers.

Bronchoalveolar lavage (BAL) of the right middle lobe was performed with 3 x 60ml aliquots of phosphate-buffered saline and immediately aspirated to ensure minimal dwell time. Endobronchial biopsies (EBBx) were taken from the (sub)segmental carinae of the right lower lobe of each patient using alligator forceps (Olympus code No. FB 15C). EBBx and BAL were transported on ice and immediately processed.

2.2.3 BAL Processing

The volume of BAL was first measured after which 10 ml of the sample was immediately set aside. The remainder was spun down (Herabus Sepatech centrifuge, 1800rpm, 15 minutes), the supernatant collected in twenty 1.5ml tubes, and frozen at -80°C. The cell pellets were snap frozen in liquid nitrogen, and then stored at -80°C. The remaining 10ml was then spun onto 30 slides using a Shandon Cytospin III (Runcork, U.K.) centrifuge (150µl, 850rpm, 10 minutes), 6 slides were put aside for differential, giemsa and mast cell cytochemical staining, the remainder were dried for 1 hour before being wrapped in aluminium foil and stored at -80°C.

2.2.4 Tissue Processing

Paraffin Sections: Biopsies were fixed in 10% neutral buffered formalin for 2 hours, then transferred to a Shandon Citadel 2000 tissue processor, where the sections were progressively dehydrated in graded ethanol before being infiltrated with wax under vacuum pressure. The total processing time was 6 hours. After processing, the tissues were mounted in paraffin wax mould on standard plastic cassettes and allowed to dry and cool overnight, before being stored at room temperature. Blocks were cut on a Leica RM2155 microtome (Leica Microsystems, Germany), at 5μm thickness, then floated onto warm (55°C) water. The cut sections were then adhered to silanised* glass slides, and dried for 30 minutes. Sections were de-waxed through graded ethanols prior to staining.

*: A 2% silane in acetone solution was used to immerse the slides for 2 minutes, followed by washing in water two more times at 2 minutes per wash, all at room temperature. Slides were then dried at 60°C for 1 hour before being ready for use.

GMA Sections: Biopsies were placed immediately into ice-cold acetone containing protease inhibitors (2mM phenyl methyl sulphonyl fluoride and 20mM iodoacetamide) and fixed overnight at -20°C. The fixative was then replaced with acetone and left at room temperature for 15 minutes, which was followed by 15 minutes in methyl benzoate again at room temperature. An infiltrating solution (5% methyl benzoate in acrylic monomer) was then used with three changes for 2 hours per cycle (3 x 2 hours) all at 4°C. The embedding solution (acrylic monomer, 2.5% N,N-Dimethylaniline and 0.2mM Benzoyl Peroxide) was then added and plastic capsules used to exclude air. Polymerisation occured overnight at 4°C after which the

solid GMA blocks were cut from the capsules and stored in airtight containers at -20°C. Blocks were cut on a resin microtome, using glass triangular knives, in thicknesses of 2-4μm. They were then floated on water and adhered to silanised (2% silane in acetone, see above) glass slides. For signal enhancement prior to staining, sections were etched with 0.5M Sodium Hydroxide (NaOH) or pure Xylene for 20 minutes at room temperature, before being digested in 0.4% Trypsin for 30 minutes at 37°C.

2.3 Immunohistochemistry and Immunofluorescence

2.3.1 Eosinophil Staining on Paraffin Sections

This protocol was done entirely at room temperature. Sections were incubated with 3% normal horse serum for 10 minutes. After tipping off the preincubate, purified monoclonal antibody for either eosinophilic granular marker 1 or 2 (EG1, EG2, Pharmacia Diagnostics, Sweden) was applied for 1 hour. The slides were then washed 2 times in PBS (2 x 5 minutes, with magnetic stirring). A biotinylated horse anti-mouse Ig antibody (VECTA Laboratories, USA) was then applied for 30 minutes. During this time a horse-raddish-peroxidase (HRP) and streptavidin mixture was incubated for at least 15 minutes before the second slide incubation period expired. Slides were then washed (2 x 5min in PBS, with stirring) and then the streptavidin-HRP mixture added for a further 20 minutes. Slides were again washed (as above), and a catalytic colour reagent (Vectastain Elite ABC Kit, VECTA Laboratories, USA) was then added for 10 minutes, the resultant brown precipitate indicating positive staining. Contrast was achieved by counterstaining with Harris Haematoxylin for 2 minutes. The slides were then washed in water for 10 minutes and dried before being

mounted in bio-mount (Dako, Denmark) mounting medium. The sections were examined under a Leika DM LB microscope.

2.3.2 GMA Immunohistochemistry

It was found that though GMA staining was possible without the etching and digestion procedure mentioned above (2.2.4), but the general enhancement of all staining performed with etching and digestion resulted in its use for all GMA staining procedures.

2.3.3 Immunofluorescence

This protocol was done entirely at room temperature. Sections were incubated with 3% normal sheep serum for 10 minutes. After tipping off of the preincubate, purified monoclonal antibody was applied for 1 hour. The slides were then washed 2 times in PBS (2 x 5 minutes, with magnetic stirring). An appropriate fluorochrome labelled secondary-conjugate was then applied for 30 minutes. The slides were then washed as before and then mounted in Fluorosafe (Dako, Denmark) mounting medium. The sections were examined under a Leika DM LB microscope. The monoclonal antibodies, the appropriate conjugates and dilutions were as follows:

Monoclonal Antibody	Dilution	Conjugate
EG1[1]	1:100	a
EG2[1]	1:50	a
Collagen IV[2]	1:400	a,b*
Collagen III [2]	1:200	a
Collagen I[2]	1:200	a
Eotaxin[3]	1:10	b
IL-5[4]	1:10	a

[1] Pharmacia, Sweden
[2] Dako, Denmark
[3] R&D, USA
[4] Pharmingen, USA

a: A FITC-conjugated $(Fab')_2$ fragment of affinity purified sheep anti-mouse Ig (DDAF; dilution 1:100; Silenus laboratories) was used.
b: An affinity purified, biotinylated horse anti-goat IgG antibody (dilution 1:100, Dako), followed by a wash step, then incubation for 20 minutes with Texas-red-streptavidin (dilution 1:200, Pharmingen). This was followed by washing and mounting (as above)
*: Note that for double labelling an alternate polyclonal goat anti-human Collagen IV (Dako) was used, thus requiring an anti-goat conjugate.

2.3.4 Double and Triple Labelling

The double fluorescent labelling protocol followed the single (above), with the following additions. A mixture containing 3% normal horse and 3% normal goat serum was used. The two primary antibodies of interest were mixed and applied together for the first incubation and the two conjugates (a and b above), mixed for the second incubation. The third incubation step containing fluorochrome labelled streptavidin was also required. For triple labelling, after the final wash step an ultra-violet fluorescing DNA dye (Bisbenzomise H33342, Calbiochem®, La Jolla, CA, USA) was applied for 1 minute, followed by a washing step in water for 10 minutes, before mounting.

2.4 Enzyme Linked Immunosorbent Assay (ELISA)

2.4.1 Commercial ELISA Kits

A commercial colorimetric ELISA kit (Quantikine Immunoassay Kit, R&D systems, USA) was purchased for detection of eotaxin in BAL. Although it proved insensitive, it formed the basis for development of the chemiluminescent eotaxin ELISA system. A RANTES chemiluminescent ELISA kit (Quantikine Immunoassay Kit, R&D, USA), was also assessed and found to be too insensitive without further in-house development.

2.4.2 IL-5 and Eotaxin Chemiluminescent ELISA

Opaque white 96 well plates (Nunc, Maxisorp, Germany), specifically charged and optimised for ELISA, were coated in mouse monoclonal antibody for either human Eotaxin (3µg/ml, Pharmingen, USA) or IL-5 (3µg/ml, R&D Systems, USA), coated in plastic wrap to avoid evaporation (this was done for all incubation steps) and incubated overnight at 4°C. The antibody was then tipped off and the plates blocked with filtered 0.3% BSA for 2 hours. 3 washes with 0.05% Tween-20 in PBS were performed using a plastic wash bottle. Randomised patient BAL samples (40 patients per plate), after thawing from -80°C to room temperature, were thoroughly mixed and added in duplicate to the plate, with doubling dilutions of standards of known concentrations (Eotaxin: 50-0.76 pg/ml, IL-5: 100-1.56 pg/ml). At these low concentrations it was vital that freshly thawed Eotaxin and IL-5 protein were used, any time at 4°C would degrade the quality of the standard curves. Plates were incubated for 4 hours at room temperature. 4 washes (as above), before a capture layer was comprised of goat polyclonal, biotinylated antibody against human Eotaxin (0.1µg/ml) or IL-5 (0.5µg/ml) was added for 2 hours, thus establishing a classic "sandwich" protein capture method. 5 washes (as above) followed. Detection of the complex was obtained via a horseradish peroxidase coated streptavidin conjugate (1:10000, Dako, Denmark), applied for 20 minutes. A final 6 washes were performed here. A specialised, luminol based, chemiluminescent substrate (Powersignal LBA, Pierce, USA) was applied for 5 minutes with gentle agitation, to generate the light-emitting signal required for chemiluminescent detection. Measurements were made on a chemiluminescent plate reader (Hewlett-Packard), set to automatic sensitivity detection with a read length of one second. As the chemiluminescent signal degrades

in 15 minutes, plates were discarded after reading. Maximum sensitivity for the assay was 0.01pg/ml for IL-5 and 0.005pg/ml for Eotaxin.

2.4.3 IL-5 and Eotaxin ELISA for Serum

As the serum detection technique was developed after the BAL protocol, many of the differences observed here are optimisations and refinements of the above protocol. This procedure followed the above method with the following changes; Plates were coated, both for eotaxin and IL-5 using 2μg/ml of primary antibody. Plate washing was performed with an automated plate washer (Model 1575, Bio-Rad Laboratories, USA), rather than by hand. Patient samples and standards were incubated overnight at 4°C as opposed to 4 hours at room temperature. The range of the known standards was 400-0.4ng (half-log dilutions) for eotaxin and 25-0.39ng/ml (serial dilutions) for IL-5.

2.5 Cell Quantitation

2.5.1 Differential Cell Counts

Cytospin slides were stained with Diff Quick (Lab Supply, Melbourne, Australia) and counted for lymphocytes, neutrophils, macrophages, eosinophils, mast cells and airway epithelial cells. Two slides from each subject were coded and scored blind as to treatment and disease status. Approximately 500 cells were counted per slide; the scores were then averaged. Results were expressed both as absolute cells numbers and as a percentage by cell type.

2.5.2 Image Analysis for Eosinophil Quantitation

Image analysis of biopsy sections for EG1 and EG2 was performed with a Leika DM LB microscope, Dage-MTI DC200 one-chip video camera and Image Pro V4.1 (Media Cybernetics, USA) software. At least 8 fields per section were captured under a 40x objective and measured via continuous digital measurement. EG1 and EG2 positive cells were counted in quantifiable lamina propria up to a depth of 150μm, excluding vessels, mucosal glands and smooth muscle. In each field, lengths of basement membrane as well total tissue area excluding any muscular or cartilaginous tissue was measured. These length and area measurements were used to standardise the absolute eosinophil numbers, thus giving eosinophils per mm of basement membrane or eosinophils per mm^2.

2.6 Molecular Biology

2.6.1 cDNA Synthesis

Extraction of RNA from snap frozen BAL cells was performed via affinity spin column purification (RNeasy, Quigen, UK) in accordance with the protocols recommended by that product for animal cells. A small portion of the extracted RNA was set aside for quantitation via spectrophotometry (Varian-Cary spectrophotometer). The remainder was immediately frozen at -20°C.

cDNA synthesis was performed using the following reagent mix:

10%	TaqMan® RT Buffer (10x)
22%	25mM $MgCl_2$
20%	2.5mM dNTPs mixture
5%	50μM Random Hexamers
2%	RNase Inhibitor (20U/μl)
2.5%	Multiscribe™ Reverse Transcriptase (20U/μl)
1%	Sample RNA (1μg)
37.5%	DEPC H_2O

A 50µl reaction was performed for each patient sample with a Peltier thermal cycler (PTC-200, MJ Research). The following parameters were used:

25°C for 10 minutes
48°C for 30 minutes
95°C for 5 minutes
4°C indefinitely

After synthesis, cDNA was again stored at -20°C.

2.6.2 Competitive RT-PCR

Competitive PCR, where an mRNA sample of unknown quantity was compared as a ratio to an artificial sequence of known quantity and sharing the same primers, was performed for the detection of eotaxin and RANTES in mRNA extracted from the cells acquired from BAL.

Eotaxin and RANTES primers and competitors were designed in-house, then manufactured and purchased commercially (Bresatec, Australia).

Optimal PCR mixes contained the following constituents:

10%	PCR Buffer (10x)
2%	20µM RANTES or eotaxin primers
0.4%	Taq Gold Polymerase
6%	25mM $MgCl_2$ (1.5mM)
2%	Upper/Lower (1:1) Competitors
20%	RT mix (see below)
59.6%	PCR water

The RT component of the PCR mixes contained the following:

10%	PCR Buffer (10x)
20%	$MgCl_2$
10%	dNTP (10mM)
4%	Rnase Inhibitor
4%	Oligo dT
4%	MulW RT
25%	extracted mRNA
23%	H_2O

A 50µl reaction was performed for each patient sample with a Peltier thermal cycler. The following parameters were used:

RT Phase: 15°C for 1 minute
42°C for 1 hour
85°C for 5 minutes
4°C indefinitely

PCR Phase: 94°C for 8 minutes
94°C for 1 minute (Start of first cycle)
68°C for 1 minute
Repeat Cycle (Total of 40 cycles)
72°C for 7 minutes
4°C indefinitely

PCR products were run on 3% (w/v) agarose gels, containing 0.1µg/ml ethidium bromide at 120 Volts, along with a Hpa II puc marker and 0.5µl of loading dye. Visualisation of PCR products was facilitated by a Fluorimager 575 (Molecular Dynamics) imaging system with ImageQuaNT (version 4.1, Molecular Dynamics, USA) software.

2.6.3 Real Time PCR

Real time PCR was performed for the detection of eotaxin and IL-5 in cDNA purified from the mRNA extracted from cells acquired from BAL.

Commercially available eotaxin and IL-5 primer and probe sequences (Applied Biosystems, USA) were used for all real time PCR procedures. These sequences were optimised for the ABI Prism 7700 Sequence Detection System instrument (Applied Biosystems) used in this protocol.

Real-time reaction mixes were made according to the following parameters:

50% TaqMan Universal PCR Master Mix (2x)
15% Eotaxin/IL-5 primer/Probe Mixture
10% Subject cDNA sample
25% Water

20µl real time PCR reactions were run on 384-well PCR plates (Applied Biosystems) in accordance with the recommended parameters for the instrument as supplied by the manufacturer:

50°C for 2 minutes
95°C for 10 minutes
95°C for 15 seconds (Start of first cycle)
60°C for 1 minute
Repeat Cycle (Total of 40 cycles).

2.7 Developmental Methods

The following sections cover, in greater detail, the development of three techniques undertaken during this project. Whilst other endeavours such as immunohistochemistry for biopsy eosinophils and differential staining and counting of subject BAL cells also took significant amounts of time, these techniques were already largely pre-established within our group. The following techniques were attempted exclusively for the detection of IL-5 and eotaxin by various methods and while several were not successful, the extensive investment of time and resources into each and experience gained, warrants their description and explanation. The techniques were:

Immunofluorescence for IL-5 and eotaxin in airway biopsies

Chemiluminescent ELISA for IL-5 and eotaxin in BAL and serum

Molecular biology for IL-5/eotaxin mRNA detection

2.8 GMA Immunofluorescence Development

2.8.1 Introduction

The initial focus for eotaxin quantitation was to develop a histological staining system that would allow for quantitation of eotaxin positive cells or tissue area via an image analysis system in endobronchial biopsies. Biopsies were taken from subjects undergoing bronchoscopy and embedded in paraffin wax and glycol methacrylate (GMA) (These protocols are presented in section 2.2.2 Bronchoscopy and specimen collection and 2.2.4 Tissue Processing). The formalin-fixed paraffin sections were primarily reserved for future *in-situ* hybridisation work and previous experience in paraffin tissues have shown that cytokines and secreted factors tend to stain poorly as compared to structural and cell-surface markers. Initial work also found a non-specific auto-fluorescence present under ultra-violet and blue light, potentially an artefact of the formalin/paraffin tissue processing protocol, which would make multiple colour immunofluorescence difficult using these tissue sections. GMA sections, with little or no background fluorescence, had greater potential for high-resolution co-localised staining of multiple factors. This potential for quantification of cytokines and their potential cell sources was a primary motivation for the development of dark field rather than light field based cytokine immunohistochemistry.

2.8.2 Glycol methacrylate Resin Sections

Glycol methacrylate (GMA) is a water soluble plastic. Structurally similar to methyl methacrylate (commonly Perspex), the plastic consists of acrylic monomers cross-linked with glycol residues. It is these water-soluble cross-linkages that allow antibody penetration into the resin (MERCK index, 11th edition, #5849). Though fluorescence based GMA immunohistochemistry was used in the early '80s (van de

Velde, 1980), it has recently seen resurgence in popularity as a medium for tissue embedding (Fraenkel, *et al*, 1994).

2.8.3 Development of Staining Protocols

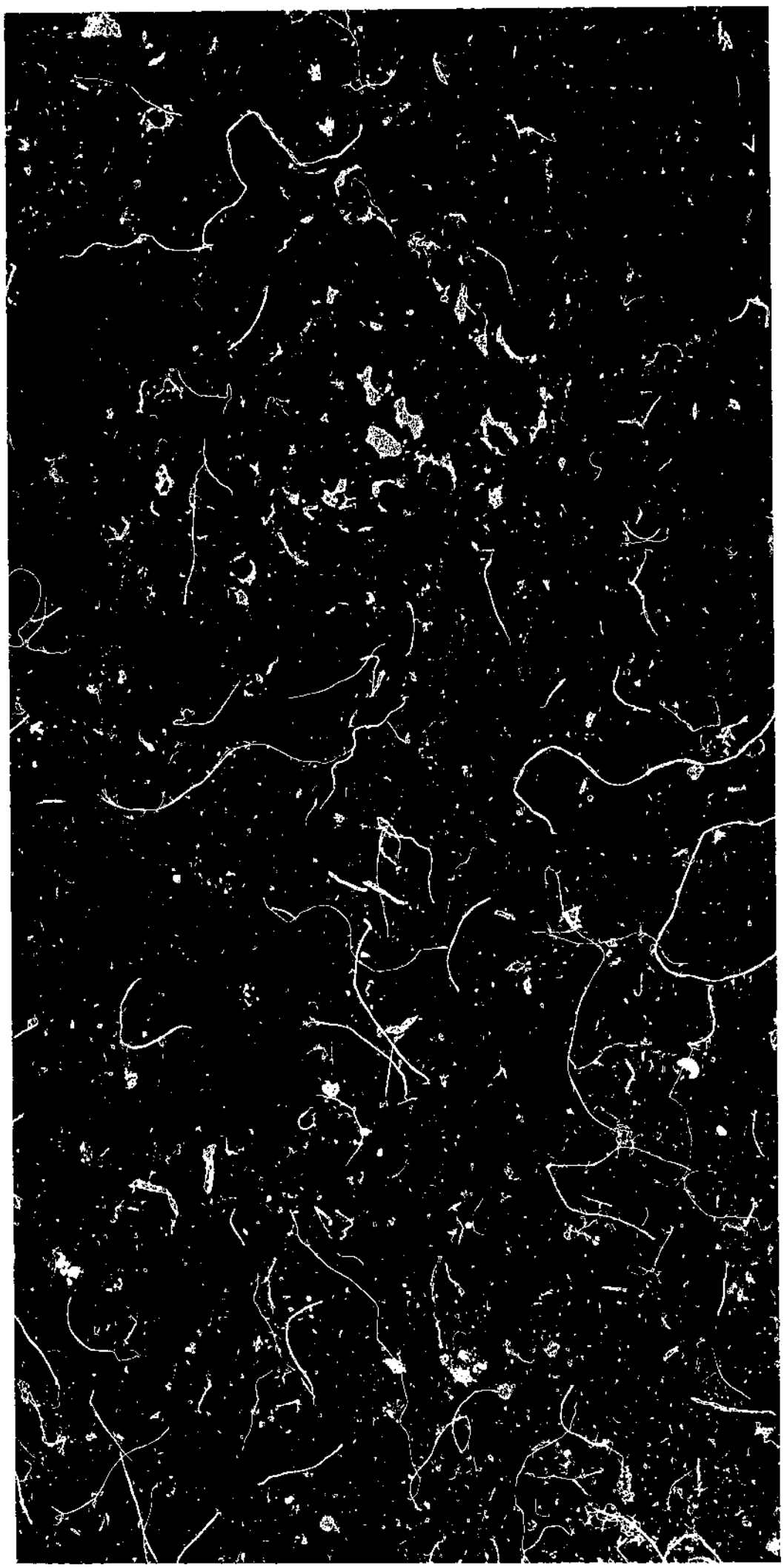
Initial work with the resin showed poor quality staining that was far weaker at equivalent concentrations on paraffin embedded tissues using ubiquitous strongly expressed antibodies, such as collagens I and IV. It was found that antigen recovery greatly enhanced all GMA immunohistochemistry in both dark and light field applications. The final developed protocol was based on a system of acetone etching and protein digestion of cut tissue sections, which was largely based on previous work by other groups (Mozdzen, *et al*, 1982), and has been described in section 2.2.4. An example of the exceptional detail provided both by the rigidity of the GMA and the lack of background fluorescence, in accordance to the final protocol, is illustrated in figure 2.8.1.

2.8.4 Results

Primary emphasis was placed on development of eotaxin staining, and while early work was promising (see figure 2.8.2), it was found that reproducibility was poor and inconsistent. The eotaxin staining was only present at very high antibody concentrations (0.25mg/ml, 0.75µg/section), and could not be achieved using alternative secondary antibodies and immunofluorescent labels. Inhibition of primary antibody was able to prevent staining, but despite this, the inconsistency of the staining and high concentration of the antibody was suggestive of a staining artefact, rather than true eotaxin staining. IL-5 staining in GMA was absent, regardless of concentration or antigen retrieval via protease digestion.

Figure 2.8.1 Cytokeratin (100x objective). A high-powered, digitally magnified field of a vessel and epithelium (top left) from an endobronchial biopsy in GMA and stained for cytokeratin.

Figure 2.8.2 Eotaxin Staining (40x objective). A confocal image showing staining for CD68, a macrophage marker (green) and eotaxin staining (red), which proved to be anomalous.



2.8.5 GMA Structural Staining

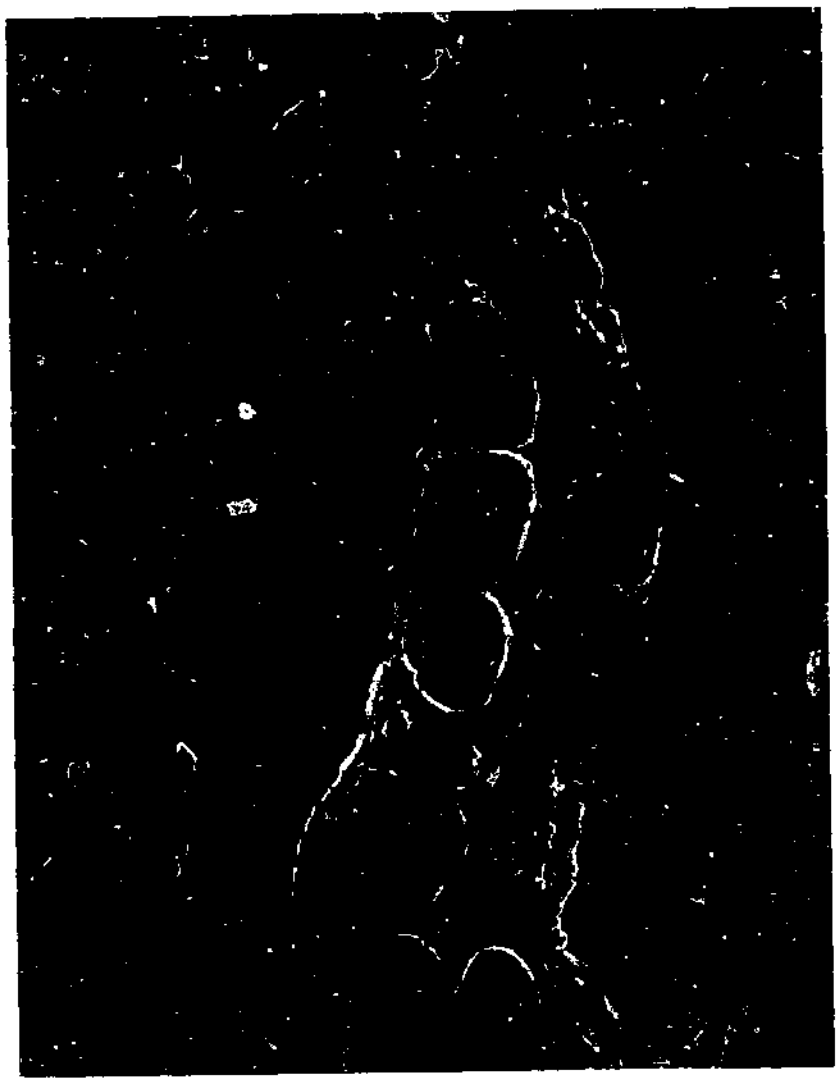
Although the GMA embedded tissue proved inadequate for cytokine staining and unreliable for cell markers, structural markers, such as collagen and cytokeratin, stained very strongly and consistently. The rigidity of the GMA sections allowed for very high-resolution images of airway tissue structure. In particular, collagen IV, a vessel and lymphatic marker, could be complemented with collagen III, which delineates the connective structures in the lamina propria, along with a nuclear dye to isolate cells (Bisbenzomise H33342, see section 2.3.4). These three-colour images provide detailed pictures of the structural and cellular components of the airways, which could be easily implemented for quantification of airway vasculature or collagen. Low and high magnification examples of the staining in airway biopsies are shown in figures 2.8.3 and 2.8.4 respectively.

2.8.6 Conclusions

Whilst development of an immunofluorescence system for the staining and quantitation of cytokines in GMA was unsuccessful, the GMA media was useful in establishment of high-resolution structural staining. Collagen staining in airways tissue was most successful, but other markers, such as von Willerbrand Factor (see figure 2.8.5), also met with some success. These staining protocols, which can be used in both multi-colour dark field and light field immunohistochemistry, have the potential to be very useful in the quantification of structural components of the airways. In fact, the protocols developed during this project have been used as a basis for airway vessel quantification by our group (Ward, *et al*, 2002).

Figure 2.8.3 Collagen III/IV Vessels (20x objective). This image shows a glandular structure, with endothelium outlined by collagen IV (red). Connective tissue is illuminated by collagen III (green) and cell nuclei are dyed (non-specifically) blue.

Figure 2.8.4 Collagen III/IV Lamina Propria (40x objective). A higher powered field of a different section of the same tissue (2.8.3). Again, collagen IV (red), collagen III (green) and nuclei (blue). Note the strong staining of the basement membrane (red, bottom right) and the large cluster of cells in the central vessel.



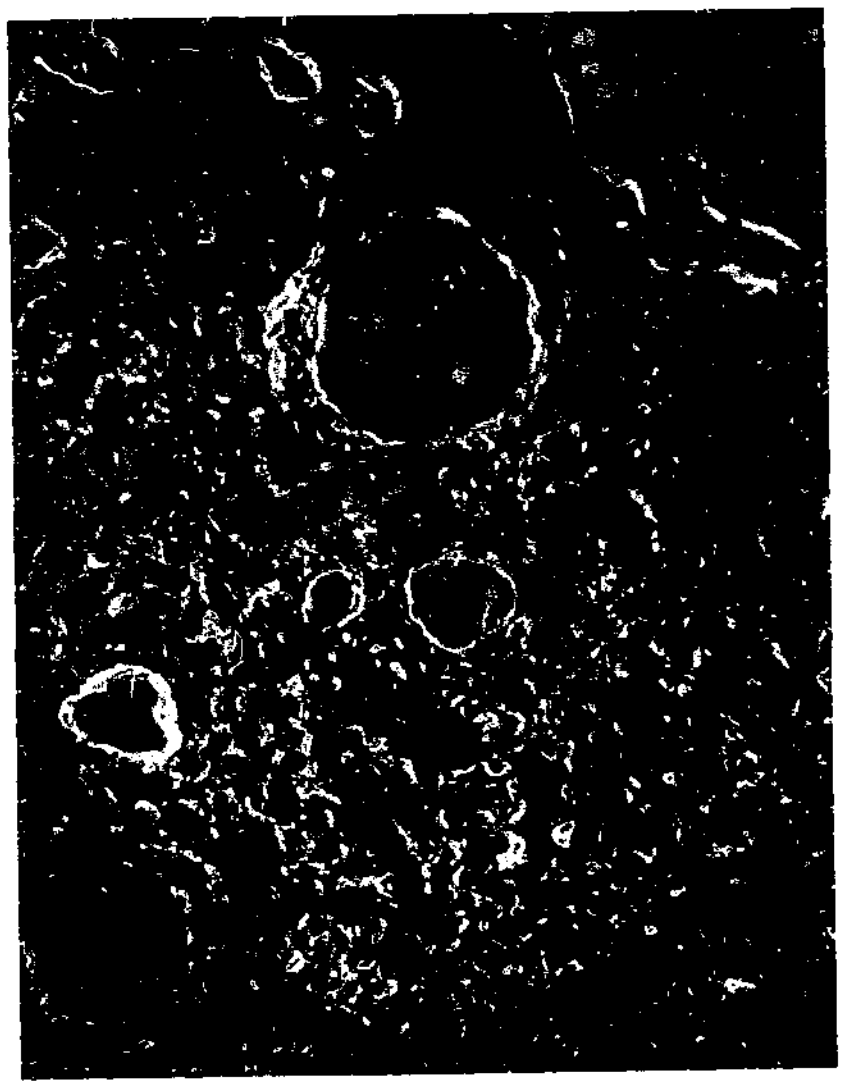
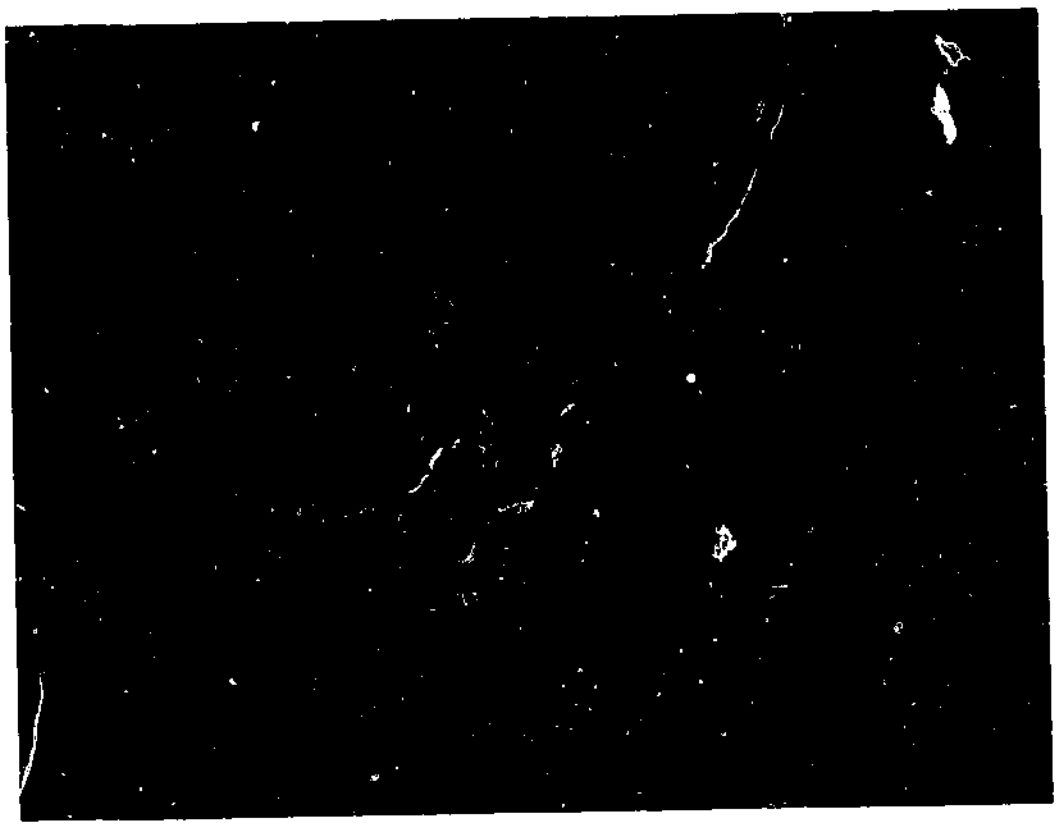


Figure 2.8.5 Double staining for collagen IV, Von Willebrand Factor (x20 objective). This field shows collagen IV^+ (green) outlining vessels that have also been stained for endothelial cells with Von Willebrand Factor (red). Nuclear material is dyed blue.



2.9 Development of Chemiluminescent ELISA

2.9.1 Introduction

While the initial strategy to quantify eotaxin was to use immunohistochemistry (see section 2.8), quantitative staining and image analysis proved difficult. Thus other strategies were presented for eotaxin quantification. Based on the available achieved tissues, cells and supernatants, quantitative PCR was assessed for eotaxin mRNA (see 2.10), which was followed by real-time PCR. Due to the quantity of cells recovered in a typical lavage (in the order of 10^6 cells), only molecular biology or flow cytometry could be performed, the low number of cells not allowing for both. Thus the decision for a PCR based assessment precluded protein measures of eotaxin via flow cytometry of lavage cells. Of the remaining materials, the bronco-alveolar lavage (BAL) fluid was available in relative abundance (20 x 1.5ml aliquots per patient, for each study time point). Though the lavage was quite dilute (3 x 60ml saline), the direct interaction between the saline and the lung epithelium, one of the primary sources of eotaxin (Rothenberg, *et al*, 1995), should have resulted in sufficient suspension of eotaxin protein to be detectable by sensitive laboratory procedures. Enzyme linked immunosorbent assay (ELISA) was chosen as a specific and sensitive technique for the detection of eotaxin protein in the BAL.

2.9.2 Initial Procedures

A commercial colour-development based ELISA kit, specific for human eotaxin (R&D, USA) was selected for initial trials. The quoted maximum sensitivity for the kit was 15pg/ml, which is very good compared to other standard chemistry or un-enhanced ELISA systems. After running 15 varied patient samples it was found that

only 30% of the analysed samples were above the 15pg/ml threshold, obviously insufficient for quantitative analysis. However, as the system was pre-optimised for detection of eotaxin in cell culture or serum, it was disappointing but not surprising to find levels in quite dilute BAL to be too low to detect. Though several different post-ELISA amplification systems are available, access to a chemiluminescent plate reader within the department, bolstered by product specification sheets stating detection levels in the order of femtograms (fg/ml, 10^{15} fg = 1g), somewhat biased the choice. Concentration of the samples and dialysis was discussed, but concerns were raised about degradation of the product and introduced artefacts, both of which could drastically effect quantitation. Though it may be possible to analyse these effects, with regards to the protocols used in this project at a later date, such an analysis is beyond the scope of this thesis.

2.9.3 First Chemiluminescent ELISA

The first experiment to assess the viability of using chemiluminescent ELISA would use the remains of the aforementioned commercial ELISA kit. Though it was not possible to change the primary antibody concentration, the kit being pre-coated, the substrate could be easily replaced with the more sensitive chemiluminescent system. The remaining problem was the plate itself. Standard chemistry ELISA uses a detection system that passes a light beam through the entire plate and measures absorbance of that light by a colour product. Conversely chemiluminescence involves emission of light by the substrate itself and detection of that emitted light. As this emission of light is omnidirectional, each well must be sealed and effectively lightproof, or the signal will mix with light from other wells and become effectively meaningless. The clear plastic commercial ELISA plate was transformed into an

opaque chemiluminescent plate by means of some white reflective paint and a fine paintbrush. Though crude, the sensitivity of the plate was increased to 3.65pg/ml, a four-fold difference. Of the 15 patient samples examined, 80% were now detectable, providing a solid basis for development of the technique.

2.9.4 Development and Optimisation

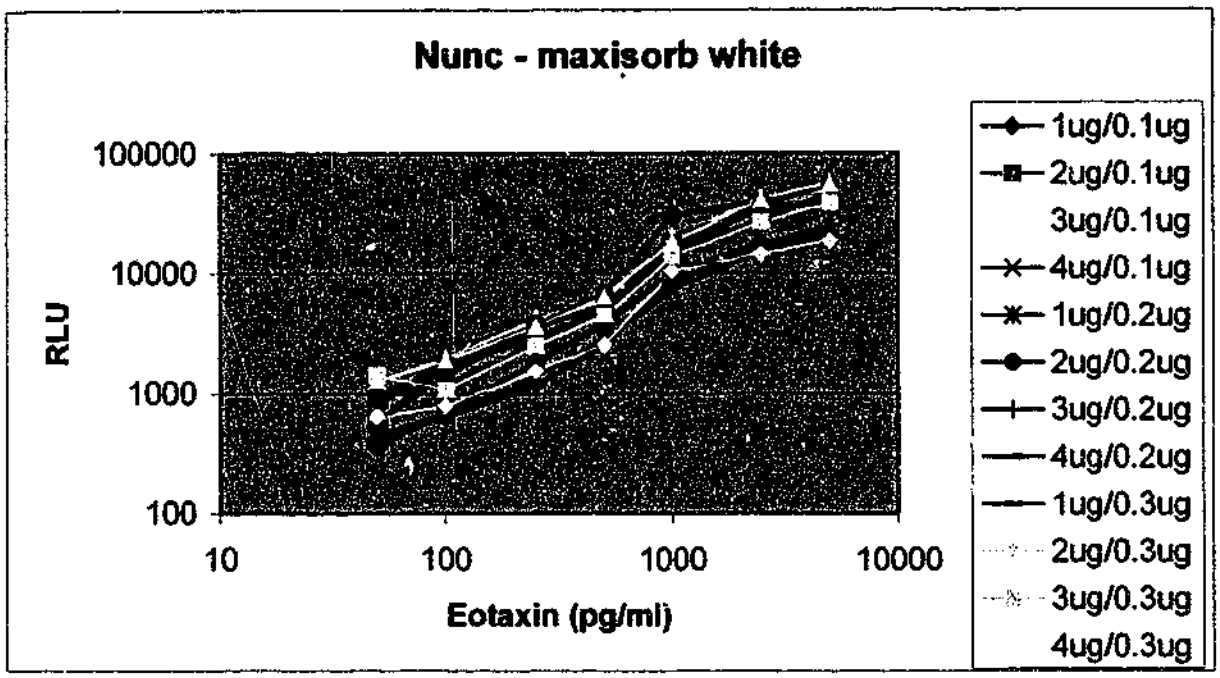
This assay used paired antibodies; a monoclonal anti-human eotaxin (R&D, USA), used to coat the plate, and a polyclonal anti-human eotaxin labelled with biotin (R&D) for detection. Both these antibodies needed to be optimised for concentration in order to maximise the assay sensitivity and specificity. As chemiluminescence systems rely on the detection of emitted light, it was expected that plate colour, whether a plate was black or white, would affect the sensitivity and background of the assay. A white plate would maximise reflection, thus all signals, specific or not, would be amplified, thus creating the highest level of sensitivity but also the highest background. Conversely, a black plate should absorb a portion of the light signal, thus the sensitivity of the system would be lower, but non-specific background would also be absorbed, making the assay cleaner.

The initial work focused on several plate coating formulations, as well as capture and detection antibody concentration. On each trial plate, 12 standard curves with titrated concentrations of capture and detection antibody were run. The first plate type was pre-optimised from ELISA and showed high sensitivity, which was demonstrated by the steep slope of the curves (see figure 2.9.1). These curves approached optimal sensitivity; with a log reduction in relative light units (RLU) accompanied by a log reduction in eotaxin concentration. Although the antibody concentrations had some

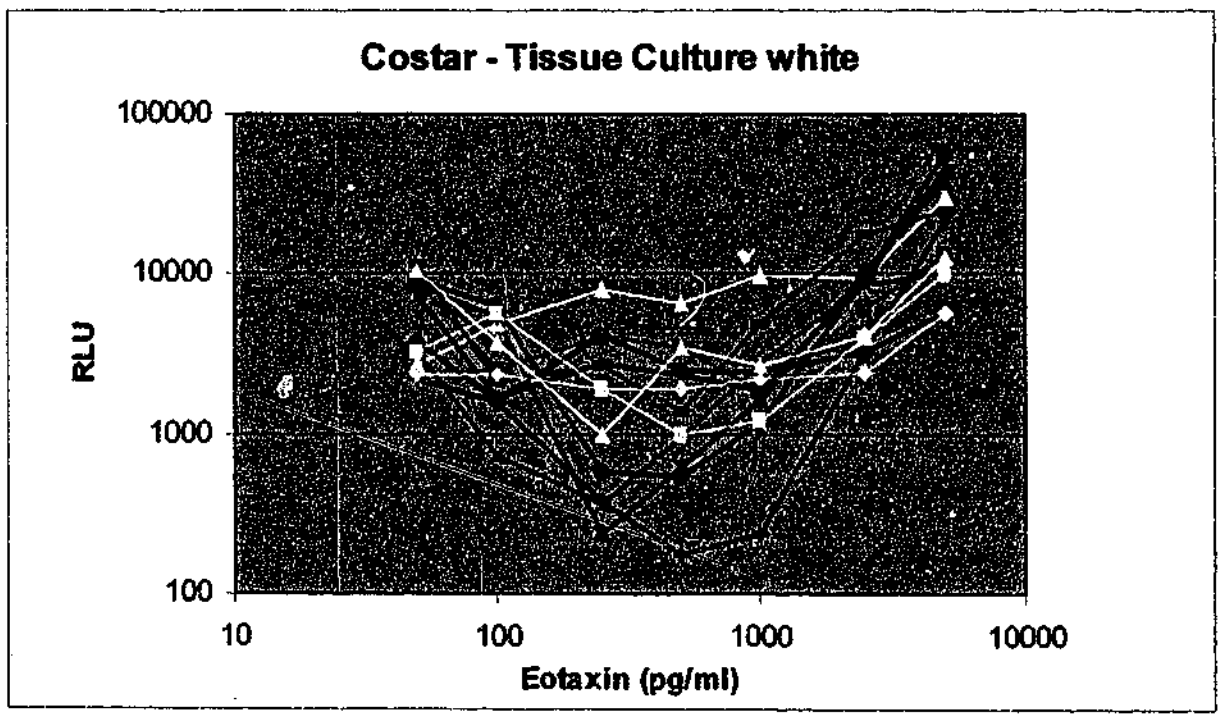
Figure 2.9.1 A standard curve checkerboard titration on ELISA optimised white plates. These plate were eventually used in our protocol. The key indicates the coating and detection antibody concentrations, and is applicable for the following three figures as well.

Figure 2.9.2 A standard curve checkerboard titration on tissue-culture optimised white plates. Note the poor reproducibility and lower slope.

hised white
the coating
wing three



nised white



effect on the slope and displacement of the standard curves, within these rang antibody concentration had less effect than plate type (see below).

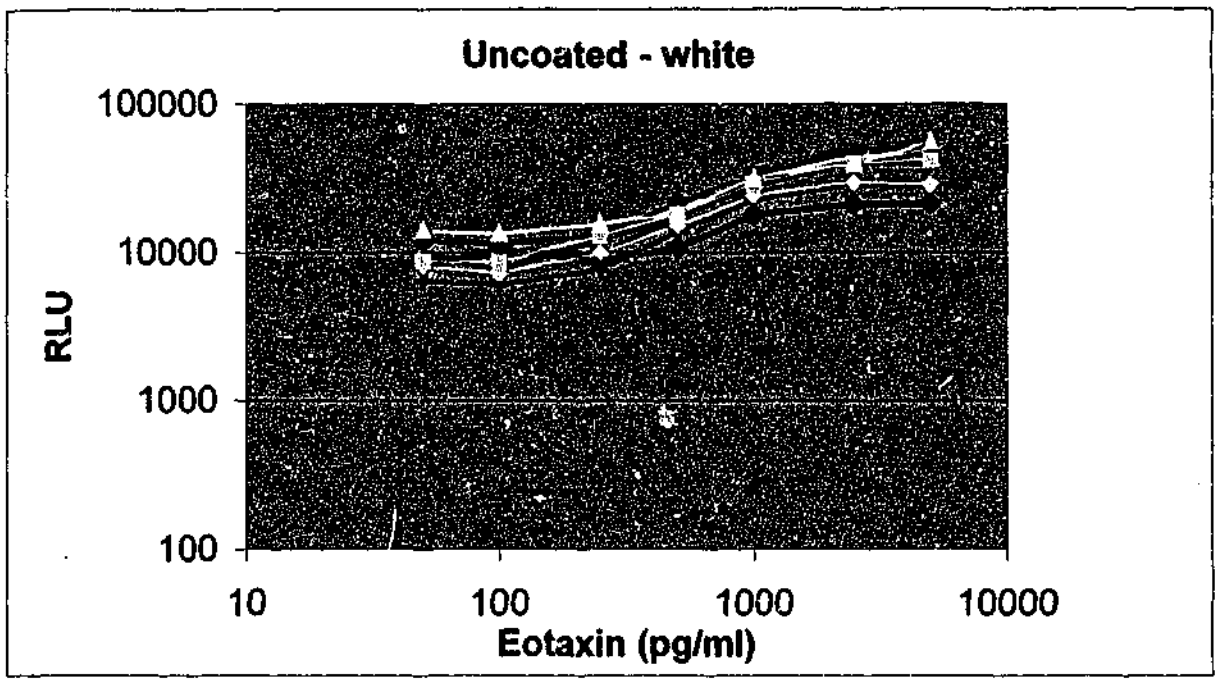
Though we were unable to ascertain from the manufacturer what degree of chargi or lack there of, or if any other coating agents were applied to the "optimised f tissue culture" plates which were trialed, the results indicate severe inconsistency capture antibody adhesion (see figure 2.9.2). The variability of the standard curv and general lack of sensitivity immediately excluded these plates. However, it may that these plates are more useful in assays measuring products in the nanogram, rath than picogram, range, as evidenced by the upward trend, and greater consistency of the standards at 5000pg (5ng). Being well above the target threshold for th development process, the nanogram sensitivity of these plates was not furth pursued.

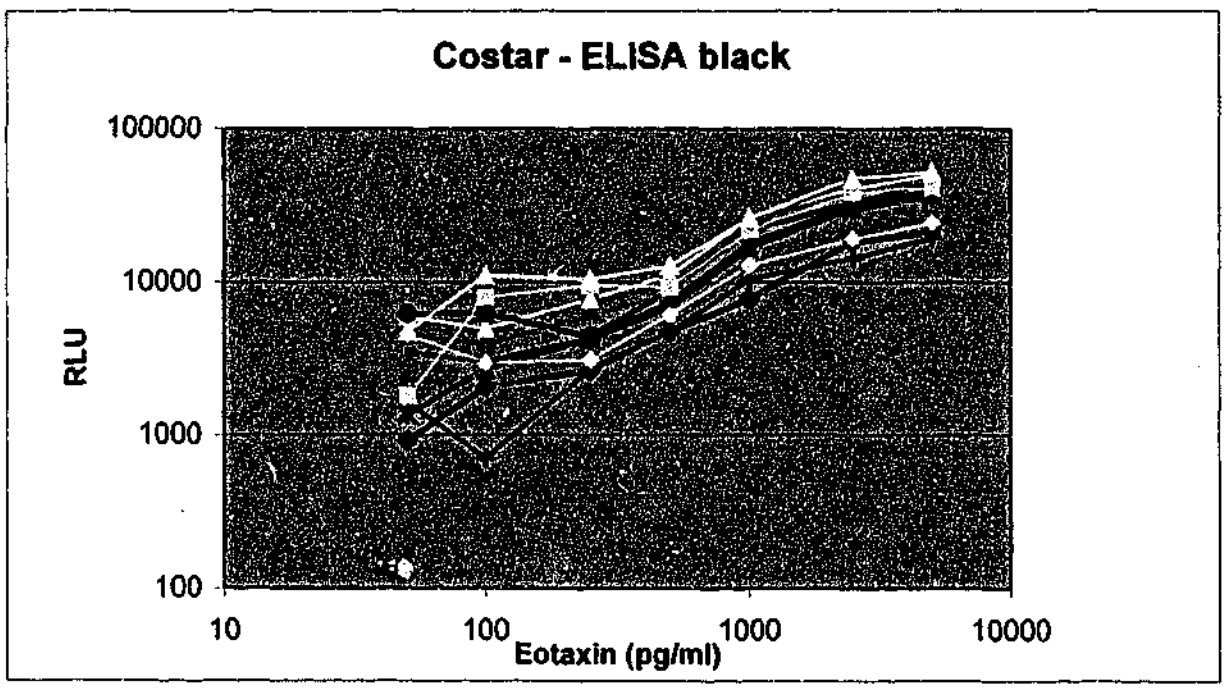
A type of uncharged, uncoated white plastic plate was also examined. While t consistency was very high between the standards (see figure 2.9.3), the sensitivity w very low, as illustrated by the poor slope on the curves, which become almost a completely flat below 100pg/ml. Though examining these plates did give some insig into the vast sensitivity enhancing effects of plate charging, uncoated plates were f too insensitive for quantitation of protein in the picogram range, at least in the conte of this system.

An important design choice for chemiluminescence ELISAs was plate colou Whereas the previous white plates, due to the reflective properties of the colou would give a stronger signal, but equally amplified background signal, a black pla

Figure 2.9.3 A standard curve checkerboard titration on uncoated white plates. While the reproducibility is good, the slope is very poor.

Figure 2.9.4 A standard curve checkerboard titration on ELISA optimised black plates. The reproducibility is very good at high concentrations, but is poor below 100pg/ml.


Uncoated - white
100000
10000
1000
100
RLU
10
100
1000
10000
Eotaxin (pg/ml)


Costar - ELISA black
100000
10000
1000
100
RLU
10
100
1000
10000
Eotaxin (pg/ml)

absorbs light and thus gives a higher signal to noise ratio, yet less intense signal. These properties were consistent with the results of testing black charge-optimised for ELISA plates (see figure 2.9.4). The slope of each of each standard curve line was considerably shallower (an average of 7.3 as compared to 8.1 for the curves in figure 2.9.1). The lower end of the curve showed considerable variation, most likely a result of approaching the detection limits of the system under these conditions. Yet at higher concentrations, the standards showed far less variation than those in figure 2.9.1. In effect giving a cleaner more consistent signal at these ranges. This low background would have made the plates ideal for an assay within a range of 200-2000pg/ml or greater, however the poor sensitivity below 100pg rendered them unsuitable for the detection of eotaxin protein in BAL.

This plate analysis clearly showed that variation was produced by both plate colour and charge. These both had a more drastic effect on the overall sensitivity and specificity of the assay than did variation in coating or detection antibody concentrations. The white ELISA-optimised plates (figure 2.9.1) were chosen for the final assay protocol, which were approximately five times the price of the uncoated white plates.

Though it was expected that the plate charge would make some difference to the sensitivity of the assay, the degree of difference was surprising. As illustrated in the upper range of the standard curves, many of the factors that influence these assays in the picogram range, such as plate colour and charge, had less or no effect on protein detection in the nanogram (thousands of picograms) range. Degradation of proteins

and antibodies after thawing and storage at 4°C was also found to cause significant degradation of the standard curves, especially for any measurements below 10pg/ml.

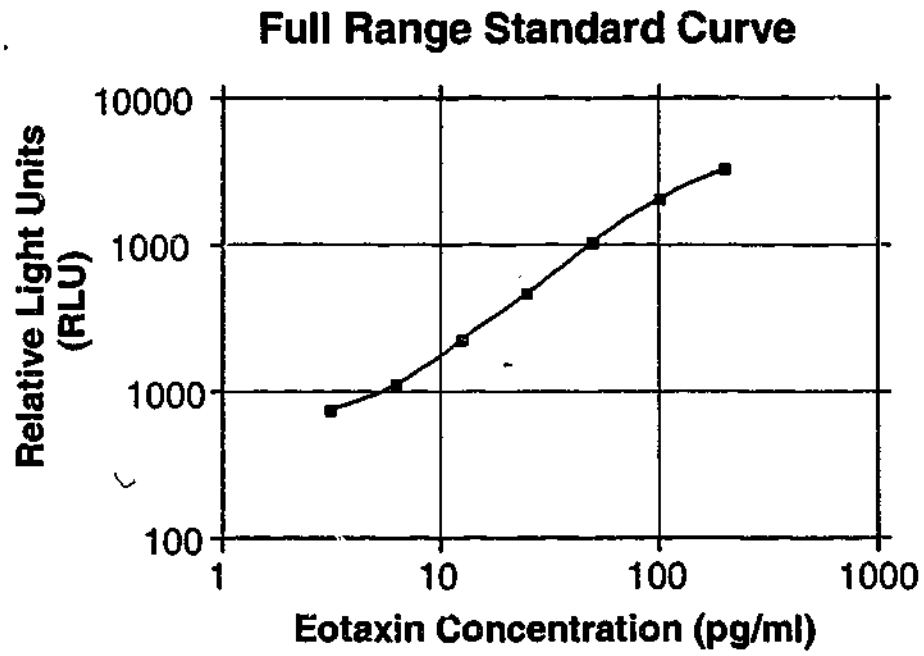
2.9.5 Optimised Standard Curves

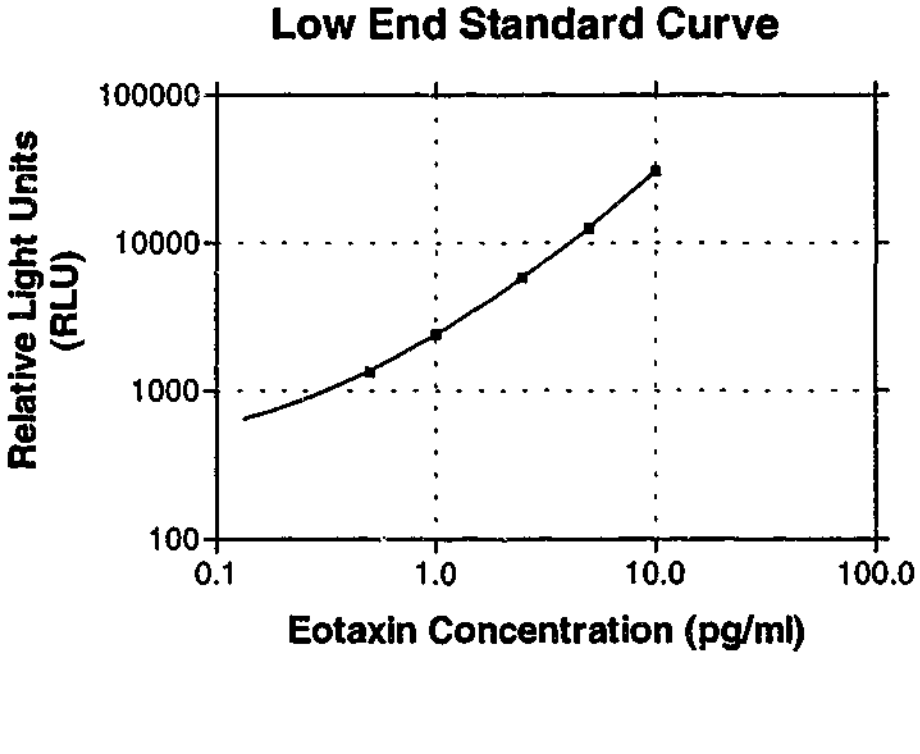
Examination of eotaxin levels in patient samples found that very few exceeded an eotaxin concentration of 100pg/ml, and a significant number were under 10pg/ml. A standard curve within the ranges of 10-1000pg/ml (see figure 2.9.5) was achievable even using thawed antibodies (antibodies stored at 4°C), a limitation not typical to ELISA but found to be relevant at the lower picogram ranges. The recombinant eotaxin protein was still found to undergo degradation significant enough to affect the assay after approximately 3 days at 4°C, facilitating an absolute necessity to store aliquots on recombinant eotaxin at -80°C to use fresh with each assay. The standard curve maintained a high degree of linearity and slope, and typically only began to taper below 5 pg/ml. While this sensitivity range accounted for approximately 75% of the typical patient sample range, it was possible to extend the curve further, to accurately assess samples measuring eotaxin levels of below 10pg/ml.

Below 2.5pg/ml the vulnerability of the assay to disrupting influences became greater still. At this extreme sensitivity all the antibodies and the recombinant protein had to be thawed fresh from -80°C for each assay to prevent degradation of the standard curve. Pipette and mixing inconsistencies also became relevant sources of error at this level, warranting extra care during assay preparation. The loss of some sensitivity at this level is depicted by the shallowed standard curve and the departure from a straight line at the lower end (see figure 2.9.6). Also of note is the relative light unit

Figure 2.9.5 A typical full range standard curve for the fully optimised chemiluminescent eotaxin ELISA.

Figure 2.9.6 A low end standard curve for eotaxin ELISA. Note that detection is still possible at 0.3pg/ml.


Full Range Standard Curve
Relative Light Units (RLU)
10000
1000
1000
100
1
10
100
1000
Eotaxin Concentration (pg/ml)


Low End Standard Curve
Relative Light Units (RLU)
100000
10000
1000
100
0.1
1.0
10.0
100.0
Eotaxin Concentration (pg/ml)

measure given by the luminometer. At these settings, which are controlled by the relative detection of the well emitting the most light, 0.5pg/ml, the lowest standard, registered 1350 RLU, and empty wells (with substrate alone) measured 400±200 RLU. It is doubtful that another two-fold eotaxin protein dilution, resulting in a bottom standard of 0.25pg/ml would be measurable with any accuracy as it would be overlapping the error range of the zero control well. Lowering the sensitivity of the instrument would only flatten the standard curve and though a black plate would certainly have reduced the zero well reading, as can be seen in the plate trial (figure 2.9.4), it is unlikely that a black plate would be sensitive enough to measure protein levels into the low picogram ranges.

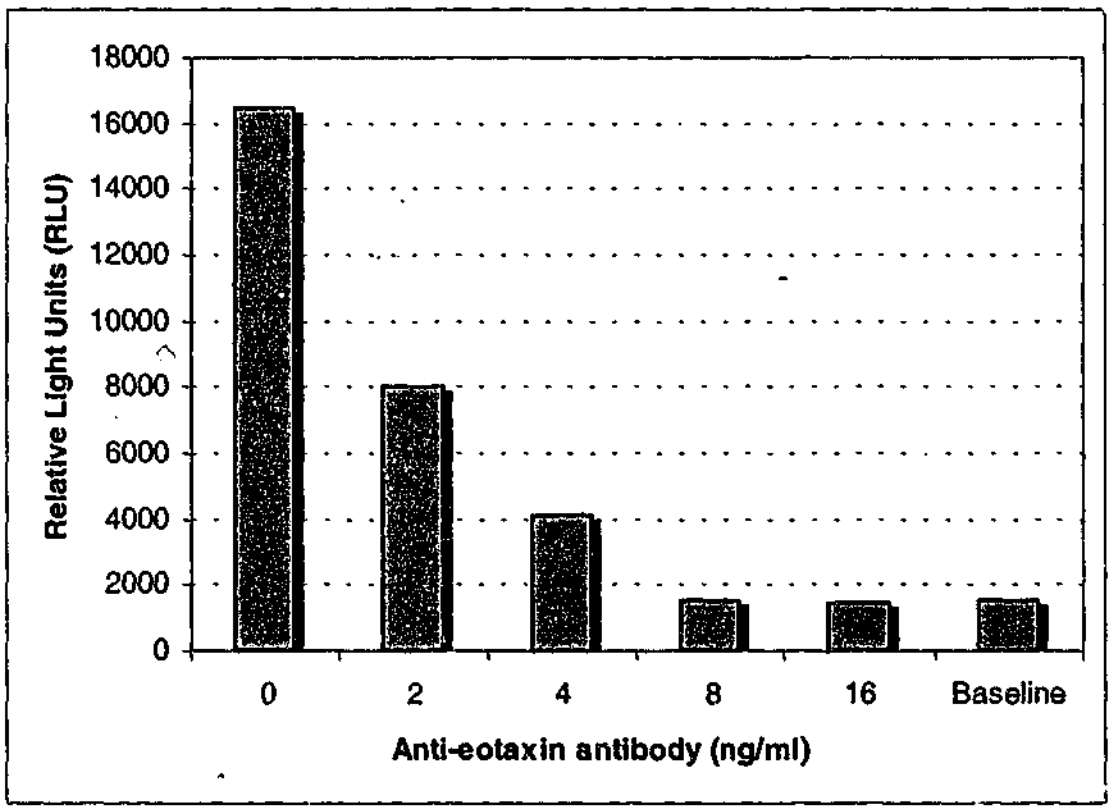
It was found after these final optimisation trials had been run that at least 90% of actual patient samples would fall within the sensitivity range of the final optimised assay. This was a vast improvement over the initial 30% of samples that were quantifiable in the commercial eotaxin ELISA kit, and would be sufficient to accurately quantify patient samples.

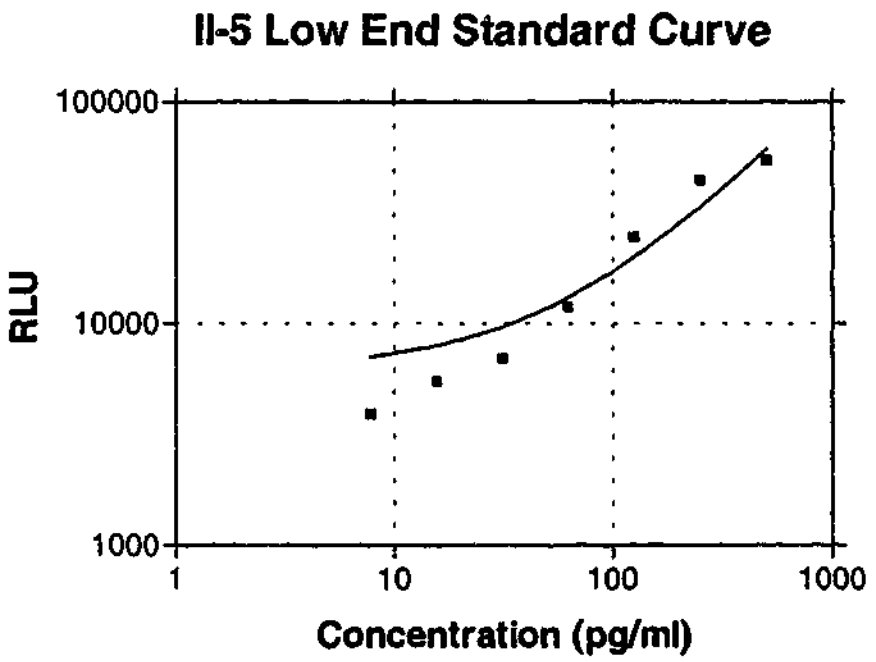
2.9.6 Validation and Reproducibility

Specificity was assessed by titrating anti-eotaxin monoclonal antibody (the same antibody used to coat the plates), into an eotaxin-spiked sample. As the concentration of free anti-eotaxin antibody increased, more eotaxin is bound, thus less binds to the plate and the final reading is correspondingly lower. The results of this experiment are illustrated in figure 2.9.7. As can be seen in the figure antibody concentrations of 8ng/ml and higher were sufficient to entirely suppress detection of eotaxin antibody.

Figure 2.9.7 An inhibition ELISA demonstration for the specificity of the assay for eotaxin. Antibody is titrated into a spiked sample, which inhibits plate binding.

Figure 2.9.8 A low end standard curve for IL-5 ELISA. Note that this assay was not as linear at low concentrations as eotaxin, being slightly less sensitive.


18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Relative Light Units (RLU)
0
2
4
8
16
Baseline
Anti-eotaxin antibody (ng/ml)


Il-5 Low End Standard Curve
100000
10000
1000
RLU
1
10
100
1000
Concentration (pg/ml)

Reproducibility was tested in two ways. Intra-plate variation was tested in one sample run across 16 wells. Inter-plate variation tested the reproducibility of 34 samples over two plates. The intra-plate coefficient of variation (CV) was 5.2%, while inter-plate reproducibility was somewhat lower, with a CV of 10.8%.

2.9.7 IL-5 ELISA Development

ELISA protocols for IL-5 detection in the BAL were developed simultaneously with eotaxin ELISA. Whilst a commercial IL-5 ELISA kit was not trialed, IL-5 ELISA underwent identical optimisation procedures to eotaxin for coating and detection antibodies, specificity and reproducibility. Whilst the sensitivity of the assay was somewhat less than eotaxin, a typical standard curve still retained some sensitivity at 5 pg/ml (see figure 2.9.8). This resulted in about 75% of subject BAL samples presenting readable levels of IL-5.

Reproducibility for the IL-5 assay was somewhat poorer than for eotaxin. Testing the optimal assay protocol resulted in an intra-plate CV of 6.5% and inter-plate CV of 14.3%.

2.9.8 Serum ELISA Development

After development of ELSA for detection of eotaxin and IL-5 in the BAL, adapting the protocols for serum detection was quite rapid. Serum levels of IL-5 and eotaxin were found to be considerably higher than those in the BAL. This resulted in a much greater reproducibility and better sample coverage in this assay. The following table summarises the salient parameters for this assay:

	Detection range	Intra-assay CV	Inter-assay CV	Samples in range
IL-5	6.3-400ng/ml	3.2%	7.9%	100%
Eotaxin	0.4-25ng/ml	2.4%	5.6%	100%

2.10 PCR Development

2.10.1 Introduction

Detection of IL-5 and eotaxin protein in airway tissue had not been successful, whereas in the BAL, ELISA for both eotaxin and IL-5 had been very successful. It was thought that the BAL data could be supported by the quantitation of IL-5 and eotaxin mRNA extracted from either biopsies or BAL cells.

Detection of IL-5 mRNA from airway cells is not novel. BAL T-cells have been shown to express elevated levels of IL-5 mRNA in allergen challenged asthmatics (Bodey, *et al*, 1999), and biopsy activated eosinophils express elevated IL-5 mRNA also after allergen challenge. Whilst these studies are largely semi-quantitative, true quantitative work from our own group has previously suggested elevated biopsy IL-5 mRNA in asthmatics (Glare, *et al*, 2002).

Less mRNA work has been undertaken for eotaxin, being a still relatively new chemokine. It has been observed that resected lung has shown elevated eotaxin mRNA in the epithelium and inflammatory cells of asthmatic subjects (Taha, *et al*, 1999) and that eotaxin mRNA production that occurs in human airway epithelial cell cultures, was inhibited by ICS in a dose dependant fashion (Lilly, *et al*, 1997).

The primary source of IL-5 in the airways is T-lymphocytes, which exist in large quantities in the BAL, and appear in smaller numbers in biopsies. For eotaxin, airway epithelium is the primary source, a large constituent of airway biopsy samples, but in relatively small numbers in the BAL. Whilst it was hoped that both cytokines could be

detected in both biopsy and BAL, it was anticipated that eotaxin mRNA may have been difficult to detect in the BAL.

2.10.2 Competitive PCR

Previous work using competitive PCR by other members of our group had resulted in a functional quantitative IL-5 mRNA detection system (Glare, *et al*, 2002). A competitor sequence had already been constructed, consisting of primer sequences for both eotaxin and RANTES, and being of intermediate size to the two, which would function for both eotaxin and RANTES, though it was untested at very low concentrations.

Initial testing of the competitor sequence for amplification with eotaxin primers was promising (see figure 2.10.1), with the system capable of detecting sequence numbers as low as 100 copies per μl once optimised (see figure 2.10.2). As illustrated in the figure, the competitor and primers worked well, though it was of critical importance to the development of the competitive PCR system that the detected subject sample mRNA be of a concentration within an order of magnitude of the competitor. This was especially important as sample ranges of much less than 100 copies per μl would be beyond the detection range of the system. When biopsy samples were tested, an unexpected problem occurred. A band of approximately three times the size of the target eotaxin mRNA was detected in every sample and was amplified preferentially to the artificial competitor sequence (see figure 2.10.3). The results of this was that the competitor band was barely detectable and the target eotaxin mRNA could not be detected.

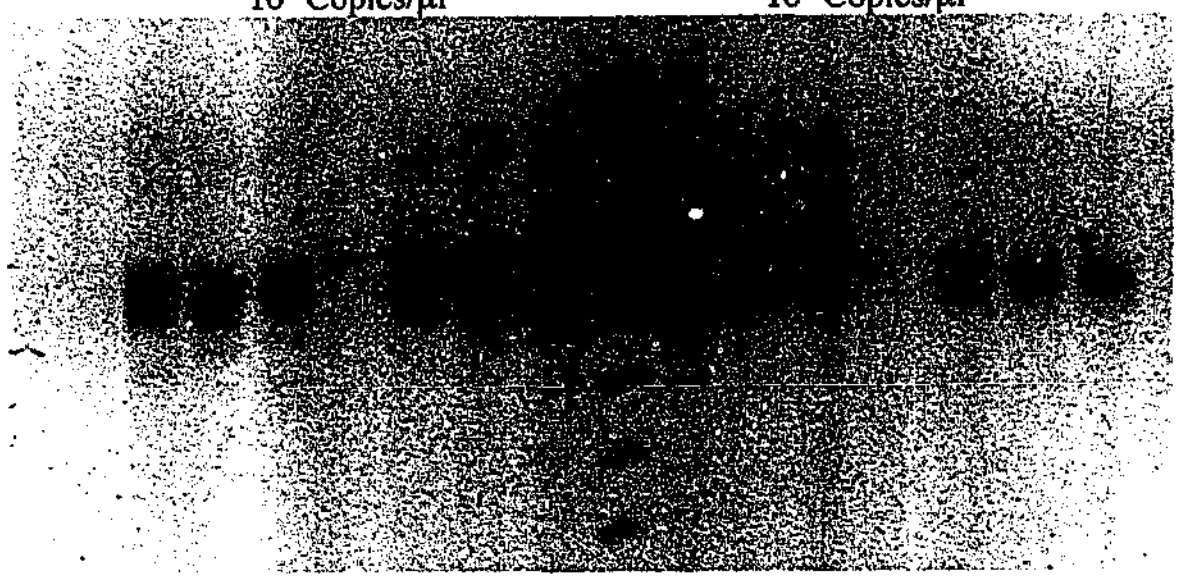
Figure 2.10.1 An agarose gel illustrating amplification of our artificial competitor sequence for use in eotaxin/RANTES competitive PCR.

Figure 2.10.2 An agarose gel illustrating amplification of our competitor sequence to maximum sensitivity (100 copies/μl).

Competitor Concentration

10^4 Copies/µl

10^3 Copies/µl



Competitor Concentration: 10^2 Copies/µl

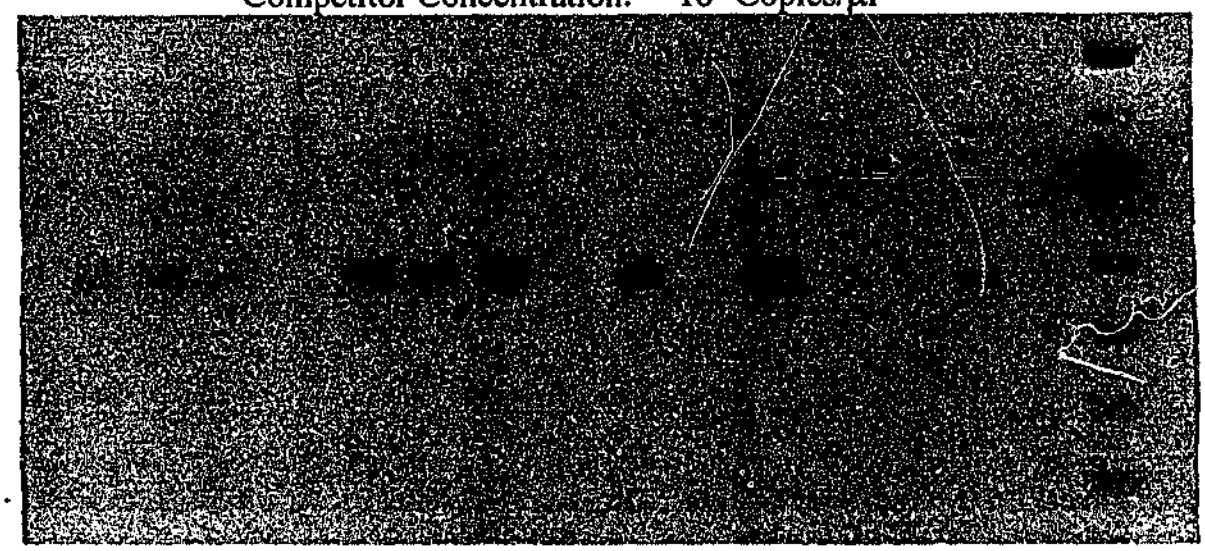
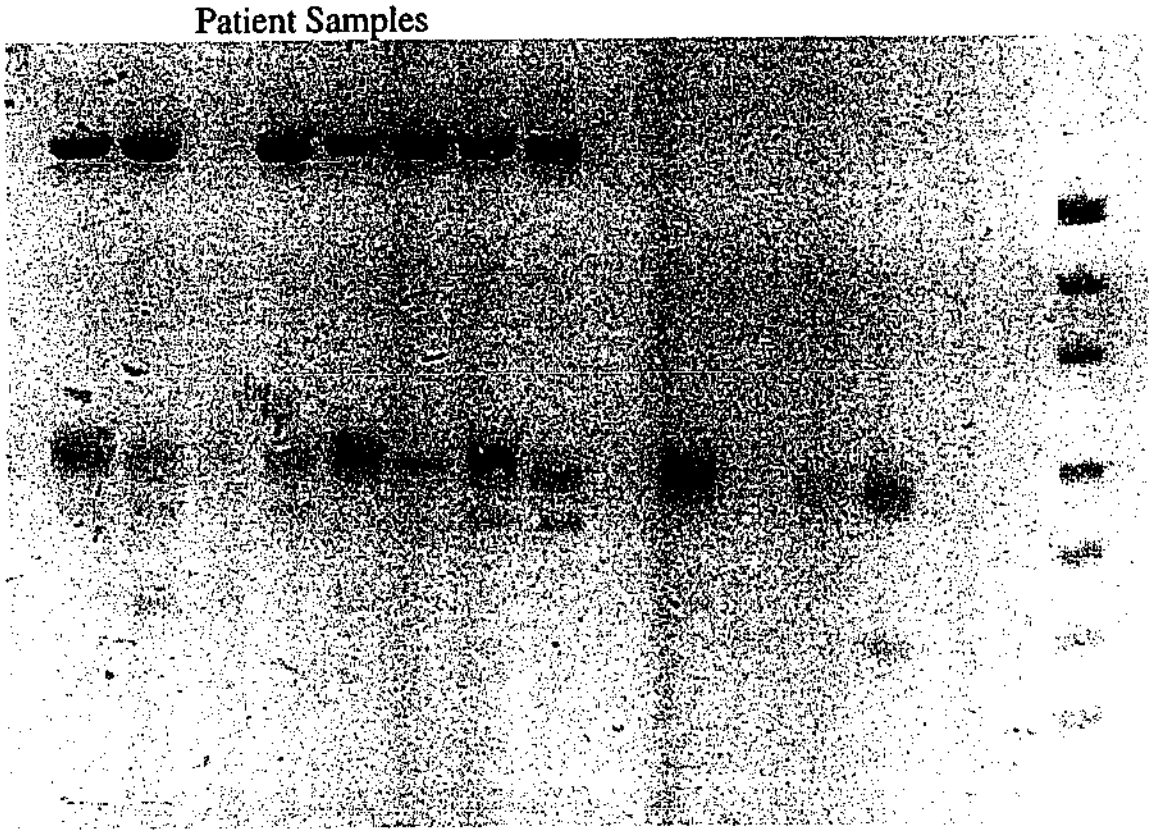


Figure 2.10.3 A patient sample run of the eotaxin competitive RT-PCR system. Note the large anomalous band that appears at the top of each patient sample.

Figure 2.10.4 A chromosomal DNA experiment. Confirming that the anomalous band seen is the patient samples originated from unknown genomic DNA. (a). competitor and genomic DNA at equal concentrations. (b). genomic DNA is excess of competitor (2:1 ratio).

Patient Samples



(a) (b)

The primers and competitor were then run with a spiked sample of human chromosomal DNA which showed the same aberrant band as seen in the samples (figure 2.10.4). As the primers did not correspond to any known DNA sequences, the only available option was to re-design the primers, shifting them by a few bases. Optimisation would need to be redone, and we still did not have a clear indication as to the viability of detecting eotaxin mRNA. With the availability of a new real time PCR machine, it was decided that the remaining developmental time would be spent on real time PCR, especially in light of commercial primers becoming available for the detection of both IL-5 and eotaxin.

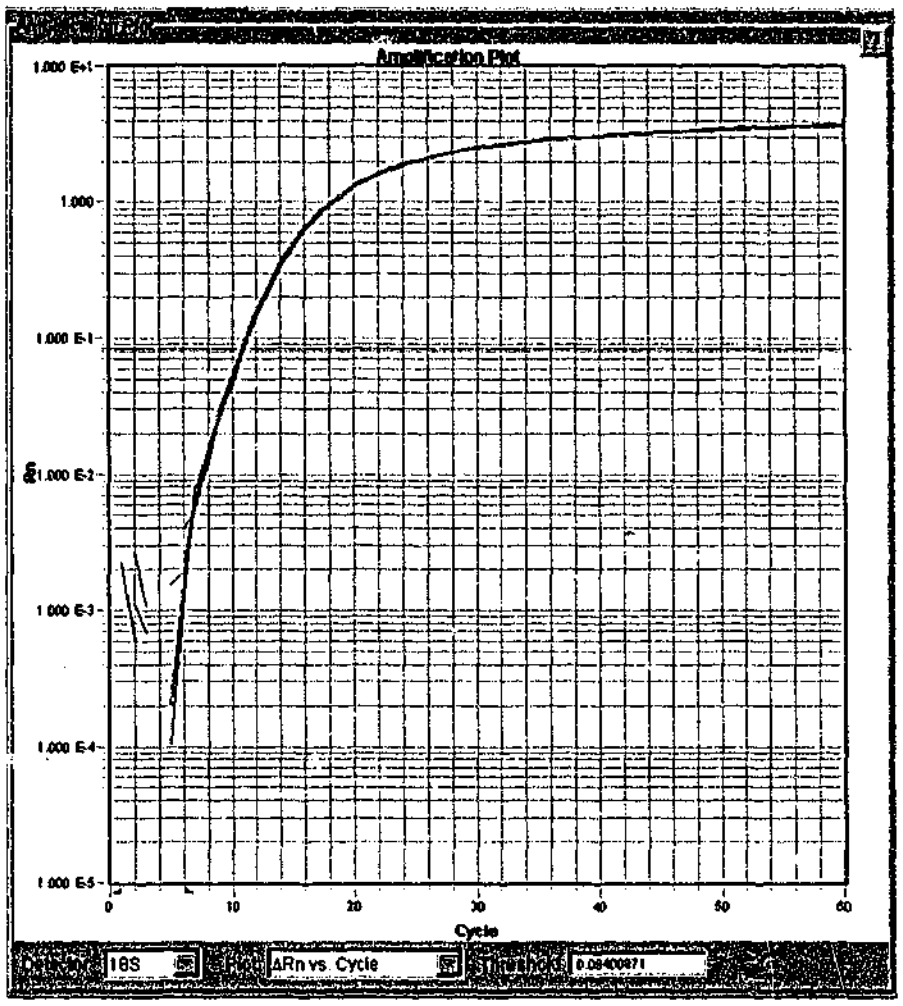
2.10.3 Real-Time PCR

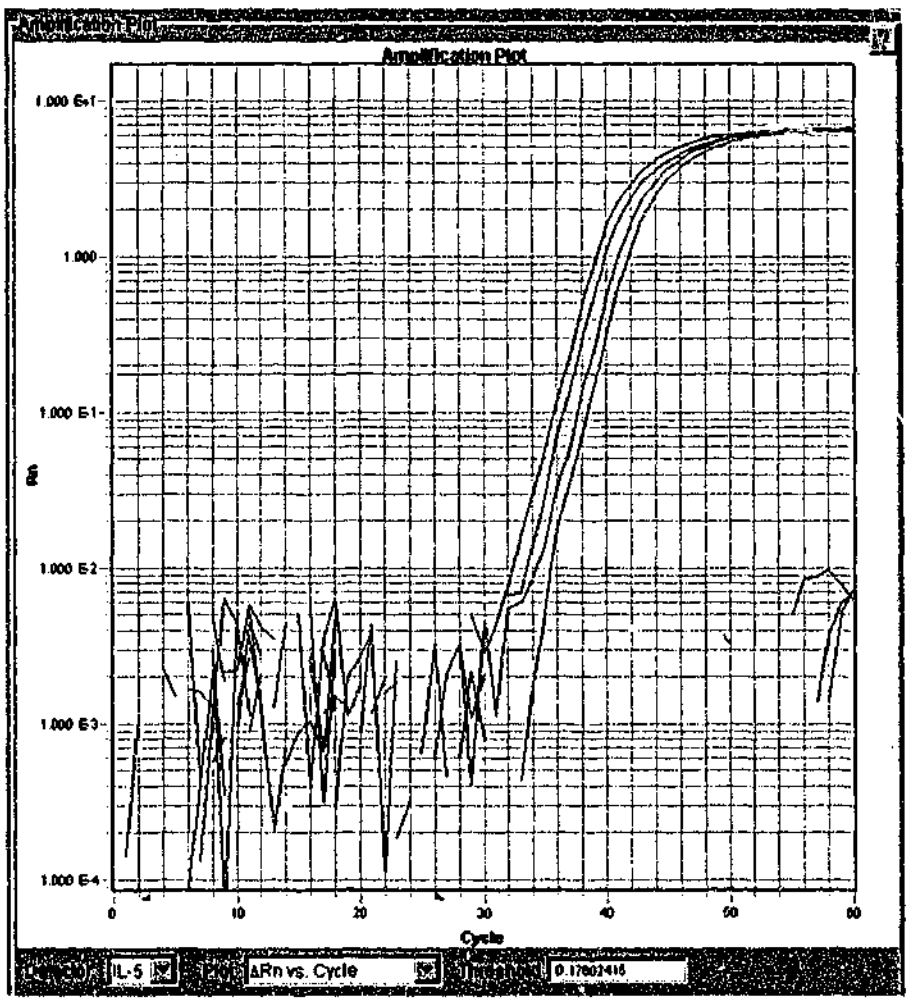
Little expertise or experience was available for the development of competitive PCR due to the novelty of the system. However, the availability of commercial primers for both cytokines, and the manufactures guarantees, promised to streamline development and speed optimisation, and it was hoped that the protocol would be functional within several weeks.

However, within the first few runs problems appeared. Though the commercial control, 18s ribosomal cDNA, performed extremely well (see figure 2.10.5), for BAL cell cDNA samples, no result was achieved for either IL-5 or eotaxin. Running the samples again with a maximal amount of cDNA (9µl of our highest sample in a 20µl reaction) for a greater number of cycles (50) still did not show any detectable levels of IL-5 or eotaxin, though 18s was still amplified consistently and proportionately.

Figure 2.10.5 A real-time PCR amplification plot showing the excellent performance of the instrument when used to measure 18s ribosomal cDNA.

Figure 2.10.6 A real-time PCR amplification plot showing the exceedingly poor performance of the instrument when used to measure IL-5 cDNA past 35 cycles.


Amplification Plot
1.000 E+1
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
1.000 E-5
Rn
0
10
20
30
40
50
60
Cycle
18S
ARn vs. Cycle


Amplification Plot
Amplification Plot
1.000 E+1
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
Rn
0
10
20
30
40
50
60
Cycle
IL-5
ΔRn vs. Cycle

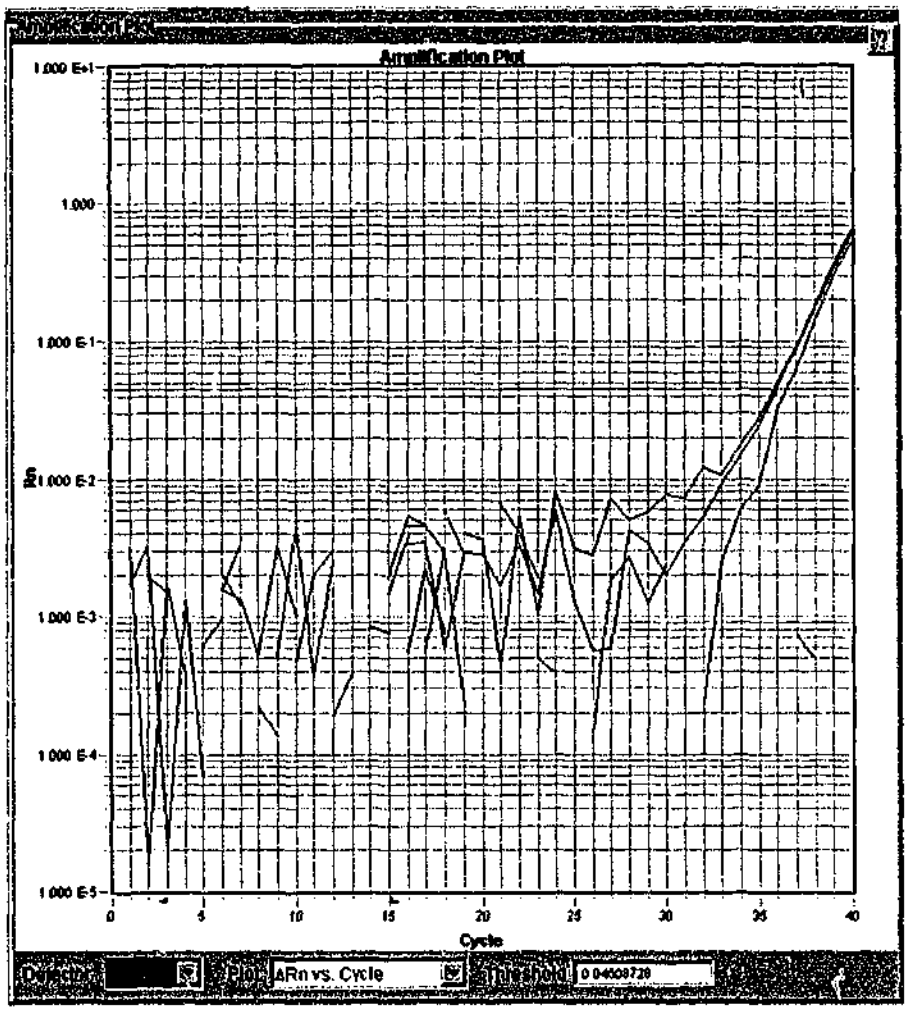
As BAL produced no solid results, mRNA extracted from biopsies was tried next, again, using maximal amounts of cDNA. This time some results were achieved for IL-5, with amplification occurring at about 35 cycles (see figure 2.10.6), but again, eotaxin was undetectable. Unfortunately, as illustrated in the figure, the triplicate reproducibility at this late cycle number was very poor, and despite some effort to resolve the problem, including extensive liasing with the manufacturer, no solution was presented within the time and budgetary constraints, despite a great deal of work.

Thus, the eotaxin primer/probe sequences had still been unable to show any results. As a final effort to verify if the primers were functional, a high eotaxin-expressing tissue sample, human endometrium (Blumenthal, *et al*, 2000), was used. The results achieved in this tissue type were very similar to IL-5 in biopsies, with eotaxin mRNA being amplified at around 35 cycles (see figure 2.10.7). Again, as it was for IL-5, the reproducibility and thus value for quantitation even in this tissue for eotaxin was very poor. The inability of the real time PCR instrument to accurately quantiate past 32 cycles does not appear to be specifically related to the primers used in amplification.

2.10.4 Conclusions

The lack of success in the real time PCR system may have been overcome with further work. We did not attempt to move beyond the manufacturers "optimised" protocols that were supposedly validated for IL-5 and eotaxin PDARs (pre-developed assay reagents), nor try to build our own primers. The development process was hampered by inexperience with the equipment we had only recently acquired, and perhaps a level of naivety in following the manufacturers recommendations. The development and trial process also proved to be far more expensive than predicted,

Figure 2.10.7 A real-time PCR amplification plot showing the poor performance of the instrument past 35 cycles, this time for eotaxin cDNA.


Amplification Plot
1.000 E+1
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
1.000 E-5
ΔRn
0
5
10
15
20
25
30
35
40
Cycle
Plot
ΔRn vs. Cycle
Threshold
0.04509729

due to the suspiciously costly proprietary reagents. Whilst achieving a quantitative eotaxin mRNA method using the real time system may be impossible, with a further investment of time and resources it may at least be possible to accurately quantitate IL-5 mRNA in the immediate future.

2.11 Statistical Methods

Statistical analysis was performed using Graphpad Prism (v3.0, Graphpad Software, USA). Data distributions throughout this thesis were tested for normality using the Kolmogorov-Smirnov (KS) test, followed by the Dallal and Wilkinson approximation to Lilliefors' method to obtain a p value. Parametric distributions were analysed with t tests for comparisons of two groups and with analysis of variance (ANOVA) followed by the Tukey posttest for multiple groups. Correlations were assessed using Pearson's test. For non-parametric data, Mann-Whitney or Kruskal-Wallis followed by Dunn's post-test was used for two or multiple comparisons respectively. Non-parametric correlations were assessed using Spearman's test.

Data in the results chapters were expressed as the mean ± the standard deviation, if parametric, and the median and 25 and 75 percentile ranges if non-parametric. Note that for parametric data this method produces much larger ranges than using standard errors, but provide a better indication of the actual distribution of the data.

Chapter 3: A Cross-sectional Analysis of Asthmatic Patients and Non-asthmatic Controls, both ICS Treated an Untreated

3.1 Introduction

Whilst this project is primarily concerned with the interaction between ICS, eosinophils, IL-5 and eotaxin, a large amount of relevant data can be acquired potentially by comparing cross-sectionally ICS treated subjects with ICS free asthmatics and contrasting these with non-asthmatic subjects. One of the primary advantages of this approach was the availability of a large number of subjects from multiple studies occurring within the department provide a vast quantity of material for this thesis. As each of these studies involved a different ICS treatment regime, the data from each subject can be compared in a numerically strong cross-sectional study. However, each subject appears only once, either under baseline treatment or when undergoing intervention treatment (In all cases an attempt was made to match levels of asthma activity, symptoms and use of reliever medication).

When examining cytokines and eosinophils in ICS treated asthmatic subjects, there are two important controls useful in both assessing changes that occur with ICS usage and establishment of a baseline from which to compare measured factors. ICS-free asthmatic subjects provide levels of eosinophils and cytokines representative of the inflamed asthmatic airways in an unsuppressed state. These subjects should have the highest level of inflammation that we would expect to see from our population of mild asthmatics, and if ICS were affecting the cytokines and cells measured, we would expect a reduction from the levels observed in this group. At the other extreme, the

non-asthmatic subjects provide a picture of the uninflamed airways, and we would expect cytokine and cellular levels in these subjects to be the lowest in the study.

Even asthmatic patients treated with relatively high levels of ICS have been demonstrated to retain a degree of inflammation in some subjects (Herwerden, *et al*, 1995), we would therefore expect that subject treated with low ICS doses would have a level of eosinophils and cytokines that lies between untreated asthmatics and non-asthmatic control subjects. For those receiving high ICS doses the difference should be further from untreated asthmatics and closer to non-asthmatics. If the ICS are not affecting the cells and cytokines in a clear dose-dependant fashion, suppression should still be observed, but there will be little difference between the high and low ICS treated subjects. There is currently no *in vivo* work examining the effects of ICS dosage on eosinophilic cytokine levels in human subjects, though *in vitro* cell culture work suggests dose-response suppression of IL-5 (Rolfe, *et al*, 1992) and eotaxin (Lilly, *et al*, 1997).

This chapter contains a comparison of the subjects recruited for the three studies in the following chapters. The subjects have been divided into ICS dosage groups and compared to data from non-asthmatic subjects. Eosinophils in both BAL and biopsy were assessed and compared with IL-5 and eotaxin protein levels from the BAL.

3.2 Materials and Methods

The procedures used in this study follow the protocols presented in chapter 2, with the following exceptions and clarifications.

3.2.1 Patients and Bronchoscopy

Patient recruitment and bronchoscopy followed the guidelines given in sections 2.2.1 and 2.2.2. While this data set was drawn from multiple studies, biopsy data was acquired in all cases from bronchial biopsies processed into formalin fixed paraffin blocks (see 2.2.4) in identical fashion.

The subject demographics were as follows:

	ICS Free asthmatics	Low ICS (<1000µg BDP) treated asthmatics	High ICS (>1000µg BDP) treated asthmatics	Non-asthmatic control subjects
Number of patients	35	27	53	24
Male/Female	22/13	12/15	32/21	15/9
Age (Years)	39±19	36±15	38±12	37±13
# of Atopics	35	27	48	12

3.2.2 BAL Differential Cell Counts

Differential cells counts of BAL cells (see 2.5.1) were performed for each subject. Eosinophils, neutrophils, lymphocytes, macrophages and airway epithelial cells were counted, with results expressed as percentages.

3.2.3 BAL ELISA for IL-5 and Eotaxin

A portion of the stored, frozen (at -80°C) BAL supernatants were thawed and used for ELISA (see 2.4.2) for both IL-5 and eotaxin for each subject.

3.2.4 Immunohistochemistry for Eosinophils

Paraffin embedded biopsies (see 2.2.4) were cut and stained for both total (EG1) and activated (EG2) eosinophils (see 2.3.1). Eosinophils were quantitated using computer image analysis and counts expressed per area of lamina propria and per mm of basement membrane (see 2.5.2).

3.3 Results

These analyses contain data from 139 subjects. Care has been taken to ensure that individuals are only included once in this cross-sectional analysis, even if alterations in treatment resulted in the subject being eligible for multiple groups.

3.3.1 BAL Cellular Differences in Asthmatics vs Controls

The following table summarises the average cell numbers calculated from differential cell counts performed on the BAL samples from each subject. Significant levels of differences between the groups, summarised below the table, were calculated by ANOVA or Kruskal-Wallis testing (depending on the normality of the data), followed by appropriate post-testing for each group.

Differential Cell count (%)	ICS-Free asthmatics	Low ICS (<1000µg BDP) treated asthmatics	High ICS (>1000µg BDP) treated asthmatics	Non-asthmatic control subjects
Macrophages	70.1±8.9	71.9±14.5	75.6±17.1	80.3±13.0
Lymphocytes	20.2±9.7	16.0 [12.2-24.8]	20.6±16.6	16.3±12.4
Neutrophils	2.2±1.6	1.5 [0.9-2.7]	2.2±1.8	2.1±2.0
Eosinophils	1.8 [0.8-4.4]	1.3 [0.3-3.7]	0.9±0.8	1.0 [0.3-1.2]
Epithelial Cells	4.0±3.7	0.9 [0.4-2.7]	0.0 [0.0-0.0]	0.0 [0.0-0.0]

Significance (Post-analyses were only done for significant distributions, non-significant post-tests are not shown):

Macrophages:	ANOVA:	**p=0.04**:
	ICS free vs non-asthmatic,	**p<0.05**
Lymphocytes:	Kruskal-Wallis test:	p=ns (0.21)
Neutrophils:	Kruskal-Wallis test:	p=ns (0.86)
Eosinophils:	Kruskal-Wallis test:	p=ns (0.08)
Epithelial cells:	Kruskal-Wallis test:	**p<0.0001**:
	ICS free vs non-asthmatic,	**p<0.001**
	ICS free vs high ICS,	**p<0.001**
	Low ICS vs non-asthmatic,	**p<0.001**
	Low ICS vs high ICS,	**p<0.001**

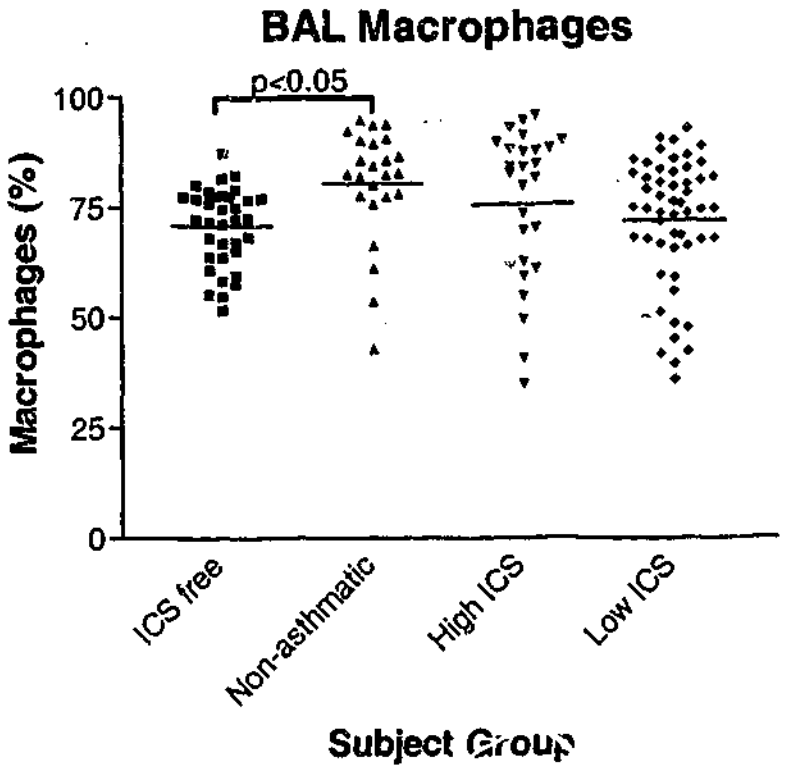
Figures 3.1 and 3.2 illustrate the significant macrophage and non-significant, but trending, eosinophil BAL cell counts respectively. The airway epithelial cells were

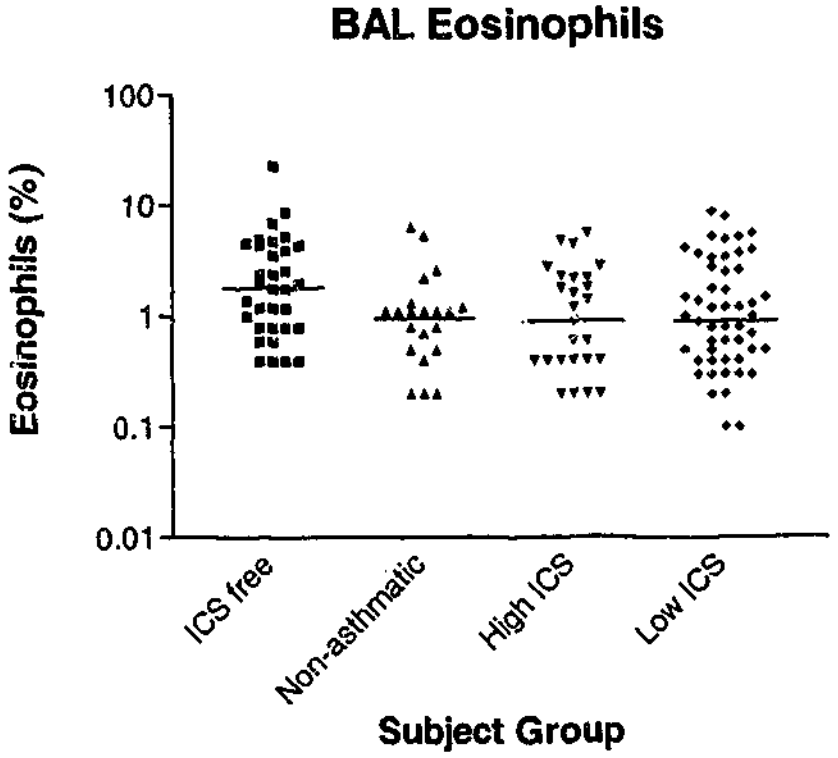
Figure 3.1 Percentage macrophages in the BAL fluid of ICS-free asthmatic subjects, ICS treated asthmatic subjects and non-asthmatic control subjects.

Figure 3.2 Percentage eosinophils in the BAL fluid of ICS-free asthmatic subjects, ICS treated asthmatic subjects and non-asthmatic control subjects. There were no significant differences between any subject groups.

subjects,

subjects,
were no





significantly different, but the comparison with the ICS free asthmatics (zero) does not warrant illustration.

3.3.2 Levels of Eotaxin and IL-5 using ELISA

BAL IL-5 and eotaxin were measured in each cross sectional group. Significant differences were observed in IL-5 between asthmatic subjects not treated with ICS and all other groups (see below). The greatest difference observed was between IL-5 levels in the ICS-free asthmatics and non-asthmatic control subjects ($p<0.001$). A similar magnitude of difference was seen in BAL eotaxin between the same groups ($p<0.001$). Whilst there was a significant elevation in BAL eotaxin in ICS free asthmatics when compared to high ICS treated asthmatics ($p<0.01$), with no difference between ICS free asthmatics and low ICS treated asthmatics. Eotaxin in low ICS treated asthmatics was higher than in non-asthmatic controls ($p<0.05$). These data and significant differences are summarised below:

	ICS Free asthmatics	Low ICS (<1000μg BDP) treated asthmatics	High ICS (>1000μg BDP) treated asthmatics	Non-asthmatic control subjects
BAL IL-5 (pg/ml)	9.0 [6.3-31.1]	3.2 [1.0-13.6]	2.3 [0.9-71.3]	0.9 [0.1-3.6]
BAL Eotaxin (pg/ml)	10.5 [5.3-18.4]	4.1 [1.4-25.6]	2.1 [0.9-5.2]	0.9 [0.4-5.7]

Significance: (Post-analyses were only done for significant distributions, non-significant post-tests are not shown):

BAL IL-5: Kruskal-Wallis test: **$p<0.0001$**
ICS-free vs Non-asthmatic, **$p<0.001$**
ICS-free vs High ICS, **$p<0.05$**
ICS-free vs Low ICS, **$p<0.01$**

BAL Eotaxin: Kruskal-Wallis test: **$p<0.0001$**
ICS-free vs Non-asthmatic, **$p<0.001$**
ICS-free vs High ICS, **$p<0.01$**
Low ICS vs normal, **$p<0.05$**

BAL IL-5 and eotaxin in each group, along with the significant differences are illustrated in figures 3.3 and 3.4 respectively.

3.3.3 Biopsy Eosinophil Numbers

Image analysis was used to quantitate both total (EG1) and activated (EG2) eosinophils in the endobronchial biopsies of all asthmatic subjects. Total eosinophils were found to be greatly elevated in ICS untreated asthmatics compared to both asthmatics treated with high ICS doses ($p<0.001$) and those treated with low doses of ICS ($p<0.001$). The same pattern was observed for activated eosinophils (see below). In subjects treated with either low or high ICS doses, no differences were observed in the numbers of total or activated biopsy eosinophils. ICS-free asthmatics were significantly higher than non-asthmatic subjects for both total ($p<0.001$) and activated ($p<0.01$) eosinophils. The table below summarises these observations:

	ICS-Free asthmatics	Low ICS (<1000µg BDP) treated asthmatics	High ICS (>1000µg BDP) treated asthmatics	Non-asthmatic control subjects[1]
Total Eosinophils (cells/mm^2)	415.2±450.0	165.0±145.3	71.55±52.85	35.2±29.5
Activated Eosinophils (cells/mm2)	200.3±218.2	45.3 [23.3-111.3]	56.53±43.62	30.9±36.0

1. These subjects comprise the non-atopic portion of the non-asthmatic subjects only (n=10)

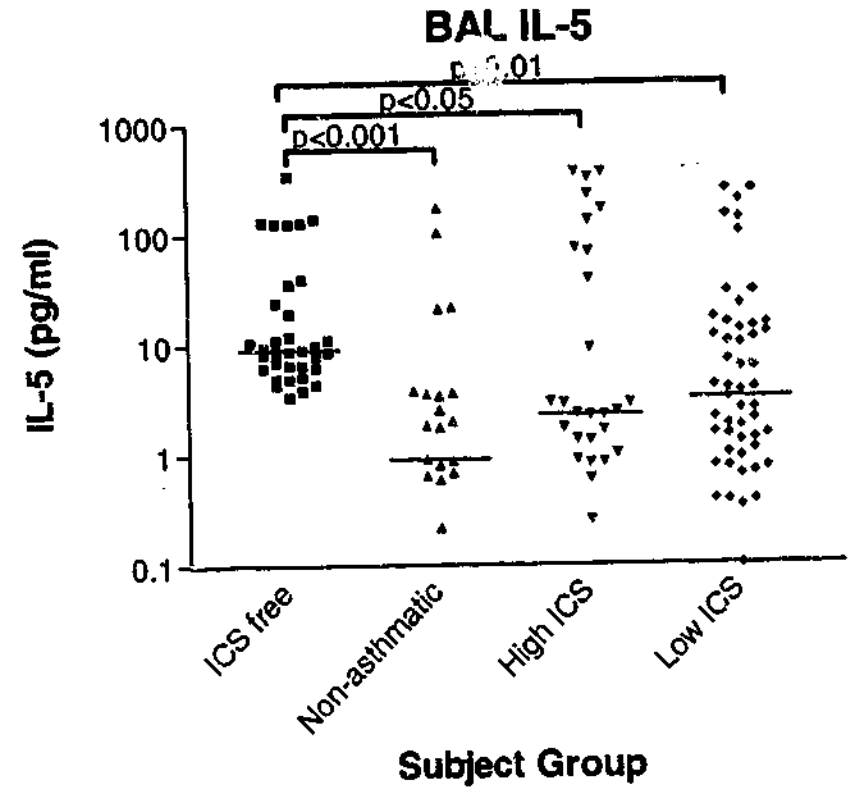
Significance (Post-analyses were only done for significant distributions, non-significant post-tests are not shown):

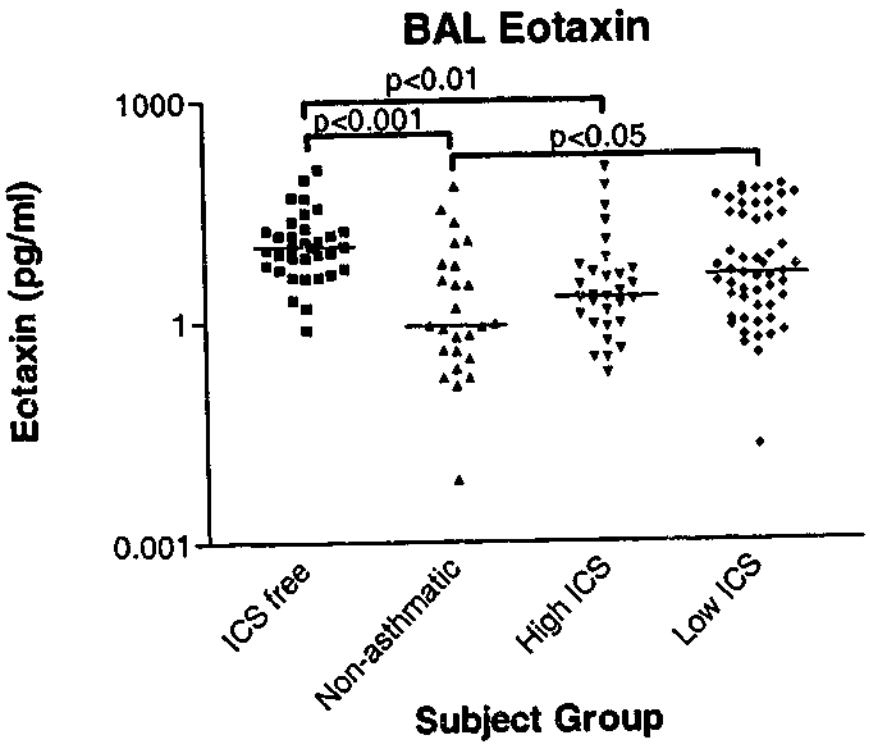
Total Eosinophils: ANOVA: **p<0.0001**
ICS-free vs Low ICS, **p<0.001**
ICS-free vs High ICS, **p<0.001**
ICS-free vs Non-asthmatics, **p<0.001**

Activated Eosinophils: Kruskal-Wallis test: **p=0.003**
ICS-free vs Low ICS, **p<0.05**
ICS-free vs High ICS, **p<0.05**
ICS-free vs Non-asthmatics, **p<0.01**

Figure 3.3 IL-5 protein measured from the BAL supernatant in ICS-free asthmatic subjects, ICS treated asthmatic subjects and non-asthmatic control subjects. Only significant differences are shown.

Figure 3.4 Eotaxin protein measured from the BAL supernatant in ICS-free asthmatic subjects, ICS treated asthmatic subjects and non-asthmatic control subjects.


BAL IL-5
p<0.01
p<0.05
p<0.001
1000
100
10
1
0.1
IL-5 (pg/ml)
ICS free
Non-asthmatic
High ICS
Low ICS
Subject Group


BAL Eotaxin
p<0.01
p<0.001
p<0.05
1000
1
0.001
Ectaxin (pg/ml)
ICS free
Non-asthmatic
High ICS
Low ICS
Subject Group

The above data are illustrated for total eosinophils in figure 3.5 and activated eosinophils in figure 3.6.

3.3.4 Cross-Sectional Correlation Comparisons

Correlation statistics for the ICS-free asthmatic subjects are given in chapter 5, high and low ICS treated asthmatics are presented in chapters 4 and 6 respectively. There were a small number of significant correlations within the non-asthmatic subject group, which are summarised below:

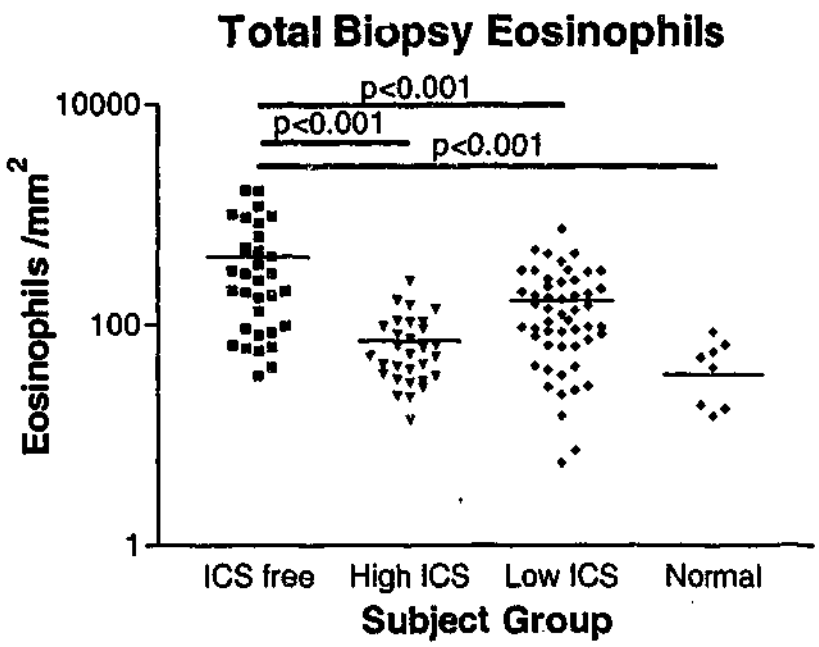
BAL eosinophils were related to BAL eotaxin ($r=0.78$, $p=0.003$), an unexpected correlation considering the relatively low levels of eosinophils present in the non-asthmatic subjects (see figure 3.7).

BAL eotaxin levels were related to serum eotaxin ($r=0.83$, $p=0.002$) and serum IL-5 ($r=0.92$, $p<0.0001$, see figure 3.8) levels. In serum, these two cytokines were themselves highly correlated ($r=0.92$, $p<0.0001$).

A correlation that was not observed (but was otherwise present in all other examined asthmatic populations) was the usually strong relationship between total and activated eosinophils in airway biopsies ($r=0.39$, $p=ns$).

Figure 3.5 Total eosinophils (EG1) measured from airway biopsies in ICS-free asthmatic subjects, ICS treated asthmatic subjects and non-asthmatic control subjects.

Figure 3.6 Activated eosinophils (EG2) measured from airway biopsies in ICS-free asthmatic subjects, ICS treated asthmatic subjects and non-asthmatic control subjects.


Total Biopsy Eosinophils
p<0.001
p<0.001
p<0.001
10000
100
1
Eosinophils /mm2
ICS free
High ICS
Low ICS
Normal
Subject Group

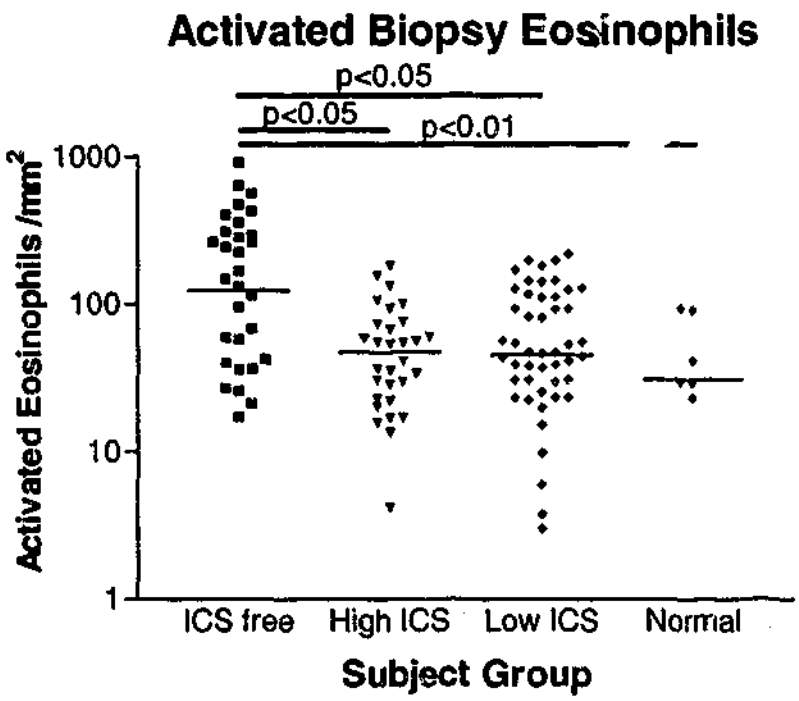
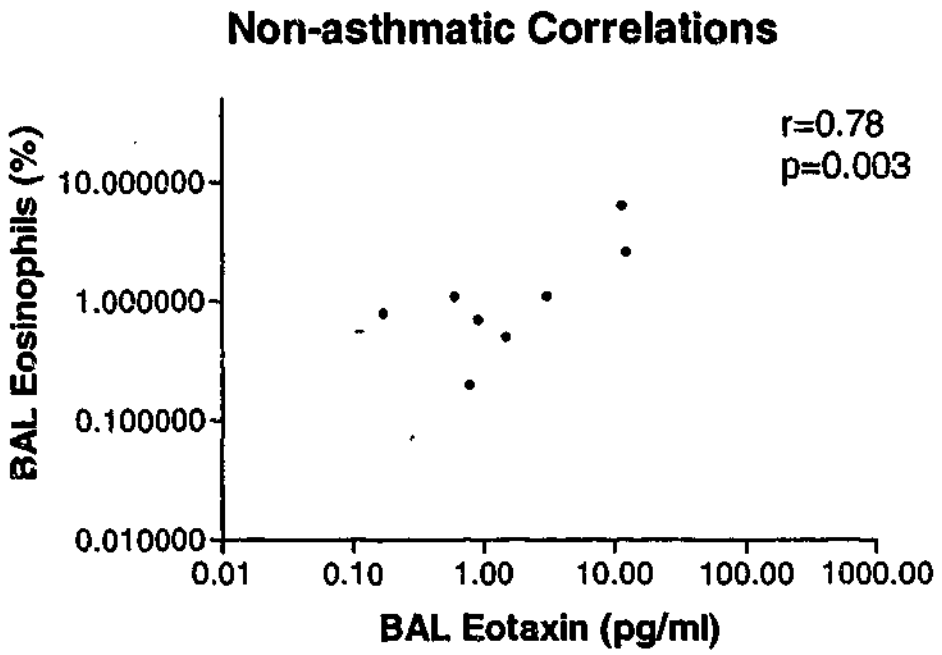

Activated Biopsy Eosinophils
p<0.05
p<0.05
p<0.01
1000
100
10
1
Activated Eosinophils /mm2
ICS free
High ICS
Low ICS
Normal
Subject Group

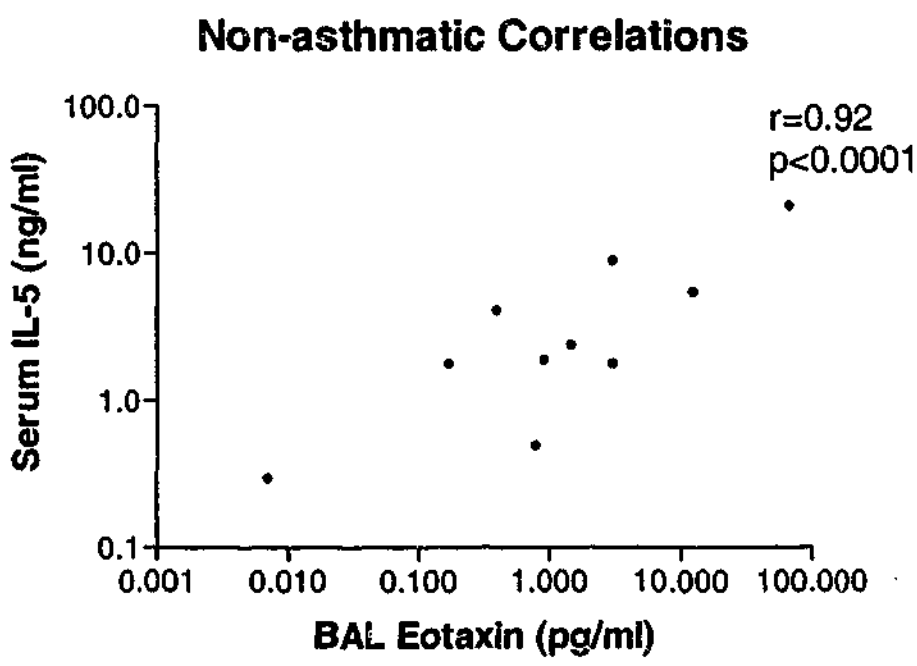
Figure 3.7 A positive relationship between BAL eotaxin and BAL eosinophils, observed in non-asthmatic control subjects.

Figure 3.8 A positive relationship between BAL eotaxin and serum IL-5, observed in non-asthmatic control subjects.

eosinophils,

observed in





3.4 Discussion

As discussed in the introduction, this chapter sought to establish two separate "controls" to which data from the ICS studies given in subsequent chapters could be compared, namely: ICS-free asthmatics and normal control, non-asthmatic subjects. The selection of these particular ICS-free asthmatics requires some further explanation. These ICS-free asthmatics subjects, at one extreme of our representative inflammatory spectrum, while symptomatic, were still considered mild in the range of asthma severity. Thus, the resolution between the inflammation in these untreated asthmatics and ICS treated subjects contained greater overlap than would be observed in cases of more severe nature. However, ethically, these asthmatic subjects whose condition was relatively stable are the most suited for studies involving manipulation of ICS treatment levels. i.e. They had similar symptoms, but were well and stable enough to be recruited for a bronchoscopic study.

In the BAL cell profiles, there was not quite a significant difference in eosinophil numbers across the four subjects groups ($p=0.08$). Without the intermediate ICS treated asthmatic subjects, the difference between normal controls and ICS-free asthmatics becomes significant ($p=0.02$), which may be an appropriate statistical interpretation considering the data is drawn from independent samplings. Since it has been suggested that eosinophil clearance may occur via eosinophil trafficking through the airway lumen (Erjefalt, *et al*, 2000), it might be expected that BAL eosinophils in ICS treated subjects would rise under these conditions, but without any research on the kinetics of such a model, speculation into the eosinophilic content of the BAL could be misleading.

The lower percentage of BAL macrophages in ICS-free asthmatics clearly illustrates that the overall number of inflammatory cells is elevated in asthmatic subjects. ICS treated asthmatics demonstrate intermediate levels of inflammation. The most drastic change across the BAL profiles was the much lower numbers of epithelial cells present in high-ICS treated asthmatics and normal controls when compared with low-ICS treated and steroid-free asthmatics. There has been interest for some time into the integrity of the bronchial epithelium in asthma, while some recent work suggests actual pathologic fragility in the epithelium of asthmatic subjects (Bucchieri, *et al*, 2002), others still suggest that epithelial shedding is a tissue staining artefact (Ordonez, *et al*, 2001). These are, of course, not necessarily mutually exclusive. Whilst we did not conduct a detailed analysis of biopsy epithelial integrity for this project, the BAL data, which is not subject to staining or processing artefact, strongly indicates increased epithelial shedding in ICS-free and also in low-ICS treated asthmatic subjects.

The measured BAL cytokines, eotaxin and IL-5, were both barely detectable in non-asthmatic subjects and at their highest levels in untreated asthmatics. The BAL IL-5 appeared more responsive to ICS, with both high and low-ICS treated groups showing lowered levels of protein relative to ICS-free asthmatics. Whilst high levels of ICS did seem to normalise BAL eotaxin, patients receiving low ICS doses had levels that were not significantly different to ICS-free subjects and still elevated above normal controls. The measured levels of BAL IL-5 and eotaxin in this study appear consistent with the findings of others, who have reported asthmatic BAL IL-5 levels at 3.0pg/ml (Teran, *et* al, 1999) and eotaxin levels at 25pg/ml (Lilly, *et al*, 2001). While these results vary somewhat from our measured levels of 9pg/ml and 11pg/ml in BAL IL-5

and eotaxin respectively, these levels are of similar magnitude and the differences are most likely due to BAL dilution variations and specific antibody sensitivities. Whilst allergen challenge models are common, studies examining BAL IL-5 and eotaxin in the context of ICS therapy, even cross-sectionally, are exceptionally rare. Thus, it is difficult to compare our BAL cytokine findings in the ICS treated subjects with those of other groups. However, with our own ICS-free asthmatics and non-asthmatic controls as benchmarks, the data from the ICS treated subjects appear consistent, biologically relevant and plausible.

The biopsy eosinophil data was as expected and consistent with published results (Bentley, *et al*, 1992). The ICS-free asthmatic subjects have a large number of tissue eosinophils, whilst those receiving ICS have lower tissue eosinophils, which seem to be reduced in a dose-dependant manner. The degree of eosinophilic inflammation is certainly much lower in high-ICS treated subjects, but does remain higher than non-asthmatic subjects, which is consistent with findings that ICS treatment does not necessarily completely abate eosinophil accumulation (Bootsma, *et al*, 1998), at least in still symptomatic individuals.

The activated eosinophils show a similar pattern to total eosinophils. However, the ratio of total to activated eosinophils changes dramatically across the groups. In ICS-free asthmatics an average of 53±7% (SEM) total eosinophils were activated whereas (perhaps paradoxically) 70±13% of eosinophils were activated in non-asthmatic subjects. Steroid treated subjects were similarly polarised, with low-ICS treated subjects showing 49±4% eosinophil activation and high-ICS treated subjects having 74±5% activated eosinophils. While many studies indicate increases in both total and

activated eosinophils in asthma, there is little data on the ratio between the two. Some recent work does suggest the terminal differentiation of eosinophils may occur at inflammatory sites within the airways (Cameron, *et al*, 2000). This may suggest that the observed increased number of inactivated eosinophils may, in active untreated asthma, in fact represent an influx of immature eosinophils. These are novel findings, and more work is necessary on the dynamics of eosinophil movement into and through the airways.

As both the ICS-treated and ICS-free asthmatic data has been drawn from the baseline of the longitudinal studies undertaken in this project, correlation analysis of those subjects has been restricted to the chapters in which those subjects appear, in order to avoid repetition. The non-asthmatic subjects, as a control group, appear solely cross-sectionally in this chapter, but serve as a valuable benchmark for the longitudinal work that follows. Direct quantitative relationships between BAL eotaxin and BAL eosinophil numbers have not been previously demonstrated. That this particular relationship was observed even in non-asthmatic subjects was surprising due to the relatively low percentage of eosinophils found in these subjects. We may have also expected a corresponding relationship between BAL eosinophils and BAL IL-5, but the slightly poorer sensitivity of the BAL IL-5 ELISA may have caused the barely detectable IL-5 levels in the non-asthmatic subjects to be less reliable. This sensitivity limitation for BAL IL-5 is also demonstrated by the strong relationship between serum eotaxin and serum IL-5, which were themselves related to BAL eotaxin but not BAL IL-5. Whether these serum cytokines have any relevance to the clinical state of the airways depends greatly on these correlations still being present in the inflamed

airways, whilst other serum eosinophilic markers such as ECP and EG2 seems to have some predictive value for bronchial hyperreactivity (Roquet, *et al*, 1996).

Interestingly, in the non-asthmatic subjects, biopsy eosinophils could not be related to any of these pro-inflammatory cytokines, nor could total eosinophils be related to activated eosinophils. Much like IL-5, the measured levels of eosinophils in the non-asthmatic airways were extremely low (35.2±29.5 cells/mm^2, or 8±7 cells/field). The potential for sampling error could have considerably hampered accurate representation of airway eosinophils. Statistically this particular measure, being only a sub-set of the non-asthmatic group (n=10), is weaker in power and has less available pairing than the other measures taken in non-asthmatic subjects.

The data in this chapter was drawn from a large set (total n=139), which has strong statistical power. There are however, several sources of potential error that require mentioning. The subject samples in each group were drawn from different studies, and though randomised within their native studies, cannot be randomised relative to each other. While every effort has been made to standardise protocols throughout the studies, it was difficult to assess any potential batching effect when pooling the data. Likewise, freezer storage time was consistent within a single study, but varied considerably across the different studies. The steroid treated subjects are the least susceptible to these sources of error, being drawn from several studies. It is unfortunate that the ICS-free subjects could not be re-run randomised to the non-asthmatic subjects, but the quantities and relative rarity (and thus preciousness) of the ICS-free subject samples dictated otherwise. Fortunately, despite these potential

confounders, the data in this cross-sectional context was plausible, consistent with the literature, and biologically relevant.

In summary, this section of the project was successful in establishing baselines for IL-5, eotaxin and eosinophil levels in ICS-free asthmatics, and non-asthmatic control subjects. It has verified the literature supporting elevated IL-5, eotaxin and eosinophils levels in untreated asthmatics. It has also been demonstrated that these cells and cytokine levels are lower by varying degrees in ICS-treated asthmatics, though not completely normalised. A change in the ratio of activated to total eosinophils was also observed and strong correlations seen in non-asthmatic subjects between BAL eotaxin and eosinophils and between serum IL-5 and eotaxin. Taken together, these observations formed a solid base to which the longitudinal studies in the following chapters will be related.

Chapter 4: A Steroid Withdrawal Study

4.1 Introduction

Steroid treatment can be of great benefit to asthmatic patients. The advent of modern ICS therapy has greatly improved the clinical benefits gained through steroid treatment whilst removing many of the systemic side effects that occurred with oral steroid use. ICS treatment is, however, far from perfect. There are still concerns regarding the potential side effects of long-term treatment with high doses causing systemic absorption of the drugs. These may include bone resorption leading to osteoporosis (Allen, 2002) and growth interference in children (Wong, *et al*, 2002). In a small selection of patients steroid treatment is simply ineffective, the reasons for which appear to be largely genetic and involve polymorphisms in the glucocorticoid receptor gene (Lane, *et al*, 1997). But the problem that affects all asthmatics on long term ICS treatment is that once treatment is withdrawn the symptoms of asthma tend to recur. Regardless of the dosage or duration of treatment, once ICS are effectively gone from the airways the patient's asthma often returns to its previous untreated state.

It is unclear why glucocorticoids (GCs) are only effective at suppressing, rather than resolving the asthmatic response. GCs act primarily on genomic binding sequences (see 1.5.1) to suppress particular genes whilst upregulating others. In particular, genes strongly associated with asthma, such as IL-5 and TNF-α, are downregulated (Rolfe, *et al*, 1992), though work directly measuring such effects in the airways is very rare. This reduction in the degree of inflammatory cytokine expression is the most likely cause of the normalisation of eosinophil numbers as well as other inflammatory cells

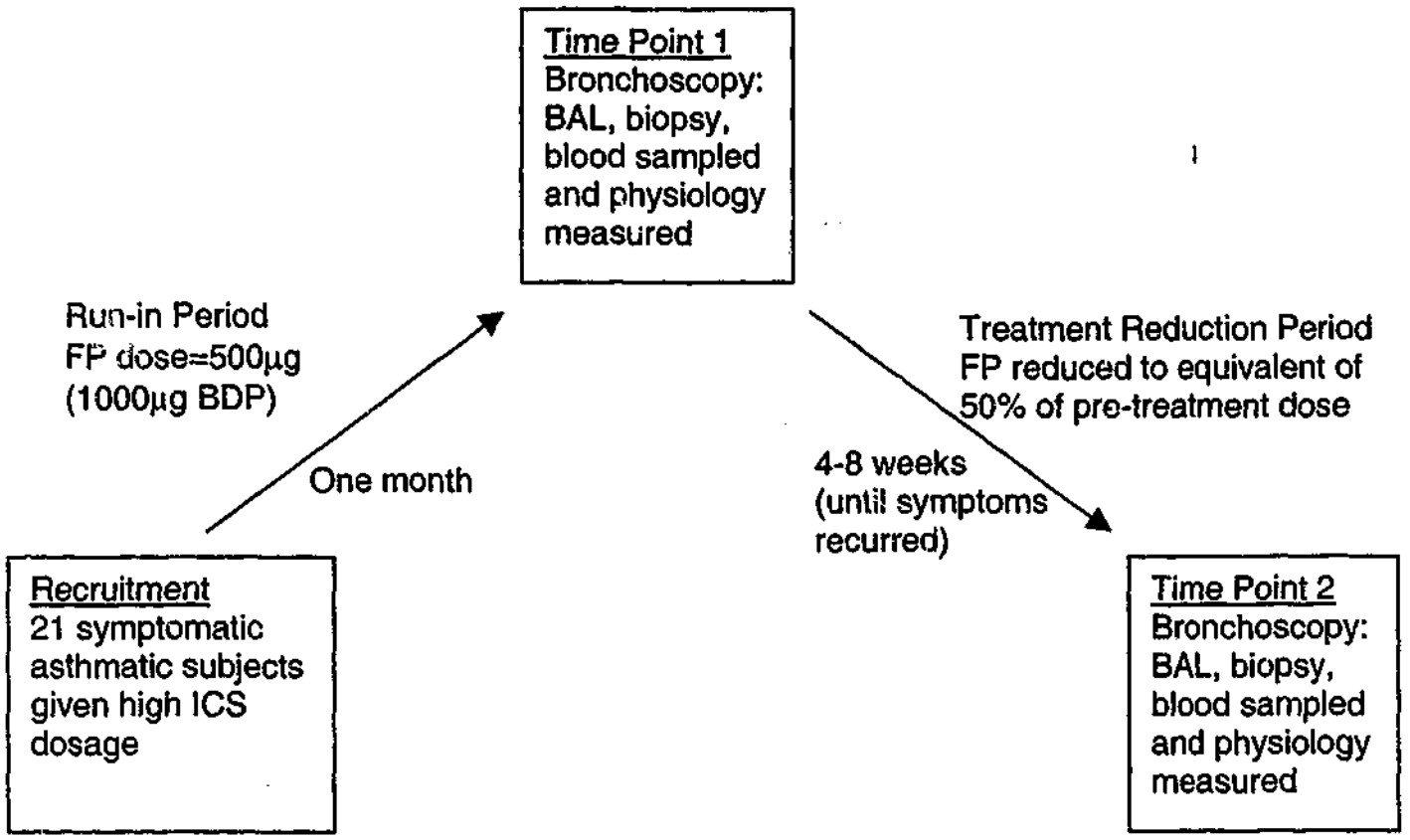
in the airways (van Herwerden, *et al*, 1995), which in turn results in improved airways hyperreactivity and improved lung function. Despite the removal of these inflammatory mediators and overall improvement in the condition of the airways, once treatment is withdrawn conditions may well return to their previous levels, including eosinophilia and presumably elevated cytokines. Two possible explanations seem immediately apparent: an immediate resurgence of the pro-inflammatory events that initiated the asthma to begin with, or a failure of the GC treatment to adequately suppress certain key aspects of the inflammatory process, so the airways are primed to reinitiate the asthmatic disease process when exposed to environmental stimuli such as allergen.

The early initiating pro-inflammatory processes that lead to the asthmatic inflammatory response are largely unknown. While early research focused on identifying external triggers, and an IgE based mast cell model, limited success and the diversity of the asthmatic syndrome has led recent efforts to re-focus and re-assess the pathological mechanisms occurring in asthma (see 1.4.1). The networks of mediators give great redundancy within the inflammatory process. This, coupled with the wide allelic variation within a human population, make analysis of cytokines and chemokines very difficult. It is not surprising that the focus of many studies is on human allergen challenge or animal models, which may not be relevant to chronic disease. Were it possible, the optimal method of observing the initiating events that are triggered in the onset of asthma would be to measure subjects before the onset of symptoms, then again as soon as they had developed asthma, in order to examine the very earliest sequence of changes.

Rather than taking individuals with asthma and studying a relatively artificial model of stimulated worsening disease (eg. the allergen challenge model), our strategy in this study was to suppress asthma in steroid responsive individuals with high doses of ICS and then cut back the dose, which would be expected to cause the return of the asthmatic symptoms. This would allow us to try and study the initiating events at a very early stage when symptoms just begin to return. This would provide novel data on the cytokine and cellular changes that occur as the effects of treatment fade, and hopefully give some indication of what may happen and in what order during the early stages of development of the asthmatic condition. Also of great interest would be the potential for defining what elements of asthma pathology are not altered even when the disease is clinically well suppressed by aggressive ICS therapy.

We thus undertook a treatment withdrawal study; which examined the effects of greatly reducing ICS treatment in a group of stable asthmatics treated with high dose ICS sufficient to completely suppress their asthma. Bronchoscopy was performed after a one month run-in period, where the subjects were stabilised on a high fluticasone propionate (FP) dose (500µg bd), then again after a reduction of their FP dosage to 50% of their original recruitment dose for an additional 4-8 weeks. We hypothesised that after this reduction in treatment, symptoms would return. Along with symptoms, we hypothesised that the eosinophilic cytokines, IL-5 and eotaxin, would increase in concentration in the airways associated with a rise in eosinophils in the blood and within the airways.

4.1.1 Study Design Diagram



Whilst the study design did not require blinding and randomisation, all laboratory measures were independently blinded and randomised to avoid bias and batching.

4.2 Materials and Methods

The procedures used in this study follow the general protocols summarised in chapter 2, with the following exceptions and clarifications.

4.2.1 Patients and Bronchoscopy

Patient recruitment and bronchoscopy followed the guidelines given in sections 2.2.1 and 2.2.2. All subjects were mildly symptomatic in spite of being on an ICS dose of 200-800 mg BDP (equivalent to 100-400mg FP).

For this study three bronchial biopsies were processed into GMA resin, three into formalin fixed paraffin blocks (see 2.2.4) and two snap frozen for molecular biology. Approximately 10ml of blood was taken from each subject for eosinophil counts and serum analysis.

The subject demographics were as follows:

Number of patients	21
Male/Female	12/9
Age (Yrs)	38±12
Duration of asthma (Yrs)	28.4±13.0
Number of Skin Prick Positives (all atopic)	2.9±1.2

4.2.2 Clinical Markers

Subject responsiveness to methacholine challenge (PD_{20}) was measured at entry into the study, and prior to each bronchoscopy. At each of these three points forced expiratory volume in one second (FEV_1), forced vital capacity (FVC) and maximal forced expiratory flows between 25% and 75% of vital capacity (FEF_{25-75}) were also recorded from expiratory flow volume loops.

Patients were given diary cards in which they recorded peak flows in the morning and in the evening from the beginning of the study run-in to the second bronchoscopy. Bronchodilator use for each day and night was also recorded over this period. Finally, subjects assessed their own health each day with a point-based system as follows:

0 No symptoms
1 Symptoms for one short period
2 Symptoms for two or more short periods
3 Symptoms for most of day (Activities unaffected)
4 Symptoms for most of day (Activities affected)

4.2.3 BAL Differential Cell Counts

Total and differential cells counts (see 2.5.1) in BAL aspirates were performed at each of the two bronchoscopic timepoints. Eosinophils, neutrophils, lymphocytes, macrophages and airway epithelial cells were counted, with results expressed as percentages. Airway epithelial contamination in this data set was negligible.

4.2.4 ELISA for IL-5 and Eotaxin in BAL Fluid

A portion of the stored, frozen (at -80°C) BAL supernatants were thawed and used for ELISA (see 2.4.2) for both IL-5 and eotaxin at each of the two bronchoscopic timepoints.

4.2.5 Blood Eosinophil Counts

Blood taken immediately prior to each bronchoscopy was assessed for eosinophils via a Coulter counter. Results were expressed as eosinophils $x10^{-9}$/dl.

4.2.6 ELISA for IL-5 and Eotaxin in Serum

Serum was isolated from subject blood samples via centrifugation on ficol gradient columns (Herabus Sepatech centrifuge, 1200rpm, 10 minutes), red blood cells and buffy coat were discarded. After storage at -4°C, serum was thawed and used for IL-5 and eotaxin ELISA (see 2.9.8).

4.2.7 Immunohistochemistry for Eosinophils in Airway Biopsies

Paraffin embedded biopsies (see 2.2.4) were cut and stained for both total (EG1) and activated (EG2) eosinophils (see 2.3.1). Eosinophils were quantitated using computer image analysis and counts expressed per area of lamina propria and per mm of basement membrane (see 2.5.2).

4.2.8 Immunohistochemistry for Eotaxin and IL-5

Immunohistochemistry, using an immunofluorescence protocol (see 2.3.3) was attempted for both eotaxin and IL-5 on both paraffin and GMA sections. In spite of a great deal of developmental time and effort, the outcomes were disappointing (see section 2.8).

4.2.9 Molecular Biology

Two methods of molecular biology were attempted to assess IL-5 and eotaxin mRNA: competitive PCR (see 2.6.1) and real time PCR (see 2.6.2). While these protocols met with only limited success, the findings have been summarised in section 2.10

4.3 Results

Of the 21 subjects initially recruited only a single dropout occurred after the first bronchoscopy. Thus, full data were available for the remaining 20 subjects for the duration of the study.

4.3.1 Clinical Markers

4.3.1.1 Diary Cards - Symptom Scores and Peak Flows

Peak Flows:

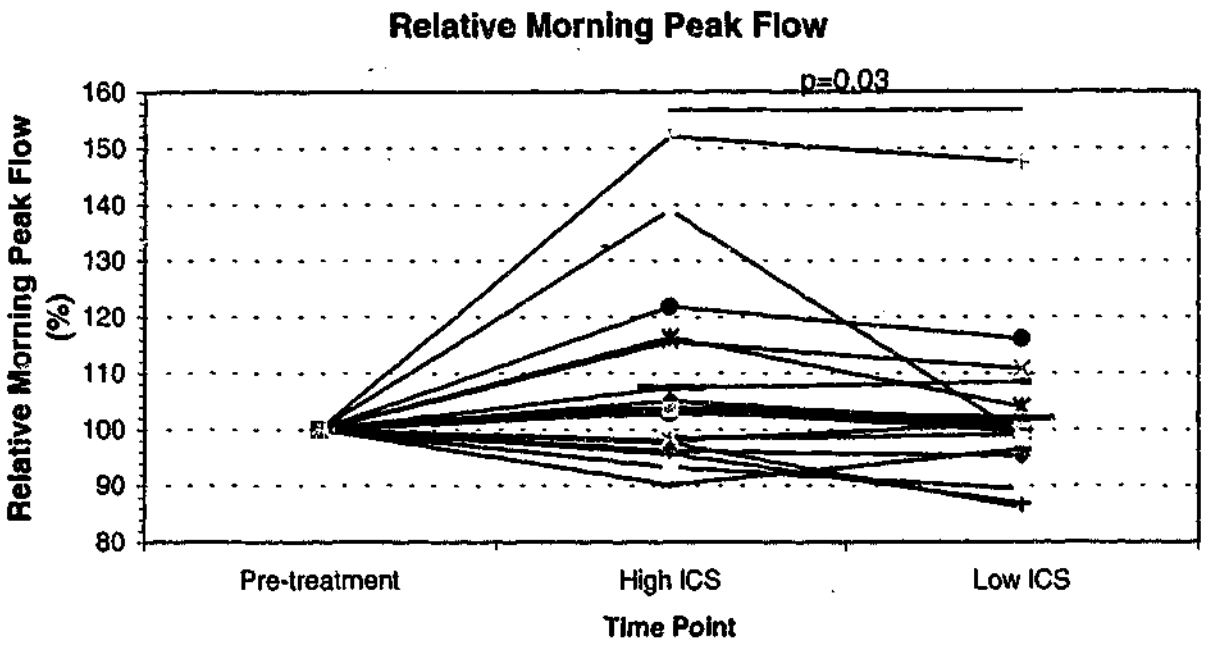
The following table summarises the peak flow data as analysed from the collated patient diary cards:

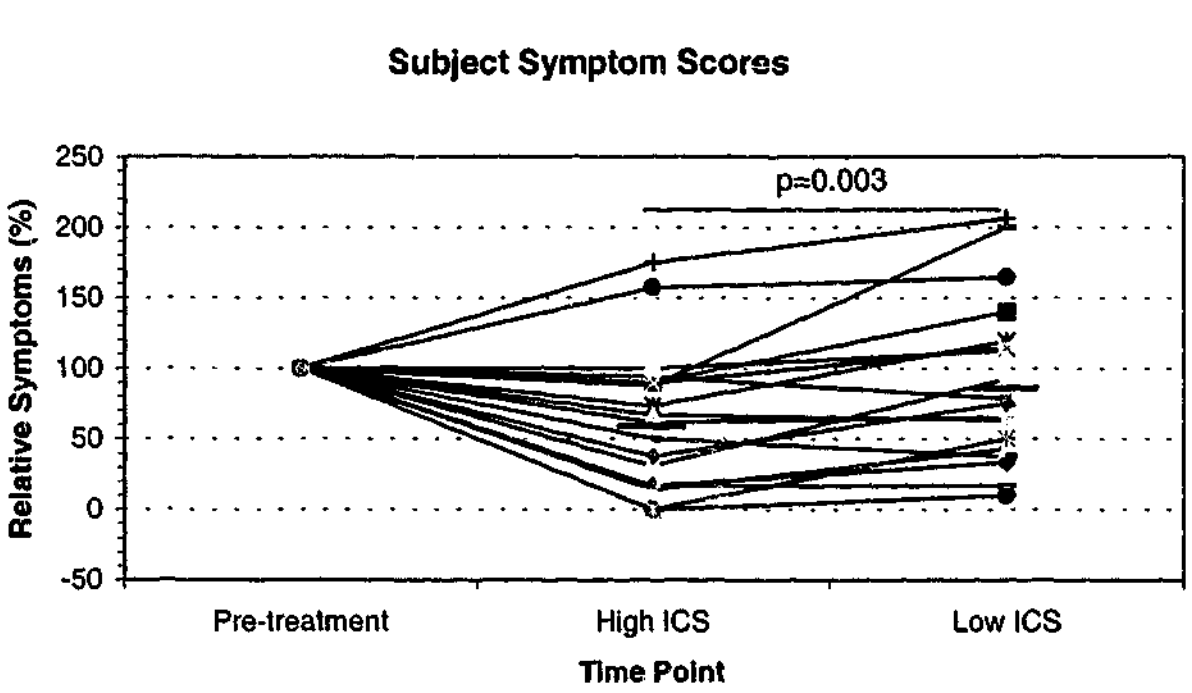
Morning Peak Flows	Run-in => 1st bronchoscopy (high ICS dose)	1st => 2nd bronchoscopy (reduced ICS dose)
Subjects with +ve PF change	13	4
Average PF improvement	13.6±15.7%	2.9±2.9%
Subjects with –ve PF change	7	16
average PF degradation	4.4±3.0%	4.1±2.8%
Net change in PF	7.3±15.4% (improvement)	4.2±7.2% (degradation)
Evening Peak Flows		
Subjects with +ve PF change	12	6
Average PF improvement	10.7±9.6	2.4±2.6
Subjects with –ve PF change	8	14
average PF degradation	5.4±4.2	7.5±8.1
Net change in PF	3.8±13.5 (improvement)	4.6±8.3% (degradation)
Morning/Evening PF agreement	No significant difference (p=0.44)	No significant difference (p=0.89)

The changes in relative peak flows were also examined. For this analysis the subjects initial morning peak flow was rated at 100% and subsequent measures compared as a ratio to this initial value. These data showed a significant reduction (p=0.03) in morning peak flow after reduction in treatment (see figure 4.1).

Figure 4.1 Subject morning peak flows, relative to pre-ICS treatment levels.

Figure 4.2 Subject symptom scores, relative to pre-ICS treatment levels. Note that a lower symptom score indicates an improvement in condition.


Relative Morning Peak Flow
p=0.03
Relative Morning Peak Flow (%)
160
150
140
130
120
110
100
90
80
Pre-treatment
High ICS
Low ICS
Time Point


Subject Symptom Scores
p=0.003
Relative Symptoms (%)
250
200
150
100
50
0
-50
Pre-treatment
High ICS
Low ICS
Time Point

Symptom Scores and Bronchodilator Usage:

Figure 4.2 illustrates the relative change in symptom scores for the subjects at pre-treatment and for both the first and second study timepoints. Each patient's initial symptoms were rated at 100% and the relative increase or decrease calculated from the change in symptoms in the week prior to each bronchoscopy. Similarly, relative bronchodilator use was also examined (figure 4.3). From pre-treatment to the first bronchoscopy, only 3 of the 20 subjects did not show an improvement in symptoms when initially treated with ICS. This was important in establishing that our subject population was actually much less symptomatic after aggressive ICS treatment. There was a significant increase in relative symptoms following reduction of ICS treatment ($p=0.003$), but no significant change in relative bronchodilator usage (p=ns, 0.09).

Lung Function Measures:

A highly significant improvement ($p<0.001$) was observed in FEV_1 after subjects were treated with high dose ICS for one month and a similar, but not as severe, reduction in FEV_1 was seen after the reduction of treatment dose ($p<0.05$, see figure 4.4). FVC showed a very similar pattern (see figure 4.5), but changed slightly less overall (FVC AVOVA: $p=0.01$ vs FEV_1, $p<0.0001$). FEF_{25-75} showed the least conformation to the pattern of patient improvement followed by deterioration, and did not significantly change overall across the entirety of the study (see figure 4.6), but this is inherently a more variable measure, subject to a greater noise to signal ratio.

Airways hyperreactivity was measured by responsiveness to methacholine challenge; the log transformed PD_{20} data indicated a significant lessening of airways

Figure 4.3 Subject bronchodilator usage, relative to pre-ICS treatment levels.

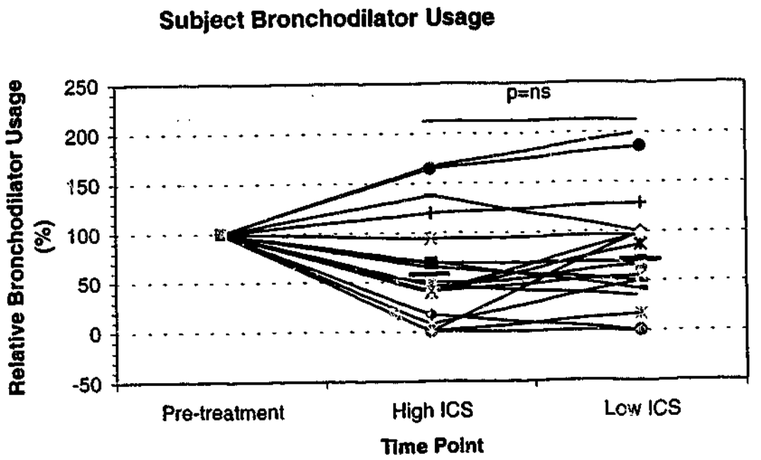
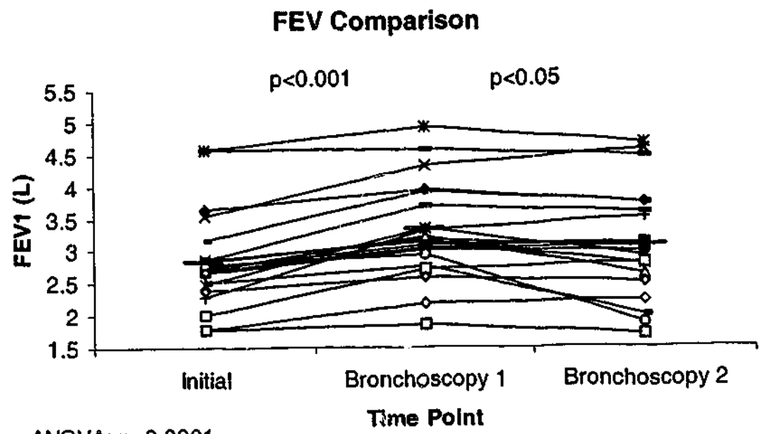


Figure 4.4 Lung function (FEV_1) in subjects before treatment, after high ICS treatment and after reduction of ICS treatment.



ANOVA: p<0.0001

Figure 4.5 Lung function (FVC) in subjects before treatment, after high ICS treatment and after reduction of ICS treatment.

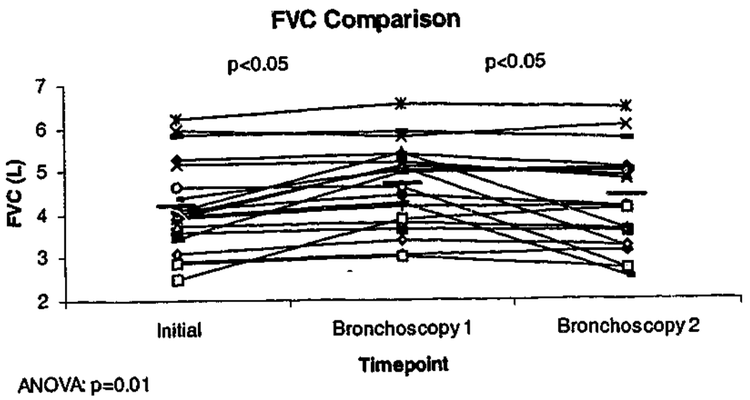
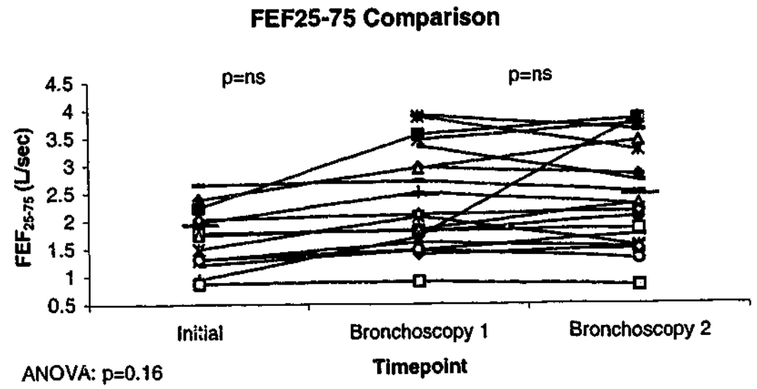


Figure 4.6 Lung function (FEF_{25-75}) in subjects before treatment, after high ICS treatment and after reduction of ICS treatment.



[illegible] reactivity ($p<0.01$) after one month of high ICS treatment. However after dose reduction, airway hyperreactivity was not changed (see figure 4.7).

4.3.2 Changes in BAL Cell Profiles After Treatment

The following table summarises the average differential cell counts performed on the BAL cells for each of the two bronchoscopies. Significance differences between the two time points were calculated by paired t tests of each cell type.

	High Dose ICS	Reduced Dose ICS	Significance High vs. Reduced ICS	Non-asthmatic Controls
Macrophages	74.1±16.4%	76.1±12.3%	ns (p=0.54)	80.3±13.0%
Lymphocytes	21.9±15.6%	20.0±11.2%	ns (p=0.51)	16.3±12.4%
Neutrophils	2.5±2.0%	2.2±1.9%	ns (p=0.50)	2.1±2.0%
Eosinophils	1.5±1.6%	1.7±2.6%	ns (p=0.74)	1.0[0.3-1.2]%
Epithelial cells	0.0±0.1%	0.0±0.1%	N/A	0.0%

4.3.3 BAL IL-5

Comparing high and reduced dose ICS treatment phases, no difference was found in the IL-5 levels in the BAL between the time points (see figure 4.8).

This data was also examined relative to the cross-sectional measures previously taken for IL-5 in the BAL (see chapter 3). These observations are tabulated below:

	High Dose ICS	Reduced Dose ICS	ICS free asthmatics	Non-asthmatic controls
Median BAL IL-5 levels (pg/ml)	2.3 [0.9-24.1]	2.5 [1.4-11.3]	12.3 [7.2-130.1]	0.9 [0.2-3.4]
Significance vs. ICS free asthmatics	p<0.05	p<0.05		
Significance vs. non-asthmatics	ns (p>0.05)	ns (p>0.05)		

Figure 4.7 bronchial Hyperresponsiveness (PD_{20}) in subjects before treatment, after high ICS treatment and after reduction of ICS treatment.

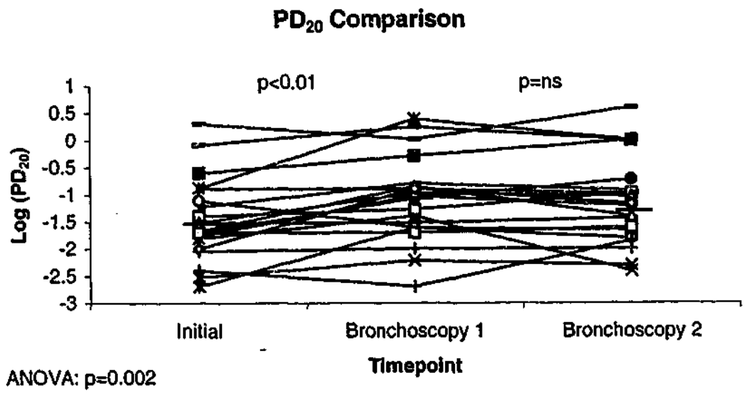
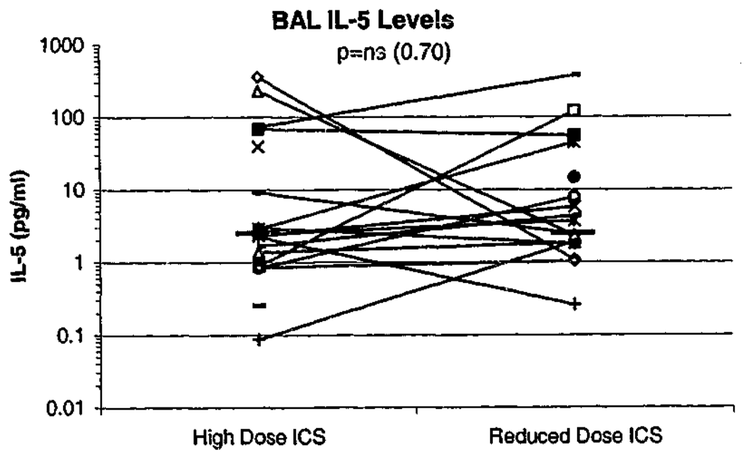


Figure 4.8 BAL IL-5 levels in patients before and after reduction in ICS treatment. No significant change was observed.



The above data indicate that the BAL IL-5 levels of the ICS treated patients in this study were significantly below the high IL-5 levels observed in untreated asthmatics and close to (not significantly different from) those of non-asthmatic subjects.

4.3.4 BAL Eotaxin

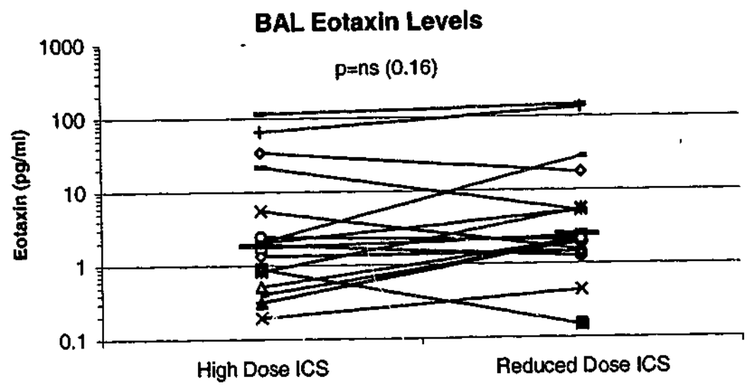
BAL eotaxin levels were also assessed for subjects at both bronchoscopic timepoints. Like IL-5, no difference was found between the BAL eotaxin in subjects receiving high dose ICS and the same subjects after their ICS dose has been reduced (see figure 4.9).

Examining the eotaxin BAL data relative to the cross-sectional data, however, gives a somewhat different picture to that observed for IL-5:

	High Dose ICS	Reduced Dose ICS	ICS free asthmatics	Non-asthmatic controls
Median BAL eotaxin levels (pg/ml)	1.9 [0.7-4.8]	2.2 [1.7-5.4]	6.4 [4.8-9.5]	0.8 [0.4-3.6]
Significance vs. ICS free asthmatics	ns ($p>0.05$)	ns ($p>0.05$)		
Significance vs. non-asthmatics	ns ($p>0.05$)	$p<0.05$		

These comparisons seem to indicate a degree of suppression of eotaxin by ICS. The asthmatics receiving high ICS doses are at an intermediate eotaxin level that was not statistically different from either ICS-free or non-asthmatic subjects. After reduction of treatment subjects' eotaxin levels were increased just sufficiently enough to differentiate them from non-asthmatic subjects ($p<0.05$).

Figure 4.9 BAL eotaxin levels in patients before and after reductio.. in ICS treatment. No significant change was observed.



4.3.5 mRNA in the BAL Cells

It was found that IL-5 mRNA could be detected in the BAL cells from these asthmatic subjects at concentrations 10^{12}-10^{15} fold less than the ubiquitous 18s ribosomal RNA subunit which was used as a control in the real-time PCR protocol (see section 2.6.2). However, at such a low concentration it was not possible to quantitate IL-5 precisely due to a lack of good reproducibility. Eotaxin quantitation was attempted both by competitive (see section 2.6.1) and real time PCR. Eotaxin mRNA in BAL cells was not detected by real time PCR, indicating very low amounts present, at least 10^{20} fold less than the 18s RNA and 10^{5} fold less than IL-5 mRNA. This is without accounting for the relative abundance of IL-5 and eotaxin expressing cells, which even together comprise a very small percentage of total BAL or biopsy cells. Competitive PCR produced an amount of unexpected DNA contamination, resulting in a large band that out-competed our target eotaxin mRNA and was specific for the developed eotaxin primers. This developmental process is detailed in section 2.10.2.

4.3.6 Total and Activated Eosinophils in Biopsies

No differences were found after reduction of ICS treatment in either total ($EG1^{+}$) or activated ($EG2^{+}$) eosinophils, when expressed as eosinophil per unit area (see figure 4.10) or per length of basement membrane (see figure 4.11). At both high and reduced-ICS doses, biopsy eosinophils were significantly lower than our established ICS-free asthmatic range ($p<0.001$, for both total and activated eosinophils) and not significantly higher than non-asthmatic controls. These data are also summarised in the table below.

Figure 4.10 Total and activated eosinophils from bronchial biopsies expressed per mm^2 of lamina propria.

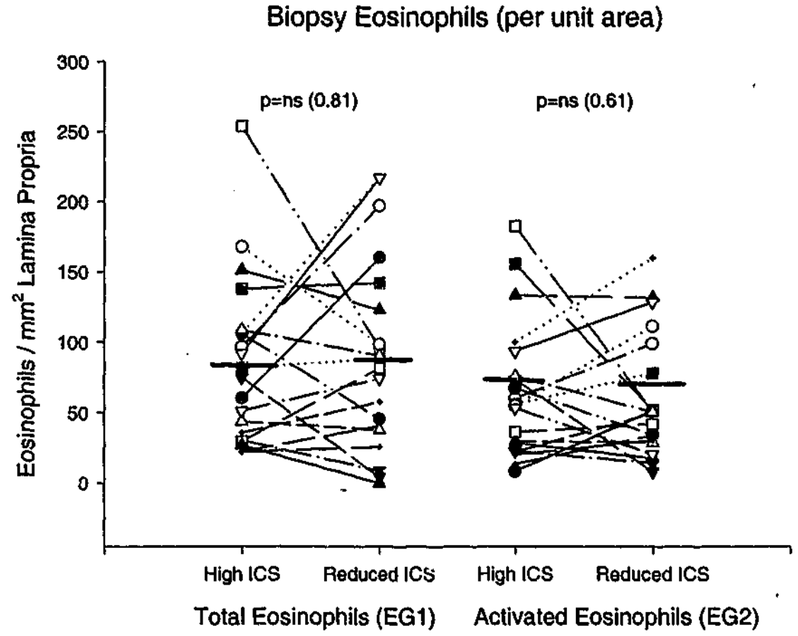
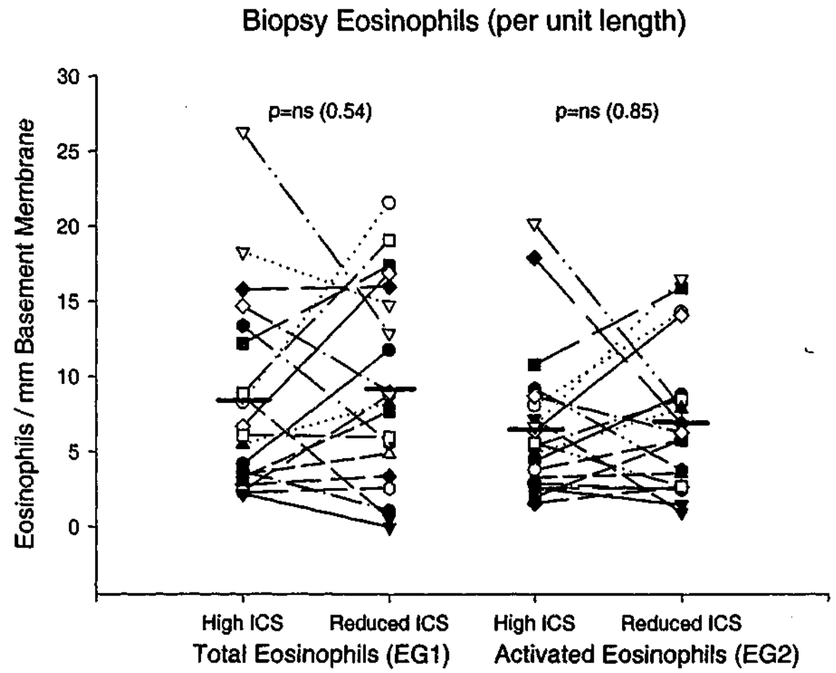


Figure 4.11 Total and activated eosinophils from bronchial biopsies expressed per mm of basement membrane.



	Total Eosinophils (EG1) / mm^2	Activated Eosinophils (EG2) / mm^2	Total Eosinophils (EG1) / mm BM	Activated Eosinophils (EG2) / mm BM
High ICS	83.8±60.6	66.2±46.8	8.5±6.5	6.7±4.9
Reduced ICS	87.5±65.9	60.4±43.8	9.4±6.6	7.0±4.9
Significance (High vs. Low ICS)	ns (p=0.81)	ns (p=0.61)	ns (p=0.54)	ns (p=0.85)
ICS-free asthmatics	415.2±450.0	200.3±218.2		
Non-asthmatics	35.2±29.5	30.9±36.0		

4.3.7 Eotaxin and IL-5 Immunohistochemistry

Immunohistochemical staining via immunofluorescence was attempted at length for both eotaxin and IL-5 in both GMA and paraffin embedded tissue, but was not successful. The GMA resin was found to be useful for high resolution, multi-colour structural immunohistochemistry (see section 2.8), but its antigenicity for cells and particularly cytokines was poor.

4.3.8 Serum Eosinophils, Eotaxin and IL-5

Eosinophils did not significantly change after reduction of ICS dose (see figure 4.12). Likewise serum levels of both IL-5 (see figure 4.13) and eotaxin (see figure 4.14) were unchanged overall after reduction of ICS treatment. The following table compares the serum IL-5 and eotaxin levels in the ICS treated subjects of this study with normal controls.

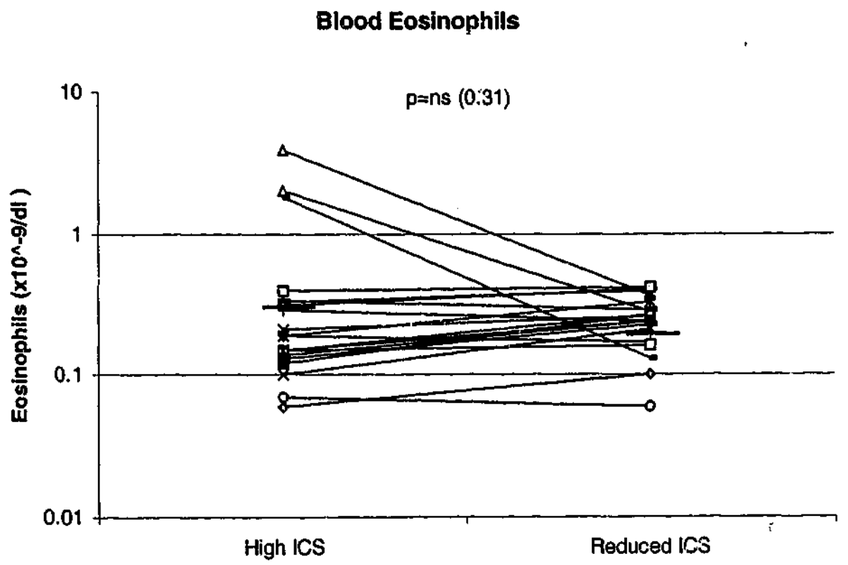


Figure 4.12 Blood eosinophil counts taken in patients before and after ICS treatment reduction.

Figure 4.13 Serum IL-5 measured in patients before and after ICS treatment reduction.

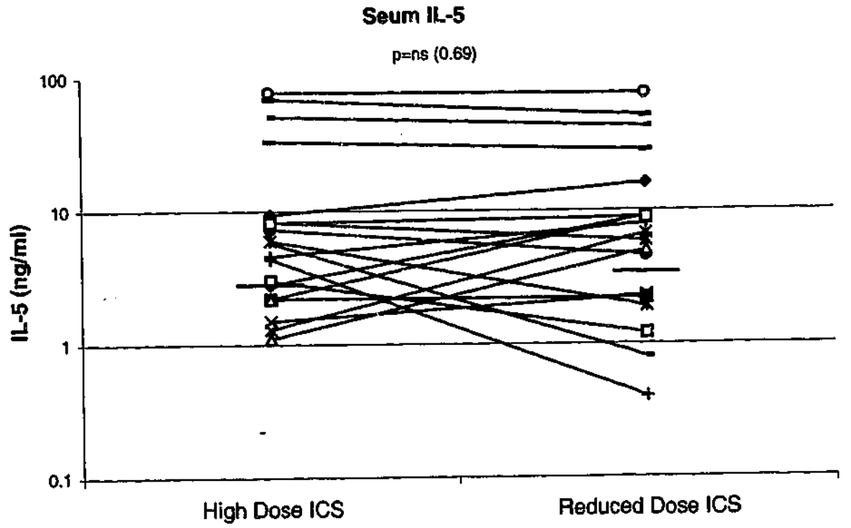
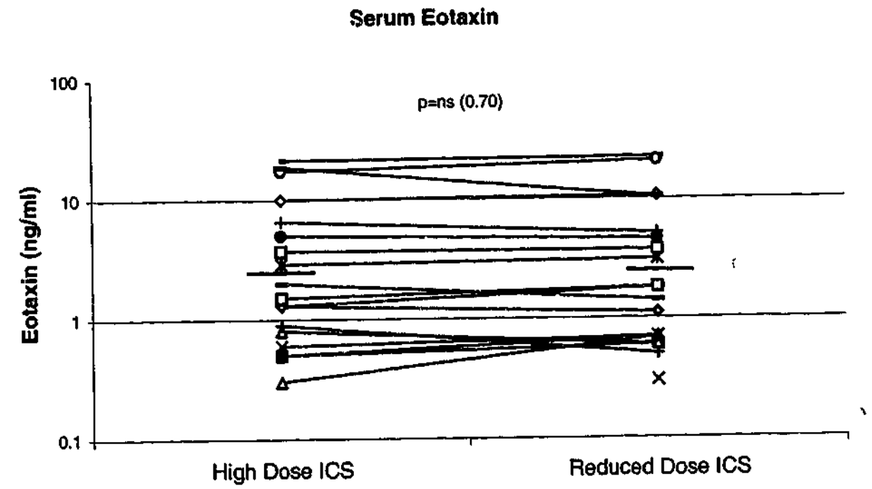


Figure 4.14 Serum eotaxin measured in patients before and after ICS treatment reduction. Note the high conservation of this measure.



	High Dose ICS	Reduced Dose ICS	ICS Free Asthmatics*	Non-asthmatic controls
Mean serum IL-5 levels (ng/ml)	5.2 [2.2-8.9]	6.3 [2.1-12.6]	9.2 [5.7-43.9]	1.8 [0.3-9.2]
Mean serum eotaxin levels (ng/ml)	4.8±6.7	4.6±6.5	6.1±7.3	5.9±7.2
Significance of IL-5 vs. ICS free asthmatics	ns (p=0.21)	ns (p=0.21)		p=0.05
Significance of eotaxin vs. ICS free asthmatics	ns (p=0.65)	ns (p=0.59)		ns (p=0.94)
Significance of IL-5 vs. non-asthmatics	ns (p=0.12)	ns (p=0.12)	p=0.05	
Significance of eotaxin vs. non-asthmatics	ns (p=0.61)	ns (p=0.53)	ns (p=0.94)	

*: Serum was not available for the entire ICS free asthmatic subject group, therefore the n for this statistical analysis was 7 for both IL-5 and eotaxin.

Correlation analyses were performed for the above serum cytokines and eosinophils in the blood. Whilst neither IL-5 nor eotaxin showed a direct relationship to blood eosinophils, serum IL-5 and eotaxin were strongly related to each other in both high (r=0.60, p=0.005, see figure 4.15) and reduced (r=0.52, p=0.01, see figure 4.16) ICS treated subjects. This relationship was weaker in non-asthmatic subjects (r=0.44, p=0.052), and was not observed in ICS free asthmatics (r=0.61, p=0.14). Note, however, that the ICS-free asthmatics were a much smaller group (n=7) and there was a greater likelihood of type II error, requiring a higher correlation coefficient (r) to achieve significance.

4.3.9 Correlations Between BAL and Biopsy Findings and Clinical Markers

The correlation analyses preformed for this study were broken into three groupings; Relationships between measures for subjects receiving high dose ICS treatment, relationships present after reduction of treatment, and relationships between factors across the two timepoints, ie. the changes over time. Due to the large number of

Figure 4.15 A positive relationship between serum IL-5 and serum eotaxin observed in subjects treated with high-dose ICS.

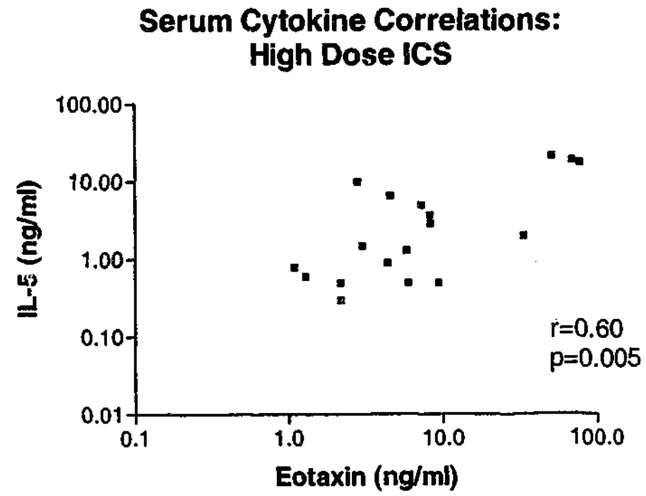
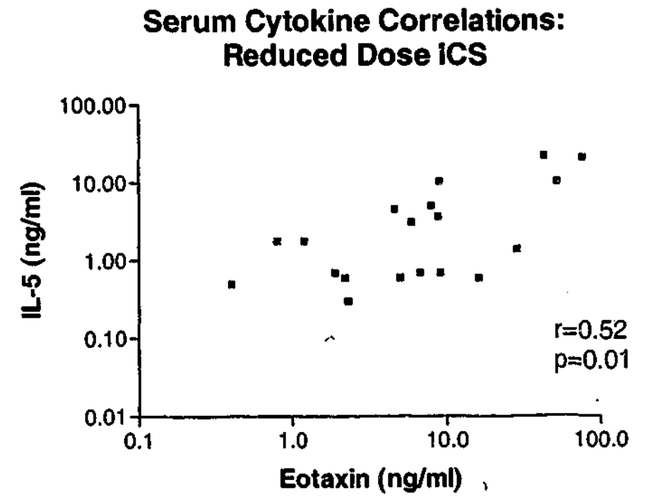


Figure 4.16 A positive relationship between serum IL-5 and serum eotaxin observed in subjects after reduction of ICS treatment.



different observations in this section, discussion of some of the relationships has been done in this section, rather than at the end of the Chapter.

Correlations in Subjects on High Dose ICS:

In endobronchial biopsies, total (EG1-staining) eosinophils correlated with activated (EG2-staining) eosinophils ($r=0.87$, $p<0.0001$). This was a good indicator that our immunohistochemistry was internally consistent, in that similar proportions of activated to total eosinophils were seen across all subjects (see figure 4.17).

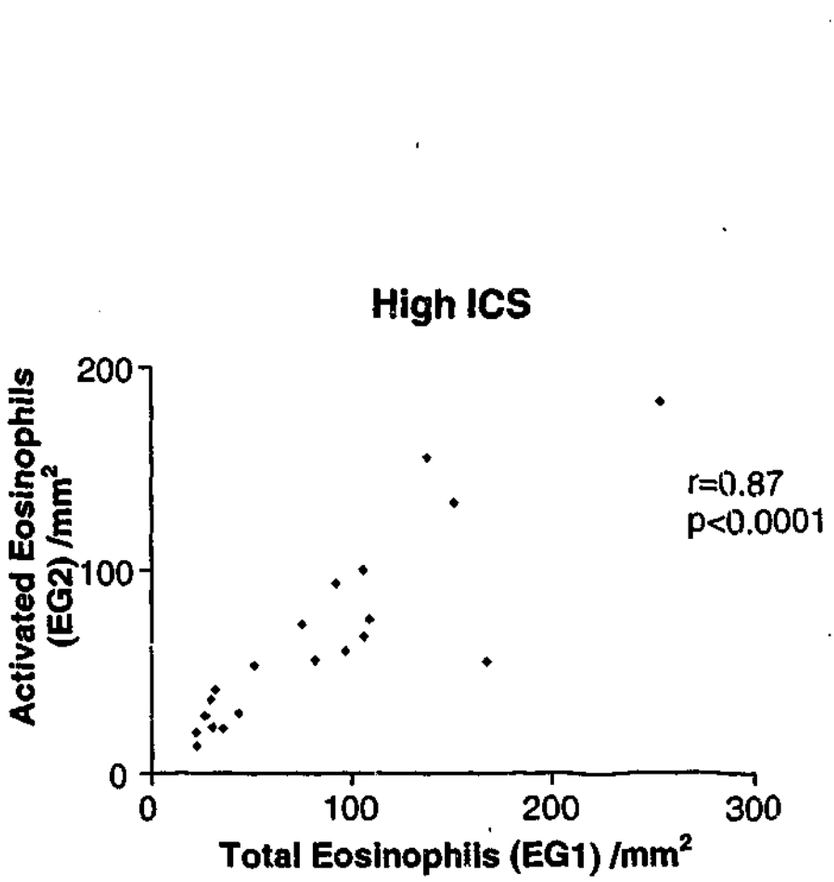
The lung function measure, FEV_1, was related to both FVC ($r=0.88$, $p<0.0001$) and FEF_{25-75} ($r=0.76$, $p<0.0001$). This was another good internal validation showing congruence between three different measures of lung function and airflow obstruction in asthma (see figures 4.18 and 4.19 respectively).

IL-5 and eotaxin in the serum showed a strong relationship ($r=0.60$, $p=0.005$). Without the presence of serum data before the subjects were placed on high dose ICS, it was not possible to determine if this relationship was a result of both these cytokines being suppressed in the serum by ICS treatment.

BAL IL-5 rather paradoxically correlated inversely with eosinophils in the BAL ($r=-0.5$, $p=0.035$). This relationship was seemingly counter-intuitive, but similar findings were found in one of the other studies in this project (see section 6.3.4). As already discussed, this may be related to the involvement of IL-5 in luminal clearance of eosinophils (see section 1.2.4).

Figure 4.17 A positive relationship between total and activated eosinophils in patients receiving high-dose ICS treatment.

Figure 4.18 A positive relationship between FEV_1 and FVC in the week before bronchoscopy, in patients receiving high-dose ICS treatment.


High ICS
Activated Eosinophils (EG2) /mm²
200
100
0
0
100
200
300
Total Eosinophils (EG1) /mm²
r=0.87
p<0.0001

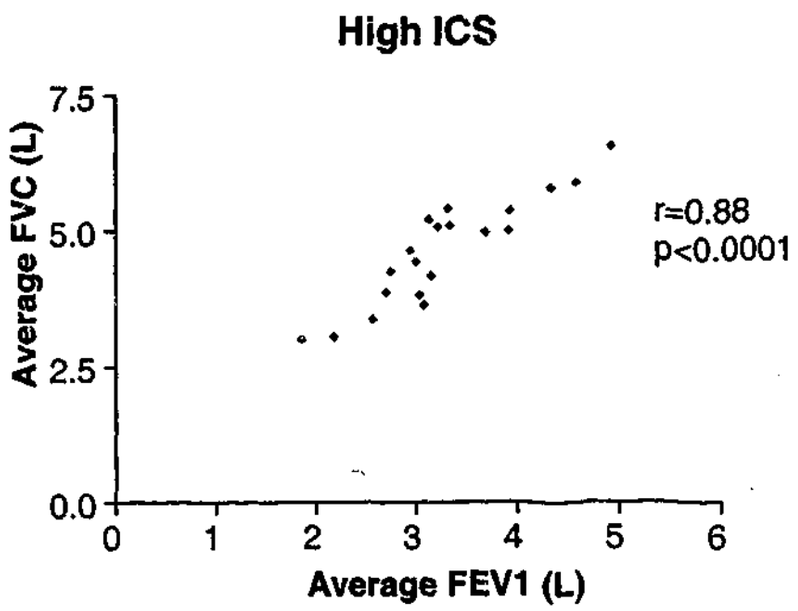

High ICS
Average FVC (L)
7.5
5.0
2.5
0.0
0
1
2
3
4
5
6
Average FEV1 (L)
r=0.88
p<0.0001

Figure 4.19 A positive relationship between FEV_1 and FEF_{25-75} in the week before bronchoscopy, in patients receiving high-dose ICS treatment.

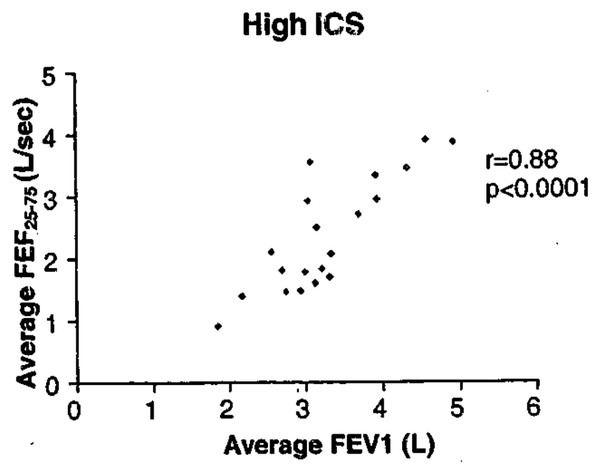
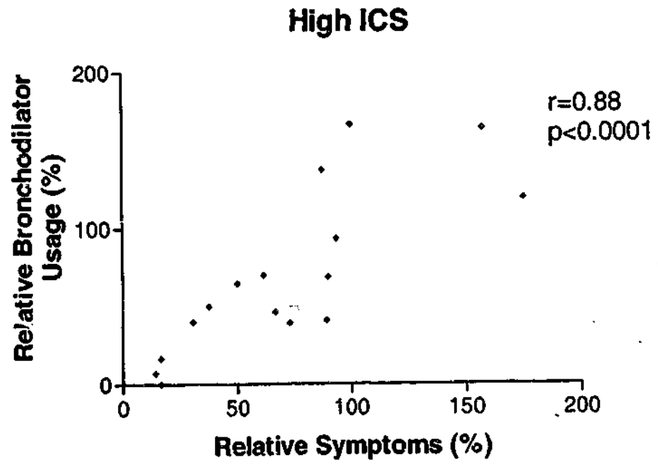


Figure 4.20 A positive relationship between change in bronchodilator usage and symptoms scores relative to pre-ICS treatment score, in patients after receiving high-dose ICS treatment.



Correlations in Subjects with Reduced ICS:

Again, relative patient symptoms related strongly to relative bronchodilator usage ($r=0.75$, $p=0.002$).

Total and activated eosinophils remained strongly related ($r=0.93$, $p<0.0001$).

FEV_1 remained consistent with the other measures of lung function FVC ($r=0.88$, $p<0.0001$) and FEF_{25-75} ($r=0.83$, $p<0.0001$).

Serum levels of IL-5 and eotaxin remained related to each other after reduction of ICS, though slightly less strongly ($r=0.52$, $p=0.018$).

Relationships Between Changes in Measures between Timepoints:

Percentage change in symptom scores related very strongly to percentage change in bronchodilator usage ($r=0.88$, $p<0.0001$), indicating that a patient's perceived well-being has, perhaps not surprisingly, a large influence on their use of reliever medication (see figure 4.20). Note that this correlation was between pre-treatment and the first bronchoscopic timepoint.

Changes in serum eotaxin after ICS-treatment reduction were inversely related to changes in PD_{20} ($r=-0.62$, $p=0.004$). Although this may be biologically plausible, ie. A decrease in a mediator related to an improvement in BHR, the serum eotaxin changes were extremely small, so caution is required.

Changes in serum IL-5 after ICS-treatment reduction correlated inversely to changes in average morning (r=-0.56, p=0.01) and evening (r=-0.62, p=0.004) peak flows in the week prior to bronchoscopy, as well as percentage changes in morning peak flows (r=-0.56, p=0.01). Again, this seems biologically appropriate, with those patients in whom serum IL-5 levels were increasing after a reduction in ICS dose becoming physiologically less stable. (see figures 4.21, 4.22 and 4.23 respectively). This may represent the very early stages of a systemic eosinophil response.

Changes in total eosinophil (EG1) numbers in biopsies after ICS-treatment reduction related to changes in activated eosinophils in the biopsies (r=0.60, p=0.005). In addition there was an apparent direct relationship with changes in morning peak flows (r=0.47, p=0.035). This correlation seems while seeming anomalous, with a decrease in inflammatory cells related to a decline in lung function, does support our other observations of an apparent dissociation between eosinophils and some physiological measures. Though, again caution is required as the changes in both these indices are small.

Figure 4.21 An inverse relationship between change in morning peak flows and change in serum IL-5 in subjects after reduction of ICS treatment.

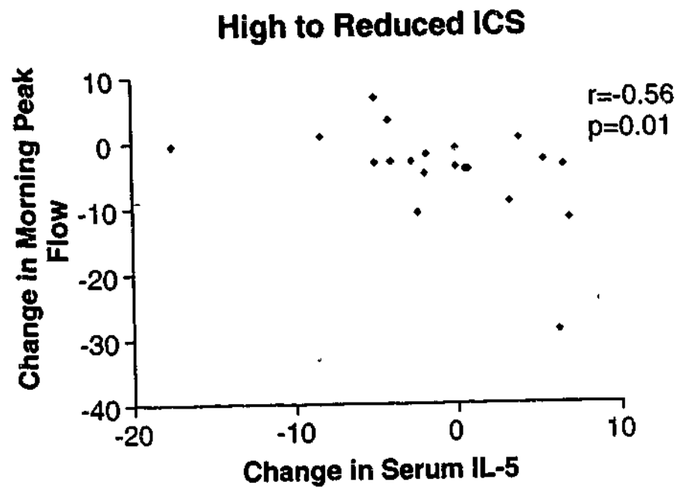


Figure 4.22 An inverse relationship between change in evening peak flows and change in serum IL-5 in subjects after reduction of ICS treatment.

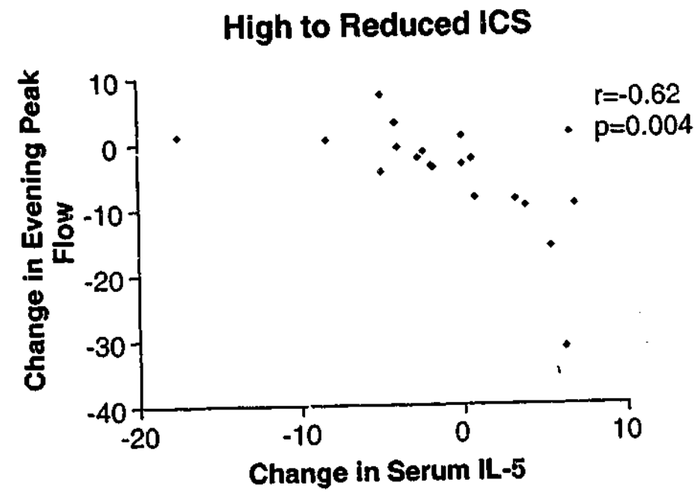
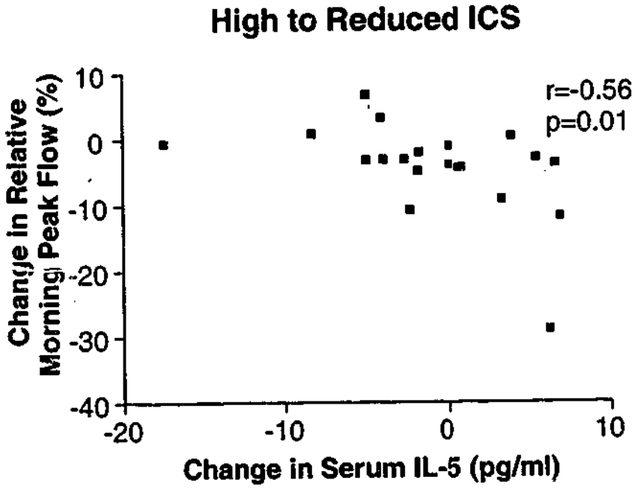


Figure 4.23 An inverse relationship between change in evening peak flows, relative to pre-treatment levels and change in serum IL-5 in subjects after reduction of ICS treatment.



4.4 Discussion

The intention of this study was to suppress asthma activity and the underlying pathological processes with aggressive ICS treatment, and then as the dosage was greatly reduced, observe the relative changes in symptoms, lung function, eosinophils and chemokines at an early stage of the clinical deterioration. By doing so, it was hoped that the true pathogenic relationships between clinical disease activity and eosinophilia, and between eosinophils and chemokines could be observed.

As bronchoscopies were not performed before the run-in stage, cellular and cytokine data was not available. However several clinical measures were taken over this period. Of these, the most significant improvement with high dose ICS was seen in FEV_1 ($p<0.001$, see figure 4.3), followed by PD_{20} ($p<0.01$) and FVC ($p<0.05$). Peak flow data showed that most subjects improved, and the average percentage improvement was positive (+7%), and only a small number of subjects' peak flows were decreased in the treatment run-in period. Symptom scores showed an average 39% reduction from their initial values after high-dose ICS treatment, reinforcing the fact that our subject population showed a large amount of clinical improvement when treated aggressively with ICS.

After reduction of ICS dosage, peak flows were reduced to almost pre-treatment levels (+2% above initial values), but were significantly lower than they were at the highest level of treatment (-5%, $p=0.03$). Patient symptoms scores also deteriorated substantially after the reduction in ICS treatment (+40% increase in symptoms, $p=0.0003$). This deterioration in condition placed patients symptoms almost exactly at the point they were before the initial high-dose ICS treatment. It seems clear from the

marked improvement in the majority of clinical markers that most subjects' asthma was substantially diminished clinically by the application of high dose ICS treatment, and as demonstrated by the subjects' symptoms scores, began to worsen upon reduction of treatment.

Based on follow-up work done after completion of one of our other studies (chapter 5), it seemed that after succession of intensive ICS treatment, approximately one month was sufficient to cause the recurrence of BHR. Another consideration was the ethics of a human drug study with a negative outcome. It was understandably difficult to gain ethics approval for a study where subjects were required to become less well, and whilst our patient group consisted of relatively mild, stable asthmatics, it would have been unreasonable not to have had a defined endpoint. While we were able to extend the ICS reduction period out to as long as 8 weeks if symptoms did not recur, any longer than this was deemed an unreasonable length of time to keep the patients in the study. Thus, the second bronchoscopy was done between 4 and 8 weeks, with the hope that this timepoint would coincide with early deterioration of the patient's physiology. In general this was precisely what occurred.

In absolute terms, most subjects recorded a decrease in peak flows after reduction in treatment; the degree of degradation averaged half the level of improvement seen after the high-dose ICS treatment. Patient-recorded symptom scores and bronchodilator usage did not rise significantly in absolute terms. However, looking at the percentage change in each index rather than the absolute change, all clinical measures (with the exception of bronchodilator use which still showed a trend ($p=0.09$)), showed

significant changes in the directions expected for a recurrence of symptoms (see above).

For the lab-based clinical indices, significant decreases, after reduction of treatment, were seen in FEV_1 ($p<0.05$, see figure 4.3) and FVC ($p<0.05$), but not FEF_{25-75} nor, importantly, PD_{20}. Thus overall, while the effects of the ICS had lasted longer than the study design had predicted, and clinically the subjects had not returned to their recruitment state, they had still undergone a mild but significant level of deterioration after reduction in ICS treatment. Though we may have initially expected the clinical data to show more dramatic differences, these more subtle changes have a very high potential value, which is discussed further below.

From the laboratory measures, it is clear that FEV_1 was the most sensitive, showing the greatest statistical significance across the three measured timepoints ($p<0.0001$). FVC was the only other measure to show significant changes between increase and reduction of ICS treatment, whereas FEF_{25-75}, being the most subject to error, remained relatively unchanged throughout the study. BHR was markedly improved after application of ICS, but did not decrease after treatment was reduced, an unexpected finding.

Interestingly, the change in BHR was statistically highly significant, only slightly less so than FEV_1, across all three timepoints ($p=0.002$). The initial increase shows PD_{20} was a very sensitive measure, but the lack of fall-off may suggest airways hyperresponsiveness is more easily influenced by ICS treatment than other lung function measures. Other studies have shown that reducing the dosage of ICS to a

maintenance level may in fact cause PD_{20} to continue to improve (Fowler, *et al*, 2002). On the other hand, removal of ICS treatment completely for at least as two weeks has been shown to be required to reduce PD_{20} to pre-treatment levels (Convery, *et al*, 2000). Our data implies that even a small dose of ICS will sustain PD_{20}, and that it seems not to be effected by the same factors as other measures of lung function.

For interpretation of our cellular data, it is important to emphasise that there was a definite, even if subtle, clinical and physiological deterioration after reduction in ICS-treatment. We had expected the significant changes in clinical indices to be reflected to some extent in the BAL. However, observations in the BAL showed that the differential cellular profile was almost identical pre and post ICS reduction. IL-5 and eotaxin in the BAL were similarly unchanged. Whereas, IL-5 was reduced to near normal levels under high ICS treatment, BAL eotaxin however was not statistically different to ICS-free asthmatics. That being said, eotaxin was also only different to non-asthmatics after reduction of ICS treatment. This may indicate a slight rise in expression, though not enough to differentiate low dose from high dose ICS BAL eotaxin levels. Thus, BAL eotaxin, though apparently affected somewhat by ICS, appears to be more resilient to high ICS dosage than IL-5. In contrast to these observations, *in vitro* data shows a high degree of dose-dependent eotaxin suppression (Lilly, *et al*, 1997), but the kinetics and *in vivo* effects of such suppression have not been previously studied.

This was the only study undertaken in this project where blood eosinophils and serum cytokines were assessed. Much like the BAL data, serum eosinophils did not significantly change after reduction of ICS dose; neither did serum IL-5 or eotaxin

levels change for the group. Cross-sectionally serum IL-5 was higher in ICS-free asthmatics when compared to normal controls, but serum IL-5 measured in both subjects receiving high or reduced ICS treatment was intermediate to both ICS-free and non-asthmatic subjects, indicating a small degree of suppression by ICS in the serum. Serum IL-5 also showed a strong relationship to peak flows (see figure 4.21), whilst serum IL-5 changed very little across the group, the figure suggests that those who experienced the greatest drops in peak flows had the highest levels of IL-5. This may be one of the initial events of the beginnings of the eosinophilic inflammatory response, and again suggests that peak flows changes occur prior to eosinophils.

Perhaps unexpectedly, serum eotaxin was not different between ICS-free asthmatics and non-asthmatic subjects, and was very highly conserved across the study period (see figure 4.12). These data would appear to indicate that the elevated eotaxin observed in ICS-free asthmatics in the BAL was restricted to the airways and did not occur systemically. There has been some amount of conflicting work by other groups, suggesting an elevation in serum eotaxin in asthmatics (Jahnz-Ro, *et al*, 2000). However, a potential confounder in serum eotaxin measurement is elevated eotaxin in other systemic allergic conditions such as atopic eczema/dermatitis syndrome, and other work suggests serum eotaxin may become more relevant in these conditions (Frezzolini, *et al*, 2002).

The biopsy eosinophil data closely reflected the eosinophils counted in BAL, with essentially no change in total biopsy eosinophils after ICS reduction. This level of biopsy eosinophils was significantly lower than that observed in ICS-free asthmatics and similar to normal controls (See section 3.3.3). Whilst the total biopsy eosinophils

increased very slightly, the number of activated eosinophils decreased slightly. Thus, the ratio of activated to total eosinophils changed from 80% in subjects receiving high ICS doses, to 69% when the dosage was reduced. Whilst the ratios between these two groups were not significant (p=0.14), they do reflect a similar trend observed in the cross-sectional analysis where ICS-free asthmatics had an increased total eosinophil number but a lower activated eosinophil-eosinophil ratio (53%), and non-asthmatic subjects had a conversely lower number but higher ratio (70%, see section 3.4). As untreated asthmatics have more total eosinophils in the airways, this increased number of inactive eosinophils may be related to untreated asthmatics having more eosinophil pre-cursors in the airways, which have been shown to be capable of differentiating at inflammatory sites (Cameron, *et al*, 2000).

Of the three longitudinal studies described in this thesis, this particular study was the most comprehensive. More lung function measures, a larger allotment of BAL supernatants and cells stored, as well as blood, was available for use. This study has been presented first chiefly because most of the technical development of techniques and a majority of the total project time was spent on this study. One of the main objectives was to study which inflammatory indices changed in asthmatic patients when ICS treatment was reduced to a point where symptoms were just beginning to recur. It was therefore initially disappointing that the measured cells (eosinophils mainly) and cytokines in the study did not significantly change within the 8 week timeframe, despite significant changes in clinical measures. Thus, the study did not meet its expected objective in answering its hypothesis, as the eosinophilic measures did <u>not</u> change with ICS treatment reduction, despite clinical deterioration. This suggests that other inflammatory mediators that were not measured may have been

involved in the recurrence of symptoms after a reduction in ICS treatment. Most studies involve large clinical changes that cause correspondingly large changes in inflammatory cells and mediators. This study, where the patient symptoms changed before any changes occurred in airway eosinophils or cytokines, suggests that eosinophilic inflammation may not be responsible for the recurrence of asthma upon withdrawal of ICS treatment. Were eosinophils initially involved in the recurrence of symptoms we could have expected a rise in eosinophil numbers in biopsy or BAL before a significant drop in lung function or symptom score, something that was not seen. While it remains possible that FEV_1, FVC and patient symptoms are extremely sensitive to very small changes in eosinophil numbers, this is not consistent with our cross-sectional observations which suggest that ICS-free asthmatics, with only somewhat poorer lung function, have vastly elevated levels of eosinophils in the airways (approximately 10-times those of non-asthmatic subjects, see section 3.3.3). In support of this, no correlation was observed in this study between lung function measures and eosinophils, either in the airways or BAL.

These data represent measures taken from subjects with the highest level of ICS suppression of cells and cytokines that we are likely to use for the treatment of mild, stable asthma. These subjects form an excellent cross-sectional group from which to compare other treatment groups, as they have an intermediate level of inflammation, which falls between ICS-free asthmatic and non-asthmatic subjects. This has already been demonstrated in Chapter 3, which includes all the patients we have studied that were treated with high-does ICS therapy. As can be seen in that section, the high ICS treated asthmatics have a level of inflammation that is consistently lower than ICS-free asthmatics and somewhat higher than normal controls.

This study has raised some fundamental questions about the importance of eosinophilic inflammation in the recurrence of asthmatic symptoms after the withdrawal of ICS treatment. These findings suggest that the interaction between ICS, eosinophils and eosinophilic cytokines may not be as important in the recurrence of symptoms than models of induced eosinophilic asthma suggest. We have shown that strong suppression of BAL and airways eosinophils as well as IL-5 and some suppression of eotaxin occurred under high doses of ICS. This suppression of inflammatory cells and cytokines continued after a reduction in treatment, in spite of deterioration in patient lung function and an increase in symptoms, for at least the 4-8 week timeframe of the project.

Chapter 5: Steroid Free Patients Placed on Long-term ICS treatment

5.1 Introduction

A direct means of studying the cellular and cytological events that occur in the airways of asthmatic patients when subjected to ICS is to sample the airways both before and after the application of ICS treatment. This section of the project involves a study of symptomatic asthmatics, not undergoing ICS treatment at baseline, who are then placed on a moderate to high dose of ICS and sampled several times over the course of one year. Whilst this design was not used to measure the immediate cellular events that occur upon the application of steroids to the airways, it was hoped that this study would allow a comparison between the untreated allergic state of the asthmatic airway and the airway after control of symptomatic asthma with ICS.

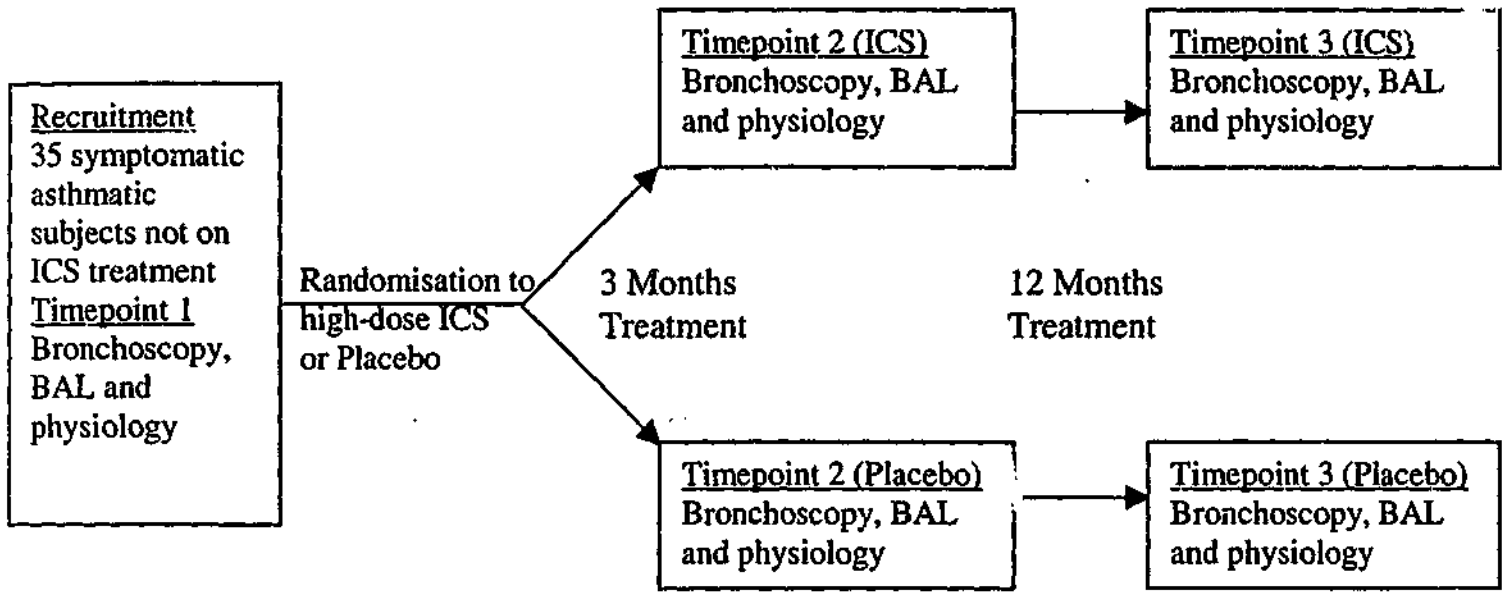
One of the primary objectives of this study was to examine the contrast between symptomatic ICS-free asthmatics and the same subjects after intensive ICS treatment. It has been observed that asthmatics who are not already undergoing ICS treatment benefit far more in terms of risk of exacerbations and asthma control than asthmatics already undergoing ICS treatment whose dosage is then increased (O'Byrne, *et al*, 2001). Many studies now have shown that for ICS treated asthmatics, additives such as salmeterol, complementing ICS treatment, are more effective (van Noord, *et al*, 1999, see also Chapter 6). From a céllular perspective, the "benefits" of ICS usage are not as clear. While it is understood that glucocorticoids affect a variety of inflammatory mediators via the GR complex (Umland, *et al*, 2002), switching genes both off and on, the entire list of up and down regulated genes remains incomplete.

What's more, among the inflammatory products which are affected, debate continues as to each of their relative importances. Yet despite the wide spread use of ICS treatment with its undeniable benefits, research involving *in vivo* examination of the interaction of ICS and cellular mediators is still uncommon.

The following study examines a group of symptomatic steroid-free asthmatic subjects who undergo bronchoscopy and physiology sampling prior to being placed on ICS or placebo treatment. The two subject groups were then sampled after both 3 and 12 months of either high-dose ICS or placebo treatment. At each of these three timepoints, eosinophils were quantitated in biopsy and BAL, and eotaxin and IL-5 protein levels assessed from BAL supernatant.

The objectives of this study were to assess the eosinophils and associated cytokines IL-5 and eotaxin in asthmatics prior to receiving ICS treatment, then to determine which of these markers of inflammation were suppressed after three months of ICS treatment. Furthermore, we hoped to observe any further improvement or reduction in eosinophilic inflammatory mediators in the same subjects after twelve months. Previous work by our group had suggested that this level of ICS treatment would significantly reduce airway eosinophil numbers (Booth, *et al*, 1995), but we did not know what effect long-term ICS treatment would have on the eosinophilic cytokines, nor if inflammation would be further reduced after 12 months of treatment. Of the three longitudinal studies undertaken in this project, this study involved the greatest amount of change in the ICS treatment regimes of its subjects and it was hoped that this contrast would allow for the strongest relationships between ICS, eosinophils and cytokines to be observed.

5.1.1 Study Design Diagram



5.2 Materials and Methods

The procedures used in this study follow the general protocols summarised in chapter 2, with the following exceptions and clarifications.

5.2.1 Patients and Bronchoscopy

Patient recruitment and bronchoscopy followed the guidelines given in sections 2.2.1 and 2.2.2. For this study three bronchial biopsies were processed into GMA resin, three into formalin fixed paraffin blocks (see 2.2.4) and two snap frozen for molecular biology.

The subject demographics were as follows (all were atopic):

	ICS Treatment Group	Placebo Group
Number of patients	18	17
Male/Female	14/4	8/9
Age (Yrs) (Range)	38 (23-62)	40 (20-70)
Duration of asthma (Yrs)	22±13	25±17

5.2.2 Clinical Markers

Subject responsiveness to methacholine challenge (PD_{20}) was measured at entry into the study, and prior to each bronchoscopy. At each of these three points forced expiratory volume (FEV_1), forced vital capacity (FVC) and forced expiratory flow between 25% and 75% of vital capacity (FEF_{25-75}) were also recorded from a flow volume curve.

5.2.3 BAL Differential Cell Counts

Differential cells counts (see 2.5.1) were performed at each of the three bronchoscopic timepoints. Eosinophils, neutrophils, lymphocytes, macrophages and airway epithelial cells were counted, with results expressed as percentages. Airway epithelial cell content in the BAL for this data set was relatively high (median=1.6 [0.6-2.8]).

5.2.4 BAL ELISA for IL-5 and Eotaxin

A portion of the stored, frozen (at -80°C) BAL supernatants were thawed and used for ELISA (see 2.4.2) for both IL-5 and eotaxin at each of the three bronchoscopic timepoints.

5.2.5 Biopsy Immunohistochemistry for Eosinophils and other Markers

Paraffin embedded biopsies (see 2.2.4) were cut and stained for both total (EG1) and activated (EG2) eosinophils (see 2.3.1). Eosinophils were quantitated using computer image analysis and counts expressed per area of lamina propria and per mm of basement membrane (see 2.5.2).

5.3 Results

Of the 35 subjects originally recruited for the study, 2 withdrew from the treatment group (one before 3 months, the other before 12 months), and 6 withdrew for the placebo group (one before three months and 5 before 12 months). This left 16 subjects in the treatment group and 11 in the placebo group for final analysis.

5.3.1 Clinical Profiles

Measures of both FEV_1 and PD_{20} were significantly improved after three months of treatment, FEV_1 then remained stable, but BHR continued to improve over the full 12 months. The following table summarises that data. Significance differences between the three time points were calculated by ANOVA, followed by Tukey post testing for each clinical marker, and is presented after the table.

	Pre-treatment	3 months	12 months	Group Significance (ANOVA)
ICS Treatment Group				
FEV_1 (L)	3.7±1.0	4.0±0.8	4.0±0.9	p=0.003
Log PD_{20}	-1.6±0.8	-0.4±1.1	0.4±1.0	p<0.0001
Placebo Group				
FEV_1 (L)	2.9±0.4	2.9±0.5	2.9±0.5	ns (p=0.89)
Log PD_{20}	-1.3±0.9	-1.3±0.9	-1.4±1.2	ns (p=0.77)

Between group analyses showed that after 12 months of treatment, the placebo and treatment groups were significantly different for both FEV_1 (p=0.009) and PD_{20} (p=0.01).

Significance (Post-analyses were only done for significant distributions, non-significant post-tests are not shown):

Within group significance for FEV_1:

Pre-treatment vs 3 months ICS treatment **p<0.01**
Pre-treatment vs 12 months ICS treatment **p<0.05**

Within group significance for PD_{20} in ICS treated subjects:

Pre-treatment vs 3 months ICS treatment	**p<0.001**
Pre-treatment vs 12 months ICS treatment	**p<0.001**
3 months vs 12 months ICS treatment	**p<0.01**

The PD_{20} data have been illustrated in figure 5.1.

5.3.2 Changes in BAL Cell Profiles after Treatment

The following table summarises the average cell numbers calculated from differential cell counts performed on the BAL cells from each subject taken at each of the three bronchoscopies. Significance differences between the three time points were calculated by ANOVA, followed by post testing for each cell type.

	Pre-treatment	3 months treatment	12 months treatment	Significance (pre-3 months)	Significance (pre-12 months)
ICS Treatment Group					
Macrophages	69.6±7.9	66.6±13.8	74.7±12.5	ns (p>0.05)	ns (p>0.05)
Lymphocytes	20.4±9.2	29.2±14.2	21.3±12.4	ns (p>0.05)	ns (p>0.05)
Neutrophils	2.3±1.3	2.1±1.6	2.0±1.8	ns (p>0.05)	ns (p>0.05)
Eosinophils	3.6±5.3	1.4±1.1	1.3±1.7	ns (p>0.05)	p<0.05
Epithelial cells	4.1±4.5	0.8±1.4	0.7±0.8	p<0.001	p<0.001
Placebo Group					
Macrophages	72.0±10.0	68.0±11.7	71.1±10.8	ns (p>0.05)	ns (p>0.05)
Lymphocytes	19.9±10.5	24.7±11.1	21.6±9.1	ns (p>0.05)	ns (p>0.05)
Neutrophils	2.0±2.0	1.6±1.4	2.5±4.0	ns (p>0.05)	ns (p>0.05)
Eosinophils	2.2±2.2	2.2±2.3	2.2±2.4	ns (p>0.05)	ns (p>0.05)
Epithelial cells	3.9±2.7	3.6±2.3	2.6±1.5	ns (p>0.05)	ns (p>0.05)

Between-group analyses showed a significant difference between BAL eosinophils in the treatment and placebo groups (p=0.05). For BAL epithelial cells, a strong trend was observed (p=0.10). The significant correlations for the above differential counts, BAL eosinophils and epithelial cells in ICS treated subjects are illustrated in figures 5.2 and 5.3 respectively. Neither eosinophils nor epithelial cells showed a significant change from 3 to 12 months.

Figure 5.1 Improvements in PD_{20} observed after 3 and 12-months treatment of patients with ICS.

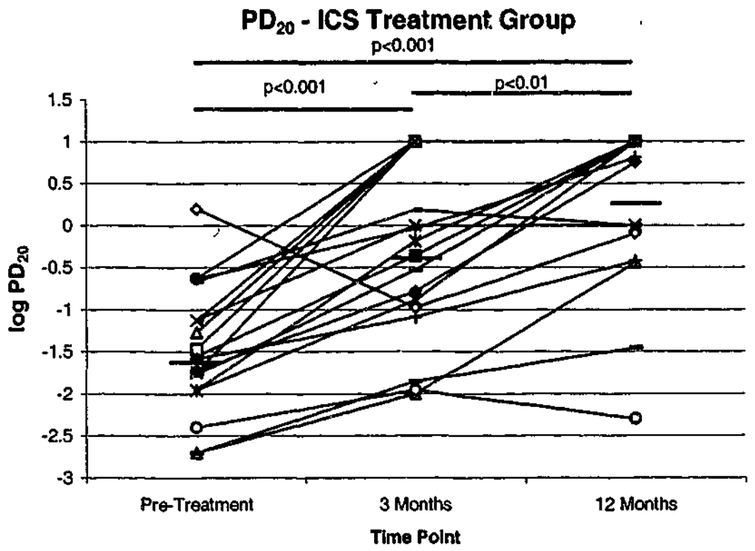


Figure 5.2 Decreases in BAL eosinophils in ICS-treated subjects after 12-months of treatment.

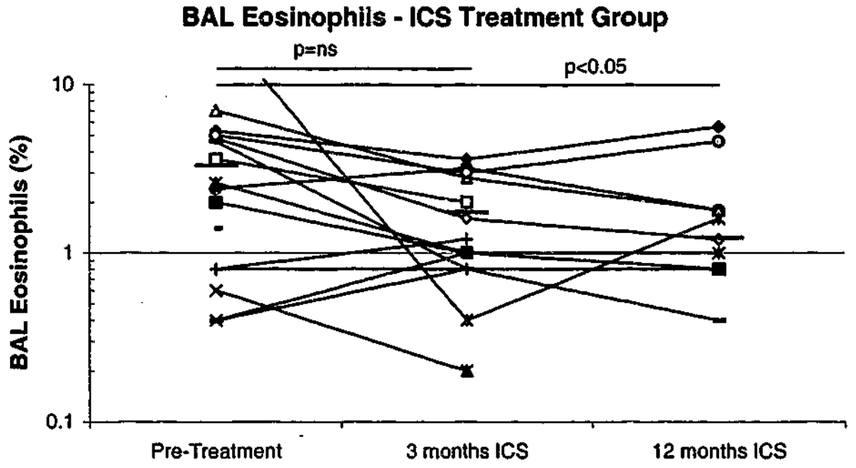
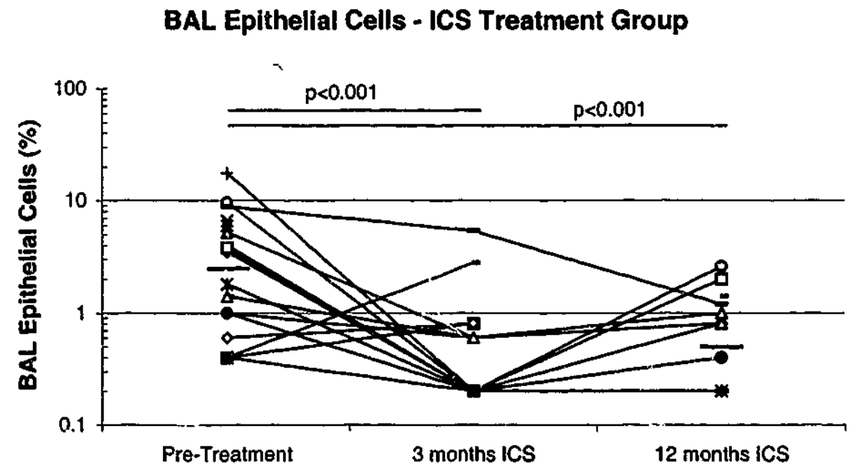


Figure 5.3 Decreases in BAL epithelial cells in ICS-treated subjects after 3 and 12-months of treatment.



5.3.3 BAL IL-5

Levels of IL-5 protein in the BAL were compared before ICS treatment and after both 3 and 12 months of treatment. In the treatment group a significant difference was seen between pre-treatment BAL IL-5 levels and both the three and 12-month treatment timepoints ($p<0.05$ at 3 months, $p<0.001$ at 12 months, see figure 5.4). The 3 and 12-month timepoints were not significantly different. The IL-5 levels were also contrasted to normal non-asthmatics and were found to be initially higher ($p<0.001$), remained somewhat higher at 3 months of treatment ($p<0.01$), but were not significantly different to non-asthmatics after 12 months of treatment. These data are summarised in the following table:

ICS Treatment Group	Pre-treatment	3 Months ICS	12 Months ICS	Non-Asthmatic controls
Median IL-5 levels (pg/ml)	15 [7.7-85.5]	7.5 [4.1-11.9]	4.2 [3.2-10]	0.9 [0.2-3.4]
Significance to ICS free asthmatics		p<0.05	p<0.001	
Significance to non-asthmatics	p<0.001	p<0.01	ns	

There was no significant difference between the 3 and 12-month time points.

The analysis of the placebo group showed no differences between BAL IL-5 levels at any of the three timepoints (see figure 5.5). When contrasted to non-asthmatic controls, all IL-5 levels were found to be higher at all timepoints ($p<0.001$ initially, $p<0.01$ at 3 months, $p<0.05$ at 12 months), though the differences became somewhat smaller in the latter part of the study. The table below summarises the data:

Placebo Group	Pre-treatment	3 Months	12 Months	Non-Asthmatic controls
Median IL-5 levels (pg/ml)	11.2 [6.7-131]	5.5 [3.9-20.5]	5.0 [3.4-15.6]	0.9 [0.2-3.4]
Significance to ICS free asthmatics		ns	ns	
Significance to non-asthmatics	p<0.001	p<0.01	p<0.05	

There was no significant difference between the 3 and 12-month time points.

Figure 5.4 Decreases in BAL IL-5 in ICS-treated subjects observed after 3-months of treatment.

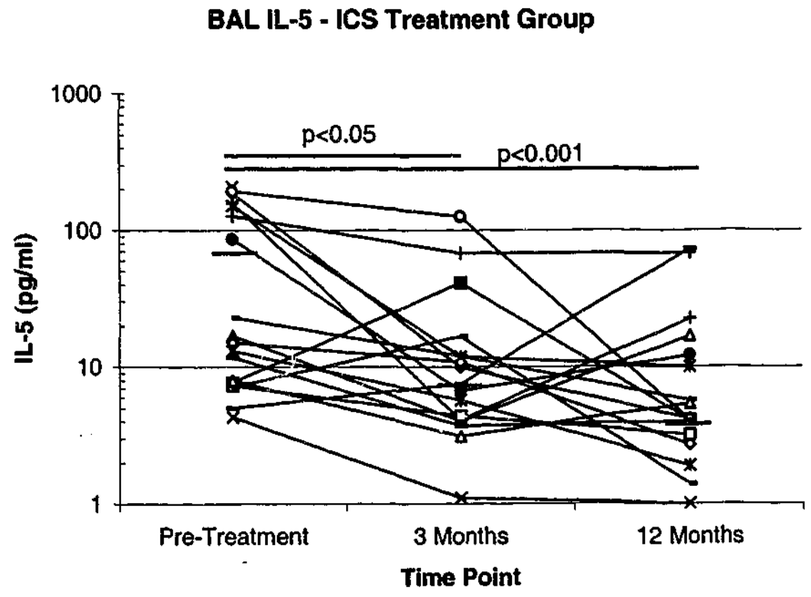
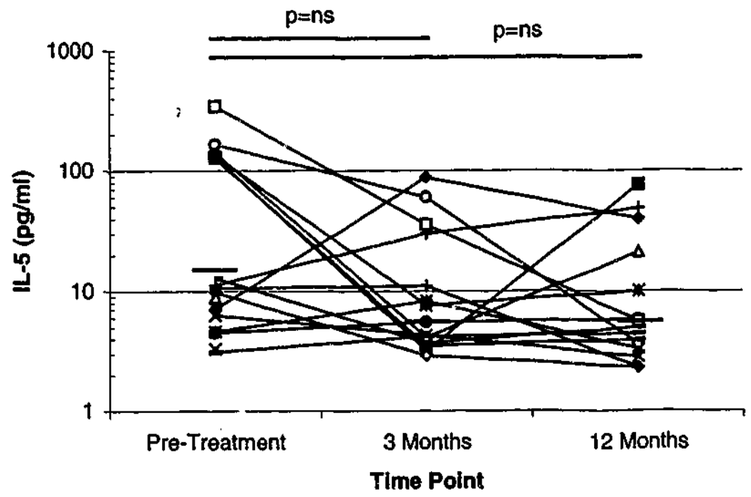


Figure 5.5 In placebo treated subjects, no significant changes were observed in BAL IL-5 after 3 or 12 months.



Between-group analyses showed that reductions in IL-5 were very similar for both the treatment and placebo subjects, and no difference was observed between the groups ($p=0.07$).

5.3.4 BAL Eotaxin

Throughout the study, BAL eotaxin was also measured. Unlike BAL IL-5, in the treatment group no differences were seen in eotaxin levels within the timeframe of the study (see figure 5.6). However, when comparing BAL eotaxin in the study to non-asthmatic controls, eotaxin levels in the asthmatic patients were greater, both before and after treatment ($p<0.01$ initially, $p<0.001$ at 3 months, $p<0.05$ at 12 months). The following table summarises the data:

ICS Treatment Group	Pre-treatment	3 Months ICS	12 Months ICS	Non-Asthmatic controls
Median Eotaxin levels (pg/ml)	6.2 [4.8-7.7]	10.5 [8.5-20]	7.1 [5.3-10.1]	0.9 [0.4-3.6]
Significance to ICS free asthmatics		ns	ns	
Significance to non-asthmatics	p<0.01	p<0.001	p<0.05	

There was no significant difference between the 3 and 12-month time points.

The placebo group showed very similar levels of BAL eotaxin across the study to the treatment group. Again, there was no significant difference between any of the study timepoints (see figure 5.7). When contrasted to non-asthmatic controls the placebo groups' levels of BAL eotaxin were higher for the duration of the study ($p<0.01$ initially, $p<0.05$ at 3 months, $p<0.05$ at 12 months). These data are summarised below:

Figure 5.6 In ICS-treated subjects, no significant changes were in BAL eotaxin observed after 3 or 12 months.

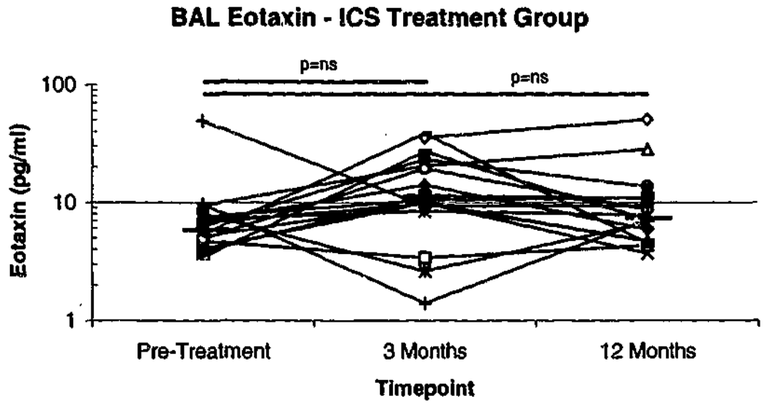
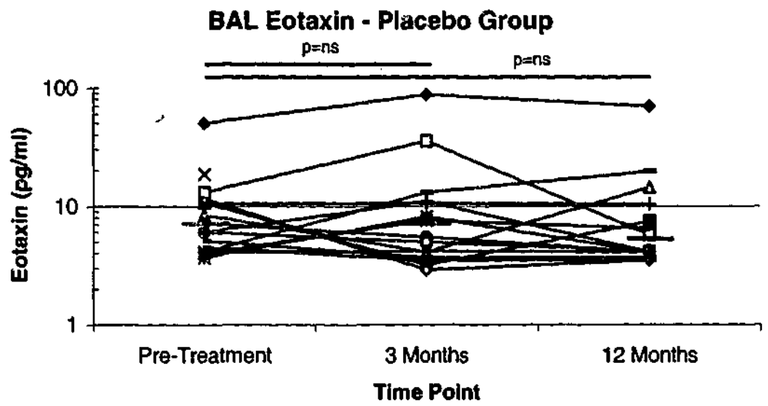


Figure 5.7 In placebo treated subjects, no significant changes were in BAL eotaxin observed after 3 or 12 months.



Placebo Group	Pre-treatment	3 Months	12 Months	Non-Asthmatic controls
Median Eotaxin levels (pg/ml)	6.3 [4.3-10.6]	6.5 [3.9-10.8]	4.5 [4.1-8.9]	0.9 [0.4-3.6]
Significance to ICS free asthmatics		ns	ns	
Significance to non-asthmatics	$p<0.01$	$p<0.05$	$p<0.05$	

There was no significant difference between the 3 and 12-month time points.

There was no significant difference between the treatment and placebo groups for BAL eotaxin.

5.3.5 Cell Markers in Biopsies

Image analysis was used to quantitate both total ($EG1^+$) and activated ($EG2^+$) eosinophils in the endobronchial biopsies of subjects before ICS treatment (ICS free) and after both 3 and 12 months of treatment with ICS or placebo. Whilst eosinophils in all groups showed some decrease in numbers after 3 months, this change was only significant in the ICS treatment group in total (but not activated) eosinophils, both per mm^2 area ($p<0.05$) and per mm basement membrane length ($p<0.05$). After 12 months ICS treatment there was a trend toward increases in eosinophil numbers, though this was not a significant increase above the 3-month data.

These data are also summarised on the table below, followed by the relevant statistical differences between the columns:

	Pre-treatment	3 Months	12 Months
ICS Treatment Group			
Total Eosinophils (EG1) /mm^2	347.3±450.6	55.8±67.6	140.0±239.4
Activated Eosinophils (EG2) /mm^2	157.3±154.4	50.7±69.3	104.1±177.1
Total Eosinophils (EG1) /mm BM	28.2±30.0	4.7±5.2	2.7 [0.9-7.7]
Activated Eosinophils (EG2) /mm BM	11.6±10.3	4.6±6.5	9.8±19.7
Placebo Group			
Total Eosinophils (EG1) /mm^2	483.2±452.6	244.0±295.8	300.1±209.6
Activated Eosinophils (EG2) /mm^2	243.3±265.3	77.3±91.4	145.8±129.0
Total Eosinophils (EG1) /mm BM	33.8±29.1	18.0±14.9	28.4±21.8
Activated Eosinophils (EG2) /mm BM	16.1±16.1	6.0±6.0	12.7±11.6

Between group analyses showed a trend for changes in total (p=0.10) eosinophils, but no difference in activated (p=0.27) eosinophils between the ICS treatment and placebo groups.

Significance (Post-analyses were only done for significant distributions, non-significant post-tests are not shown):

Significance of /mm^2 measures:

Total eosinophils:

Treatment group: ANOVA: **p=0.02**
ICS free vs. 3 months ICS, **p<0.05**
Placebo group: ANOVA: p=0.60

Activated eosinophils:

Treatment group: ANOVA: p=0.11

Placebo group: ANOVA: p=0.71

Significance of /mm BM measures:

Total eosinophils:

Treatment group: ANOVA: **p=0.03**
ICS free vs. 3 months ICS, **p<0.05**
Placebo group: ANOVA: p=0.40

Activated eosinophils:

Treatment group:	ANOVA:	p=0.30
Placebo group:	ANOVA:	p=0.63

The data distributions and significances for eosinophils per unit area are illustrated for each group as follows: Total eosinophils in the ICS treatment group (figure 5.8) and placebo group (figure 5.9), followed by activated eosinophils in the ICS treatment (figure 5.10) and placebo (figure 5.11) groups.

5.3.6 Correlations between factors in the BAL, Biopsies and Clinical Markers

The correlations for this study were divided into two categories; pre-treatment correlations examine the relationships observed in steroid-free asthmatic subjects. Post-treatment correlations examine the relationships between changes in measures from before treatment to after 12 months of ICS and placebo treatment. This becomes important because there were large and probably real changes in the placebo subjects as patients spontaneously improved or were removed from the study.

Pre-treatment Correlations (ICS Free Asthmatics):

BAL eosinophils were related to both total (r=0.36, p=0.04) and activated (r=0.51, p=0.002, see figure 5.12) eosinophils from airway biopsies.

Figure 5.8 Biopsy total eosinophils were significantly reduced after 3-months of ICS treatment.

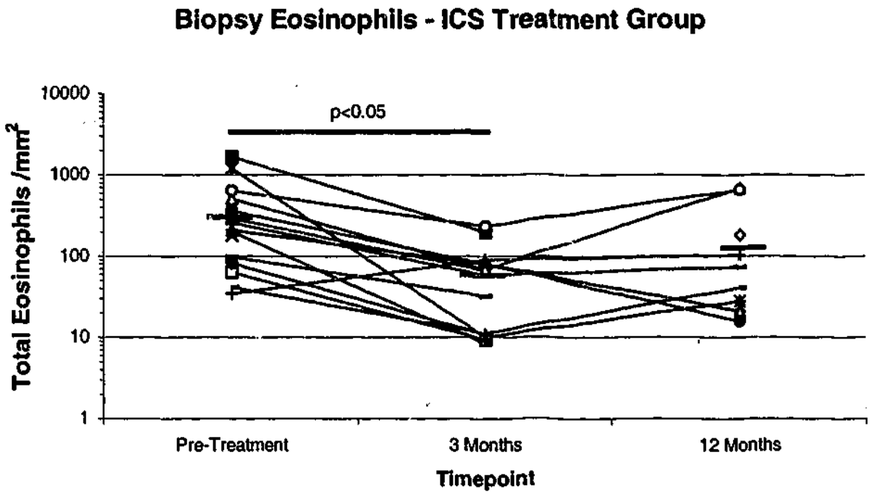


Figure 5.9 Biopsy total eosinophils were not significantly reduced after 3 or 12-months in the placebo group.

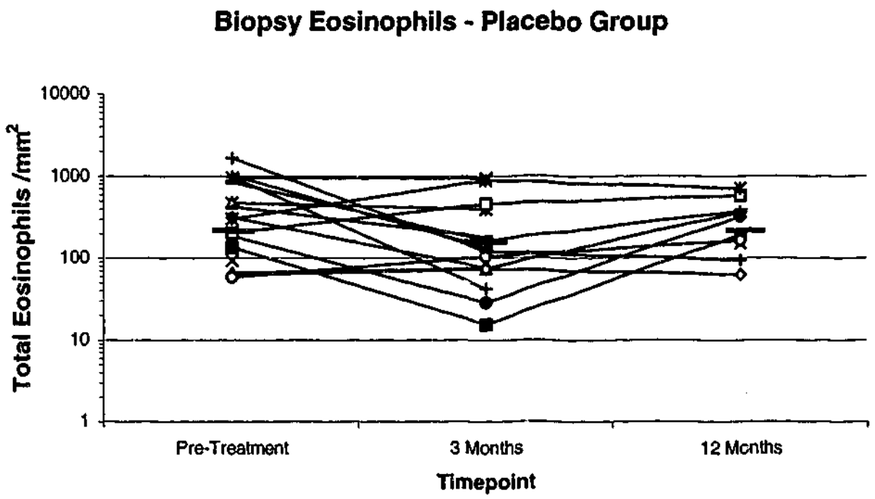


Figure 5.10 Biopsy activated eosinophils did not change after 3 or 12-months of ICS treatment.

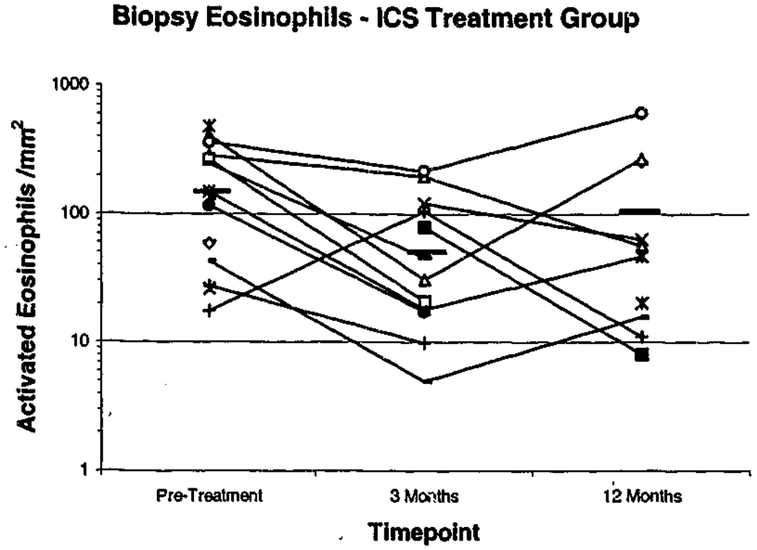


Figure 5.11 Biopsy activated eosinophils did not change after 3 or 12-months of placebo treatment.

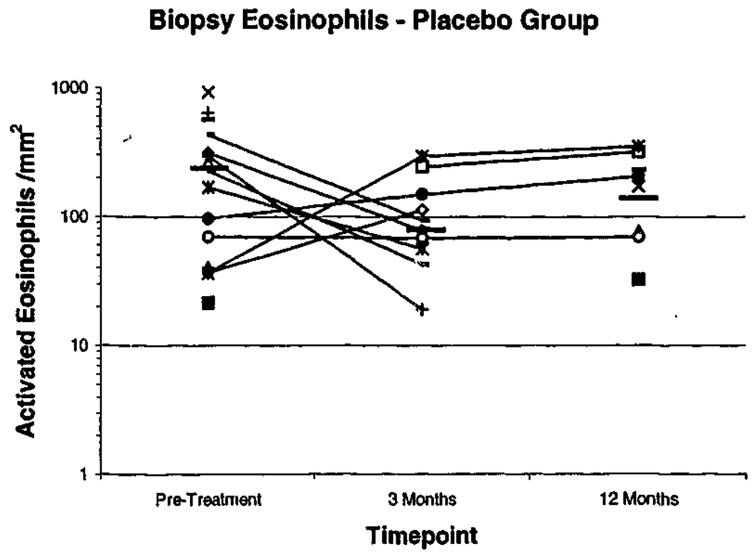


Figure 5.12 A positive relationship between biopsy activated eosinophils and BAL eosinophils, observed in subjects prior to treatment.

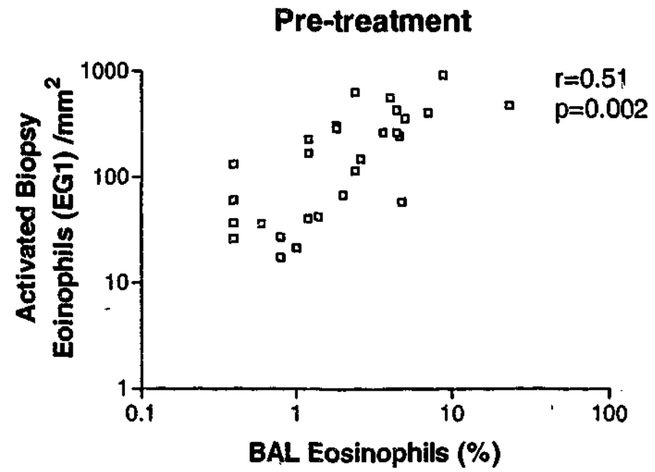
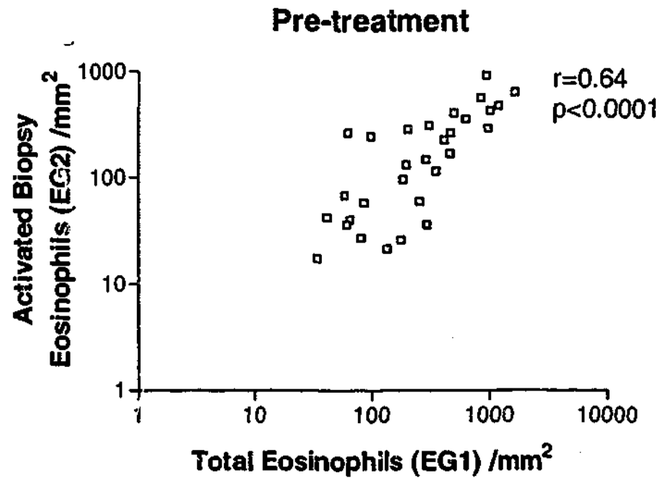


Figure 5.13 A positive relationship between activated and total biopsy eosinophils, observed in subjects prior to treatment.



Total biopsy eosinophils were related to activated eosinophils (r=0.64, p<0.0001, see figure 5.13) and inversely related to log PD_{20} (r=-0.44, p=0.009, see figure 5.14).

Activated eosinophils were also inversely related to log PD_{20} (r=-0.43, p=0.01).

Importantly, given the basic rationale for this study, no correlations were observed between levels of BAL IL-5 or eotaxin and any other factors.

Post-treatment Correlations (ICS Free Asthmatics after 12 months ICS treatment):

Treatment Group:

ΔBAL eosinophils were related to ΔActivated biopsy eosinophils (r=0.65, p=0.02), though the changes in BAL eosinophils numbers were quite small.

ΔActivated biopsy eosinophils were inversely related to Δlog PD_{20} (r=-0.75, p=0.005, see figure 5.15).

ΔBAL IL-5 was related positively to both ΔBAL eotaxin (r=0.65, p=0.006, see figure 5.16), and ΔBAL epithelial cells (r=0.67, p=0.004, see figure 5.17).

Placebo Group:

ΔTotal biopsy eosinophils were related to ΔActivated biopsy eosinophils (r=0.94, p<0.0001), but changes in both these measures were very small.

No other significant relationships were observed in these placebo subjects.

Figure 5.14 An inverse relationship between log PD_{20} and total biopsy eosinophils, observed in subjects prior to treatment

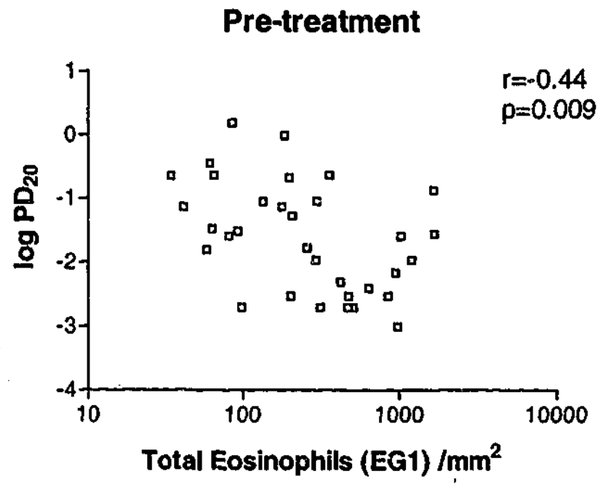


Figure 5.15 An inverse relationship between the change in log PD_{20} and the change in activated biopsy eosinophils, observed in ICS treated subjects.

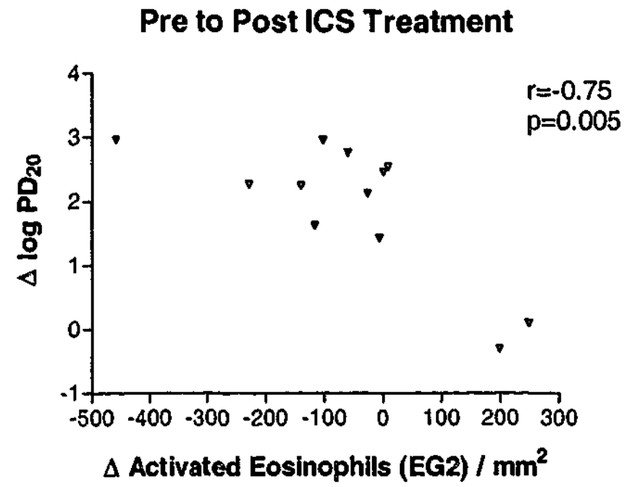


Figure 5.16 A relationship between the change in BAL eotaxin and the change in BAL IL-5, observed in ICS treated subjects.

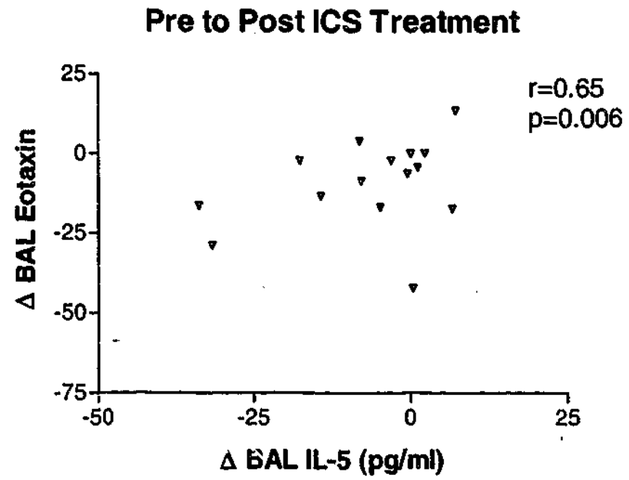
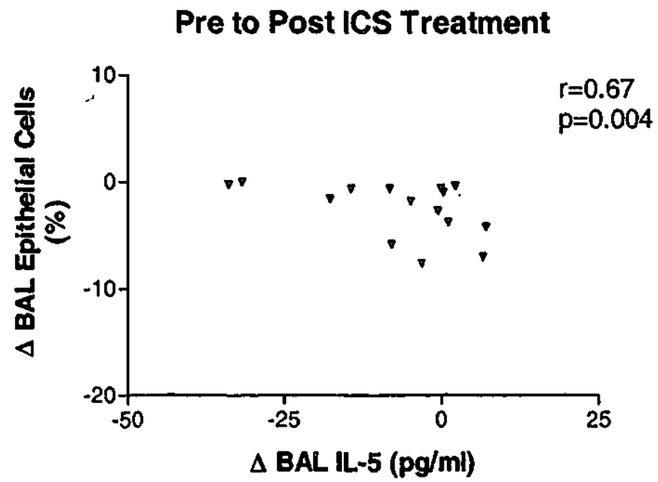


Figure 5.17 A relationship between the change in BAL epithelial cells and the change in activated BAL IL-5, observed in ICS treated subjects



5.4 Discussion

Three longitudinal studies were undertaken in this thesis. This study, which took steroid free asthmatics and placed them on an ICS or placebo treatment regime for 12 months, showed the greatest degree of contrast from beginning to end in patient symptoms, inflammatory cells and cytokine levels. Even before analysis of the clinical data, it was clear, after unblinding of the data, that the withdrawal rate in the placebo group was very high (6 of 17 subjects) compared with the treatment group (2 of 18 subjects), illustrating clearly differences in the health between the two groups. This was reflected in the clinical data where ICS treated subjects showed marked improvements in both lung function and BHR (see section 5.3.1), a finding that supports the work of others (Barnes, 1990). We also would have expected a decrease in the physiological measures in those subjects not receiving treatment, but those that became unwell were those who were withdrawn from the study, and thus the physiology in the remaining placebo subjects did not change after 12 months. The fact that the placebo group did not deteriorate despite lack of treatment suggests that our inclusion criteria for remaining in the study may have selected for those patients who spontaneously improved, thus creating a mild bias in the placebo group which could explain why that group mirrored the ICS treatment group for some indices of inflammation.

For the inflammatory indices examined, many significant changes were observed in this study that were not seen in the other studies examined in this thesis. Our cross-sectional data seemed to indicate that BAL differential cell counts were somewhat insensitive in detecting differences in eosinophilic inflammation in asthmatic patients (see section 3.3.1). The data presented for our steroid treated asthmatics supports this

to some degree, showing that a full 12 months of ICS treatment was required before a significant decrease could be shown in BAL eosinophil numbers, though there was a definite trend after three months of ICS treatment. Another observation only seen in this study was the amount of epithelial cell shedding into the BAL. Asthmatics not treated with ICS, showed about 3.5% epithelial cells in the BAL. However, when treated with ICS, the number of epithelial cells in the BAL decreased to less than 1%. While this decrease was not significantly different relative to the placebo changes in this study (p=0.10), after treatment, BAL epithelial cell levels were very similar to those seen in the other studies, all of which consisted of asthmatics being treated with similar levels of ICS. Our observations are consistent with those of other groups, who have shown a difference in the number of epithelial cells in the BAL between asthmatic and normal subjects (Oddera, *et al*, 1996), and earlier work has also demonstrated improvements in airway biopsy epithelial cell integrity after long-term ICS treatment (Lundgren, *et al*, 1988). Whilst it was not pursued further within the context of this thesis, these epithelial findings would form an excellent basis for the examination of longitudinal factors affecting epithelial integrity in a future study.

IL-5 in the BAL showed a substantial decrease after both 3 and 12 months of treatment, and while there was no significant difference in IL-5 between the 3 and 12-month timepoints, at 12 months BAL IL-5 was suppressed to essentially non-asthmatic levels. Unfortunately, in the placebo group, BAL IL-5 also decreased somewhat, though not significantly. It does seem however, that those untreated patients with the highest levels of IL-5 were also those who were withdrawn from the study, thus this downward trend in BAL IL-5 may have been due to selection against those whose IL-5 levels remained high. BAL eotaxin levels remained elevated above

non-asthmatic subjects after 12 months regardless of the presence or absence of ICS treatment. In fact, while the eotaxin levels in ICS treated asthmatics did not change over 12 months, those in the placebo group experienced a slight downward trend. This downward shift in untreated asthmatics resembles, but is not as severe, as that seen in BAL IL-5 and is again probably due to selection against higher cytokine expressing untreated asthmatics being withdrawn from the study. A correlation was also seen between changes in BAL IL-5 and changes in BAL eotaxin in ICS treated subjects when comparing pre to post ICS treatment ($r=0.67$, $p=0.004$). This observation could suggest that a level of eotaxin suppression occurs with ICS treatment, which to a limited extent parallels the more marked reduction in BAL IL-5.

In the airway biopsies, total eosinophils were greatly reduced after 3 months of ICS treatment, while no further reduction was seen at twelve months. This change was not seen in the placebo subjects though again a slight downward trend was observed (as above). Activated eosinophils were not significantly reduced in ICS-treated subjects, but a trend was detected ($p=0.11$). This reduction in tissue eosinophils after ICS treatment was confirmation of our expectations that 12-months of ICS treatment would substantially reduce airways inflammation. The ratios of total to activated eosinophils were initially 57%, and while it did increase to 63% after treatment with ICS, this change was not significant, nor as high as the ratio of activated to total eosinophils observed in ICS treated asthmatics (70-80%) in other studies in this thesis (see sections 3.4 and 4.4). As discussed previously, this may be related to eosinophil pre-cursors in the airways, which have been shown to be capable of differentiating at inflammatory sites (Cameron, *et al*, 2000).

There were several relationships observed in the steroid free asthmatics that were not seen in the other studies. BAL eosinophils were related to biopsy eosinophils, and while we may have expected this correlation to be present for other cells, the relatively high levels of eosinophils and lack of ICS may have made the compartmental link between BAL and biopsy eosinophils more apparent in this study. BHR was also related to activated eosinophils in untreated asthmatics at baseline, but as no similar relationship was seen with total eosinophils, it is difficult to validate this observation. However, patients treated with ICS also showed a strong correlation between changes in activated eosinophil numbers and changes in BHR, and while there was still no correlation with total eosinophils, it is of great interest that in this study where eosinophils significantly changed so did BHR. This is complementary to our steroid withdrawal study (see Chapter 4), where neither eosinophils nor BHR changed.

This study has shown that ICS treatment for a period of 3 to 12-months is sufficient to significantly lower airway and BAL eosinophils, cause a significant reduction in epithelial cells present in the BAL, lower BAL IL-5 and improve BHR and lung function. This level of ICS treatment, however, did not cause a significant reduction in BAL eotaxin levels. This study illustrates the clear relationship between ICS treatment and reduction of markers of inflammation, specifically eosinophils and IL-5 as a primary eosinophilic cytokine. The lack of reduction in eotaxin after ICS treatment was unexpected, as eotaxin expression is at least partially suppressible by corticosteroids *in vitro* (Pang, *et al*, 2001). The relationship observed between BHR and biopsy eosinophils, though consistent with an improvement in health and reduction in inflammation, is also interesting in that it contrasts with our steroid

withdrawal study where patients also improved in lung function but not in BHR or airway eosinophils.

These data represent significant observations into the relationship between asthmatic ICS treatment and eosinophilic inflammation. Examination of these eosinophilic cytokines and their direct measurement in the context of a human ICS study has not, to our knowledge, been performed before. While the repression of IL-5 by ICS was largely expected, the failure of ICS to suppress eotaxin expression is also a novel finding and raises questions as to this cytokine's importance in regulation of eosinophils in the airways.

Chapter 6: The Effects of Long Acting Beta Agonist in Combination with Low Dose ICS vs. High Dose ICS

6.1 Introduction

Whilst previous sections of this thesis have involved studies in which subjects were withdrawn from ICS treatment (Chapter 4) or placed on ICS after being steroid-free (Chapter 5), this section deals with supplementing the treatment of subjects already receiving ICS treatment, and is an extension of a previously published study (Li, *et al*, 1999). In this study, we attempted to gain insight into the underlying mode of action in airway inflammation when a long acting beta agonist (LABA) is added to ICS, in contrast to doubling the dose of ICS. Whilst there are many potential agents that could be used in conjunction with ICS, such as antileukotriene drugs and immune inhibitors such as cyclosporine A, this study was concerned with the more typical forms of asthma treatment supplementation. This study examined the effects, on eosinophilic cytokines, of doubling ICS doses in a patient sub-set, whilst supplementing another with ICS plus long acting β_2 agonist, and comparing both these groups to subjects maintained on the original unsupplemented ICS dose.

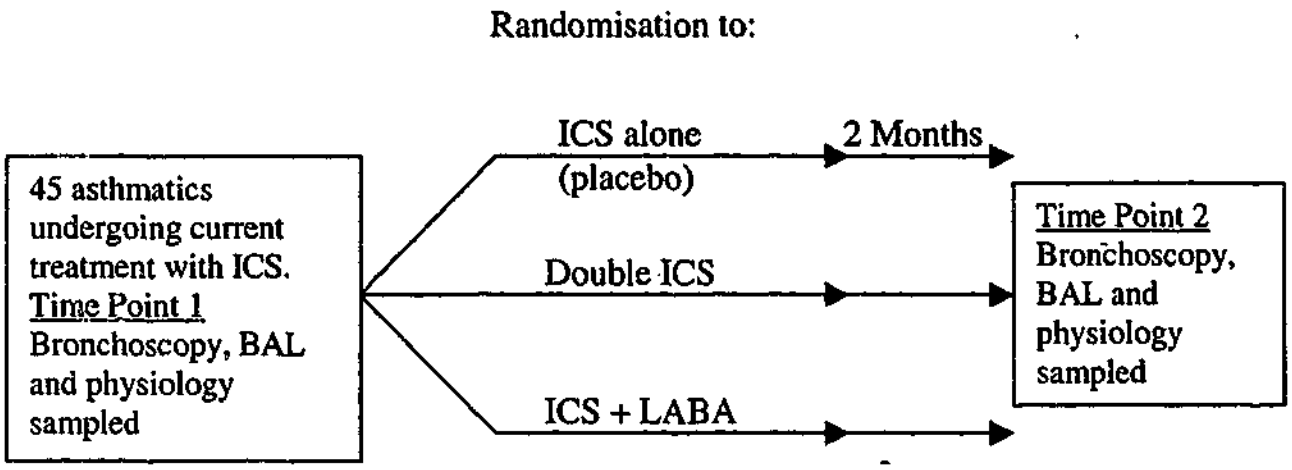
There has been strong evidence that, at least therapeutically, combined treatment with long acting β_2 agonists (LABA) and ICS significantly improves peak flows and reduces symptom score (Woolcock, *et al*, 1996), although this positive patient outcome did not correspond to alteration in BHR or increasing exacerbation rates. This and other studies (Greening, *et* al, 1994) have suggested that although an increase in ICS dose results in improved peak flows, combined treatment with ICS and LABA provides even more improvement. Despite the apparent therapeutic value

of such combined treatment regimes, the underlying cytochemical events that occur in the airways under such circumstances are largely undocumented.

Though studies with gluco-corticosteroids (CS) have shown some inhibitory effect on IL-5 and eotaxin production in cell culture *in vitro* (Lilly, *et al*, 1997), very little human or animal *in vivo* data exist relating CS treatment to cytokines levels or eosinophil numbers. Longitudinal data for this area of research are notably absent in spite of the widespread use of inhaled corticosteroids (ICS) in asthma therapy. Similarly, there are no data of which we are aware documenting the effect of LABA on eotaxin or IL-5 levels in humans in spite of the popularity of LABA in asthma management and some suggestion of an anti-inflammatory effect (Li, *et al*, 1999). Though many elements of the "β_2 agonist debate" about safety of regular β-agonist use have been resolved (Lipworth, 1997), there is still concern about the possible adverse effects of LABA on airway inflammation in asthma (Mcivor, *et al*, 1998).

This work builds upon our previously published data (Li, *et al*, 1999) that consisted of a prospective, longitudinal, parallel group, placebo-controlled study in which supplementary fluticasone or salmeterol was given to symptomatic asthmatics in spite of already receiving low dose ICS (beclomethasone, BDP). The study showed significant reductions in biopsy eosinophil numbers after combined ICS and LABA treatment. To extend that work we sought to specifically examine the quantities of eotaxin and IL-5 in the BAL fluid of these subjects, to assess whether these cytokines could be related to the anti-eosinophilic effect that we had already observed. We hypothesised that this reduction in eosinophilic inflammation may be mediated through IL-5 and/or eotaxin.

6.1.1 Study Design Diagram



6.2 Materials and Methods

The procedures used in this study followed the protocols presented in chapter 2, with the following exceptions and clarifications.

6.2.1 Patients and Bronchoscopy

Patient recruitment and bronchoscopy followed the guidelines given in sections 2.2.1 and 2.2.2. For this study three bronchial biopsies were processed into formalin fixed paraffin blocks (see 2.2.4) and three snap frozen.

The subject demographics were as follows:

	ICS (Control) Group	Supplemented ICS Group	ICS + β-agonist group
Number of patients	16	16	13
Male/Female	7/9	11/5	8/5
Age (Years)	38±14	40±12	39±12
# of Atopics	13	11	13

6.2.2 Clinical Markers, BAL Cell Differential Counts and Immunohistochemistry for Cell Markers

These clinical and cellular measures were taken from the published study (Li, *et al*, 1999) and used to compare cytokine measures to. Full details can be found in the paper. In brief, subject responsiveness to methacholine challenge (PD_{20}) and forced expiratory volume (FEV_1), was measured prior to each bronchoscopy. Differential cells counts of BAL cells were performed at each of the two bronchoscopic timepoints. Eosinophils, neutrophils, lymphocytes, macrophages and airway epithelial cells were counted, with results expressed as percentages. Paraffin embedded biopsies were cut and stained for both total (EG1) and activated (EG2) eosinophils. Eosinophils were quantitated using computer image analysis and counts expressed per area of lamina propria and per mm of basement membrane.

6.2.3 BAL ELISA for IL-5 and Eotaxin

A portion of the stored, frozen (at -80°C), BAL supernatants were thawed and used for ELISA (see 2.4.2) for both IL-5 and eotaxin at each of the two bronchoscopic timepoints.

6.3 Results

47 subjects were originally enrolled in this study, and only 2 withdrew after the first bronchoscopy, leaving 45 subjects that followed the study to completion. Results below for clinical markers, BAL cell and biopsy eosinophil counts have been taken from the original paper and integrated with the cytokine data (Li, *et al*, 1999). To maintain internal consistency, the same statistical methods used throughout this thesis have been applied to the data. Thus, there is a small degree of deviation from how the data was originally presented.

6.3.1 Changes in Clinical Markers

The original study showed that lung function measured by percent predicted FEV_1 was found to improve significantly only in subjects treated with additional ICS (p=0.006) though a trend was observed in LABA treated subjects (p=0.15). Analysis of the amount of change in FEV_1 between groups, however, showed no significant differences (p=ns (0.50)). These data are illustrated in figure 6.1. Airways hyper-reactivity was decreased (represented by an increase in log PD_{20}) in subjects treated with ICS and additional LABA (p=0.02), though a very strong trend was present in additional ICS treated subjects (p=0.051). Again, there was no difference in the amount of change between groups (p=ns (0.85)). See figure 6.2.

6.3.2 Changes in BAL Cell Profiles after Treatment

The following table summarises the average cell numbers calculated from differential cell counts performed in the original study on the BAL cells from each subject taken

Figure 6.1 Lung function (FEV_1) before and after supplemental treatment in the three patient groups.

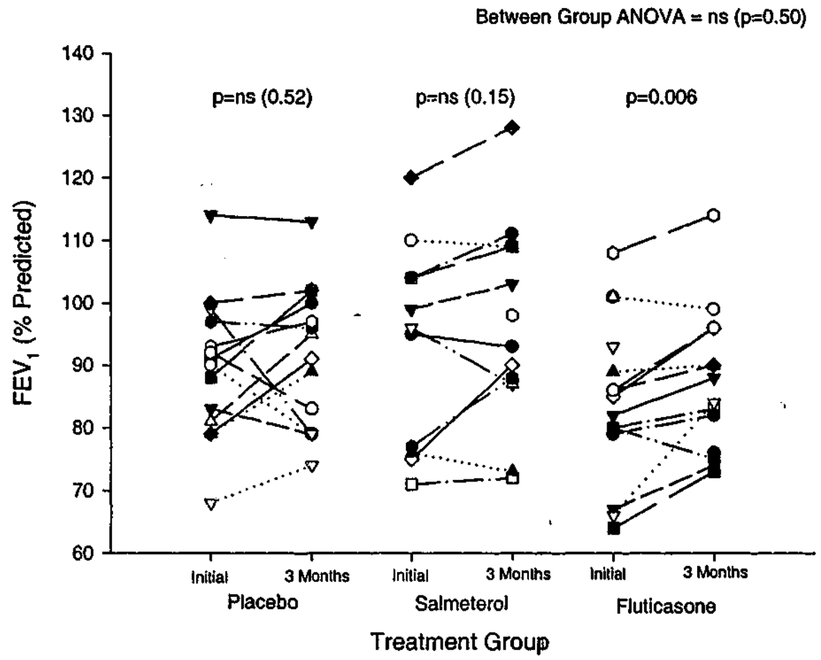
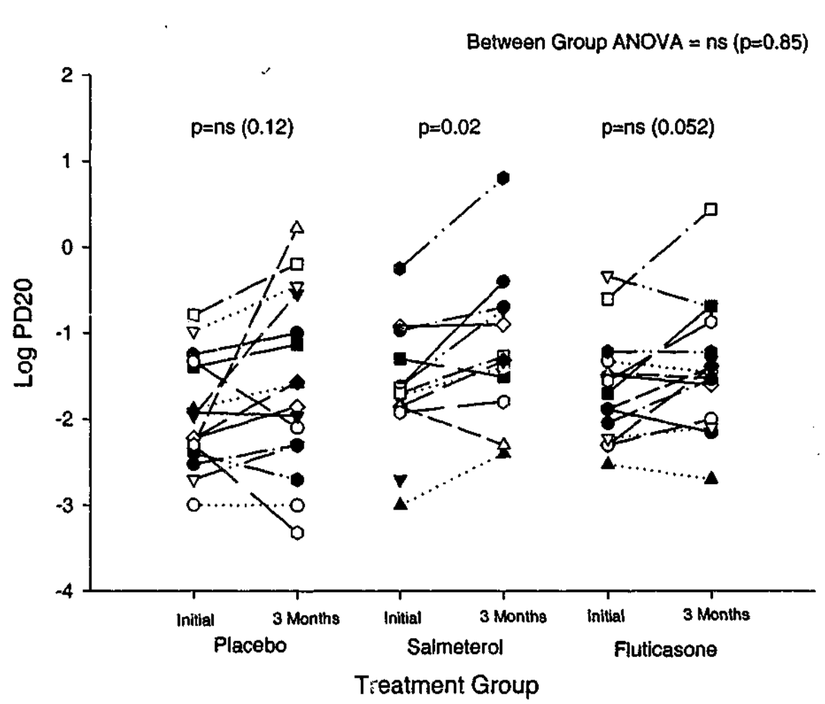


Figure 6.2 Airways hyperresponsiveness (PD_{20}) before and after supplemental treatment in the three patient groups.



at the two bronchoscopic timepoints. Significance differences between the two time points were calculated by paired t-tests for each cell type.

	Initial ICS Treatment	3 Months Supplemental Treatment	Significance (Initial – 3 months)
ICS (Control) Group			
Macrophages	75.1±10.1	71.0±14.1	ns (p=0.22)
Lymphocytes	15.4±5.8	18.3±10.3	ns (p=0.21)
Neutrophils	2.5±2.3	4.1±4.7	ns (p=0.23)
Eosinophils	1.8±1.4	1.7±1.9	ns (p=0.97)
Epithelial cells	5.2±6.1	4.8±5.6	ns (p=0.82)
Supplemented ICS Group			
Macrophages	71.0±16.7	71.4±12.7	ns (p=0.91)
Lymphocytes	22.6±16.0	17.7±8.7	ns (p=0.20)
Neutrophils	4.0±9.3	7.3±12.4	p=0.001
Eosinophils	2.2±2.9	1.9±2.2	ns (p=0.38)
Epithelial cells	1.4±1.1	2.1±3.0	ns (p=0.36)
ICS + LABA group			
Macrophages	72.7±12.9	65.7±19.3	ns (p=0.22)
Lymphocytes	22.0±14.4	27.9±18.6	ns (p=0.33)
Neutrophils	2.9±2.0	2.3±1.9	ns (p=0.22)
Eosinophils	1.9±2.0	1.5±1.8	ns (p=0.35)
Epithelial cells	2.6±2.1	2.6±3.7	ns (p=0.98)

6.3.3 BAL IL-5

After 3 months of treatment, only subjects treated with fluticasone and supplemental LABA showed a significant change, which was an increase in BAL IL-5 levels. ($p<0.01$, see figure 6.3). These data, along with a comparison of BAL IL-5 levels to non-asthmatic control subjects, are summarised in the table below:

	BAL IL-5 (pg/ml)		Significance		
	Initial	3 months	Initial to 3 months	Initial to normal	3 months to normal
ICS alone	6.6±8.3	3.5±3.5	ns (p=0.19)	ns (p=0.32)	ns (p=0.98)
Supplemented ICS	6.9 [0.9-23.3]	3.7 [1.2-23.9]	ns (p=0.33)	ns (p=0.17)	ns (p=0.18)
ICS + LABA	4.5±6.3	27.2±31.7	p=0.03	ns (p=0.46)	p=0.008
Normal Range	1.5 [0.8-6.2]				

Figure 6.3 BAL IL-5 levels before and after supplemental treatment in the three patient groups.

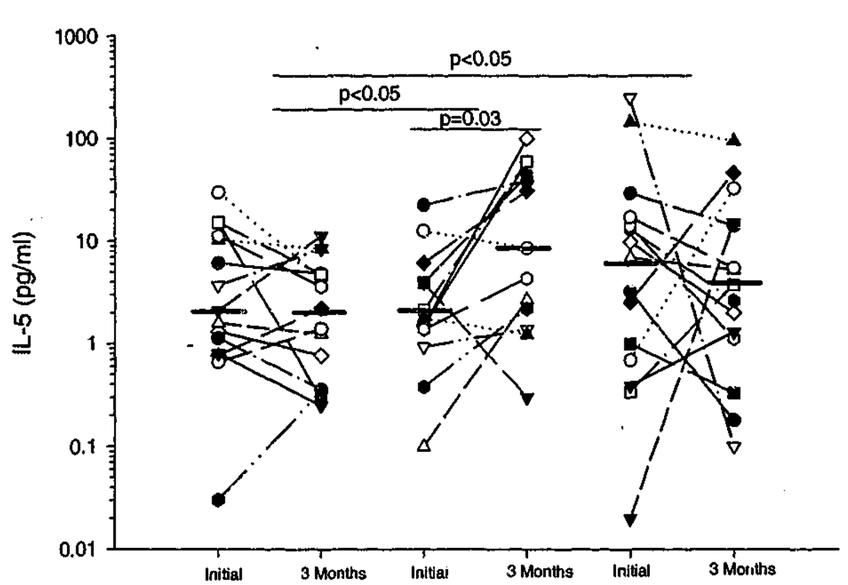
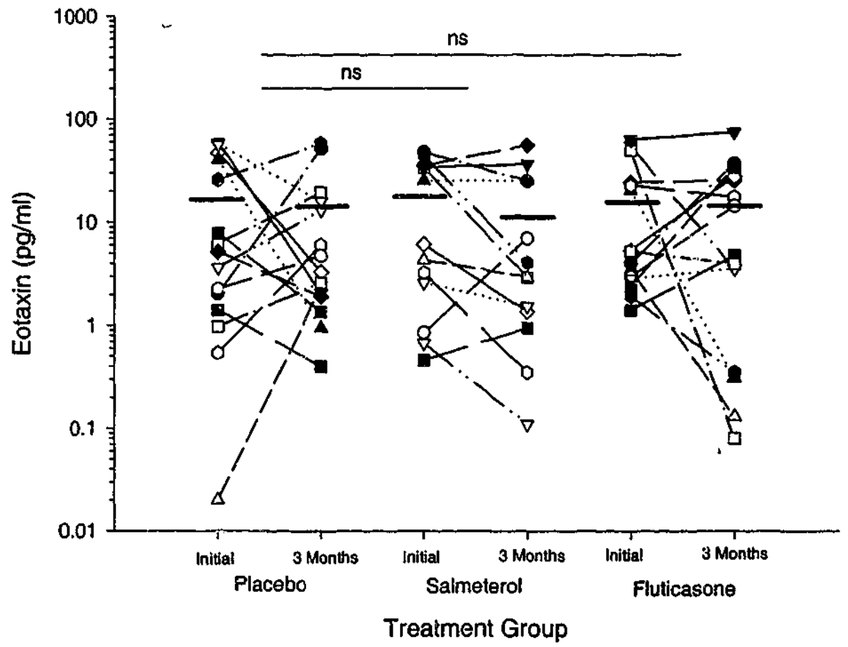


Figure 6.4 BAL eotaxin levels before and after supplemental treatment in the three patient groups.



Between-group significance (non-significant post-tests are not shown):

Between-group Kruskal-Wallis test: **p=0.008**
ICS alone vs. ICS + LABA: **p<0.05**
ICS alone vs Supplemented ICS vs: **p<0.05**

6.3.4 BAL Eotaxin

BAL eotaxin levels did not significantly change in subjects in any treatment group, and did not significantly vary between groups (see figure 6.4). The mean BAL eotaxin levels for each group are presented below with calculated significance both within each treatment arm and relative to the established normal range:

	BAL Eotaxin (pg/ml)		Significance		
	Initial	3 months	Initial to 3 months	Initial to normal	3 months to normal
ICS alone	17.2±22.0	12.3±18.4	ns (p=0.85)	ns (p=0.58)	ns (p=0.62)
Supplemented ICS	17.7±21.6	16.5±20.7	ns (p=0.98)	ns (p=0.06)	ns (p=0.22)
ICS + LABA	18.4±18.6	12.6±17.7	ns (p=0.24)	ns (p=0.18)	ns (p=0.96)
Normal Range	0.95 [3.2-39.1]				

Between group ANOVA: p=ns (0.86)

6.3.5 Total and Activated Eosinophils in Biopsies

The initial study showed that subjects treated with ICS and additional LABA showed a significant decrease in total, but not activated, eosinophils, both normalised to total area of lamina propria (p=0.01, see figure 6.5) and per mm of basement membrane (p=0.02). The following table summarises that data, indicating the mean and significant differences for biopsy eosinophils:

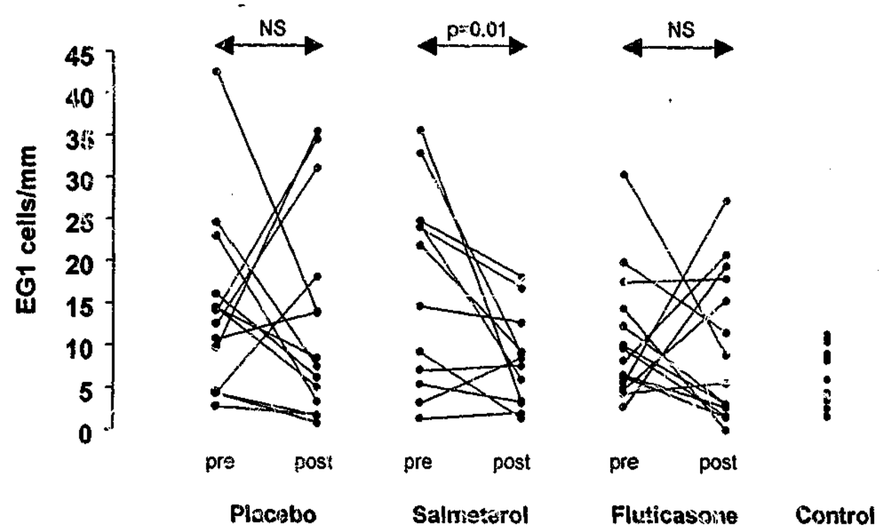


Figure 6.5 Airway eosinophil numbers before and after supplemental treatment in the three patient groups. (From Li, *et al*, 1999).

	Total Eosinophils (EG1) / mm^2		Activated Eosinophils (EG2) / mm^2		Total Eosinophils (EG1) / mm BM		Activated Eosinophils (EG2) / mm BM	
	Initial	3 months	Initial	3 months	Initial	3 months	Initial	3 months
ICS alone	180.2 ±108.5	163.9 ±156.6	68.7 ±72.0	65.9 ±63.1	13.45 ±10.5	13.0 ±12.4	5.6 ±7.0	5.6 ±6.2
Significance	ns (p=0.57)		ns (p=0.68)		ns (p=0.79)		ns (p=0.83)	
Supplemented ICS	116.5 ±85.5	123.8 ±126.8	52.81 ±38.1	51.79 ±81.4	10.3 ±7.7	9.4 ±8.7	4.6 ±3.6	3.6 ±4.8
Significance	ns (p=0.86)		ns (p=0.98)		ns (p=0.77)		ns (p=0.60)	
ICS + LABA	274.8 ±216.1	110.4 ±73.9	78.8 ±67.2	46.9 ±42.6	17.1 ±11.7	8.1 ±5.3	6.1 ±6.4	3.7 ±3.6
Significance	p=0.01		ns (p=0.14)		p=0.02		ns (p=0.22)	

Between-group statistics showed no significant difference in the change in total eosinophil numbers after any form of treatment (ANOVA = ns (p=0.054))

6.3.6 Correlations between Factors in the BAL, Biopsy and Clinical Markers

Correlation analyses for this study have been divided into two sections. First, measured factors in the study groups have been pooled and compared, before randomisation. Then each treatment arm has been analysed separately for both simple relationships and change in factors before and after supplemental treatment. These results have been analysed by Pearson's (parametric) or Spearman's (non-parametric) correlation analysis, as appropriate for the distribution of the data.

Pre-randomised ICS treated subjects:

BAL IL-5 correlated with bronchoscopy return volume (r=0.32, p=0.01). Though potentially confounding, this relationship has not been demonstrated in any other treatment or study group and is probably artefactual.

BAL eotaxin was positively related to BAL airway epithelial cells (r=0.33, p=0.03), which may refect that primary eotaxin expression is by airway epithelium.

Biopsy total eosinophils per mm^2 were strongly related to activated eosinophils per mm^2 (r=0.73, p<0.0001), as well as BAL airways epithelial cells (r=0.38, p=0.01, see figure 6.6) and BAL neutrophils (r=0.32, p=0.04).

BAL eosinophils were found to be related to biopsy total (r=0.36, p=0.02) and activated (r=0.32, p=0.04) eosinophils per mm basement membrane, but not per mm^2.

ICS-alone treated subjects (placebo):

In subjects given no supplemental treatment, changes in both IL-5 and eotaxin did not relate to the small variations and any other measures within this group. BAL eosinophil changes also could not be related to other factors. It was reassuring that the more important correlations seen in the other groups did not appear in the placebo analyses, were most correlations would be due to random noise.

Additional ICS treated subjects:

BAL IL-5 levels in subjects treated with additional ICS were found to be negatively related to BAL lymphocytes (r=-0.59, p=0.02, see figure 6.7). Flow cytometry data from the same study showed that BAL $CD3^+$ T cells also correlated inversely to BAL

Figure 6.6 A positive relationship observed between BAL epithelial cells and biopsy total eosinophils in subjects before supplemental treatment.

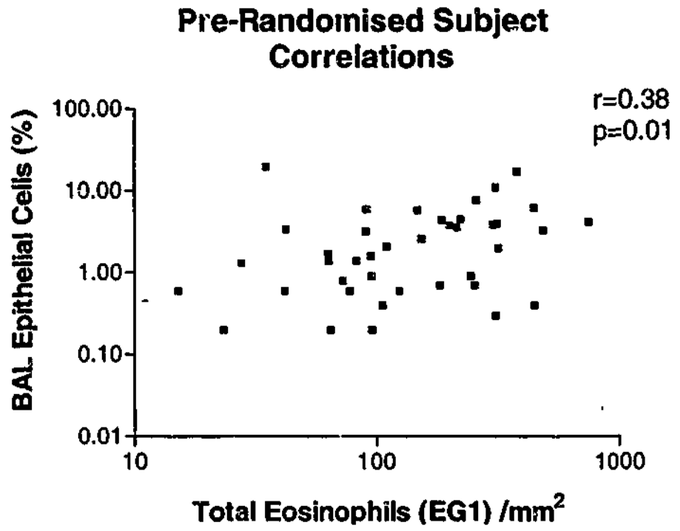
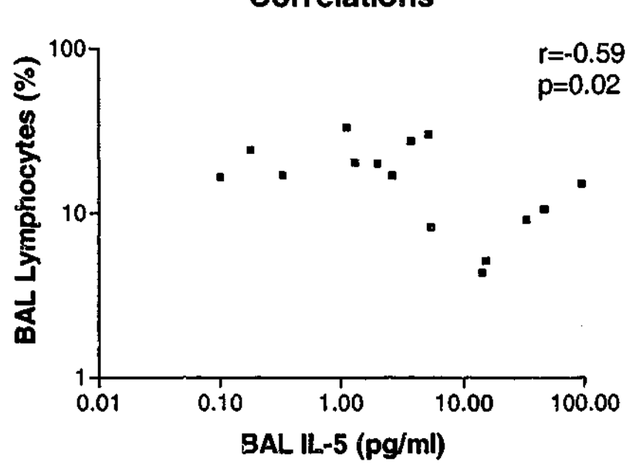


Figure 6.7 An inverse relationship observed between BAL lymphocytes and BAL IL-5 in subjects treated with additional ICS.



IL-5 (r=-0.58, p=0.03, see figure 6.8). As T cells are a primary source of IL-5, the direction of this relationship was unexpected and apparently paradoxical.

BAL eotaxin showed a positive correlation with activated eosinophils per mm^2 in biopsies (r=0.59, p=0.03, see figure 6.9). Only these ICS supplemented patients showed a direct positive relationship between eotaxin and eosinophils, a correlation that perhaps may have been expected to be more frequent.

Biopsy total eosinophils per mm^2 were again related to activated eosinophils (r=0.76, p=0.001). However, BAL eosinophils showed no significant relationships in this subject group.

Additional ICS treated subjects, relative to pre-treatment:

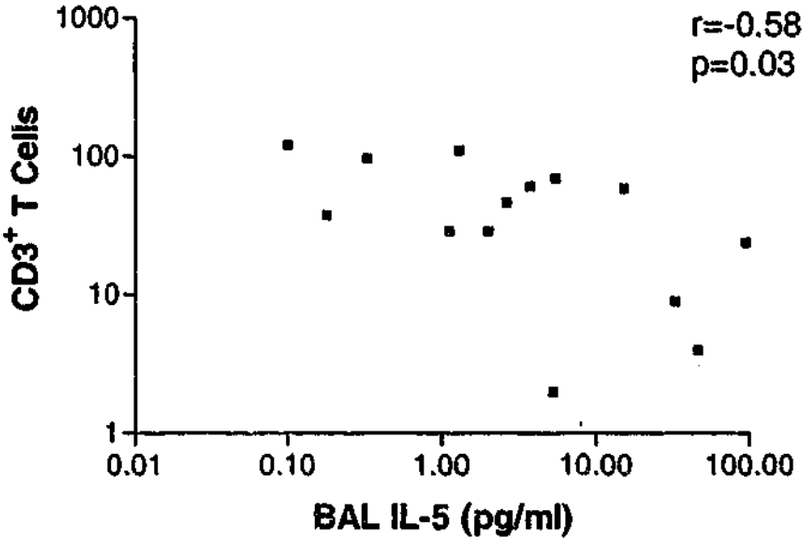
The change in BAL IL-5 levels was inversely related to the change in number of biopsy eosinophils per mm^2 (r=-0.67, p=0.005). However, no relationship was observed between change in BAL IL-5 and change in BAL lymphocytes or eosinophils. The relative change in biopsy eosinophils was also very small.

Changes in the levels of BAL eotaxin were directly related to changes in both biopsy eosinophils (r=0.62, p=0.01, see figure 6.10) and activated eosinophils (r=0.69, p=0.003) per mm^2. These strong relationships support some degree of eosinophil regulation by eotaxin, but were only observed in this subject group.

Figure 6.8 An inverse relationship observed between BAL T cells and BAL IL-5 in subjects treated with additional ICS.

Figure 6.9 A positive relationship observed between biopsy activated eosinophils and BAL eotaxin in subjects treated with additional ICS.

Additional ICS Subject Correlations



Additional ICS Subject Correlations

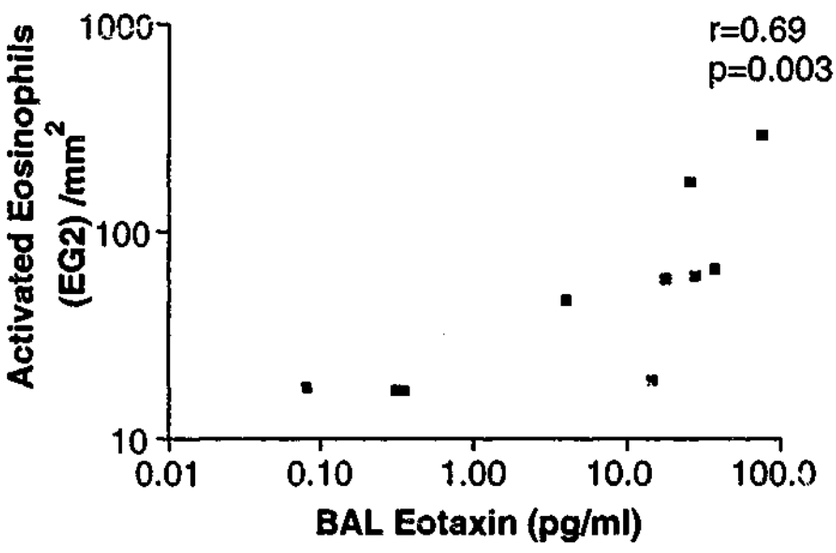


Figure 6.10 A positive relationship observed between change in total biopsy eosinophils numbers and changes in BAL eotaxin in subjects after treatment with additional ICS.

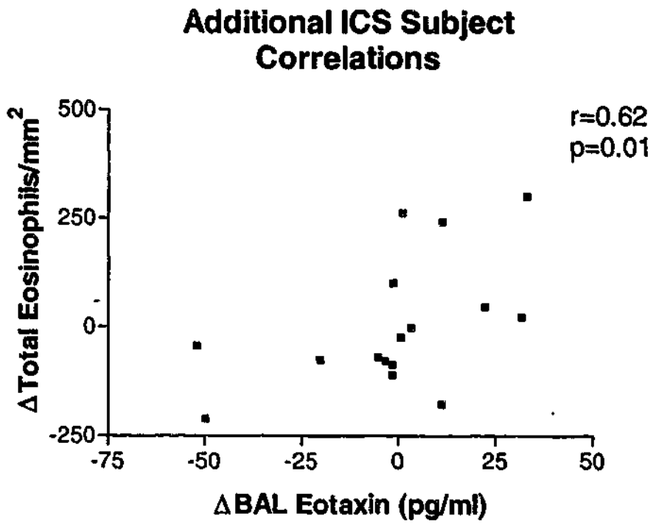
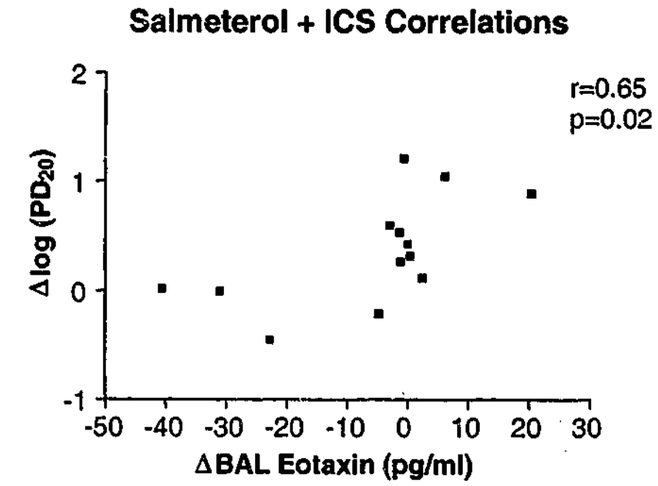


Figure 6.11 A positive relationship observed between change in PD_{20} and changes in BAL eotaxin in subjects after treatment with supplemental LABA.



Changes in total biopsy eosinophil numbers per mm^2 were, unlike in all other treatment groups, unrelated to changes in activated eosinophil numbers, which may suggest changes in the ratio of total to activated eosinophils, when patients are treated with higher ICS doses.

LABA supplemented subjects:

In subjects treated with ICS supplemented with LABA, BAL IL-5 did not correlate with any measures of inflammation. BAL eotaxin was found to be related to BAL airway epithelial cells ($r=0.67$, $p=0.01$), reflecting the findings in the unsupplemented (placebo) subjects. This could perhaps indicate that epithelial cells in the BAL were contributing to eotaxin levels.

Total biopsy eosinophils were related to activated biopsy eosinophils ($r=0.76$, $p=0.003$) per mm^2, but neither correlated with any other factors.

BAL eosinophils were not found to relate to any factors in this treatment group.

LABA Supplemented Subjects, relative to pre-treatment:

Changes in BAL IL-5 levels showed no relationship to changes in any other measured indices in subjects treated with ICS supplemented with β-agonist.

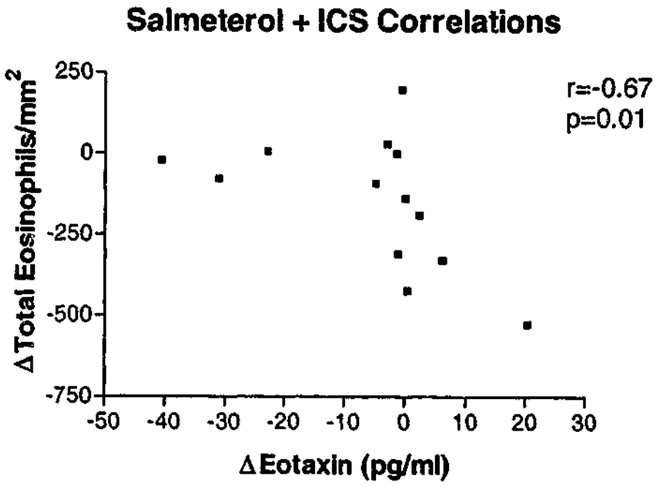
Changes in BAL eotaxin levels were directly related to decreasing airways hyperresponsiveness (increases in log PD_{20}, $r=0.65$, $p=0.02$, see figure 6.11) and inversely related to changes in total biopsy eosinophils ($r=-0.67$, $p=0.01$, see figure

6.12). Taken together these observations suggest that as eosinophil numbers are reduced, perhaps in association with a decrease in airways hyperresponsiveness, eotaxin may actually increase.

Biopsy total eosinophils were found to change relative to biopsy activated eosinophils per mm^2 ($r=0.59$, $p=0.04$).

Changes in BAL eosinophils were unrelated to changes in other factors, which was surprising in this group where a number of eosinophil and eotaxin relationships had already been observed.

Figure 6.12 An inverse relationship observed between change in biopsy total eosinophils and changes in BAL eotaxin in subjects after treatment with supplemental LABA.



6.4 Discussion

This study sought to relate the differences in the eosinophilic cytokines, IL-5 and eotaxin, to previously observed decreases in airway eosinophilia and improvements in physiology when patients already undergoing ICS treatment were supplemented with additional ICS or LABA (Li, *et al*, 1999). It was hoped that improvements in subjects asthma could be related to decreases in airway eosinophils and these in turn to cytokine levels. This study was able to demonstrate improvements in subject lung function and BHR when increased or supplemented treatment was applied, allowing an assessment of whether eosinophils or eosinophil-related cytokines are implicated as underlying these positive clinical and physiological changes.

While the clinical data was presented in the original study, several observations in these patients are relevant to other studies in this thesis. For FEV_1, improvement was only significant in subjects treated with additional ICS, though there was a trend for improvement in subjects treated with ICS and LABA (see figure 6.1). In contrast, PD_{20} showed the reverse (see figure 6.2). This contrast was also observed in our steroid withdrawal study (see Chapter 4), where PD_{20} was far more susceptible to ICS dosage than FEV_1. Furthermore, when analysing the change between all three treatment types, there was no significant difference between any of the groups. This suggests a level of continued improvement even to a small extent in patients whose treatment has not been supplemented, and is consistent with findings that far lower ICS doses are sufficient to maintain high PD_{20} (Fowler, *et al*, 2002) and in some cases may cause PD_{20} to continue to improve.

Again, supporting our observations in other studies undertaken in this thesis, the BAL cellular data, from differential cell counts, changed very little in all subject groups. While the original work showed significant changes in BAL percentage ($CD4^+$) T-cells by flow cytometry, the only significant change by differential counting was a neutrophilia in additional ICS treated subjects, an observation not supported in any of our other groups or studies. Even an increase in macrophage percentage, normally a sign of relative decrease in inflammatory cells, was not observed. This is wholly consistent with the observations from studies in this thesis (See sections 4.3.2 and 5.3.2), which also show a low sensitivity in BAL cell counts, despite large changes in airway eosinophil numbers.

For the BAL cytokines, treatment with additional fluticasone had no further significant effect on IL-5 levels in the BAL, suggesting the initial beclomethasone dosage was sufficient to fully suppress IL-5 production. This was supported statistically, with patients in this study having IL-5 levels not significantly different from normal controls before supplemental treatment was given. The long-term kinetics of IL-5 suppression by ICS *in vivo* in human subjects has not been previously examined. This strong BAL IL-5 suppression by ICS has been observed both cross-sectionally and longitudinally in all ICS treated subjects throughout this project.

Treatment with ongoing low dose ICS plus added salmeterol, appeared to increase levels of IL-5 in the BAL, relative to other treatment types. It may be relevant that these were the only subjects who, after treatment, demonstrated significantly lower airway wall eosinophil levels (see below) as well as being overall the most clinically improved, a finding supported by previous work by other centres (Dente, *et al*, 1999).

Statistical analysis was also performed on the pre-treatment IL-5 data, which showed no significant differences between the three subject groups prior to treatment ($p=0.64$), lessening the likelihood that the rise in BAL IL-5 is merely regression to the mean. This observation must still be tempered by the lack of a relationship in regression analysis between the change in IL-5 levels in the BAL fluid and the decrease in biopsy eosinophils. However, it remains quite a striking finding and the dynamics of airway lumen eosinophil clearance are likely to be very complex.

Although a rise in BAL IL-5 with salmeterol may seems paradoxical, recent work suggests that luminal entry may be a more viable explanation for the clearance of eosinophils from the airways than apoptosis (Erjefalt, *et al*, 2000). Whilst apoptosis was once considered the most likely fate of eosinophils in the airways, it is now considered less likely and indeed salmeterol may actually prevent eosinophil apoptosis in the airways (Kankaanranta, *et al*, 2000). Understanding of the mechanisms for clearance of eosinophils via luminal entry may be of great assistance in understanding the need for airway epithelium to constitutively express eotaxin (Rothenberg, *et al*, 1995) and may assist in explaining why eotaxin appears difficult to suppress *in vivo*. The interesting baseline relationship between epithelial cells in BAL and BAL eotaxin may be relevant to this, pointing perhaps to a source of the eotaxin. Whilst it might be expected that a corresponding rise in BAL eosinophils would occur, work in this area is also sparse, and without any research data available currently on the kinetics of such a model, speculation into the eosinophilic content of the BAL could be misleading. Future work on apoptosis of eosinophils in the BAL may provide further insight.

Eotaxin remained at a fixed level in spite of intense ICS therapy or salmeterol. Though *in vitro* data (Lilly, *et al*, 1997) suggests that eotaxin in cells is easily suppressible with steroids, there are little data available *in vivo* in the airways. It is possible that airway eotaxin is readily suppressed by ICS, but returns to baseline levels rapidly, or remains at a fixed high level in an asthmatic airway. There is currently no other human *in vivo* data, other then this current work, as far as we are aware on BAL eotaxin levels and ICS interaction. It seems clear, however, that these higher than normal levels of eotaxin are suggestive of an ongoing constitutive abnormality that is not effectively dampened by current treatments. This may explain why in asthma recurrence of symptoms and repopulation of the airways by eosinophils can occur rapidly when ICS treatment is withdrawn (Humbles, *et al*, 1997). Although genetic polymorphisms within the eotaxin gene could create a disposition toward higher levels of eotaxin expression, no such variation has yet been described. A high degree of variation in the 3'-untranslated region of the eotaxin gene (Bartels, *et* al, 1996) has been identified, but no functional significance has yet been assigned.

The biopsy eosinophil data showed no changes in the unsupplemented and double dose-ICS treated subjects, but a significant reduction in subjects treated with combined ICS and LABA. Activated eosinophils were not significantly reduced in any of the groups, but again ICS and LABA treated subjects showed a reasonably strong trend ($p=0.14$). Analysis of the baseline data showed that the biopsy eosinophils counted in the ICS and LABA treated subjects were significantly higher (ANOVA: $p=0.02$) than both the ICS supplemented and unsupplemented subjects. Unlike the BAL IL-5, where no difference was observed between the subjects pre-

treatment, the eosinophil data suggests the possibility that the observed differences could be due to regression to the mean after treatment, as no statistical difference between the total eosinophil counts post-treatment were observed (ANOVA: p=0.51).

No simple direct relationship was detected overall between BAL eotaxin and BAL IL-5 levels, or between either cytokine and airway eosinophils. However, a significant positive correlation was observed between BAL eotaxin and biopsy eosinophils in subjects treated with supplemental fluticasone (see figures 6.10 and 6.11). This was the only situation in which we found this relationship that we had hypothesised to be more generally present. This eotaxin/eosinophil relationship was not preserved, and was actually reversed, after combined treatment with low dose ICS and salmeterol (see figure 6.13). This may support our hypothesis on luminal clearance of eosinophils, in that increase in airway epithelial eotaxin expression could be a prerequisite for chemotaxis of eosinophils into the lumen under the right combination of circumstances.

The subjects showing the strongest and most numerous cellular and cytokine relationships are those whose steroid levels were approximately doubled. It may be that the influence of the high ICS dose in this group was sufficient to inhibit most typical inflammatory processes and thus expose the underlying eotaxin/eosinophil relationship that may be difficult to detect under more complex, competing and confounding influences.

In summary, the two principal findings of this study were that BAL eotaxin levels remained elevated and unchanged in both fluticasone and salmeterol treated subjects

over a number of months, and that IL-5 increased after salmeterol was added to low dose ICS. This may then assist in luminal clearance of eosinophils, as it was concurrent with an apparent decrease in airway biopsy eosinophils. Both these findings are novel for *in vivo* human asthma research, and the relative steroid tolerance of eotaxin compared to IL-5 are supported by the findings of our other studies. The many highly complex relationships in a system such as asthma serve to complicate *in vivo* clinical studies such as this to an extent that it can be difficult to differentiate signal from noise or confounders. Taking two instantaneous points in time and generating only a few specific pieces of data can only show us a limited picture. Yet, the fact that we see any differences in these cytokines at all, despite the noise and complexity, only stresses the likely relevance and potential importance of such investigations, which in time may allow decisive intervention in the disease process.

Chapter 7: Summary and Conclusions

7.1 Overview

This thesis has made several novel observations into the interaction between ICS treatment, eosinophils and eosinophilic cytokines in the asthmatic airways. The eosinophil has long been associated with allergic disease, but its precise role in asthma is still not clearly understood and is currently under some revision (see section 1.2). The cytokines of interest were: IL-5 (see section 1.3.2), able to modulate a number of eosinophil developmental, activation and survival processes; and eotaxin (see section 1.3.3), a chemokine unique is its ability to induce local chemotaxis in eosinophils exclusively.

Inhaled corticosteroids have come to prominence in their ability to effectively suppress asthma, but despite this, their precise *in vivo* actions, particularly with regards to the immune system, are not well understood (see section 1.5). While the interaction between eosinophils, cytokines and ICS treatment is doubtlessly complex and contains many levels of redundancy, we have endeavoured to examine a few important effects of specific cytokines to eosinophil function. By doing this, we hoped to be able to further explore the role of the eosinophil in the asthmatic airway, and related anti-eosinophil actions of ICS treatment. The role of IL-5 and eotaxin in asthma has been poorly explored in human *in vivo* studies, and it is our hope that this thesis will provide a substantial contribution to the understanding of these interactions.

We had acquired a large base of asthmatic subjects from three longitudinal bronchoscopic studies, as well as a number of patients on a range of ICS-dosages, available for cross-sectional studies, and a large number of non-asthmatic control subjects. For each subject, lung physiology, BAL and biopsies were collected (see section 2.2), and blood was also collected in several of the studies. Physiological measurements included FEV_1 and BHR (PD_{20}). Eosinophils in BAL were measured by total and then differential cell counting and in biopsies using immunohistochemistry (see section 2.3) for the granular markers EG1 (total eosinophils) and EG2 (activated eosinophils). Several methods were attempted extensively to accurately quantify IL-5 and eotaxin. Immunofluorescence in biopsies (see section 2.8) and molecular biology for mRNA detection in both biopsy and BAL cells (see section 2.10) were unfortunately unsuccessful, even after a great deal of time, effort and expenditure of resources. However, we were able to develop a sensitive chemiluminescent ELISA system, which was able to measure both IL-5 and eotaxin in the BAL fluid (see section 2.9).

7.2 Studies

There were four principle results sections in this thesis, a cross-sectional analysis of the data (see Chapter 3), from various treatment groups, and then three longitudinal studies. The first of these studies, on which the greatest amount of developmental time and resources of this thesis was spent, was a steroid reduction study (see Chapter 4). In this study, patients were treated with a high-ICS dose to comprehensively suppress their symptoms, and then treatment was reduced to an extent where asthmatic symptoms just remerged, the objective being to catch the eosinophil and cytokine changes at the point of symptom recurrence. The second study (see Chapter 5)

involved examining symptomatic ICS-free asthmatics and then placing them on long-term ICS to measure the changes in eosinophils and cytokines as symptoms became suppressed. The final study (see Chapter 6) involved examination of the cytokines in a group that we already knew had airway eosinophils significantly reduced through the supplementation of ICS treatment with LABA (Li, *et al*, 1999) in an attempt to tie the cellular changes to changes in cytokine levels. The major findings in each of these studies are summarised below.

7.2.1 Cross-sectional Data

The cross-sectional analyses undertaken in this thesis established an important framework from which to examine the longitudinal data. For the BAL no significant differences were seen in eosinophil numbers, however, a trend (p=0.08) toward higher numbers of eosinophils in ICS-free asthmatics compared to non-asthmatics was observed. Airway epithelial cells in the BAL were very high in ICS-free asthmatics when compared to those treated with ICS and non-asthmatics, reflecting a level of epithelial disruption in the untreated asthmatic subjects. Macrophages, as a percentage, were also lower in untreated asthmatics than normal controls, indicating an increase in the total proportion of inflammatory cells in the BAL of asthmatic patients (see section 3.3.1).

The sensitivity of BAL differential cell counts to distinguish airway inflammation in asthmatics, even untreated with ICS, seemed marginal and for a majority of factors there was no difference between symptomatic untreated asthmatics and non-asthmatic control subjects. This was particularly highlighted for eosinophils, where we would have expected a large difference between asthmatics and non-asthmatics. The

exception to this was in the epithelial cell shedding data, which was very interesting and probably warrants further investigation.

In contrast to the BAL cellular data, the IL-5 and eotaxin protein data from the BAL were both shown to be very high in untreated asthmatics when compared to normal controls (see section 3.3.2). In patients treated with low doses of ICS, IL-5 was significantly lower ($p<0.01$) than in untreated asthmatics, whereas eotaxin remained elevated, and did not significantly differ, from untreated asthmatics. Patients treated with high-ICS doses had significantly lower levels of both IL-5 ($p<0.05$) and eotaxin ($p<0.01$) than untreated asthmatics. As both cytokines are steroid responsive *in vitro*, the reductions observed in ICS-treated patients were not unexpected, though the requirement for a higher ICS dose to significantly reduce eotaxin was a novel finding. The relative ICS insensitivity of eotaxin could provide a basis for the immediate chemotaxis of eosinophils into the airways when ICS treatment is withdrawn and why rapid deterioration in asthma activity seems generally to occur when ICS are withdrawn.

Biopsy eosinophils were much more sensitive and reflective, than BAL cell counts, of the inflammatory condition of the airways at different levels of treatment (see section 3.3.3). Eosinophil counts in airway biopsies were almost 10-fold higher in untreated asthmatics than in non-asthmatic controls. Patients treated with both high and low-dose ICS treatment showed intermediate numbers of eosinophils, lower than untreated asthmatics, but higher than non-asthmatic subjects. There was no significant difference between eosinophil levels in the low and high-dose ICS treated subjects. While the kinetics of airway wall clearance of eosinophils into the lumen are not

clearly understood, it would seem that the number of eosinophils in the BAL remains relatively fixed, and does not directly reflect the vastly increased eosinophils in the airways of asthmatic patients.

7.2.2 Steroid Withdrawal Study

It was hoped that eosinophils and cytokines could be examined close to the beginning of disease reactivation with early symptom recurrence. Subjects showed large improvements in lung function, BHR, symptom scores and peak flows when placed on high-dose ICS treatment (see section 4.3.1). When ICS levels were then reduced, patients experienced a worsening of lung function (FEV_1 and FEF_{25-75}, but not FVC), peak flows and symptom scores, but there was no change in BHR (PD_{20}). The observed divergence of lung function and BHR is novel and may reflect that the subjects in this study were indeed very much at a very early point of disease activity re-emergence. This point at which the ICS-treatment effect has only just begun to fade is potentially very important for facilitating the detection of pathologic mechanisms. Thus it may be possible to identify what measures are important to the re-initiation of inflammation and therefore potentially important in the initiation of asthma.

The BAL cellular data in this group showed no significant changes or trends in any of the measured cell types after reduction of treatment (see section 4.3.2). Thus, as indicated by the cross-sectional data, BAL cell measurements proved to be quite insensitive in the measurement of eosinophils.

In the airways, tissue eosinophil numbers also did not significantly change (see section 4.3.6). The cross-sectional data suggest that airway eosinophils are a very

sensitive measure, and greatly affected by ICS. The lack of change in eosinophils with reduction in ICS treatment, and definite disease deterioration suggests that eosinophils may be involved in a later stage of asthmatic disease rather then its initiation. This distinction does not preclude the eosinophil for being important in exacerbation of asthma or later changes such as airway remodelling, but suggests that clinical deterioration occurs in asthma before eosinophils are involved.

The IL-5 and eotaxin levels in the BAL likewise did not change after the reduction of ICS treatment. This was surprising, as we had hypothesised that an increase in eosinophilic cytokines would occur at a very early stage of the inflammatory process. From these observations it seems clear that IL-5 and eotaxin are not vital to the establishment of symptoms in asthma, at least not initially. This does not mean these cytokines are not important in the recruitment of eosinophils, but merely that events can occur in the airways that cause a worsening of asthma which precedes the upregulation of IL-5 and eotaxin.

In this study, blood was taken for IL-5 and eotaxin measurement from the serum (see section 4.3.8), as this was the only study in this thesis that included serum data, cross-sectional analysis of the serum cytokines was also performed here. Serum IL-5 was found to be elevated in ICS-free asthmatics (from the small pool of available serum) above normal controls ($p=0.05$). In ICS-treated patients, serum IL-5 was intermediate between these groups. Longitudinally, reduction of ICS dose did not cause a significant rise in IL-5, though there was perhaps a slight trend ($p=0.12$). Serum eotaxin was not different in any asthmatics and normal controls cross-sectionally, and was remarkably conserved longitudinally, barely changing after reduction in ICS

treatment. Blood eosinophils also did not change. These data seem to indicate that eotaxin is largely a localised chemokine, rather than being systemically inducable. While serum IL-5 also did not change, it was closely related to patient peak flows (see section 4.3.9) and those patients whose peak flows were most reduced after ICS reduction were those who had the most increased levels of serum IL-5. This suggests that we were beginning to see changes in systemic IL-5, presumably at a very early stage before the effects on circulating eosinophils occur.

7.2.3 Effects of Long Term ICS

We observed the changes in eosinophils and cytokines when symptomatic patients were aggressively treated with ICS over a period of several months. We hypothesised that eosinophils, IL-5 and eotaxin would be suppressed by treatment and would relate to each other and also to improvements in lung function or BHR. This was our most straightforward study in design and had the greatest contrast in the condition of subjects between beginning (ICS-free) and end (treated aggressively with high-ICS dosages).

Physiological improvement in the ICS-treated patients was substantial after 3 months (see section 5.3.1). After 12-months BHR continued to improve, whereas lung function (FEV_1) did not. This observation suggests that lung function reached its maximum level of improvement by 3-months, whereas BHR changed more gradually. This divergence was reminiscent to what was seen in the ICS withdrawal study (see above), and again may indicate that lung function cannot be directly related to BHR.

In the BAL cells, eosinophils were reduced in ICS treated subjects, but only after 12-months of treatment; airway epithelial cells also showed great reduction even after 3-months of ICS treatment (see section 5.3.2). This was the only time a significant change was seen in eosinophils in the BAL. Even these changes were quite small compared to the changes in biopsy eosinophils, further reinforcing what we had already observed cross-sectionally. ie. BAL differential cell counts were poor indicators of the eosinophil numbers in the airways. The very significant ($p<0.001$) reduction in epithelial cells in the BAL was again reflective of our cross-sectional observations and this study group would be very useful for a future study of the assessment of epithelial integrity in airway biopsies.

BAL IL-5 levels were significantly reduced after 3-months of ICS treatment, but were only slightly lower than this after 12-months. In contrast, BAL eotaxin in the ICS-treated patients did not significantly change over the entire study (see sections 5.3.3 and 5.3.4). While the BAL IL-5 changes were much as we had hypothesised, ie. that ICS-treatment would reduce eosinophilic inflammation and cytokines, the lack of change in BAL eotaxin was unexpected. Whilst some apparent effect of ICS treatment was observed cross-sectionally on BAL eotaxin levels, no changes were seen in any of our longitudinal studies, including this one, while all other eosinophilic and physiological measures had changed after 12-months of treatment. It seems clear that the responsiveness of eotaxin in the airways to ICS treatment is quite low.

In airway biopsies, in contrast to BAL, ICS-treated subjects showed a large reduction in both total and activated eosinophil numbers (see section 5.3.5) after 3 months. No further reduction was seen at 12-month of treatment. This reduction in eosinophil

numbers, while concurrent with drops in BAL IL-5 and lung function, only showed a significant direct relationship with PD_{20}. This again seemed to emphasise a close relationship between eosinophils and BHR, whereas lung function could not be easily related to other measures. The data also emphasise the lack of correlation between airway wall eosinophils and BAL eosinophils, although the relationship between them may be complex and not detectable by simple time-point sampling.

7.2.4 LABA/ICS Supplemental Study

Our final study took patients already undergoing ICS treatment and supplemented that treatment by doubling the ICS dose, or adding additional LABA (see Chapter 6). A portion of this work had already been published (Li, *et al*, 1999), and showed a decrease in the biopsy, but not the BAL eosinophil numbers in patients receiving ICS and additional LABA. We aimed to add to this work by looking at the eosinophilic cytokine levels and relating them to the observed changes in eosinophil numbers and physiology.

The physiological changes in this study were subtler than in the other studies (see section 6.3.1). Some improvement in lung function was seen with both supplemental ICS and LABA, but only the ICS group showed significant improvement. PD_{20} also improved in both groups and while the increase was only significant in the LABA group ($p=0.02$), there was also a strong trend in patients treated with supplemental ICS ($p=0.052$). However, the increase in PD_{20} after LABA may have been an acute inflammatory effect, rather than reflecting a change in the underlying disease status. From the clinical data alone it was difficult to determine which group was most improved, but interestingly it was only the supplemental LABA group that

demonstrated significantly reduced eosinophils in the airway wall. We also did not observe the divergence between lung function and BHR that was seen in the other longitudinal studies, though the physiological changes in this study were quite small.

Due to the baseline use of ICS, levels of BAL IL-5 were suppressed to near non-asthmatic concentrations at the beginning of the study (see section 6.3.3). It was therefore not surprising that supplemental ICS treatment did not further reduce IL-5 levels. Supplemental LABA, however, did have an unexpected effect: BAL IL-5 levels in this group were elevated after 3-months of treatment. This was not something we had observed in other studies, cross-sectionally or longitudinally, and may be an effect related to the clearance of eosinophils from the airway wall. Investigation of IL-5 sources in the airways, such as T cells or epithelial cells, might also assist in explaining the increased BAL IL-5. BAL eotaxin may have shown a level of suppression prior to supplemental treatment (see section 6.3.4), enough to make eotaxin levels not significantly different to those of non-asthmatic controls in this group, compared with other studies. However, supplemental treatment with ICS or LABA had no additional effect on eotaxin levels. These initially low eotaxin levels may suggest that ICS had some effect on eotaxin *in vivo*, though perhaps only over a very long time. Unfortunately, our long term (12-months) ICS study did not show decreases in BAL eotaxin, which may suggest even longer ICS exposure is required.

7.3 Conclusions

This thesis hypothesised that there would be quantifiable relationships between eosinophils, eotaxin and IL-5, that would relate to physiological measures and be affected by ICS treatment. Several such interactions have been successfully shown.

We have shown that IL-5 was readily influenced by ICS treatment, even over short terms and while not directly related to eosinophil number in the airways, IL-5 had a tendency to change when airway eosinophils changed. Likewise, when eosinophils were constant, IL-5 did not vary much.

Conversely, eotaxin, which we had expected to be closely related to IL-5, remained unchanged throughout all our longitudinal studies, regardless of ICS or LABA treatment. Cross-sectional data suggested that eotaxin was mildly influenced by ICS treatment, but such influences, based on our longitudinal observations, seem very small.

In relating these cells and cytokines to physiological measures, it was demonstrated that eosinophils and IL-5 had a much closer association with BHR than with lung function. In fact, while BHR related to several measures of eosinophils and BAL and serum IL-5 across several studies, none of our measured factors showed any strong relationship to lung function, which seemed independent of our factors of interest. We also observed a divergence between BHR and lung function, which though in some circumstances are closely related, changed at different rates in several studies. Of particular note was an observation that lung function, upon reduction of ICS treatment, deteriorated before BHR or eosinophils changed, which very strongly implies that eosinophilic factors may not be vital, at least to the initiation of asthmatic symptoms.

Eotaxin was not readily affected by ICS, did not change even when eosinophils changed greatly, and could not be easily linked to physiological measures. It is thus

difficult to conclude that eotaxin plays an important role in the pathology of asthma. Even if anti-eotaxin therapy was developed, it is likely that the redundancy within the eosinophil chemotactic network would result in upregulation of other chemotactic factors such as MCP or eotaxin-2, and perhaps ultimately have little effect on eosinophil numbers in the airways. From our observations, IL-5 in the airways, readily influenced by ICS treatment, seemed to be more important in the regulation of eosinophils. IL-5 seems a far better therapeutic target, being directly involved in many aspects of eosinophil function. Unfortunately, recent clinical trials (Flood-Page, *et al,* 2002) have shown disappointing results with anti-IL-5 antibody therapy, leaving airway eosinophils persisting to some extent. The question of the role of the eosinophil in asthma pathology remains. A key might be the link between recruitment of eosinophils to the airways and the clearance of them into the lumen. The data with LABA may be important and illuminating in this regard.

While this thesis has only looked at a small aspect of eosinophil regulation, this work shows that lung function deterioration occurs significantly before changes occur in eosinophils, eosinophilic cytokines or even BHR. This is a novel finding. This work shows that these latter measures do change in the treatment of asthma with ICS, but that they may change after lung function improves. It is therefore difficult, with these observations, to conclude that the eosinophil is ultimately responsible for the development of asthmatic symptoms. However, until the role of the eosinophil in asthma can be completely excluded, which seems unlikely, elucidation of the cytokine cascades involved in eosinophil activation and chemotaxis will remain important. However, the direction of our groups' asthmatic research in the near future, as a result of the findings of this thesis, will be directed at determining the other cellular events

that might precede the changes in these eosinophilic cytokines, which we hope may be able to determine what, if not the eosinophil, is responsible for the early initiation of asthmatic symptoms.

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